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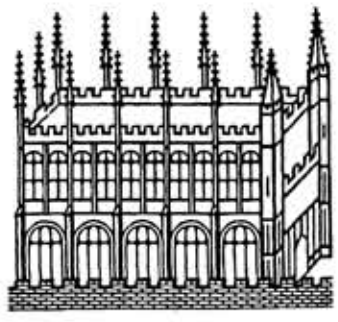

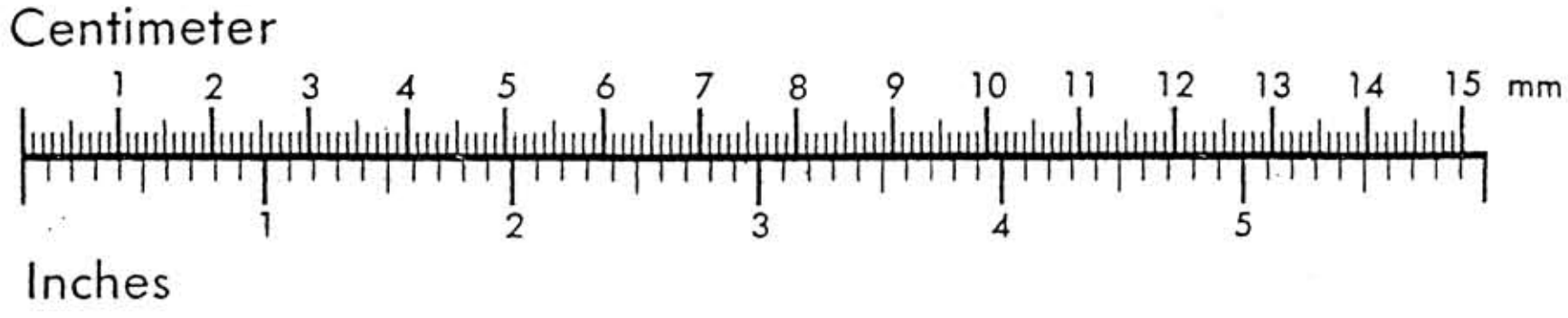

ROLE OF THE NMB0342-NMB0348 LOCUS IN MENINGOCOCCAL PATHOGENESIS AND INVESTIGATION OF NMB0345 AS A VACCINE CANDIDATE

A thesis submitted for the degree of Doctor of Philosophy

of the University of Oxford

by

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Abstract

Neisseria meningitidis is the most common cause of bacterial meningitis in the Western world and the second leading cause of mortality in 1-5 year-olds in the United Kingdom. A signature-tagged mutagenesis screen of approximately 3,000 insertional mutants of a serogroup B isolate of *N. meningitidis*, C311^{+}, identified 73 genes required for pathogenesis in an infant rat model of meningococcal septicaemia. Homology-based searches indicate that two of the genes identified, NMB0342 and NMB0345, have homologues in other pathogenic bacteria and exist within a potential operon of seven genes (NMB0342-NMB0348). NMB0342 is a homologue of *ispA* (intracellular septation protein) of *Shigella flexneri*, required for effective intercellular spread and plaque formation in epithelial cells. NMB0345 is a homologue of *cbf*, a *Campylobacter jejuni* gene that encodes a 29 kDa protein which is a major antigenic peptide. PCR and Southern analyses of genes in the NMB0342-NMB0348 locus show that they are conserved across a wide range of pathogenic isolates and serogroups of *N. meningitidis*. However, these genes are present in only a sub-set of commensal strains. *N. meningitidis* mutants with transposon insertions in NMB0342 and NMB0345 are highly attenuated when directly competed with the wild-type bacterium in the infant rat model of infection. Mutations have been introduced into other genes within the locus and the resulting mutants analyzed for their ability to cause systemic disease. Mutants with transposon insertions in the NMB0343 and NMB0344 genes are significantly attenuated, while mutants with insertions in NMB0347 and NMB0348 are attenuated to a lesser degree. Complementation of the NMB0342 mutation by ectopic chromosomal integration of a wild-type copy of NMB0342 almost completely restores the virulence of the bacterium. *In vitro* cell-culture analyses and microscopic analysis of mutants in association with host cells demonstrate that a mutant with a transposon insertion in NMB0345 is significantly reduced in its adherence to Chang epithelial cells compared to the wild-type bacterium. Whole blood and serum-sensitivity assays show that the NMB0342 and NMB0345 mutants are highly serum-sensitive compared to the wild-type bacterium. Preliminary experiments have demonstrated that immunization of mice with recombinant NMB0345 protein confers protection against challenge with the serogroup B isolate MC58.

In vitro assays demonstrate that recombinant NMB0345 is an active peptidyl prolyl *cis-trans* isomerase (PPIase). Together, the results from these investigations support a role for genes of the NMB0342-NMB0348 locus in the pathogenesis of *N. meningitidis* infections. Further investigation is needed to assess the potential of NMB0345 as a vaccine candidate.

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List of Abbreviations

amp	ampicillin
APS	ammonium persulphate
bp	base pairs
BBB	blood brain barrier
BSA	bovine serum albumin
carb	carbenicillin
CFU	colony forming units
CNS	central nervous system
CTAB	cetyl-trimethyl-ammonium bromide
DAPI	4′,6-Diamidino-2-phenylindole
DIC	differential interference contrast microscopy
DIC	disseminated intravascular coagulopathy
DNA	deoxyribonucleic acid
dCTP	2′-deoxycytosine 5′-triphosphate
dH_20	distilled water
ddH_20	double distilled water
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EtBr	ethidium bromide
EtOH	ethanol
g, mg, μg, ng, pg	gram, milligram, microgram, nanogram, picogram
xg	times gravity (relative centrifugal force)
HUVECs	human umbilical vein endothelial cells
IP	intraperitoneal
kan	kanamycin
kb	kilobase pairs
kDa	kilodalton
L, ml, μl	litre, millilitre, microlitre

LB	Luria Bertani
LPS	lipopolysaccharide
MAC	membrane attack complex
M, mM, μM, nM	molar, millimolar, micromolar, nanomolar
NaOH	sodium hydroxide
NHS	normal human serum
nm	nanometer
nt	nucleotide
OD	optical density
ORF	open reading frame
OMP	outer membrane protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPIase	peptidyl prolyl *cis-trans* isomerase
PVDF	polyvinylidene fluoride
rev	revolutions
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TAE	tris acetate EDTA
TE	tris-EDTA
TEMED	tetramethyl-1-,2-diaminomethane
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
V	volts

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Introduction

1.1 Microbiology

The genus *Neisseria* is a member of the Neisseriaceae family of Gram-negative bacteria. *Neisseria meningitidis* (meningococcus) and *Neisseria gonorrhoeae* (gonococcus) are the two pathogenic species of this genus and are closely related at the nucleotide level. The only known host for both species is man (Guibourdenche *et al.*, 1986). *N. meningitidis* is an aerobic diplococcus, 0.6-1.0 μm in diameter, carried in the human nasopharynx, which is the only reservoir for this bacterium, and dissemination from person to person is thought to occur via respiratory droplets (Apicella, 1990). *N. meningitidis* is normally cultured in a peptone-blood base medium at 37°C in the presence of 5-10% CO_2. The organism is highly susceptible to temperatures either above or below 37°C and following death, undergoes rapid autolysis both *in vivo* and *in vitro* (Morse, 1996).

1.2 History of meningococcal disease

Accounts of patients with symptoms resembling meningococcal septicaemia and/or meningitis (inflammation of the membranes surrounding the brain and spinal cord) have been recorded from as early as 460-360 B.C. Hippocrates described a condition that featured headache, tinnitus, inflammation of the brain, and a high rate of mortality. Claudius Galen (C 129-216 A.D.) and Rhazes (al-Rhazi) (C 865-925 A.D.) also described conditions with symptoms similar to those of meningitis. However, the Swiss physician Vieusseux is recognized as the first to provide a detailed description of an outbreak of the

disease that occurred in Geneva in 1805. Symptoms recorded, include violent headache, vomiting, stiffness of the spine, and livid patches on the skin (Vieusseux, 1806). In 1879, the German scientist Albert Neisser, who was studying gonorrhea, named the genus of the causative organism *Neisseriae*. Isolation and culture of the meningococcus from patients was unsuccessful until 1887, when Anton Weichselbaum, a scientist working in Vienna, isolated the bacterium from six out of eight meningeal exudates. Weichselbaum subsequently named the organism *Diplococcus intracellularis meningitidis* (Heubner, 1896). Eight years later, the use of lumbar puncture allowed the isolation of meningococci from the cerebrospinal fluid (CSF) of patients with meningitis.

Prior to the development of antibiotics, the rapid onset and high mortality associated with meningococcal disease, combined with the absence of any defined treatment caused considerable fear and anxiety. The first successful treatment for meningococcal disease was developed in 1906 by a German bacteriologist, Jochmann, who used horse serum raised against meningococci to treat patients with meningococcal meningitis. Twelve of the seventeen patients treated with this serum survived (Jochmann, 1906). Soon after, Simon Flexner and James Jobling of the Rockefeller Institute developed an effective anti-meningitis serum that was used to treat four hundred cases from Europe and the U.S.A., reducing mortality from 70-80% to 25%. In addition, they were able to demonstrate the usefulness of intra-spinal immunotherapy, a route of treatment used until the 1930s. Sulphonamides were developed between 1935 and 1940 and proved to be an effective treatment for meningococcal disease, reducing mortality to 10% (Schwentker *et al.*, 1937). These drugs inhibit the growth of the meningococcus and thereby allow the establishment of

an antibody response. Sulphonamides continued to be used until the introduction of penicillin in 1939. Today, β-lactam antibiotics including penicillin, ampicillin and the third generation cephalosporins, ceftriaxone and cefotaxime, are the main drugs used to treat meningococcal disease. Despite the use of these antibiotics, mortality resulting from meningococcal disease today remains at approximately 10% (Havens *et al.*, 1989). However, a five-year study of 331 children with meningococcal disease by Booy *et al.* demonstrated that a program of improvements in healthcare management and delivery was able to reduce mortality to 2% (Booy *et al.*, 2001).

1.3 Classification

Conventionally, meningococcal strains have been defined using antibodies that recognize surface-exposed epitopes present on either the capsule or the outer membrane. *N. meningitidis* strains are designated by one of 13 different serogroups (A, B, C, E-29, H, I, K, L, M, W_{135}, X, Y, and Z) based on the structural differences in their capsular polysaccharides (van Deuren *et al.*, 2000). A common feature of the disease-associated serogroups B, C, Y, and W_{135}, is the presence of sialic acid in their capsular polysaccharide (Jarvis and Vedros, 1987). The non-sialic acid capsule of group A *N. meningitidis* is composed of *N*-acetyl mannosamine-1-phosphate (Liu *et al.*, 1971). Isolates can be further divided into serotypes based on the identification of class 2/3 outer membrane protein (OMP) antigens (PorB) (Frasch *et al.*, 1985). Serosubtyping is based on sequence variation in PorA, the class 1 OMP only expressed by *N. meningitidis* (Feavers and Maiden, 1998) (Frasch *et al.*, 1985). *N. gonorrhoeae* is the only other *Neisseria* species known to have a

porA gene. However, the gene is not expressed due to the presence of frameshift and promoter mutations (Derrick *et al.*, 1999). Isolates can also be designated one of 13 L-types or immunotypes, based on the antigenic properties of lipopolysaccharide (LPS).

Multilocus enzyme electrophoresis (MLEE) has been used for the identification of clones particular to disease isolates by assigning alleles electrophoretic types (ET) according to the electrophoretic mobility of the enzymes they encode. A difficulty encountered with this method is that isolates with different alleles at the nucleotide level may be indistinguishable due to similar electrophoretic mobilities. A recently developed method, multiple locus sequence typing (MLST), allows the direct assignment of alleles based on the nucleotide sequence of seven house-keeping genes, and is therefore able to distinguish a larger number of alleles per locus than MLEE (Maiden *et al.*, 1998).

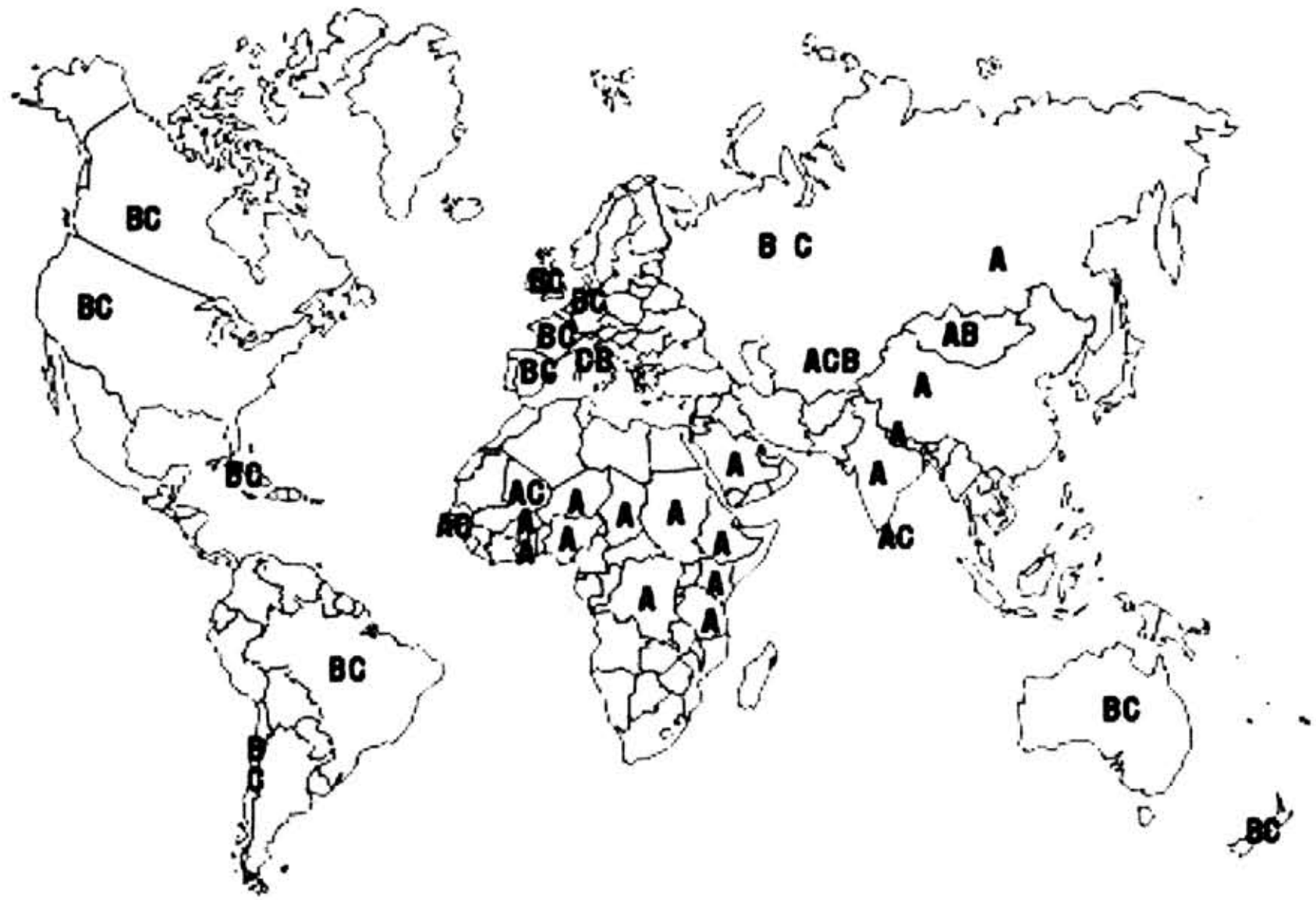
1.4 Epidemiology

N. meningitidis is the most common cause of bacterial meningitis in developed countries (Jones, 1995), and the second leading cause of mortality in children between the ages of one and five in the UK after road traffic accidents (Ramsay *et al.*, 1997). The method traditionally used to estimate rates of carriage of *N. meningitidis* in the general population is nasopharyngeal swabbing which gives a rate of approximately 10% (Griffiss, 1995). However, a study comparing the rates of detection of *N. meningitidis* in tonsillar tissue by swabbing and by immunohistochemistry found that swabbing detected only about a quarter of the carriers identified by immunohistochemistry (Sim *et al.*, 2000).

The incidence of meningococcal disease recorded annually ranges from 0.5 to 10 per 100,000 persons, but can be higher than 400 per 100,000 persons during epidemics (Zollinger, 1997) (Schuchat *et al.*, 1997). Serogroup B and C isolates are responsible for the majority of infections in industrialized countries, whereas serogroup A infections, and to a lesser extent serogroup C, dominate in third world countries (Fig. 1.1). Serogroup B isolates cause approximately 50-90% of cases in Europe and are particularly prevalent in Norway, the Netherlands, Germany, and Denmark (Cartwright *et al.*, 2001). The remainder of cases are largely due to serogroup C strains. The incidence of infections caused by serogroup B isolates is highest in infants of less than one year of age (Jodar *et al.*, 2002).

The majority of serogroup B disease isolates are members of four clonal complexes; ET-5, lineage III, cluster A4, and ET-37 (van Deuren *et al.*, 2000). *N. meningitidis* strains belonging to the ET-37 complex cause up to 40% of meningococcal disease in Europe and the United States and are commonly associated with institutional outbreaks of serogroup C disease. Members of the ET-5 complex were responsible for increased incidences of hyperendemic meningococcal disease in Western Europe during the 1970s and 1980s.

Fig. 1.1 Worldwide distribution of predominant *N. meningitidis* serogroups A, B, C, Y, and W135 (1996-1997)



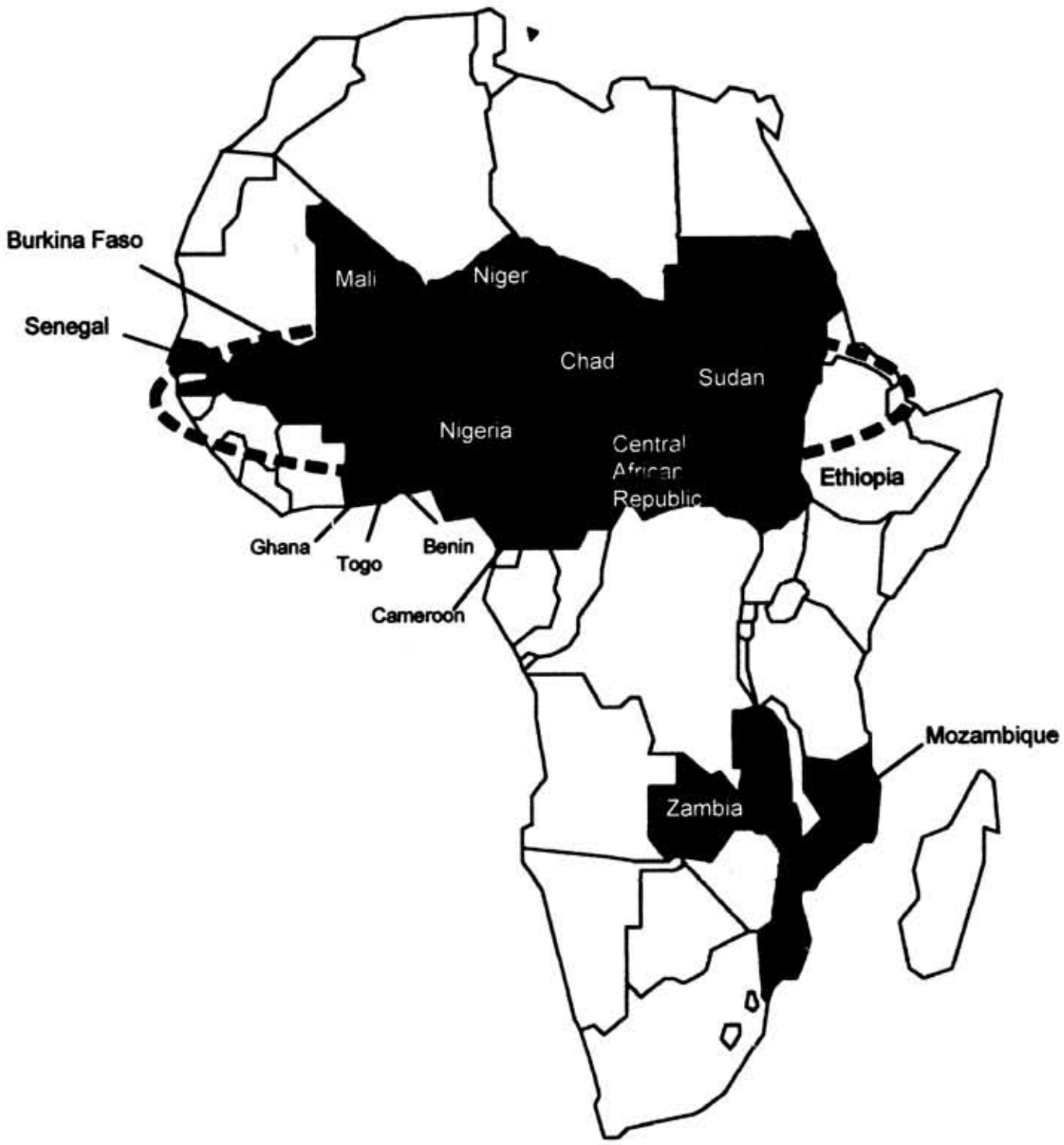
Tikhomirov *et al.*, 1997

However, these strains were largely replaced as a major cause of meningococcal disease in many countries by strains belonging to the clonal complex, lineage III. The reasons for such shifts in the prevalence of particular clonal complexes are not clear, but it is thought that ET-5 complex and lineage III strains, for example, may have prolonged carriage compared to meningococcal strains belonging to the subgroups of serogroup A (Blakebrough *et al.*, 1982).

Meningococcal epidemics resulting from serogroup A infections predominate in sub-Saharan Africa (Moore, 1992). This region, termed the meningitis belt, comprises 15 countries, where epidemics caused by serogroup A strains occur in 8-14 year cycles, with the number of cases rising at the end of the dry season, and declining rapidly after the start of the rainy season (Fig. 1.2). Infections caused by serogroup A isolates are also responsible for the majority of cases of meningococcal disease in China where epidemics occur approximately every 8-10 years (Caugant, 1998).

Recent years have seen a global rise in infections caused by serogroup W_{135}. The majority of these infections have been associated with disease outbreaks that occurred during the Hajj pilgrimage to Mecca, Saudi Arabia in 2000 and 2001 (Wilder-Smith, 2003; Wilder-Smith *et al.*, 2003).

Fig. 1.2 The African meningitis belt



Countries of Africa in which serogroup A infections predominate are shown in blue. Countries comprising the meningitis belt, where epidemics caused by serogroup A *N. meningitidis* occur in 8–14 year cycles, are indicated within a dashed line (WHO, 2001).

While it remains unclear why particular serogroups predominate in certain parts of the world, the occurrence of invasive meningococcal disease is thought to be determined by a combination of exposure to a new virulent strain, environmental factors that may enhance transmission, and the susceptibility of the population (van Deuren *et al.*, 2000).

1.5 Risk factors for carriage

Carriage rates of *N. meningitidis* can be influenced by numerous factors including age, sex, smoking, occupation, and meningococcal vaccination (Cartwright, 1995). Studies in Western communities have shown that nasopharyngeal carriage of meningococci is uncommon in infancy and childhood. However, carriage rates rise steadily during the first two decades reaching a peak of approximately 20-25% during the late teenage years and early adult life, then gradually declines (Cartwright *et al.*, 1987) (Caugant *et al.*, 1994) (Fraser *et al.*, 1973) (Gold *et al.*, 1978b) (Marks *et al.*, 1979). While some studies have found no difference in carriage rates between the sexes (Greenfield *et al.*, 1971) (Gold *et al.*, 1978b) (Blakebrough *et al.*, 1982) (Ronne *et al.*, 1993), others have shown that rates of carriage are higher in males than in females (Cartwright *et al.*, 1987) (Olsen *et al.*, 1991) (Caugant *et al.*, 1994). Studies have shown a correlation between smoking and increased rates of carriage (Stuart *et al.*, 1989). An association between passive smoking in the home and increased rates of carriage has also been shown (Caugant *et al.*, 1994) (Blackwell *et al.*, 1990) (Thomas *et al.*, 1991) (Blackwell *et al.*, 1992). While immunization with serogroup C (Gotschlich *et al.*, J Exp Med, 1969; 129: 1385-95) or serogroup A+C polysaccharide vaccines reduced carriage rates of serogroup C *N. meningitidis* strains among military

recruits, immunization with a serogroup A polysaccharide vaccine in Africa was not found to affect rates of carriage of serogroup A strains (Broome, 1986) (Blakebrough *et al.*, 1983) (Hassan-King *et al.*, 1988). High rates of carriage are observed within families and close contacts of cases of meningococcal disease (Marks *et al.*, 1979) (Olcen *et al.*, 1981). Increased rates of carriage may also be observed in closed or semi-closed communities such as military establishments, schools, and prisons (Aycock and Mueller, 1950) (Fraser *et al.*, 1973).

1.6 Pathogenesis of *N. meningitidis* infections

Up to 20% of the general population carry *N. meningitidis* as a harmless commensal (Brandtzaeg, 1995). However, in a small percentage of carriers, the bacterium is able to enter the bloodstream where it may lead to septicaemia and/or enter the cerebrospinal fluid (CSF) to cause meningitis (Apicella, 1990). The observations that *N. meningitidis* is detected in the blood of patients prior to detection in CSF (DeVoe, 1982), and that bacteria can be recovered from the CSF of rhesus monkeys following intravenous inoculation (Amoss and Eberson, 1919) indicate that *N. meningitidis* enters the CSF via infection of the bloodstream. This requires that the bacterium is able to cross the blood brain barrier (BBB). The blood brain barrier is composed of two different structures; endothelial cells of brain capillaries which have tight junctions restricting paracellular movement, and the choroidal plexus which is located in the cerebral ventricles and is the main site of CSF synthesis. Endothelial cells of the choroidal plexus do not have tight junctions and the BBB in this region is created by tight junctions at the ventricular surface. It is not known whether

bacteria invade the CSF by breaching the tight junctions between cells of the BBB or whether they are transported through cells (Nassif, 1999; Nassif *et al.*, 1999).

1.7 Invasive meningococcal disease

Prerequisites for invasive disease include exposure to a pathogenic strain, invasion of the nasopharyngeal mucosa, and crucially, survival of the meningococcus in the bloodstream, as without bloodstream infection, invasive disease will not occur (van Deuren *et al.*, 2000). Our current knowledge of the factors required for carriage and progression to invasive disease is incomplete.

Although, *N. meningitidis* can cause a variety of syndromes including meningitis, septicaemia, conjunctivitis, pericarditis, epiglottitis, pneumonia, and arthritis, meningococcal disease most commonly presents as meningococcaemia, sepsis, septic shock, and/or meningitis (Steven and Wood, 1995) (van Deuren *et al.*, 2000). The development of invasive disease may be influenced by numerous host factors including age, sex, season, exposure to smoking, socio-economic factors, contact with a case, and host immunity, and genetic susceptibility (Cartwright, 1995).

A number of bacterial factors, mostly located at the cell surface, are known to contribute to the pathogenic process. Pathogenic isolates of *N. meningitidis* are enclosed by a polysaccharide capsule that is attached to an outer membrane containing lipids, outer membrane proteins (OMPs), and lipopolysaccharides (LPS) (Fig. 1.3).

1.8 *N. meningitidis* virulence determinants

1.8.1 Lipopolysaccharide

Previous studies have examined the involvement of meningococcal LPS (endotoxin) in the pathogenesis of *N. meningitidis* infections (Mandrell and Zollinger, 1977), (Scholten *et al.*, 1994). The main components of LPS are Lipid A, an inner core region, and two short oligosaccharide chains of variable length (Rietschel *et al.*, 1991). Lipid A is the main toxic component of LPS (Brandtzaeg *et al.*, 2001). The LPS expressed by *N. meningitidis* differs from that of enteric Gram-negative bacilli. *N. meningitidis* LPS does not express a repeating O-antigen such as is present in *E. coli* LPS (Vogel *et al.*, 1999; Vogel *et al.*, 2000). Instead, the saccharide portion of *N. meningitidis* LPS is composed of an inner core from which extends two short chains of sugar molecules, the α and β chains. The inner core comprises two heptose sugars linked to lipid A via two 2-keto-3-deoxyoctulosonic acid (KDO) residues. The α-chain of meningococcal LPS can be sialylated (Estabrook *et al.*, 1997) (Tsai and Civin, 1991) (Poolman *et al.*, 1985). Addition of sialic acid to the terminal galactose residue of LPS is performed by an α-2, 3, sialyltransferase (Gilbert *et al.*, 1996). Endogenous sialylation of the terminal Galβ1-4GlcNAcβ1-3Galβ1-4Glc (lacto-N-neotetraose or LNnt) epitope of LPS confers serum-resistance to the bacterium (Mandrell *et al.*, 1991) (Erwin *et al.*, 1996). Sialic acid is also present on the glycosphingolipids of human cells. The majority of disease isolates express the LNnt epitope which is found in isolates with immunotypes L2, L3, L5, L7, and L9. In contrast, among isolates from asymptomatic individuals, the L1 and L8 immunotypes predominate (Jones *et al.*, 1992).

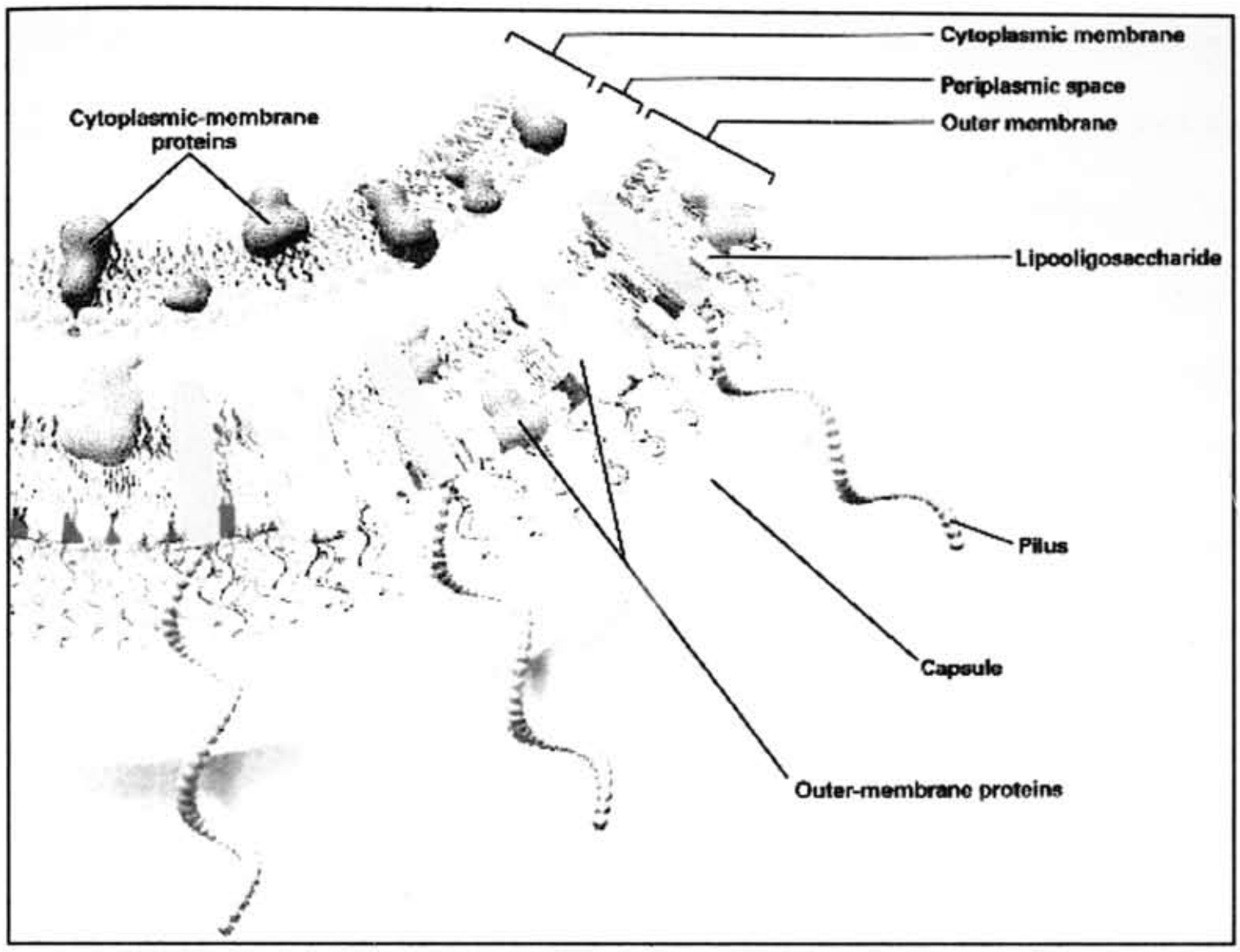
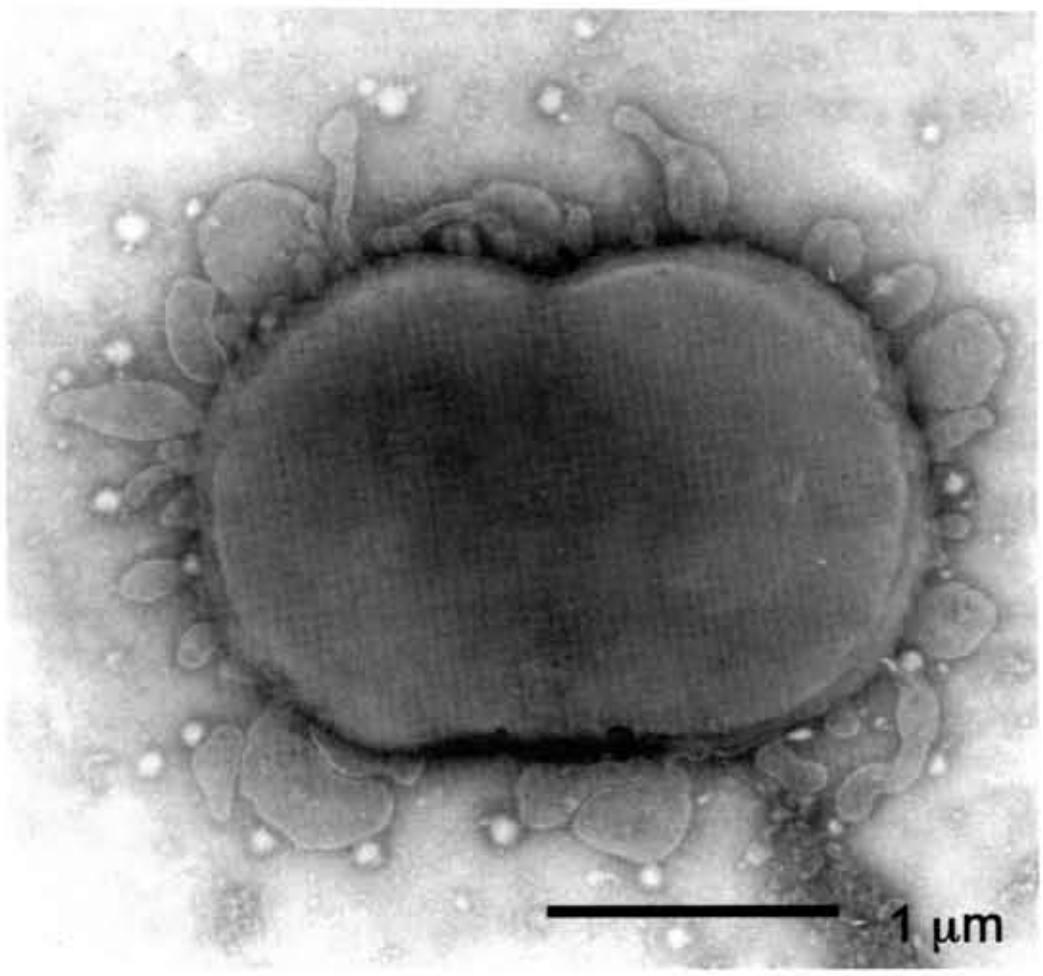


Fig. 1.3 Major components of the *N. meningitidis* cell membrane (Rosenstein *et al*, 2001).

The sialic acid present in the LPS of *N. gonorrhoeae* has been shown to bind a specific site on factor H (Ram *et al.*, 1998a; Ram *et al.*, 1998b) and exogenous sialylation of LPS in *N. gonorrhoeae* is known to render the bacterium serum-resistant. However, the influence of LPS sialylation on the serum-resistance of *N. meningitidis* remains the subject of debate (Estabrook *et al.*, 1992) (Jarvis and Vedros, 1987). It is thought that sialylation of LPS in *N. meningitidis* enhances serum-resistance by masking the LNnt epitope (Estabrook *et al.*, 1997). Mutation of the *galE* gene, which encodes UDP-glucose-4-epimerase required for the addition of galactose to the α chain, results in truncation of the LPS molecule, and *galE* mutants are highly serum-sensitive despite having a polysialic acid capsule (Vogel *et al.*, 1999). However, sialylation of LPS is not indispensable for meningococcal serum-resistance, as mutation of the *lst* gene which encodes the α2,3-sialyltransferase does not render the bacterium serum-sensitive (Vogel *et al.*, 1999).

During the growth and lysis of meningococci, endotoxin is released in the form of vesicular outer membrane structures or 'blebs' that are made up of 50% LPS, and 50% OMPs, lipids, and capsular polysaccharides (Fig. 1.4). The release of blebs is thought to play a major role in the induction of LPS-mediated septic shock (Poolman, 1995), (van Deuren *et al.*, 2000). Release of LPS in the bloodstream leads to the initiation of a cascade of inflammatory responses, (Morrison and Ulevitch, 1978). Elevated levels of cytokines including tumour necrosis factor alpha (TNFα), and interleukins IL-1, IL-6, and IL-8 are associated with the severity of shock, multiple organ failure, and disseminated intravascular coagulation (DIC).

Figure 1.4 Membrane blebbing



Electron micrograph of meningococci showing blebbing of the outer membrane (x70,000 magnification, bar, 1 μm) (K. Cartwright, Meningococcal Disease).

TNFα is the main cytokine associated with endotoxin-induced shock. The onset of shock is associated with poor prognosis (Waage *et al.*, 1989). Higher levels of endotoxin are recovered from the blood of patients with septic shock than from those with lower levels of bacteraemia resulting in a milder 'meningococcaemia' (Prins *et al.*, 1998). A 10-fold increase in LPS levels in plasma from 10 to 100 endotoxin units/ml results in >80% increase in mortality due to shock (Brandtzaeg *et al.*, 2001). While under-activation of the cytokine response results in an insufficient immune response, over-activation can be highly deleterious, leading to endothelial damage and capillary leakage. The effects of over-activation of the cytokine response include the development of purpuric skin rash and shock. During the acute stage of meningococcal infection, levels of TNFα, IL-1, IL-6, and IL-10 are frequently elevated. Increases in the levels of TNFα, IL-6, and IL-10 are associated with risk of mortality (van Deuren *et al.*, 2000).

1.8.2 Capsular polysaccharides

Of the 13 known serogroups of *N. meningitidis*, serogroup A, B, C, Y, and W_{135} strains are most commonly associated with invasive disease. The capsule is an essential virulence determinant of *N. meningitidis*. The presence of capsular polysaccharide protects the bacterium from a number of host immune defences, including phagocytosis, opsonization, and complement-mediated killing, and is critical for bacterial survival during invasive disease (Frosch *et al.*, 1992; Frosch and Muller, 1993) (Swartley and Stephens, 1994). It is thought that the presence of capsule may protect the bacterium by preventing complement binding and in particular, the insertion of the membrane attack complex (MAC) into the

bacterial outer membrane (Ram *et al.*, 1999). The capsular sialic acid residues of serogroup B and C meningococci facilitate the binding of the complement regulatory protein factor H to C3b, thereby preventing the binding of factor B to C3b and subsequent activation of the alternate pathway (Estabrook *et al.*, 1997) (Estabrook *et al.*, 1992) (Kahler *et al.*, 1998). The alternative pathway of innate immunity does not require antibodies and is activated through the interaction of C3 with factor B and factor D to generate C3b (Emonts *et al.*, 2003).

The majority of *N. meningitidis* isolates recovered from either the blood or the cerebrospinal fluid of patients with meningococcal disease are encapsulated (van Deuren *et al.*, 2000), while mutants lacking capsular expression are serum-sensitive and non-pathogenic. Conversely, the expression of capsule is thought to inhibit initial interactions between the bacteria and host cells and is down-regulated during colonization of the nasopharynx and attachment to epithelial and endothelial cells (Deghmane *et al.*, 2002) (Tzeng *et al.*, 2001).

1.8.3 Pili

Pili are filamentous projections composed of repeating polypeptide subunits (pilins) that extend from the cell surface of most Gram-negative bacteria. The type IV pili of *N. meningitidis* are dynamic structures with numerous functions including bacterial aggregation, adhesion to host cells, and DNA transformation (Merz and So, 2000). Type IV pili are found in many Gram-negative bacterial pathogens including *Moraxella bovis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (Seifert *et al.*, 1990). The importance of

these structures is demonstrated by the observation that primary cultures of pathogenic *N. meningitidis* and *N. gonorrhoeae* isolated from human mucosal surfaces and meningococci isolated from blood and cerebrospinal fluid are consistently piliated. The involvement of pili in the initial adhesion to epithelial cells has been demonstrated *in vitro* (Nassif *et al.*, 1994). Expression of pili is down-regulated during the entry of meningococci into epithelial cells (Taha *et al.*, 1998).

Pili are composed of multimers of PilE (~17-20 kDa), the major pilus subunit which is encoded by the *pilE* locus (Stephens *et al.*, 1985). PilE is synthesized as a precursor that is processed by PilD, the prepilin peptidase. PilD cleaves off the first seven amino acids from the N-terminal domain of PilE (Strom *et al.*, 1993). The C-terminal domain of PilE contains variable regions that generate antigenic variation in pili (Nassif *et al.*, 1993) resulting from either homologous recombination between expressed pilin loci (*pilE*) and variant silent pilin loci (*pilS*) or intragenic recombination in the *pilE* genes (Gibbs *et al.*, 1989). Pilin has been shown to undergo post-translational modifications such as O-glycosylation and phosphorylation (Marceau *et al.*, 1998), (Forest *et al.*, 1999). However, the role of these modifications in piliation and pilus-mediated adhesion are not clear.

The PilC protein (110 kDa) is encoded by two genes, PilC1 and PilC2 and expression of at least one of these is required for pilus biogenesis and competence for transformation (Nassif *et al.*, 1994). PilC is located at both the pilus tip and the surface of the outer membrane (Jonsson *et al.*, 1991), (Rudel *et al.*, 1995). PilC1 is critical for bacterial adherence to host cells and studies have shown that PilC1$^+$ bacteria are adhesive regardless of the expression

of PilC2 (Nassif *et al.*, 1994), (Rahman *et al.*, 1997). Purified PilC has been shown to bind to human epithelial cells *in vitro* (Rudel *et al.*, 1995). PilC undergoes phase variation due to changes in the length of a poly-G tract present in the *pilC* genes (Jonsson *et al.*, 1991).

The cytoplasmic, nucleotide-binding protein, PilT, is required for twitching motility, DNA transformation, and pilus retraction (Merz and So, 2000). The PilT protein is also required for the induction of intimate attachment to host cells (Pujol *et al.*, 1999). While *pilT* mutants are able to assemble pili efficiently, they are not competent for DNA uptake (Merz and So, 2000).

Pili show a high degree of antigenic diversity. Two different classes of type IV pili are expressed by meningococci-class I and class II, which differ both antigenically and structurally. Class I pili are closely related to the pili of *N. gonorrhoeae* and bind the monoclonal antibody SM1. Class II pili have smaller subunit molecular weights than gonococcal and meningococcal Class I pili and do not react with SM1 (Virji *et al.*, 1989) (Aho *et al.*, 1997). Variations in pili structure significantly influence the adherence of *N. meningitidis* and *N. gonorrhoeae* to epithelial cells (Virji *et al.*, 1992). Neisserial pili undergo rapid phase shifts during infection and Neisserial strains may express more than one pilin type (Tinsley and Heckels, 1986).

1.8.4 Outer membrane proteins

Five different classes (1-5) of *N. meningitidis* major outer membrane proteins (OMPs) have been defined according to differences in molecular weight. Class 1 PorA and Class 2 PorB

are porins, which facilitate transport of ions through the cell membrane and exhibit cation- and anion-selectivity, respectively (Massari *et al.*, 2003b). The genes encoding PorA and PorB are homologous to known *E. coli* porin genes. PorA is frequently recognized by bactericidal antibodies and has been investigated as a vaccine candidate (Toropainen *et al.*, 2001; van der Voort *et al.*, 1997). However, the development of PorA-based vaccines has been complicated as although the transmembrane regions of these proteins are highly conserved, their surface–expressed loops exhibit sequence variation due to genetic recombination and events mediated by multiple phase variation mechanisms (Saukkonen *et al.*, 1989) (van der Ende *et al.*, 2000). *N. gonorrhoeae* isolates expressing the Por1A isotype have been shown to resist complement-mediated killing through the binding of an exposed loop of this protein to factor H, a regulator of the complement pathway (Ram *et al.*, 1998a; Ram *et al.*, 1998b).

The Class 5 OMPs, Opa and Opc, play a role in adhesion to, and invasion of, host cells. These proteins share homology with the opacity-associated proteins (Opas) of *N. gonorrhoeae* (Merz and So, 2000). Following initial attachment mediated by type IV pili, further contact is mediated by the class 5 OMPs, Opa and Opc. In non-capsulated strains, Opa and Opc can mediate interactions with host cells. This interaction is influenced by the sialylation of LPS, which strongly inhibits interactions with both endothelial and epithelial cells (Nassif, 1999). Four copies of *opa* genes are present in the *N. meningitidis* genome. Opa proteins vary in the number expressed (between 0 and 4), size, and antigenicity. Expression of the Opa proteins is regulated by altering the number of CTCTT repeats within the signal sequence-encoding part of the gene (Belland *et al.*, 1997). The *opc* gene, present

in a subset of strains, undergoes phase variation resulting from mutations in the poly-C tract in its promoter region which modulate or eliminate expression (Sarkari *et al.*, 1994). Opa binds to carcinoembryonic antigen (CEA) CD66 receptors (Virji *et al.*, 1996). Opc binds to heparan sulfate proteoglycan (HSPG) receptors (de Vries *et al.*, 1998). HSPGs, together with the extracellular matrix proteins, vitronectin and fibronectin, act as receptors for a subset of Opa proteins and Opc. Interaction with these receptors induces uptake of meningococci by epithelial cells.

1.8.5 Autotransporters

Autotransporters are a recently identified group of outer membrane proteins that do not require any other protein for expression on the bacterial surface. These proteins normally have an N-terminal signal sequence and a protease domain that cleaves the mature protein from the pro-protein (Henderson *et al.*, 1998).

N. meningitidis expresses IgA1 protease, an autotransporter that cleaves human IgA1 at its hinge region. IgA1, which binds to bacteria and plays a role in mucociliary clearance, is the most common immunoglobulin isotype of the upper respiratory tract (Henderson *et al.*, 1998). Production of the IgA1 protease is thought to be a virulence determinant of *N. meningitidis*. Clinical isolates of *N. meningitidis* have been shown to have higher IgA1 protease activity than carriage isolates (Vitovski *et al.*, 1999). Furthermore, the IgA1 protease of *N. gonorrhoeae* cleaves a site in the lysosomal/phagosomal protein, h-lamp-1. Lamps (lysosome-associated membrane proteins) are thought to protect the membrane of

mammalian cells from degradation (Hauck and Meyer, 1997). Antibodies against IgA1 protease have been detected in the sera of patients recovering from meningococcal disease and these antibodies have been found to persist following recovery from infection (Brooks, 1992).

1.8.6 Superoxide dismutase

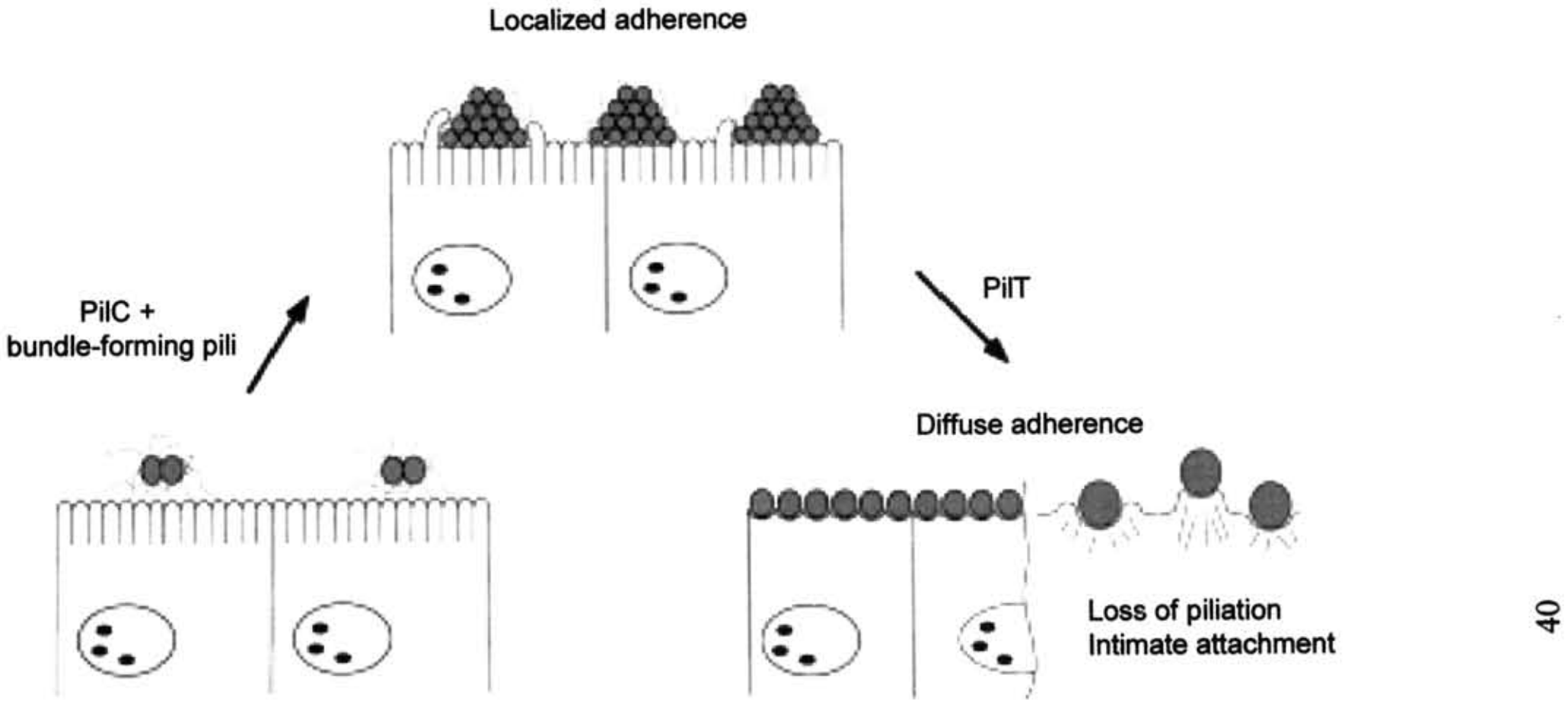
Phagocytic cells are able to kill microorganisms by producing small-molecule oxidants via respiratory burst in the phagolysosome. Bacteria express a [Cu,Zn] superoxide dismutase (SOD) which belongs to a family of proteins that convert superoxide radicals into hydrogen peroxide and oxygen. The [Cu,Zn] SOD of *N. meningitidis* protects the bacterium from the deleterious effects of superoxide *in vitro*, and a *sodC* mutant is attenuated in a murine model (Wilks, 1998). However, the mechanism underlying the attenuation of this mutant is unclear. Although bacteria unable to express [Cu,Zn] SOD are phagocytosed by human monocyte-derived macrophages more avidly than wild-type *N. meningitidis*, they do not appear to be at a disadvantage once internalized (Dunn, 2003).

1.9 Interaction of *N. meningitidis* with host cells

Based on studies of the adherence of *N. meningitidis* to cultured epithelial cells, a two-step model of adhesion has been proposed (Fig. 1.5). Initially, bacteria attach to the surface of host cells, localizing in small colonies on the apical surface. The bacteria then disperse forming a monolayer. This step, termed initial attachment, is pilus-mediated and requires

the involvement of PilT. During the second step, termed intimate adhesion, bacteria adhere firmly to the apical membrane and may be found intracellularly. The *crgA* gene (contact-regulated gene A) of *N. meningitidis* has recently been shown to act as a transcriptional regulator whose expression is induced upon cell contact. Binding sites for CrgA have been identified within the promoter regions of the *pilE* and *sia* genes, which are required for the biosynthesis of pili and capsule respectively (Deghmane *et al.*, 2000). It is thought that *crgA* may regulate transition from initial to intimate adhesion by down-regulating pili and capsule expression (Deghmane *et al.*, 2002). The complement-regulatory protein, membrane cofactor protein or CD46, has been identified as a pilus receptor for pathogenic *Neisseria* (Kallstrom *et al.*, 1998).

Fig. 1.5 Initial and intimate adhesion



Attachment of *N. meningitidis* to an epithelial monolayer involves two steps. Pilus-mediated adhesion is thought to be critical during the initial step of localized adherence and requires the presence of one PilC allele. A pilin variant responsible for the formation of bundled pili is also required for the formation of colonies on the surface of cells. The second step involves diffuse adherence. At this stage, *N. meningitidis* has lost its pili. The loss of piliation and induction of intimate attachment is facilitated by the pilin subunit PilT (Nassif *et al.*, 1999b).

CD46 is a transmembrane glycoprotein expressed in all human cell types with the exception of erythrocytes (Liszewski *et al.*, 1991). CD46 is thought to determine the host specificity of *N. meningitidis*. Studies have shown that while *N. meningitidis* is unable to adhere to cells of non-human origin such as Chinese hamster ovary cells (CHO), the bacterium is able to adhere to CHO cells expressing CD46 (Kallstrom *et al.*, 1997). A recent study has shown that transgenic C57BL/6 mice expressing human CD46 are more susceptible to meningococcal disease than wild-type mice (Johansson *et al.*, 2003). Interaction between pili and CD46 triggers a signal transduction response in the host cell that leads to a rise in cytosolic calcium. This is thought to be required for the establishment of intimate contact between the bacterium and the host cell as well as for bacterial uptake and invasion. The induction of signal transduction pathways leads to a rearrangement of the host cell cytoskeleton including the depositing of phosphotyrosine, actin, ezrin, and a number of transmembrane glycoproteins at sites of bacterial attachment (Merz and So, 1997; Merz *et al.*, 1999) (Merz and So, 2000). However the pilin subunit/s required for this interaction and the signalling pathways involved have yet to be identified.

1.10 Phase and antigenic variation

During the stages of colonization and invasion, a number of cell-surface structures of *N. meningitidis* including pili, class 5 and other OMPs, capsule, and LPS, are known to undergo rapid and reversible 'on/off switching' or phase variation. This process is thought to enhance the bacterium's survival within the host at multiple stages of infection and allows evasion of host immune mechanisms (van Deuren *et al.*, 2000). The mechanism of

slipped strand mispairing alters the length of repetitive nucleotide sequences in either the promoter or coding region of a gene during replication. It is thought that over 100 such repetitive sequences, termed contingency loci, in the serogroup B strain MC58 genome may be under this type of genetic control (Snyder, 2001). Numerous *N. meningitidis* virulence factors including PorA, pili and Opa proteins undergo antigenic variation, a process that causes changes in sequence that can affect the structure of antigens. Phase and antigenic variation of virulence factors involved in attachment, invasion and serum-resistance are mechanisms that allow the bacterium to evade the host immune response. The high frequency of variation (10^{-2}-10^{-4} per cell per generation) and number of determinants that undergo such variation mean that bacteria within a single colony may express many different surface phenotypes (Tzeng and Stephens, 2000).

1.11 Iron acquisition

The ability of bacteria to acquire iron is essential for metabolic processes such as nucleotide biosynthesis and energy production (Bridges and Seligman, 1995). Unlike most pathogenic bacteria, *N. meningitidis* does not utilize siderophores, which are small ferric iron-binding molecules that chelate iron from host molecules (West and Sparling, 1985), to acquire iron. Instead, the organism has a system of iron uptake that is dependent on surface receptors for host iron-containing glycoproteins, such as transferrin and lactoferrin (Gray-Owen and Schryvers, 1996) (Schryvers and Morris, 1988b). Transferrin is a serum glycoprotein, while lactoferrin is present in mucosal secretions and is produced by leucocytes at sites of inflammation. The surface-exposed proteins TbpA and TbpB, encoded by *tbpA* and *tbpB*

respectively, comprise the transferrin receptor of *N. meningitidis* and specifically bind human, and not rodent, transferrin (Legrain *et al.*, 1993). The lactoferrin receptor is also composed of two proteins; LbpA and LbpB, encoded by the genes, *lbpA* and *lbpB* (Schryvers and Morris, 1988a) (Bonnah *et al.*, 1998) (Pettersson *et al.*, 1998). Unbound or free iron is at low concentration in the body. However, these glycoproteins efficiently chelate iron from both mucosal surfaces and the blood. The TonB, ExbB, and ExbD proteins comprise a transport system that provides energy for the dissociation of iron from the Tbp and Lbp proteins and transport into the bacterial cytoplasm (Braun, 1995). The transport of iron across the outer membrane by TbpA or LbpA is TonB-dependent (Stojiljkovic and Srinivasan, 1997). In addition, *N. meningitidis* is capable of utilizing haemoglobin, haemoglobin-haptoglobin, and haem as sources of iron. The HmbR outer membrane protein binds haem and haemoglobin (Stojiljkovic *et al.*, 1996), while the HpuA and HpuB receptors bind haemoglobin and haemoglobin-haptoglobin (Lewis *et al.*, 1997). The importance of iron during infection is highlighted by the observation that the addition of inorganic iron enhances the virulence of meningococci when injected into mice (Calver *et al.*, 1976). Analysis of a library of signature-tagged *N. meningitidis* mutants in an infant rat model of septicaemia identified attenuated mutants with transposon insertions in *tonB*, *exbB*, and *exbD* (Sun *et al.*, 2000).

1.12 Host response and genetic susceptibility

Invasion of the bloodstream by *N. meningitidis* results in the activation of three main cascade pathways. These are the complement system, the inflammatory response, and the

coagulation and fibrinolysis pathway. These processes do not exist independently, but interact with each other at various stages. A number of genetic polymorphisms in components of these pathways appear to affect the susceptibility, severity, or outcome of meningococcal disease (Emonts *et al.*, 2003).

1.12.1 Innate immunity

Innate immunity is key to the initial recognition of invading pathogens. Pathogen associated molecular patterns (PAMPs) present in the invading pathogen are recognised by aspects of host innate immunity. Examples of this type of recognition include the MBL pathway, LPS binding protein (LBP), bactericidal permeability protein (BPI), soluble CD14, C reactive protein and serum amyloid P (Emonts *et al.*, 2003). However, it is the host response to LPS that is the main trigger of the three cascade pathways; the complement system, the inflammatory response, and the coagulation and fibrinolysis pathway. Each of these has been shown to be involved in recognition of *N. meningitidis* (Wright *et al.*, 1990) (Levin *et al.*, 2000). (Blondin *et al.*, 1997) (Serino and Virji, 2000) (Noursadeghi *et al.*, 2000).

Recently identified Toll-like receptors (TLRs) are involved in the recognition of molecules produced by a wide range of pathogens. TLR-4 is thought to be the main receptor for Gram negative microorganisms and is responsible for sensing of LPS primarily through the recognition of lipid A (Zughaier *et al.*, 2004) (Ingalls *et al.*, 2000). Polymorphisms in TLR-4 lead to a deficiency in the recognition of LPS thereby preventing activation of an adequate

inflammatory response (Underhill, 2002) (Akira, 2001). Prior to binding to TLR-4 via CD14, LPS forms a complex with LBP. A study has shown that polymorphisms in LBP (Cys98Gly and Pro436Leu) may be associated with poorer prognosis of bacterial infections in men (Hubacek, 2001).

The increased incidence of recurrent meningococcal disease in patients with deficiencies of the complement pathway highlights the importance of this system for the clearance of meningococci (Figueroa *et al.*, 1993). The complement system is central to host defence against *N. meningiti*dis and complement-mediated bactericidal activity of human sera is a fundamental mechanism preventing the replication of *N. meningitidis* in the blood (van Deuren *et al.*, 2000). Following the entry of meningococci into the bloodstream, all three complement pathways (classical pathway, alternative pathway, and mannose-binding lectin (MBL) pathway) are activated. The three complement pathways converge at C3, producing the molecules, C3a and C3b. C3 deficiency leads to defective opsonization and phagocytosis of bacteria thereby increasing the risk of infection by *N. meningitidis* and other bacteria. Properdin, or Factor P, of the alternative complement pathway, stabilizes C3 convertase. Three types of properdin deficiency have been described. In type I deficiency, Factor P is absent from plasma. This is normally due to a mutation that leads to the expression of a truncated form of Factor P (Spath, 1999). Two different polymorphisms have been described for type II deficiency which is characterized by low levels of Factor P in plasma (Westberg, 1995). In type III deficiency, although normal levels of Factor P are present, the protein is dysfunctional due to a single base substitution (T>G in exon 9) resulting in Try387Asp (Fredrikson, 1996). All three types of deficiency correlate with an

increased susceptibility to meningococcal disease. However, the severity of meningococcal disease differs in patients with different types of properdin deficiency. Patients with late complement deficiency (C5-C9) are particularly susceptible to *Neisseria* infections and suffer recurrent infection (Fijen *et al.*, 1998). This increase in susceptibility principally results from the inability to form a membrane attack complex (MAC), a complex formed by the fusion of five terminal complement proteins C5, C6, C7, C8, and C9 that creates a transmembrane pore leading to the lysis of the target cell (Esser, 1994). Deficiencies in complement factor D of the alternative pathway are also thought to increase susceptibility to *N. meningitidis* infection (Biesma *et al.*, 2001).

1.12.2 Acquired immunity

Bactericidal antibodies capable of inducing complement-mediated lysis play a crucial role in providing resistance to *N. meningitidis* infections. Elevation of antibody titres resulting from nasopharyngeal colonization with meningococci has been demonstrated and longitudinal studies of serogroup C infection amongst U.S. military recruits demonstrated that individuals developed bactericidal activity following colonization with the bacterium. Individuals with serum bactericidal activity (SBA) titres of greater than 1:8 were protected against invasive disease and the introduction of a successful serogroup C polysaccharide vaccine was shown to elicit bactericidal activity (Goldschneider 1969a, 1969b, Gotschlich, 1969a). The importance of bactericidal antibodies is further highlighted by the success of recent vaccination campaigns with serogroup C conjugate vaccine in the U.K. to

dramatically lower the annual number of cases resulting from serogroup C infection (Ramsay *et al.*, 2001).

To date, the SBA is considered to be the gold standard for measuring protection given by capsular polysaccharide-based meningococcal vaccines. In this assay, *N. meningitidis* is lysed by serum antibody in the presence of human or animal complement. SBA titres are represented as the reciprocal serum dilution resulting in ≥50% or 90% killing compared to the number of bacteria present prior to incubation with serum and complement (Vermont *et al.*, 2002). While conjugate A+C polysaccharide vaccines inducing bactericidal activity have been shown to be highly effective (Fairley *et al.*, 1996) (Anderson *et al.*, 1994), similar conclusive evidence is lacking for bactericidal activity as the immunological correlate of immunity against serogroup B infection. A trial performed to assess the efficacy of two outer membrane protein (OMP)- based vaccines found that the use of the SBA as a correlate of protection may underestimate efficacy (Perkins *et al.*, 1998). Therefore, the usefulness of this assay for assessing the effectiveness of serogroup B vaccines is under debate. Other assays used to measure immunity to serogroup B *N. meningitidis* include the whole blood assay, the opsonophagocytosis assay, measurement of T cell responses, and models of passive protection (Ison *et al.*, 1999, Naess *et al.*, 1998, Saukkonen *et al.*, 1989).

The whole blood assay (WBA) can be used to measure the killing capacity of human blood following either vaccination or infection and is thought to be a more sensitive measure than the SBA as it measures both complement-mediated antibody killing and the opsonophagocytic activity of blood (Ison *et al.*, 1999). The WBA is further described in section 1.16.2. However, the correlation between killing in the whole blood assay and

protection against meningococcal disease is not known. Furthermore, logistically, performing the whole blood assay is more difficult than the SBA as it is dependent on using blood collected no more than two hours before performing the assay (Vermont *et al.*, 2000).

The opsonophagocytosis assay measures serum opsonic activity using freshly isolated human polymorphonuclear cells (PMNs). In these assays, the phagocytosis of either ethanol-fixed or heat-killed *N. meningitidis* can be measured by either flow cytometry, chemiluminescence or by measuring oxidative burst. However, the opsonophagocytosis assay has not been tested in an efficacy trial (Martinez *et al.*, 2002).

T-cells play a key role in the regulation of the immune response. They are required for the establishment of immunological memory and promote the activation of phagocytic cells thereby enhancing the uptake of meningococci. T-cell proliferation assays can be used to measure human T-cell responses induced by vaccination and have been used to demonstrate that T-cell responses to OMV, PorA, and PorB were induced following vaccination of individuals with the Norwegian OMV vaccine (Naess *et al.*, 1998). However, this assay is not routinely used to the efficacy of meningococcal vaccines.

An infant rat model of passive protection has been used extensively to assess the ability of monoclonal antibodies raised against meningococcal antigens to protect against challenge with *N. meningitidis* strains (Schmidt *et al.*, 2001). Animal models used in the study of meningococcal pathogenesis are described further in section 1.16.1.

The importance of antibody-mediated immunity is reflected by the age distribution of infection which peaks at the ages of 6 months to 2 years and again, although to a lesser extent, in late teenage years (Ramsay *et al.*, 1997). The initial peak in childhood corresponds with loss of maternal antibody, while the peak in late teenage years coincides with entrance into university and military communities and subsequent exposure to new strains. Adaptive immunity to *N. meningitidis* may be induced in response to exposure to *N. meningitidis* strains prevalent in the community or as a result of cross-protective antibodies raised against *N. lactamica* (Olsen *et al.*, 1991).

Immunity to invasive meningococcal disease is considered to be largely dependent on serum immunoglobulin G (IgG) which acts together with complement to elicit bactericidal activity (Jarvis, 1995). Fc gamma receptors (FcγRs) are present on leucocytes, macrophages, and neutrophils and bind IgG. There are three receptor subtypes of FcγR and polymorphisms have been described in each. Studies indicate a polymorphism in FcγRIIa (a single point mutation resulting in an arginine in place of histidine at amino acid position 131 of the receptor), is associated with increased disease severity and complications (van der Pol, 2001) (Domingo, 2002).

1.12.4 Cellular immunity

T cell responses are thought to play an important role in acquired and vaccine-induced protective immunity through the induction of antibody responses. T-helper cells are required for the generation and maturation of antibody responses directed against T-cell

dependent antigens to provide immunological memory. A cellular immune response is therefore important for both inducing and maintaining protective immunity. Cellular immune responses are observed following both vaccination with meningococcal serogroup B vaccines and meningococcal infection (Pollard and Frasch, 2001).

1.12.4 Coagulation pathway

Meningococcal sepsis stimulates the coagulation and fibrinolysis pathways. Although these two processes result in different end-products (fibrin clots or fibrin degradation productions), the factors involved at the start of both cascades are the same. Infection leads to inflammation, and the above pathways are activated by cytokines. It is the over-activity of PAI1 (plasminogen activator inhibitor) and the low concentrations of activated protein C (APC) and Protein S, thrombin, and thrombomodulin which result in a net pro-coagulant state. This leads to thrombotic lesions and disseminated intravascular coagulation. An association has been shown between specific PAI1 polymorphisms and increased levels of PAI1 and increased risk of death (Hermans, 1999) (Eriksson, 1995) (Westendorp, 1999).

1.12.5 Cytokines

High serum levels of TNFα are associated with increased severity. Several polymorphisms of TNFα have been identified. Although TNFα production is affected by host genetic factors, no clear correlation has been shown between these polymorphisms and the outcome of meningococcal disease (Hackett, 2001) (Nadel, 1996). Polymorphisms in interleukins

including IL-1, IL-6, and IL-10 have also been identified. Studies have shown that a combination of polymorphisms in different members of the IL-1 family may have a synergistic effect that adversely affects survival (Read, 2003).

1.13 The clinical problem

Meningococcal meningitis and sepsis are devastating diseases that, without rapid antibiotic treatment, result in high rates of mortality in children and young adults (Apicella, 1990) (Gotschlich *et al.*, 1969). The rapid onset of the disease, followed by the fulminant course of the infection in some cases, creates a medical emergency (Cartwright, 1995). The mortality rate resulting from meningitis is between 5%-15% (Naess *et al.*, 1994) rising to 20-80% when associated with septicaemia. 50% of deaths occur within 24 hours of presentation of the initial symptoms. 10-20% of survivors may undergo amputations or plastic surgery, (van Deuren *et al.*, 2000) while 8-20% of survivors of meningococcal meningitis suffer adverse neurological effects such as sensorineural deafness, mental retardation, spasticity, seizures, and concentration deficits (Baraff *et al.*, 1993) (Djupesland and Gedde-Dahl, 1983) (Edwards and Baker, 1981) (Erickson and De Wals, 1998) (Pomeroy *et al.*, 1990) (Schildkamp *et al.*, 1996).

1.14 Diagnosis and treatment

The 'gold standard' for diagnosis of meningococcal disease involves bacteriological culture. However, this approach can be complicated by prior antibiotic treatment of patients. Other

methods used for identification include Gram-staining of CSF (Dunbar *et al.*, 1998) detection of polysaccharide antigens in CSF, and enzyme-linked immunosorbent assays (ELISA). However, antigen tests can be unreliable for diagnosis, particularly in the case of serogroup B infections (Zollinger *et al.*, 1997) (Carlone *et al.*, 1997).

PCR analysis allows rapid serogroup identification and avoids the necessity for viable bacteria and has been used for diagnostic purposes in the U.K. since 1996 (Rosenstein *et al.*, 2001b). Early diagnosis of the disease is crucial as fulminant meningococcal septicaemia (FMS) can result in death within a few hours of onset. Early symptoms, which are not present in all cases, can include myalgia, chills, and fever and may resemble those of flu. Consequently, despite increasing awareness of the symptoms of meningococcal disease among medical practitioners and the public, delays and difficulties in initial diagnosis often occur. Frequently, it is the suspicion and concern of family members and doctors that plays the most important role in early detection of the disease (Granier *et al.*, 1998) (Riordan *et al.*, 1996). Six to twelve hours after onset of FMS, haemorrhagic skin lesions including purpura and petechiae (haemorrhages or haemorrhagic spots in the skin) may appear (van Deuren *et al.*, 2000) (Fig. 1.6). Symptoms include photophobia, irritability, neck stiffness, vomiting, and loss of consciousness. Early symptoms of meningococcal meningitis are similar to those of FMS.



Fig. 1.6 **Purpuric rash of meningococcal septicaemia**
(nhsdirect.nhs.uk)

During acute meningococcal sepsis, production of activated protein C (APC) by endothelial cells is impaired (Faust, 2001). Activated protein C has both anti-thrombotic and anti-inflammatory activity and it is thought that reduced expression of this molecule may be responsible for the intense cytokine activation and disseminated intravascular coagulation associated with severe meningococcal disease. Activated protein C has been successfully used as an adjunctive therapy in the treatment of sepsis (Bernard, 2001) and is likely to be beneficial for the treatment of certain individuals with meningococcal septicaemia.

Beta-lactam antibiotics are the treatment of choice for meningococcal disease. Due to their low minimum inhibitory concentration (MIC) and excellent penetration into the CSF, cefotaxime or ceftriaxone are commonly used where finances allow. Penicillin and ampicillin are effective in treating *N. meningitidis* meningitis, with penicillin being the drug of choice. However, rare isolates expressing β-lactamase have high-level resistance to these antibiotics (Arreaza *et al.*, 2004). In patients who do not respond to treatment with penicillin, the antibiotic ceftriaxone can be used. Where beta-lactams are unavailable, chloramphenicol remains an effective antibiotic for treatment of meningococcal infections (Quagliarello and Scheld, 1997). As it is relatively inexpensive and effective, chloramphenicol continues to be used for the treatment of meningococcal infections in developing countries despite its high level of toxicity (Rosenstein *et al.*, 2001b).

1.15 Vaccines

The rapid progression of meningococcal disease means that despite ongoing advancements in treatment, infections caused by *N. meningitidis* continue to be associated with significant

mortality and morbidity. As a result, ongoing research is strongly focused towards the development of vaccines that are able to confer long-lasting protection against each of the known pathogenic serogroups. A vaccine made from purified meningococcal A, C, W_{135}, and Y polysaccharide is available. Although polysaccharide vaccines are generally well tolerated, they are poorly immunogenic in infancy, serogroup-specific, and do not induce long-term immunological memory as they do not elicit a T-cell dependent response (Frasch, 1995).

1.15.1 Conjugate vaccines

The success of *Haemophilus influenzae* serotype b (Hib) and pneumococcal conjugate vaccines demonstrates that the immunogenicity of polysaccharide antigens can be enhanced by conjugation to a protein carrier. These conjugate vaccines elicit T-cell-dependent immune responses, giving long-lasting protection (Rosenstein *et al.*, 2001a). Furthermore, polysaccharide-protein conjugate vaccines have been shown to be both safe and immunogenic in infants. In addition, immunization with these vaccines reduces nasopharyngeal carriage. The recent introduction of a serogroup C conjugate vaccine in the U.K. has resulted in a 92% reduction in cases of meningitis resulting from serogroup C infection in young children and 95% in teenagers (Ramsay *et al.*, 2001). The occurrence of capsular group replacement by isolates belonging to serogroups that are not included in the vaccine has been recorded in infants and toddlers immunized with a multivalent pneumococcal conjugate vaccine (Dagan *et al.*, 1997) (Lipsitch, 1999) (Eskola *et al.*, 2001) and is therefore a concern with the use of such vaccines. Capsule switching between serogroup B and C strains resulting from horizontal genetic exchange of genes involved in

capsule synthesis has been reported (Swartley *et al.*, 1997) (Vogel *et al.*, 2000). However, to date, there has been no evidence of capsular group replacement following use of the monovalent serogroup C conjugate vaccine (Trotter *et al.*, 2004). Clinical trials of bivalent A + C polysaccharide conjugate vaccines have been found to be immunogenic in infants, toddlers, and adults. Clinical trials of conjugate vaccines incorporating serogroup A, C, Y, and W_{135} polysaccharides conjugated to carrier proteins are currently in progress. Multivalent meningococcal polysaccharide-protein conjugate vaccines are likely to be available in the USA and Europe within a few years (Jodar *et al.* 2002).

1.15.2 Serogroup B vaccines

1.15.2.1 Capsular polysaccharide–based serogroup B vaccines

At present, there is no effective vaccine available for serogroup B meningococcus which accounts for over 60% of disease isolates in the U.K. (MacLennan, 2001). While polysaccharide vaccines have been effective in preventing disease caused by serogroups A and C, the serogroup B polysaccharide capsule, which consists of a linear homopolymer of α2→8 N-acetyl neuraminic acid, is poorly immunogenic (Frasch, 1995). This is thought to be due to the development of immunotolerance to poly-sialylated glycoproteins, present in host tissues (Finne *et al.*, 1983; Finne *et al.*, 1987). Research into the development of serogroup B polysaccharide vaccines has been limited due to the possibility that such vaccines may lead to autoimmunity. To circumvent this problem, Jennings and colleagues proposed a strategy in which the native N-acetyl groups present on the serogroup B polysaccharide are replaced with N-propionyl groups followed by conjugation to a protein carrier (Jennings *et al.*, 1986; Jennings *et al.*, 1987). This vaccine was found to be highly

immunogenic in mice generating bactericidal IgG antibodies that confer passive protection against serogroup B infection (Ashton *et al.*, 1989). However, as immunization of laboratory animals with this vaccine results in the generation of a subset of antibodies that react with host polysialic acid, concerns over the safety of serogroup B polysaccharide-based vaccines remain (Fusco *et al.*, 1997) (Granoff *et al.*, 1998). Therefore, a number of alternative strategies for the design and development of vaccines conferring protection against serogroup B infection are currently under investigation.

1.15.2.2 Lipopolysaccharide

Due to its integral role in the pathogenesis of meningococcal infections, extensive work has been carried out to investigate LPS as a vaccine candidate that may provide protection in a non-serogroup specific manner. The serogroup B L3,7,9 immunotype is frequently associated with disease-causing isolates (Jones *et al.*, 1992). Studies have demonstrated that vaccines composed of detoxified LPS or L3,7,9-toxoid conjugates induce opsonic antibody responses (Verheul *et al.*, 1993a; Verheul *et al.*, 1993b). In addition, murine monoclonal antibodies raised against conserved inner core epitopes of LPS have been shown to elicit protective immunity in laboratory animals (Plested *et al.*, 2003). Clinical trials to assess the efficacy of LPS-based vaccines are anticipated.

1.15.2.3 Outer membrane vesicle (OMV)-based vaccines

The outer membrane proteins (OMPs) of serogroup B *N. meningitidis* have been investigated as potential vaccine constituents. In order to enhance immunogenicity by presenting the proteins in a more natural conformation, a number of OMP vaccines consisting of OMPs contained within spheres of bacterial outer membrane, termed outer membrane vesicles (OMVs) have been developed (Sierra *et al.*, 1991, Zollinger *et al.*, 1979, Frasch *et al.*, 1988, Bjune *et al.*, 1991, Cartwright *et al.*, 1999, Vaccine). Outer membrane vesicles (OMVs) are prepared from *N. meningitidis* cells (Fig. 1.4). These vesicles contain the major porin proteins as well as other proteins (60-100 kDa), and LPS (Poolman, 1995). To counter repeated outbreaks resulting from serogroup B *N. meningitidis* infection in Cuba and Norway, OMV-based vaccines were developed by these countries (Sierra *et al.*, 1991) (Bjune *et al.*, 1991). The vaccine developed by the Finlay Institute in Cuba has been registered in 19 countries mainly in Latin America. Brazil, Chile, Colombia, and Iceland have all carried out trials of the Cuban OMV vaccine developed at the Finlay Institute. The results of the Brazilian study suggest that the Cuban-produced vaccine may be effective for the prevention of serogroup B meningococcal disease in older children ($\geq$ 48 months) and adults (de Moraes *et al.*, 1992, Tappero *et al.*, 1999). A Norwegian OMV vaccine has also been tested in Chile. However, the Chilean study concluded that neither the Cuban nor the Norwegian vaccine was likely to confer protection during epidemics caused by heterologous strains of *N. meningitides* (Tappero *et al.*, 1999). The protective efficacy of the Cuban vaccine has also been tested in Iceland in a study involving 408 fifteen to twenty-year olds. The results of the Norwegian trial of the Cuban vaccine suggest that found that after two doses of the Cuban vaccine, the proportion increase in antibody levels (Perkins *et al.*, 1998).

Currently the Cuban OMV vaccine is only used routinely in Cuba. Although these vaccines have demonstrated efficacies of 50-80%, they do not give any significant protection in the age group most at risk from serogroup B infection, children less than four years of age. The majority of antibodies elicited by OMVs are against PorA and to a lesser extent against Opc (Rosenqvist *et al.*, 1995). In infants, bactericidal antibody responses elicited by OMVs are mainly directed against PorA (Tappero *et al.*, 1999). Antibody responses are largely directed against the variable regions in loops 1 and 4 of PorA resulting in a serosubtype-specific response. Therefore, OMV vaccines prepared from a single *N. meningitidis* strain will not protect against all group B infections. However, as epidemics caused by serogroup B strains are largely caused by single strains, OMV vaccines may be useful for targeting single strains under these circumstances. An OMV vaccine is being developed in response to recurrent serogroup B epidemics in New Zealand caused by a single strain (Jodar *et al.*, 2002). In order to improve the level of protection afforded against multiple serogroup B strains, hexavalent OMV vaccines prepared from two *N. meningitidis* strains each expressing three different PorA proteins have been developed in the Netherlands. However, bactericidal antibody responses in infants following two to three injections of this vaccine were found to be minimal. A further problem associated with OMV vaccines is the possible emergence of pathogenic PorA-deficient *N. meningitidis* strains.

1.15.2.4 Transferrin binding protein B

Owing to difficulties encountered in the design of vaccines based on polysaccharide and OMVs, recent efforts in the development of vaccines for the prevention of serogroup B

infections have focused on selecting conserved antigens that are capable of eliciting bactericidal antibody responses likely to provide long-term protection against a wide range of *N. meningitidis* isolates. An example of a protein under investigation as a vaccine, is transferrin binding protein B (TbpB), a surface-exposed lipoprotein required for iron-acquisition by *N. meningitidis*. Despite the occurrence of frequent amino acid variations in the TbpB protein sequence, the immunization of laboratory animals with recombinant TbpB elicits high levels of bactericidal antibodies that have bactericidal activity against the majority of serogroup B *N. meningitidis* strains (Danve *et al.*, 1993). However, a recent clinical trial conducted by Aventis Pasteur found that while adults immunized with the TbpB vaccine generated high levels of serum IgG antibody against the protein, only a small proportion of this antibody had bactericidal activity (Danve, B., *et al*, 1998:53). Attempts to enhance the immunogenicity of the TbpB vaccine have included the addition of the TbpA protein to the vaccine, and the use of adjuvants (West *et al.*, 2001).

1.15.2.5 Neisserial surface protein A

Neisserial surface protein A (NspA) is a highly conserved membrane protein of unknown function that has been selected as a vaccine candidate. Monoclonal antibodies (mAbs) raised against NspA detect expression of this protein by all meningococcal strains tested to date (250 strains including representations of all of the pathogenic serogroups of *N. meningitidis*). Although NspA is weakly immunogenic during natural infection, a vaccine based on this protein is currently under development by BioChem Vaccins, Canada. Immunization of laboratory animals with recombinant NspA elicits bactericidal antibodies

in laboratory animals that confer passive protection against challenge with serogroup B *N. meningitidis* (Martin *et al.*, 1997) (Cadieux *et al.*, 1999). However, these antibodies were only found to be bactericidal against approximately 50% of serogroup B *N. meningitidis* isolates tested (Moe *et al.*, 1999). Furthermore, significant differences have been observed in accessibility to the NspA protein among *N. meningitidis* strains (Moe *et al.*, 1999; Moe *et al.*, 2001). Progress in the development of NspA as an effective vaccine requires further studies aimed at determining the basis of resistance to killing by anti-NspA antibodies by some strains and improving the immunogenicity of the NspA antigen.

1.15.2.6 *Neisseria lactamica*

Studies indicate that colonization with the commensal *Neisseria* species, *N. lactamica*, contributes to natural immunity to pathogenic *Neisseria* spp. (Wilson and Overman, 1976). *N. lactamica* does not possess a polysaccharide capsule, but shares antigens common to *N. meningitidis* (Cann and Rogers, 1989) (Kim *et al.*, 1989). A study by Gold *et al.* showed that 66% of carriers of *N. lactamica* developed antibodies that were bactericidal to *N. meningitidis* strains of serogroups A, B, and C (Gold *et al.*, 1978a). *N. lactamica* has only very rarely been associated with invasive disease (Wilson and Overman, 1976). Therefore, *N. lactamica* is currently being investigated as a vaccine candidate. Immunization with *N. lactamica* killed whole cells, outer membrane vesicles, or outer membrane protein pools has been shown to confer protection against challenge with serogroup B *N. meningitidis* in an animal model (Oliver *et al.*, 2002). Clinical studies are now awaited to assess the suitability of *N. lactamica* as a vaccine for the control of *N. meningitidis* disease.

1.15.2.7 Genome sequence-based approaches

Analysis of the recently published *N. meningitidis* MC58 (serogroup B) genome sequence identified 2158 potential open reading frames. Using an approach termed 'reverse vaccinology', computer algorithms were used to identify proteins that are likely to be surface-located (25% of all gene products). Over 500 such proteins were designated as potential vaccine candidates. These proteins were cloned and expressed in *E. coli* and mouse sera subsequently raised against 350 recombinant proteins. 28 of these genome-derived antigens (GNAs) were found to elicit antibodies effective against serogroup B *N. meningitidis*. These antibodies were then tested for bactericidal activity as well as their ability to bind to the surface of *N. meningitidis* (Pizza *et al.*, 2000). GNA33 is an example of a genome-derived antigen that has been selected as a vaccine candidate. GNA33 encodes a highly conserved *N. meningitidis* lipoprotein that has homology to the *E.coli* lipoprotein, MltA, a membrane-bound transglycosylase. Although GNA33 elicits bactericidal antibodies in laboratory animals, it is thought this activity may be due to cross-reaction with loop 4 of PorA in a subset of *N. meningitidis* strains. Several other GNAs including the NadA protein and GNA1870 are currently under investigation as vaccine candidates (Comanducci *et al.*, 2002) (Masignani *et al.*, 2003).

1.15.2.8 *In-vivo* gene expression

As a result of phase and/or antigenic variation, numerous *N. meningitidis* antigens are known to vary in their expression, with some antigens expressed only under *in vivo* conditions (Jodar *et al.*, 2002). As described above, variability in the expression of antigens

can prove to be a significant problem when selecting antigens primarily on sequence-based prediction of surface-location and conservation across pathogenic strains. Methods such as *in vitro* expression technology (IVET) (Chiang *et al.*, 1999) signature tagged mutagenesis (STM) (Sun *et al.*, 2000), and microarray analysis (Grandi, 2003) offer an important advantage over bioinformatic-based methods for antigen selection such as 'reverse vaccinology'. Antigens are selected based on knowledge of their expression during infection. IVET allows genes to be identified by the induction of their promoters under specific environmental conditions encountered during infection. STM is a method in which mutations are labeled by DNA tags, allowing the identification of genes required for survival in the host. This method is described in detail in a later section. Microarray technology has also been used to identify putative vaccine candidates by analyzing gene expression during adhesion to or invasion of epithelial cells by *N. meningitidis*. Up to 40% of the genes identified by this approach were not previously recognized as being involved in pathogenesis and were of unknown function. Five of the 12 adhesion-induced surface-located antigens identified by the study were found to induce bactericidal antibodies in mice (Grifantini *et al.*, 2002).

1.16 Models of infection

N. meningitidis is an exclusively human pathogen, and therefore a major difficulty that arises in the study of *N. meningitidis* pathogenesis and virulence is the question of how closely the pathogenesis of meningococcal disease observed using animal models of infection resembles that in humans. However, several *in vitro* models of infection have

been developed to study the interaction of *N. meningitidis* with the host (Virji *et al.*, 1992 and 1993, Grifantini *et al.*, 2002). These models aim to mimic the diverse environments encountered by *N. meningitidis* at each of the known stages of meningococcal pathogenesis and include the use of cultured human epithelial and endothelial cells, infection of human nasopharyngeal explants, and *in vitro* assays using human whole blood and serum. In addition, a number of *in vivo* models of infection using laboratory animals have been developed (Nassif *et al.*, 1992, Mackinnon *et al.*, 1993).

1.16.1 Animal models

A number of animal models have been used to study defined stages of meningococcal pathogenesis. These models provide a system in which the interaction of the bacterium with elements of the host immune response and systemic circulation can be examined. In addition, the pathophysiology of meningococcal disease including disease progression, severity, and outcome may be followed. An intranasal infant mouse model was developed in order to examine nasopharyngeal colonization and mucosal immune responses (Salit *et al.*, 1984) (Mackinnon *et al.*, 1993) (Mackinnon *et al.*, 1992). However, as achieving reproducible levels of bacteraemia using this model have proved to be difficult, animal models using the intraperitoneal challenge route have also been developed. Both infant rat and mouse models have been used successfully to establish bacteraemia and meningitis via the intraperitoneal challenge route (Nassif *et al.*, 1992) (Vogel *et al.*, 1996) (Johansson *et al.*, 2003). Infant rats have also been used to demonstrate the ability of monoclonal antibodies raised against meningococcal antigens to confer passive protection against subsequent challenge with *N. meningitidis* strains (Schmidt *et al.*, 2001). Novel *N.*

meningitidis vaccine candidates are commonly tested in mice to determine whether antibodies elicited by immunization with these antigens can effectively protect against bacterial challenge. Models of infection using genetically defined 'knock-out' animals allow the role of specific host factors in *N. meningitidis* pathogenesis to be investigated. However, such models have been used in only a few studies of meningococcal pathogenesis (Peiser *et al.*, 2002) (Massari *et al.*, 2002).

Although animal models have made a significant contribution to our understanding of meningococcal pathogenesis, the results of such studies must be treated with caution, as it cannot be assumed that these results can be extended to humans. Furthermore, it is recognized that aspects of the experimental conditions under which these infections are established such as route of inoculation and size of inoculum differ from natural infection. Animal infection models normally involve a large inoculum that limits the potential for growth of the bacteria *in vivo*. Furthermore, supplementation with iron compounds such as iron dextran, is required for establishing infections in mice (Holbein, 1981).

1.16.2 Whole blood model

An *ex vivo* whole blood model of meningococcal bacteraemia was developed by Ison and colleagues to allow measurement of the bactericidal activity and immune response of human whole blood. This model can also be used to examine neutrophil activation, cytokine responses, and the expression of specific meningococcal antigens (Ison *et al.*, 1995) (Morley *et al.*, 2001). The whole blood model has been used to measure both survival of

the bacteria in the blood, and levels of cytokines such as TNFα, IL-1β, Il-6, IL-8, and IL-10 and appears to reflect cytokine responses observed in patients with meningococcal septicaemia (Kragsbjerg *et al.*, 1998) (Robinson *et al.*, 2002) (Nolte *et al.*, 2002).

1.16.3 Organ culture model

The mucosa of the nasopharynx is the usual site of meningococcal colonization and the site at which the bacteria traverse the mucosal barrier and enter the bloodstream. The bacterium is occasionally found in the urogenital tract and anal canal (McGee *et al.*, 1983) (Stephens, 1985). Human nasopharyngeal explants inoculated with *N. meningitidis* have been used to study the ability of *N. meningitidis* to attach to and enter cells of the upper respiratory tract. These studies have shown that following the attachment of meningococci to non-ciliated columnar cells, the bacteria are endocytosed by these cells. Furthermore, there is a correlation between the invasiveness of bacteria in the organ culture model and the virulence of the clonal lineage to which they belong (Read *et al.*, 1999) (Townsend *et al.*, 2002).

1.16.4 Cell culture models

A number of *in vitro* assays using cells of human origin have been developed to study the interaction of *N. meningitidis* with host cells. These assays have been used to study three main pathogenic events of meningococcal pathogenesis; mucosal cytotoxicity, attachment to host cells, and host cell invasion. Epithelial cells isolated directly from mucosal sites of

colonization such as oropharyngeal and tonsillar sites have been used in studies of meningococcal attachment. However, cells obtained can vary widely in their degree of maturation, viability, and size (Stephens, 1989). Primary cell lines derived from cells isolated from uterine, endometrial, or conjunctival tissues overcome many of these difficulties. However, as primary cell lines are often difficult to establish and maintain, immortalized cell lines, mainly of tumor origin, have been used extensively in studies of *N. meningitidis* pathogenesis. Commonly used cell lines include HeLa cells (human cervical carcinoma cell line), Hep-2 cells (human laryngeal carcinoma cell line), and Chang conjunctival cells (Raza *et al.*, 1999) (Massari *et al.*, 2003a) (Virji *et al*, 1992 and 1993). 16HBE14 epithelial cells (derived from respiratory tissue) have recently been used to analyze gene expression of in response to contact with epithelial cells using DNA microarrays (Grifantini *et al.*, 2002). Studies of the interaction of meningococci with cells of the blood brain barrier rely on the use of *in vitro* cell culture systems using endothelial cells. Endothelial cells such as human umbilical vein endothelial cells (HUVECs) and human brain-derived endothelial cells (HBMECs) have been used in both adhesion and invasion assays (Virji *et al.*, 1991) (Nassif *et al.*, 2002) (Unkmeir, 2002). The interaction of *N. meningitidis* with human meningeal cells *in vitro* has been examined using either fresh sections of human brain tissue or cultured cells derived from human meningiomas. The results of these studies suggest that piliated meningococci are more likely to attach to leptomeninges and meningeal blood vessels than to the cerebral cortex (Hardy *et al.*, 2000).

The main advantages of using cell lines such as Chang, 16HBE14, HeLa, and Hep-2 are that these cell lines are well-characterized and well-established and have been used extensively

for the study of the interaction of *N. meningitidis* with eukaryotic cells (Virji *et al.*, 1992 and 1993, Grifantini *et al.*, 2002, Raza *et a*l., 1999, Massari *et al.*, 2003a). The main disadvantage of using such cell lines is that they are not derived from nasopharyngeal tissue, the normal site of colonization by *N. meningitidis* and therefore may not express markers normally present on cells derived from nasopharyngeal tissue that may be important for the interaction of the bacterium with these cells. Furthermore, the mucus layer normally encountered by bacteria colonizing the nasopharynx is not present in these cell culture models and the cells are not polarized. However, as Chang cells (derived from conjunctival cells) and 16HBE14 cells are considered to be derived from respiratory tissue, these cells are often used in preference to HeLa or Hep-2 cell lines which are derived from human cervical carcinoma cells and human laryngeal carcinoma cells respectively (Stephens, 1989, Merz and So, 2000, Dehio *et al.*, 2000, Grifantini *et al.*, 2002).

1.17 Genetic analysis of pathogenic *Neisseria*

The study of pathogenic *Neisseria* has been limited by the lack of genetic tools for constructing libraries of mutants with insertional inactivation of random or selected genes. Previous approaches include transposon mutagenesis, (Kathariou *et al.*, 1990) (Nassif *et al.*, 1991) shuttle mutagenesis (Seifert and So, 1991), and insertion:duplication (Hamilton *et al.*, 2001). However, each of these techniques has its limitations.

1.17.1 Transposon mutagenesis

The conjugative transposons, Tn*916* and Tn*1545Δ3* have been used to generate insertional mutants of *N. meningitidis*. However, the number of independent mutations, and the range of isolates in which these transposons can be used, is limited. Both are large genetic elements that are difficult to manipulate. In addition, the genes encoding the transposases in Tn*916* and Tn*1545Δ3* (Kathariou *et al.*, 1990) (Nassif *et al.*, 1991), are located within the transposon ends, making these elements potentially unstable; Tn*1545Δ3* insertions have been shown to be unstable in *N. gonorrhoeae* (Crooke *et al.*, 1998). Tn*916* has, however, been used successfully to study genes involved in lipopolysaccharide (Kahler *et al.*, 1996) and capsule biosynthesis in an isolate of *N. meningitidis* (Swartley and Stephens, 1994).

1.17.2 Shuttle mutagenesis

Shuttle mutagenesis utilizes the natural competence of *Neisseria* spp. for DNA uptake and its efficient system of homologous recombination (Seifert and So, 1991). In this method, fragments of Neisserial genomic DNA (3-4 kb) are ligated into *E. coli* plasmid vectors and subjected to transposon mutagenesis in *E. coli*. The mutated fragments are then introduced into *Neisseria* by transformation. There are two main difficulties with this method. Firstly, the presence of a large number of restriction:modification systems in *Neisseria* spp. (Stein *et al.*, 1995) results in an unrepresentative cloning of Neisserial genomic DNA in *E. coli* (Tang, C. & Mehr, I., unpublished data). Secondly, a number of high rate of transformation (*hrt*) loci are present in the *N. meningitidis* genome, at which the rate of recombination can be two orders of magnitude greater than for other sequences of similar length (Claus *et al.*,

1998). As a result, attempts to use shuttle mutagenesis *for N. meningitidis* signature-tagged mutagenesis (STM) were unsuccessful (Claus *et al.*, 1998). STM is a powerful genetic tool, employing unique DNA tags that are incorporated into genomic DNA allowing the screening of mutants within pools in *in vivo* models of infection (Hensel *et al.*, 1995). To date, this method has been used to identify genes required for virulence by more than 15 bacterial pathogens including *S. pneumoniae*, *E. coli* K1, and *M. tuberculosis*, in a wide range of hosts (Mecsas, 2002).

1.17.3 Insertion:duplication

In insertion:duplication mutagenesis, fragments of *Neisseria* DNA are ligated into an *E. coli* suicide vector. The pools of plasmid are transformed into *Neisseria,* allowing a single cross-over event leading to gene-disruption. The difficulties encountered with this method are similar to those in shuttle mutagenesis, although, the fragments of genomic DNA used are usually <1 kb in length resulting in a lowered probability of 'hot-spotting' of integrations than in shuttle mutagenesis. This method has been used successfully for *Streptococcus pneumoniae* STM (Polissi *et al.*, 1998). However, the requirement for the individual cloning of genomic fragments into vectors to create a library of insertional mutants makes this both a time-consuming and labour-intensive strategy.

1.17.4 *in vitro* mutagenesis

A method of *in vitro* transposition using Tn*10* was developed to generate a library of 2,850 insertional mutants of $C311^+$, an ET-5 serogroup B isolate of *N. meningitidis* from a patient with invasive meningococcal infection (Virji *et al.*, 1991). The $C311^+$ isolate was selected as preliminary experiments comparing the level of bacteraemia achieved by inoculation of five-day-old rats with either strain MC58 or strain $C311^+$, demonstrated that higher levels of bacteraemia could be achieved and maintained over a 20-24 hour period following inoculation with strain $C311^+$ than with strain MC58. Furthermore, a ten-fold lower inoculating dose was required when inoculating with strain $C311^+$ than when inoculating with strain MC58 (C. Tang, personal communication). A vector, pSTM115, was constructed for *in vitro* transposition of *N. meningitidis* and includes the 70 bp outside ends (o) of Tn*10* flanking a kanamycin resistance cassette (kan^R), a copy of the *Neisseria* DNA uptake sequence (NUS), and the origin of replication from pACYC184 (Fig. 1.7).

The method of *in vitro* transposition avoids the necessity for cloning of DNA sequences prior to mutagenesis and can also be used to mutate PCR products for the construction of single mutants. In order to create a library of signature tagged transposon mutants of *N. meningitidis*, 96 different pSTM115 derivatives, each containing unique signature tags, were used to perform *in vitro* transposition genomic DNA isolated from strain $C311^+$ in 96 separate reactions. The mutated genomic DNA was subsequently used to transform the wild-type strain, $C311^+$ in 96 separate transformations. The resulting transformants were used to construct a library of 2,850 signature tagged transposon mutants of strain $C311^+$ (Sun *et al.*, 2000).

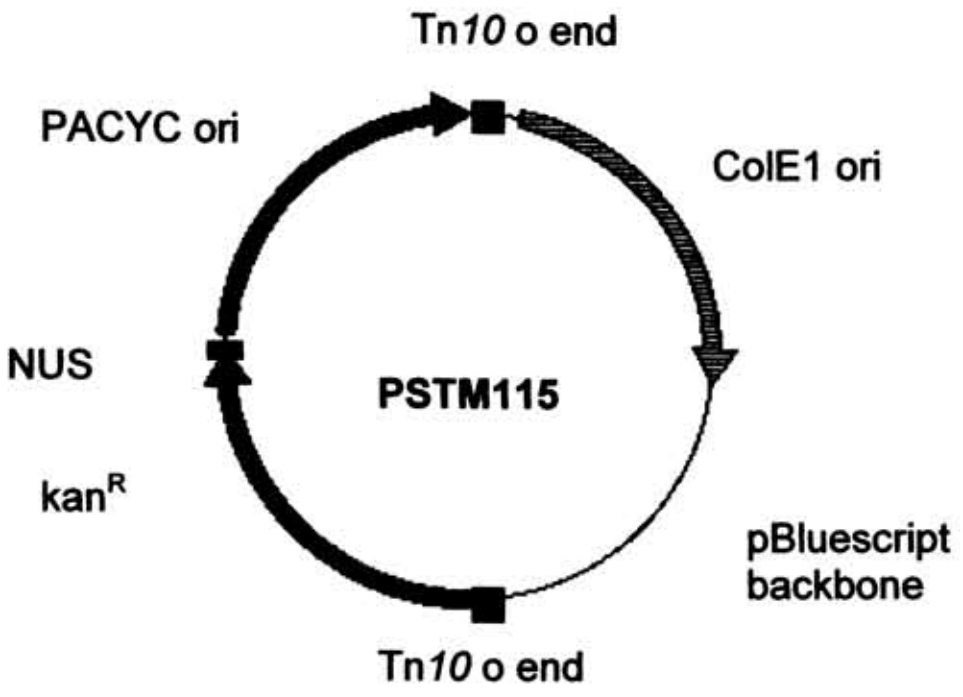


Fig. 1.7 Map of pSTM115

The plasmid contains the 70 bp outside (o) ends of Tn*10* flanking the kanamycin resistance marker (kan^R), a copy of the *Neisseria* DNA uptake sequence (NUS), and the origin of replication (ori) from pACYC184. The backbone of the plasmid is derived from pBluescript (Stratagene), from which the β-lactamase gene has been deleted.

Screening of the library in an infant rat model of meningococcal septicaemia allowed the identification of 73 genes required for the establishment of systemic disease (Fig. 1.8). Eight insertions were in genes encoding known pathogenicity factors. Involvement of the remaining 65 genes in meningococcal pathogenesis had not been demonstrated previously. Approximately 20% of the genes identified were of unknown function (Sun *et al.*, 2000).

1.18 *ispA* and *cbf*

Homology-based searches of the nucleotide sequence of two genes identified in the STM screen of *N. meningitidis* strain C311^{+}, NMB0342 and NMB0345, against the serogroup B genome sequence (Tettelin *et al.*, 2000) revealed that the two genes are 622 bp apart and exist within a potential operon of seven genes (Fig. 1.9, Table 1.1).

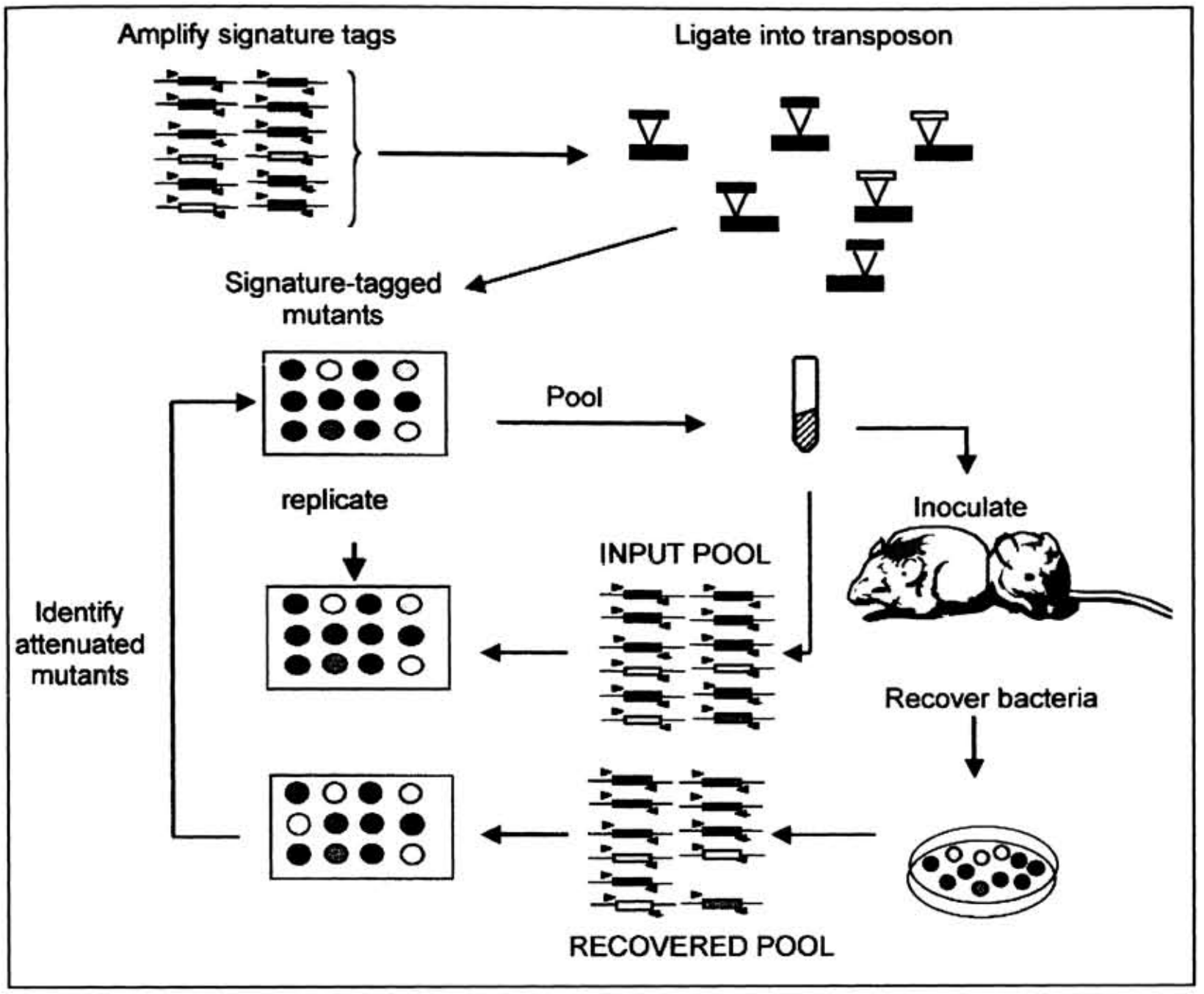
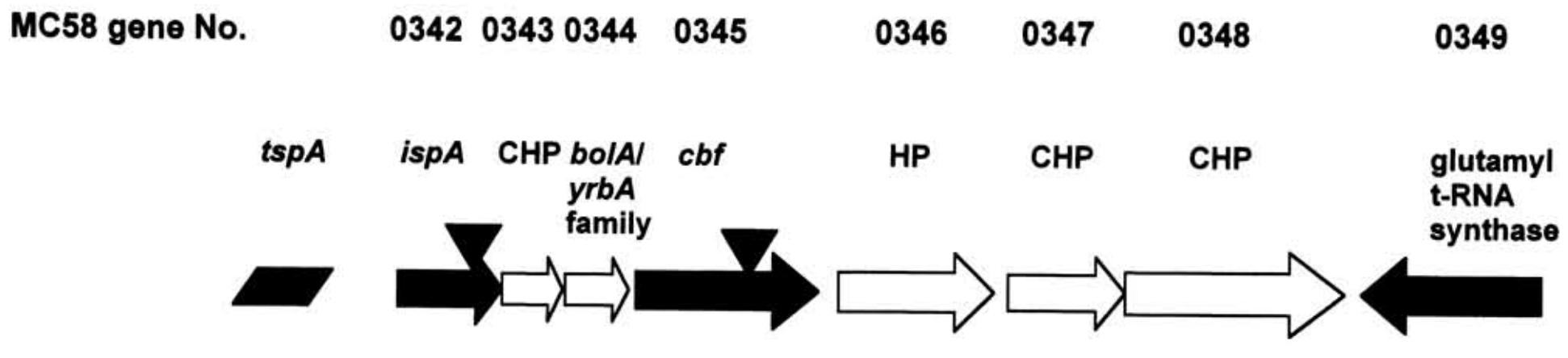


Fig. 1.8 Schematic representation of signature tagged mutagenesis

Fig. 1.9 The NMB0342-NMB0348 locus



The NMB0342-NMB0348 locus showing the relative positions of the Tn*10* insertion sites of mutants 4E3 (insertion in NMB0342) and 25E5 (insertion in NMB0345) which were identified in an STM screen of the serogroup B isolate, C311^{+}. The NMB0342 and NMB0345 genes are shown in blue.

Locus	Name	Cellular role (s)	Coordinate in MC58 genome sequence	Orientation with respect to origin	Length (nt)
NMB0341	*tspA*	Unknown : General	352415 to 349791	reverse	2625
NMB0342	*lspA* homologue	Cell division	352621 to 353148	forward	528
NMB0343	CHP	Hypothetical: Conserved	353166 to 353441	forward	276
NMB0344	*bolA/yrbA* family	Unknown: General	353444 to 353719	forward	276
NMB0345	*cbf* homologue	Cell envelope	353771 to 354634	forward	864
NMB0346	HP	Unknown	354708 to 355463	forward	756
NMB0347	CHP	Hypothetical: Conserved	355539 to 356027	forward	489
NMB0348	CHP	Hypothetical: Conserved	356061 to 357068	forward	1008
NMB0349	GSP	Unknown: General	358028 to 357144	reverse	885

Table 1.1 Details of the NMB0342-NMB0348 locus and flanking ORFs of serogroup B *N. meningitidis* strain MC58

Key:

tspA **= T-cell stimulating protein A**

ispA **= intracellular septation protein A**

CHP = conserved hypothetical protein

cbf **= cell-binding factor, putative**

HP = hypothetical protein

GSP = glutamyl-tRNA synthetase-related protein

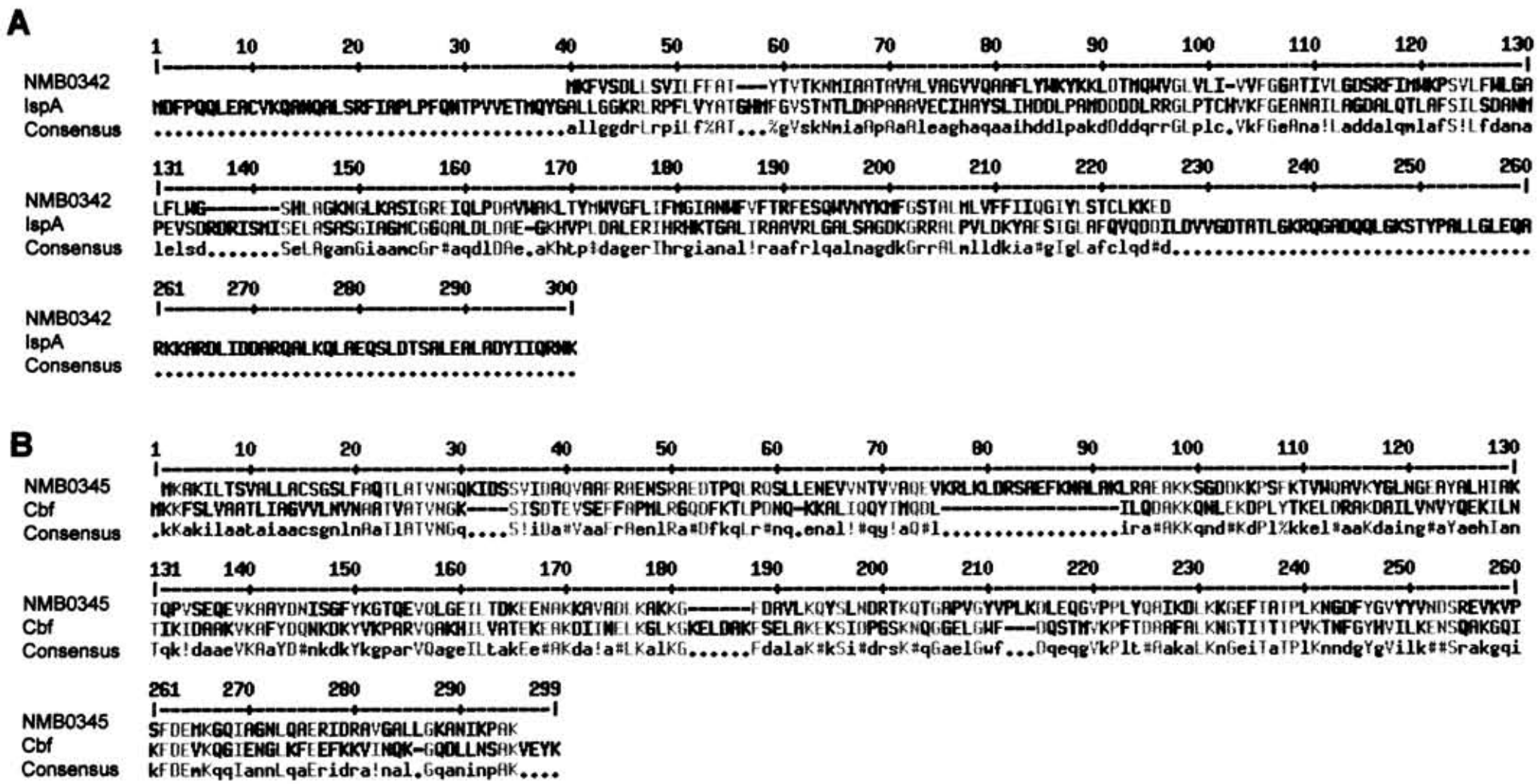
nt = nucleotide

NMB0342 is a homologue of the *ispA* gene of *Shigella flexneri* which is required for effective intercellular spread in epithelial cells (Hong *et al.*, 1998) (Mac Siomoin *et al.*, 1996) (Fig. 1.10, Panel A). *S. flexneri* is a Gram-negative bacillus that causes shigellosis, or bacillary dysentery, in humans (Sansonetti, 2001). In order to cause disease, *S. flexneri* must invade, multiply, and spread in colonic epithelial cells (Hong *et al.*, 1998). A study by Mac Siomon *et al.*, 1996, identified an avirulent Tn*10* mutant of *S. flexneri* that was incapable of spreading throughout an epithelial cell monolayer, and formed long, filamentous bacteria without septa. The mutation also affected the ability of the bacteria to polymerize host cell actin and was found to be in a gene designated *ispA* (Mac Siomoin *et al.*, 1996). Another *ispA* mutant was independently isolated by Hong *et al.*, 1998, using Tn*phoA* mutagenesis. The *S. flexneri ispA* gene encodes a 56 kDa hydrophobic protein with multiple membrane-spanning domains (Mac Siomoin *et al.*, 1996).

NMB0345 is a homologue of *cbf-2*, a *Campylobacter jejuni* gene that encodes a 29 kDa protein (PEB4) and is a major antigenic peptide of this organism (Fig. 1.10, Panel B). *C. jejuni*, a Gram-negative motile rod, is one of the most common causes of gastroenteritis worldwide (Allos, 2001). PCR analysis of a collection of *C. jejuni* strains indicates that *cbf* is highly conserved. Screening of a $\lambda gt11$ genomic library of an epidemic *C. jejuni* strain in *E. coli*, using an antibody to purified PEB4 (a previously identified *C. jejuni* protein) led to the identification of a clone containing two complete open reading frames (ORFs). The first ORF was *cbf-2*. A study by Kervella *et al.* identified two proteins, designated as Cbf-1 and Cbf-2 (cell-binding factor-1 and cell-binding factor-2), that co-purify upon glycine extraction and correspond to the PEB1 and PEB4 proteins respectively, originally purified

by Pei *et al.* (Pei *et al.*, 1991) (Kervella *et al.*, 1993). However, while Cbf-1 is thought to be required for adherence to epithelial cells, Cbf-2 is not a cell-binding factor (Burucoa *et al.*, 1995). The first 22 codons of *cbf*-2 encode a signal peptide sequence required for export to the periplasmic space where it is thought to be involved in the folding of secreted proteins (Burucoa *et al.*, 1995).

Fig. 1.10 Alignments of NMB0342 and NMB0345



Translated MC58 genome sequences of NMB0342 and NMB0345 were aligned with IspA of *S. flexneri* and Cbf of *C. jejuni* respectively using the Multalin multiple sequence alignment program. Amino acids that are highly conserved are shown in red. Amino acids that are weakly conserved are shown in blue. All other residues are shown in black. All positions in each sequence that are identical to the consensus are given in upper case. All other residues are given in lower case. Positions with no conserved residues are represented by a dot in the consensus line. The consensus symbol '!' refers to amino acids I or V, the symbol '%' refers to either F or Y, and the symbol # refers to any one of the following residues: NDQEBZ.

1.19 NMB0343 and NMB0344

NMB0343 and NMB0344, located between the *ispA* and *cbf* homologues of *N. meningitidis*, encode a conserved hypothetical protein and a BolA/YrbA family protein, respectively. Overexpression of *bolA* in *E. coli* results in morphological changes that cause cells to take on a 'round' morphology. This is dependent on the expression of *ftsZ*, a gene involved in the initial steps of cell-division. These morphological changes result in a reduction in surface area exposed to environmental stress factors (Aldea *et al.*, 1989).

Aims of work in this thesis

The isolation of transposon mutants with insertions in NMB0342 and NMB0345 in an STM screen of *N. meningitidis* established the requirement for these genes in *N. meningitidis* pathogenesis. These sequences have homologues in other known human pathogens. NMB0342 and NMB0345 exist within a potential operon of seven genes. The main focus of this study is to determine the specific roles of the NMB0342-NMB0348 locus in *N. meningitidis* pathogenesis. The following aims were established.

1. To establish whether the genes of the NMB0342-NMB0348 locus are conserved in other bacterial pathogens and to establish whether the locus is conserved across a range of pathogenic and non-pathogenic isolates of *Neisseria* spp.

2. To determine whether genes adjacent to NMB0342 and NMB0345 are involved in *N. meningitidis* pathogenesis and to confirm whether the attenuating phenotype is a result of the transposon insertion in NMB0342 and NMB0345.

3. To investigate the involvement of the NMB0342 and NMB0345 genes at multiple steps of pathogenesis.

4. To investigate the biological role of NMB0345.

5. To assess the potential of the NMB0345 gene product as a vaccine candidate.

2 Materials & Methods

2.1 Bacterial strains and growth conditions

(See Appendix A for complete list of bacterial strains)

Neisseria strains were cultured in BHI broth (Oxoid, 37 g dissolved in 1 L dH_2O with 1 g soluble starch, BDH, Merck) or on BHI agar (1% wt/vol) supplemented with Levinthal's base (500 ml defibrinated horse blood, 1 L BHI broth autoclaved in 2 L conical flask, 1 g NAD, Sigma, U.K.) (Alexander, 1965). Media was inoculated from frozen stocks of bacteria stored in media containing 15% glycerol, at -80°C or in liquid nitrogen. Cultures were incubated at 37°C with 5% CO_2 for 16-18 hours. *E. coli* was grown in Luria-Bertani Broth (LB) (20 g LB medium (Gibco, BRL) dissolved in 1 L distilled dH_20 and autoclaved) at 37°C with shaking, or on LB agar (Agar No. 1, Oxoid, Basingstoke, Hampshire) added to LB broth at a final concentration of 1%.

2.2 Antibiotics

Antibiotics were used at the following concentrations: kanamycin (kan) (50 μg/ml) for both *E. coli* and *N. meningitidis*, carbenicillin (50 μg/ml) for *E. coli*, and erythromycin at 200 μg/ml and 2 μg/ml for *E. coli* and *N. meningitidis*, respectively. Stock solutions of carbenicillin (50 mg/ml) and kanamycin (100 mg/ml) were prepared in ddH_2O and sterilized

using 0.22 μm filter units and stored at -20°C. Erythromycin (100 mg/ml) was prepared in ethanol and stored at -20°C. All antibiotics were purchased from Sigma, U.K.

2.3 Isolation of genomic DNA from *N. meningitidis*

A loopful of bacteria (10 μl loop) was harvested from an agar plate and suspended in 567 μl T E buffer (10mM Tris, pH 7.6, 1mM EDTA), 30 μl of 10% SDS (wt/vol) and 3μl of 20 mg/ml proteinase K (Sigma, U.K.). The mixture was then incubated for 1 hour at 37°C. 100 μl of 5M NaCl and 80 μl of cetyl-trimethyl-ammonium bromide/NaCl (CTAB/NaCl) (10% CTAB; 0.7M NaCl) (CTAB, Sigma, U.K., NaCl, BDH, Merck) were added and the solution incubated at 65°C for 10 minutes. One volume of chloroform/isoamyl alcohol (24:1) (BDH, Merck) was added to the solution and vortexed, then centrifuged at 16,000 *xg* for 10 minutes (Heraeus instruments 'Pico' Biofuge). The aqueous layer was then transferred to a 1.5 ml tube and one volume of phenol pH 7.5/chloroform/isoamyl alcohol (25:24:1) (BDH, Merck) added and vortexed. The solution was then centrifuged and the aqueous layer taken off as before. The DNA was precipitated by the addition of 0.6 volumes of isopropanol (BDH, Merck), centrifuged at 16, 000 *xg* for 20 minutes (Heraeus instruments 'Pico' Biofuge) and the pellet washed with 70% ethanol (BDH, Merck) and air-dried. Finally, the DNA pellet was resuspended in TE buffer containing RNAse (100 μg/ml; Sigma, U.K.).

2.4 Isolation of bacterial plasmid DNA

For the purification of plasmid DNA, the QIAprep®Spin Miniprep kit was used by following the protocol provided by the supplier (Qiagen).

2.5 Polymerase chain reaction (PCR)

All PCR amplifications of *N. meningitidis* genomic DNA were performed in a Perkin Elmer GeneAmp® System 9700. The reaction mixture contained 1 pmol/μl of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2U of Taq DNA Polymerase, and 0.1 volume of 10x PCR buffer (Gibco BRL) in a 25 μl volume. As controls, one reaction containing no Taq Polymerase and one reaction containing no target DNA were included each time. Typical thermal cycling conditions were as follows: an initial denaturation step of 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1-3 minutes (depending on the length of the fragment being amplified), and a final extension step at 72°C for 7 minutes. In general, an annealing temperature of 55°C was used for PCR amplifications of *N. meningitidis* genomic DNA. However, the annealing temperature was increased in some cases depending on the optimal annealing temperature for the primer pair used.

2.6 Southern transfer

Restriction endonuclease-digested genomic DNA was separated on 0.8% agarose gels and transferred to nitrocellulose membranes (Hybond N+, Amersham) as described by

Sambrook *et al*, 1989. DNA was UV-fixed to membranes using a UV transluminator (UVP).

2.7 Labelling of DNA probes

DNA fragments were labelled with [^{32}P]-dCTP (Amersham) using an NE Blot kit (New England Biolabs). 5-15 ng of DNA was added to a total volume of 33 µl dH_2O and denatured by heating to 100°C for 5 minutes, then cooled rapidly on ice. 5 µl 10x labelling buffer, 6 µl ATG mix, 3 µl α^{32}P- dCTP and 1 µl DNA Polymerase (Klenow fragment) were then added and mixed. The reaction was incubated for 2 hours at 37°C. To stop the reaction, 1 µl of 0.5 M EDTA was added. 7.5 µl of 2 M NaOH was added to the reaction and left at RT for 5 minutes to denature the probe.

2.8 Hybridizations

Blots were pre-hybridised at 42°C in 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 50% formamide (Sigma, U.K.), and 7% SDS for 10 minutes following which the hybridisation buffer was replaced with fresh hybridisation buffer, to which the denatured probe was added. Hybridisations were performed overnight at 42°C in a Hybaid oven, and blots washed at 65°C twice for 20 minutes in Wash 1, and then twice for 20 minutes in Wash 2.

Wash Solutions	Wash 1	Wash 2
0.5 M Na_2HPO_4	250 ml	40 ml
500 mM EDTA, pH 8.0	2 ml	2 ml
10% SDS	200 ml	100 ml
dH_2O to 1L	548 ml	858 ml

For low-stringency hybridizations, 30% formamide was used in hybridisation buffer and washes were carried out at 53°C. To strip bound probes before reusing membranes, the blots were washed with 0.5 M NaOH for 30 minutes, then with 2x SSC, 1% SDS for a further 30 minutes at RT.

2.9 Autoradiography

Blots were sealed and placed in X-ray cassettes with X-OMAT film (Kodak, U.S.A.) and exposed at -80°C for an appropriate length of time.

2.10 Restriction endonuclease digestion of DNA

Restriction digests were performed with 0.1 volumes of 10x reaction buffer, 0.01 volumes of BSA, restriction enzyme(s) and either plasmid or genomic DNA. All reagents were obtained from New England Biolabs. Reactions were incubated at the appropriate temperature for 2 hours for plasmid DNA, or 5 hours to overnight for genomic DNA.

2.11 Gel electrophoresis and purification of DNA from agarose gels

DNA was combined with gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and fractionated on 0.8-1.0% agarose (Gibco, BRL) gels in x1 Tris Acetate EDTA (TAE) buffer (Tris, 0.4 M, EDTA-Na2-salt 0.01 M, acetic acid 12.01 g/L 0.2 M). DNA was visualized by UV illumination using a transluminator (UVP). Gel fragments were excised using a scalpel and purified using the QIA quick Gel Extraction Kit (Qiagen) according to the protocol provided by the supplier.

2.12 Ligations of DNA

DNA fragments were ligated in two different ways. PCR products were ligated into the pCR 2.1 TOPO vector (Invitrogen) according to the protocol provided by the supplier. All other ligations were performed using a 1:5 ratio of insert to vector, 4 μl 5x T4 DNA ligase buffer and 1 μl T4 DNA ligase (1 unit/μl) made up to a final volume of 20 μl with dH_20 (Sigma, U.K.). The mixture was incubated at 16°C for 1.5 hours.

2.13 Transformation

2.13.1 Heat-shock transformations (*E. coli*)

10 μl of a ligation reaction was added to 50 μl of DH5α competent cells (Gibco Life Technologies) and kept on ice for 30 minutes. After a heat-shock at 37°C for 45 seconds, the mixture was left on ice for 2 minutes, then 450 μl of SOC medium. SOC medium was prepared by dissolving 20 g bacto-tryptone (Gibco, BRL), 5 g bacto-yeast extract (Gibco,

BRL), and 0.5 g NaCl (BDH) in 950 ml dH_20. 10 ml of a 250 mM KCl (BDH) solution was then added and the volume adjusted to 1 L and the solution autoclaved. 5 ml of a sterile solution of 2M $MgCl_2$ was added to the final solution. The mixture was then incubated at 37°C with continuous shaking (200 rpm) for 1 hour (kanamycin selection) or 30 minutes (carbenicillin selection). The bacteria were then plated to the appropriate selective medium.

2.13.2 *Neisseria* transformations

N. meningitidis was grown on BHI agar for 16-18 hours, then a suspension of bacteria made in PBS, and 10 µl spotted onto BHI agar and allowed to dry. 5-10 µl DNA was then added to the bacterial suspension and allowed to dry. Plates were incubated at 37°C with 5% CO_2 for 4 hours, after which time bacteria were harvested from the plates using a sterile loop, and spread onto selective media.

2.14 Quantification of DNA

DNA concentration was determined by measurement of the absorbance at 260 nm using a Cecil spectrophotometer. An OD of 1.0 at 260 nm is taken to be equivalent to 50 µg/ml double stranded DNA (Sambrook *et al*, 1989).

2.15 DNA sequencing

DNA sequencing was performed using 1 µl primer (3.2 pmol/µl), 2 µl of 5x sequencing buffer, 4 µl Big Dye mix, 500 ng DNA, and dH_2O (Sigma, U.K.) to make up to a final volume of 20 µl. Sequencing was performed using the following thermal cycling conditions: An initial denaturation step at 96°C for 1 minute followed by 30 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. The reaction was precipitated by the addition of 2 µl of 3M NaAc and 55 µl of 96% ethanol and storage at -20°C for one hour. The reaction was then centrifuged for 30 minutes at 16,000 *xg*, the pellet washed with 76% ethanol, then centrifuged for a further 30 minutes at 16,000 *xg*. The supernatant was removed and the pellet dried at 96°C for 1 minute. Automated sequencing was performed by the Department of Biochemistry, University of Oxford.

2.16 *in vitro* transposition

in vitro transposition using the EZ::ZN™<KAN-2> Insertion Kit was performed according to the protocol provided by the supplier (Cambio). Details of the EZ::TN™ <KAN-2> transposon are given in Appendix G.

2.17 Assessment of the ability of *N. meningitidis* to cause bacteraemic infection

2.17.1 Infection of infant rats

Neisserial strains were grown on BHI agar at 37°C with 5% CO_2 for 16-18 hours. Suspensions of each strain were made in PBS. An aliquot of this suspension was lyzed in

0.1 M NaOH, 1% SDS, and the OD measured; for *N. meningitidis*, an OD of 1.8 at 260 nm is equivalent to 10^9 colony-forming units/ml (CFU/ml). Suspensions of 2 x 10^6 CFU/100 µl were made for each mutant and for the wild-type strain. Inoculae were prepared by combining suspensions of mutant and wild-type bacteria in a 1:1 ratio. Groups of 3-5 five day-old Wistar (Harlan) were each inoculated with 100 µl of the inoculum I.P. Following inoculation, dilutions of the remaining inoculum were made in BHI and plated to both BHI and BHI medium containing kanamycin to allow the percentage of mutant to wild-type present in the inoculum to be confirmed for the calculation of competitive indices (C.I.s). 5 µl of blood was obtained by a tail vein bleed at 20-22 hours post-inoculation and diluted in 100 µl of BHI medium. 20 µl of each of the following dilutions made in BHI (neat, 1:10, 1:100, and 1:1000) and plated to both BHI and BHI kan_{75} medium. Following incubation of the plates overnight at 37°C, the bacteria were enumerated, and the competitive index (C.I.) calculated.

Calculation of C.I.:

$$\text{C.I.} = \frac{\text{(ratio of mutant to wild-type in the output (recovered from animals)}}{\text{(ratio of mutant to wild-type in the input (inoculum)}}$$

2.17.2 Infection of wild-type and C3 deficient mice

Two groups of five six week-old C57BL/6 and C57BL/6 C3-/- female mice (Professor Marina Botto, Hammersmith Hospital) were inoculated with 5 x 10^4 CFU/0.5 ml of a 1:1 ratio of $C311^+$ and either the NMB0342 or NMB0345 mutant prepared in BHI medium and

supplemented with 0.5% (wt/vol) iron dextran (Sigma, U.K.). Mice were inoculated with 0.5 ml given I.P. 3 µl of blood was obtained by a tail vein bleed at 21 hours post-inoculation and diluted in 100 µl of BHI medium. 20 µl of each of the following dilutions made in BHI (neat, 1:10, 1:100, and 1:1000) and plated to both BHI and BHI kan_{75} medium. Plates were incubated at 37°C overnight and the competitive index (C.I.) was calculated.

2.18 Web-based searches

The following websites were used for database searches, identification of conserved domains, primer design, and sequence alignments:

http://www.tigr.org/tdb/CMR/gnm/htmls/SplashPage.html	*N. meningitidis* strain MC58 genome database.
http://www.genome.ou.edu/gono_blast.html	University of Oklahoma *Neisseria gonorrhoeae* strain FA1090 genome sequencing project.
http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi	Simultaneous BLAST searches against multiple microbial genomes.
http://www.sanger.ac.uk	The Wellcome Trust Sanger Institute.
http://www.sanger.ac.uk/Software/Pfam/search.shtml	Protein families database of alignments and HMMs.
http://www.sbc.su.se/~miklos/DAS/maindas.html	DAS transmembrane prediction server.
http://www.cbs.dtu.dk/services/LipoP/	LipoP 1.0 Server for the prediction of lipoproteins and signal peptides in Gram-negative bacteria.

http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer	*Saccharomyces* Genome Database (SGD™) site for primer design.
http://prodes.toulouse.inra.fr/multalin/multalin.html alignment site	MultAlin multiple sequence alignment (Corpet *et al.*, 1988).

2.19 Bacterial growth curves

Bacterial strains were grown on BHI agar for 16 hours at 37°C, 5% CO_2. Inoculae were prepared by making suspensions of each strain in phosphate buffered saline (PBS) (Gibco BRL). Lysates of each suspension were made in P2 solution (Qiagen) and the OD of each lysate was measured at 260 nm. 10^9 CFU of each bacterial strain was inoculated into 10 ml of proteose peptone broth (proteose peptone (Difco) 15.0 g, K_2HPO_4, 4.0g, KH_2PO_4, 1.0 g, NaCl 5.0 g with 2% vitox supplement (Oxoid) in 75 cm^2 tissue culture flasks with filter caps (Nunc™) in triplicate. The flasks were placed into a large plastic box that was sealed with parafilm and rocked at 10 rev/min. The OD was measured at 600 nm by transferring 1 ml from each flask to a plastic cuvette with a plug-in cap (Elkay). The OD was measured every hour over 5 hours.

2.20 Adhesion of bacteria to cultured human cells

Chang epithelial cells (ECACC) were grown from frozen stock in RPMI (Sigma, U.K.) containing glutamine (2 mM) and 10% fetal bovine serum (FBS) (Sigma, U.K.) and penicillin/streptomycin (Sigma, U.K.) (used at 1 unit/ml penicillin, 0.1 μg/ml streptomycin (Sigma, U.K.), stock pen/strep solution 10,000 units penicillin, 10 mg/ml streptomycin per

ml in 0.9% NaCl) at 37°C with 5 % CO_2. Cells were grown to near confluence in 75 cm^2 tissue culture flasks (Nunc™). Media was removed from the flask and the cells washed twice with 10 ml sterile PBS. Cells were trypsinized using 1 ml trypsin (Invitrogen) and incubated for 2-3 minutes at 37°C with 5 % CO_2. 10 ml of RPMI containing glutamine (2 mM), 10 % FBS, and penicillin/streptomycin was added and the cells were resuspended in this medium using a 10 ml pipette. 5 ml of the resuspended cells was added to 20 ml of RPMI containing glutamine (2 mM), 10 % FBS, and penicillin/streptomycin. This flask was used to seed 1 ml Chang cells into each of 10 wells of a 24 well plate (Nunc™) and grown overnight to a monolayer of 10^5 cells. An extra well was seeded for enumeration. Cells were counted using a haemocytometer (BDH). Just prior to the adhesion assay, media was removed from each well and the wells were washed three times with RPMI containing glutamine (2 mM). For all steps using RPMI, the medium was pre-warmed to 37°C.

Bacterial strains were grown on BHI agar for 16 hours at 37°C with 5 % CO_2. Bacteria were resuspended in 1 ml of PBS. 20 µl of this suspension was added to 980 µl lysis buffer (P2 buffer, Qiagen) and an OD of the lysate taken at 260 nm. Suspensions were made up to 10^8 CFU/ml in RPMI containing glutamine (2 mM) and human serum (10 %). 1 ml of each suspension was added to each well. Three wells were inoculated per strain. The plate was centrifuged at 110 *g* for 5 minutes, then incubated for 3 hours at 37°C with 5 % CO_2. Following this incubation, non-adherent bacteria were removed and each well was washed three times with RPMI containing glutamine (2 mM). 1 ml of 1% saponin in PBS (filter sterilized using a 0.22 µm filter unit (Millipore)) was added to each well and the plate incubated at 37°C with 5 % CO_2. The contents of each well were then re-suspended by

pipetting up and down five times. Cells were then scraped from the surface of the plate. The suspension was then mixed by pipetting up and down 15 times. This was then transferred to a 1.5 ml tube and 1:100, 1:200, 1:500, 1:1000, and 1:10, 000 dilutions made in PBS in a 96 well microtitre plate. 10 µl drops of each dilution were plated in triplicate onto BHI agar, and plates incubated overnight at 37°C with 5 % CO_2. Bacteria were then enumerated by counting the colonies on plates the following day.

2.21 Expression and purification of the NMB0345 protein

2.21.1 Cloning of the *N. meningitidis* gene NMB0345 for expression in *E. coli*

NMB0345 was amplified by PCR from genomic DNA from the serogroup B isolate, MC58 using BioXact polymerase (Bioline) and primers NG337 and NG338, designed according to the published genome sequence of MC58 (TIGR) under standard conditions. The PCR product was run on a 0.8% gel, then excised from the gel and purified. The PCR product was ligated into pCR® T7/CT-TOPO® (Invitrogen) and transformed into *E. coli* strain DH5α. (Please refer to Appendix G for details of the pCR® T7/CT-TOPO® vector). Plasmids isolated from transformants were checked for the presence of the correct insert by PCR using primers NG337 and NG338 and employing the same cycling conditions as described above. Plasmids from transformants of interest were restriction mapped using *Xba*I/*Hind*III to confirm the presence of an insert and *Ssp*I/*Hind*III to check the orientation of the insert. Plasmid DNA was isolated from the transformant of interest as described above. This plasmid was designated as pSB5. Plasmid pSB10 was generated using the

same method as that described for the cloning of pSB5 except that primers NG337 and NG375 were used to amplify the NMB0345 gene lacking the last seven amino acids.

2.21.2 Expression of recombinant NMB0345 protein

For expression of recombinant NMB0345 protein in *E. coli*, 10 ng of pSB10 was used to transform *E. coli* BL21 (DE3) pLysE competent cells by heat shock transformation. SOC rescue was performed at 37°C with shaking (225 rpm) for 1 hour. The entire transformation was then inoculated into 50 ml of LB, carbenicillin (100 μg/ml), chloramphenicol (34 μg/ml) and grown overnight at 30°C with shaking (225 rpm) in a 200 ml flask. The OD of the overnight culture was measured at 600 nm and the culture diluted to an OD of approximately 0.118 in LB containing carbenicillin and chloramphenicol in a final volume of 250 ml in a 1 litre flask and incubated with shaking (225 rpm) at 30°C. The culture was grown to an OD of approximately 0.4 –0.6 at 600 nm. Expression of recombinant NMB0345 protein was induced by the addition of IPTG (1 mM final concentration, prepared in dH_20 (Sigma, U.K.) filter-sterilized using a 0.22 μm filter unit, Millipore) and incubated at 30°C with shaking for 2-3 hours. The culture was then centrifuged at 2740 *g* at 4°C for 10 minutes. The supernatant was discarded and the pellet stored at –20°C until required for protein purification.

2.21.3 Purification of recombinant NMB0345 protein

Purification of recombinant NMB0345 protein was performed under non-denaturing conditions using a Talon™ metal affinity resin (Clontech) according to instructions provided by the manufacturer (Talon™) with the exception that lysozyme was not added to the cell pellet as this step was not found to be necessary for efficient purification and sonication, (using a Sonopuls sonicator) (Bandelin) was carried out for three cycles of 1 minute each rather than for 10 seconds with 1 minute intervals between cycles rather than 30 second intervals in order to achieve a more homogenous lysate. The resin was centrifuged at 700 *xg* for five minutes instead of two minutes.

2.22 Estimation of protein concentration using a Bradford assay

The concentration of purified recombinant NMB0345 protein was estimated using a Bradford assay. The spectrophotometer (UV-1201, Shimadzu) was calibrated at A_{595} using Bradford reagent (Sigma, U.K.) and elution buffer (Talon™, Clontech). Dilutions of bovine serum albumin (BSA) (100-2000 µg/ml) were prepared in 1 x elution buffer from 10 mg/ml BSA (NEB). Dilutions of purified protein were also made in 1 x elution buffer. 50 µl of each sample was added to 3 ml of Bradford reagent in a plastic cuvette and mixed. OD readings of each BSA sample were taken at 595 nm to generate a standard curve. Protein concentration (µg/ml) was estimated by measuring the OD of samples of purified protein at 595 nm correcting for the dilution, and plotting these values against the standard curve.

2.23 Preparation of *N. meningitidis* whole cell lysates for SDS-PAGE

For *N. meningitidis* whole cell lysates, bacteria were grown for 16 hours on BHI agar, then harvested from half an agar plate and resuspended in 0.5 ml dH_2O (Sigma, U.K.) in 2 ml screwcap tubes (Sarstedt). Samples were then boiled for 5 minutes and centrifuged for 5 minutes at 15,700 *g*. Bacterial pellets were then re-suspended in 150 µl dH_20 and combined with an equal volume of 1 x SDS-PAGE sample buffer (1.2 ml 0.5 M Tris pH 6.8, 1 ml 10% SDS, 0.1 ml beta mercaptoethanol, 1 ml glycerol, 0.001 g bromophenol blue). Prior to loading, all samples were boiled for 5 minutes. Samples were then centrifuged briefly (15-20 seconds) and 10-20 µl analysed by SDS-PAGE.

2.24 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE analyses of protein samples were performed according to methods described by Sambrook *et al.*, 1989. Polyacrylamide gels were prepared using a BioRad Mini-Protean II gel apparatus and were run in (Tris Borate EDTA) TBE buffer (Tris 0.89 M, EDTA-Na2-salt 0.02 M, Boric acid 0.89 M) at either 90 V for 2-3 hours or at 15 V overnight (Sambrook *et al.*, 1989).

Running Gel:

3 ml 40% Acrylamide/Bis solution (BioRad)

2.5 ml 1.5 M Tris-HCl (BDH), pH 8.8

100 µl 10% SDS (BDH)

4.35 ml ddH_2O (Sigma, U.K.)

15 µl TEMED (BDH)

50 µl 10% APS (Sigma, U.K.)

final volume: 10 ml

Stacking Gel:

3.2 ml dH_2O (Sigma, U.K.)

50 µl 10% SDS (BDH)

500 µl 40% acrylamide/bis solution (BioRad)

1.25 ml 0.5M Tris (BDH) pH 6.8

10 µl TEMED (BDH)

25 µl 10% APS (Sigma, U.K.)

Final volume: 5 ml

2.24.1 Staining and de-staining of SDS-PAGE mini gels

Polyacrylamide gels were stained by placing into approximately 100 ml of Coomassie Blue Stain (0.2% Coomassie Blue R-250, 40 % methanol (v/v), 10% glacial acetic acid (v/v), 50% ddH_2O), and were then either heated in a microwave on a high setting for 1 minute,

then allowed to stand for 5 minutes at room temperature, or left in stain overnight at room temperature. The staining solution was then replaced with 100 ml Destain 1 solution (40 % methanol (v/v), 10% glacial acetic acid (v/v), 50% ddH_2O) and the gel heated in a microwave on a high setting for 1 minute, then allowed to stand for 10 minutes. Destain 1 solution was replaced with 100 ml of Destain 2 solution (10% glacial acetic acid (v/v), 4% glycerol (v/v), 86% ddH_2O) and the gel was placed on an orbital shaker (HS 250 Basic IKA Labortechnik) overnight.

2.25 Western transfer

Protein samples were run on a polyacrylamide gel with a pre-stained marker (6.4-250 kDa) (BioRad). An Immobilon P polyvinylidene fluororide (PVDF) membrane (Millipore) was pre-wet with methanol. The polyacrylamide gel and PVDF membrane were both pre-equilibrated in equilibration buffer (48 mM Tris base, 39 mM glycine, pH 8.3) for 30 minutes. Ten sheets of Whatman paper cut to the size of the polyacrylamide gel were pre-wet in equilibration buffer. Five sheets of pre-wet Whatman paper were laid on top of each other, ensuring no bubbles were present between layers. The PVDF membrane was placed on top of the Whatman paper. Next, the polyacrylamide gel was placed on top of the PVDF membrane which was then overlaid with five more sheets of pre-wet Whatman paper again ensuring that no bubbles were present between layers. Western transfer was performed at 15 V for 20 minutes using a Trans-blot® SD Semi-Dry transfer cell (BioRad). Following transfer, the PVDF membrane was blocked in 5% milk (skimmed milk powder (Sigma, U.K.) dissolved in PBS) for 1 hour at room temperature or at 4°C overnight with gentle

shaking. Prior to incubation with the primary antibody, the PVDF membrane was rinsed once in PBS.

2.25.1 Incubation with primary antibody

The antibody was diluted in PBS and incubated with the PVDF membrane for 1-2 hours followed by 3 washes with PBS of 5 minutes each. Murine monoclonal Anti-Polyhistidine Peroxidase Conjugate Clone HIS-1 (Sigma) was used at a 1:2000 dilution in PBS. Anti-NMB0345 antibody (mouse or rabbit) was used at either a 1:500 or 1:750 dilution in PBS. Following incubation with the primary antibody, the PVDF membrane was washed with PBS three times for five minutes each.

2.25.2 Incubation with secondary antibody

The antibody was diluted in PBS and incubated with the membrane for 1-2 hours followed by three washes with PBS for five minutes each. Murine monoclonal Anti-Polyhistidine Peroxidase Conjugate Clone HIS-1 (Sigma, U.K.) was used at a 1:2000 dilution in PBS. Goat anti-rabbit peroxidase conjugate monoclonal antibody (Sigma, U.K.) was used at a 1:500 dilution.

2.25.3 Development

Solutions A (20 µl 30% H_2O_2 (v/v), BDH), 30 ml dH_2O (Sigma, U.K.) and B (30 mg 4-chloro-1-napthol (Sigma, U.K.), 10 ml methanol (BDH) were mixed just prior to addition to the membrane and left for 1-5 minutes to allow colour to develop. The solution was then discarded and the membrane washed in ddH_2O 2-3 times for 5 minutes, and allowed to dry on 3MM filter paper (Whatman).

2.26 Analysis of *N. meningitidis* lipopolysaccharide

2.26.1 Lipopolysaccharide extraction

Cells were grown to late log phase in proteose peptone broth at 37°C to an OD of 1.0 at 540 nm, and concentrated to an OD of at 540 nm 1.5 in PBS. The resultant cell pellet from 500 µl of sample was resuspended in 100 µl dH_2O. An equal volume of 2X sample buffer (6% SDS; 6% 2-mercaptoethanol; 10 mM DTT; 46% glycerol; 60 mM Tris, pH 8.0; 0.1% bromophenol blue) was added and the samples boiled for 10 minutes. Protein was digested by the addition of proteinase K to a final concentration of 50 µg/ml at 37°C overnight. Samples were again boiled for 10 minutes and a second volume of proteinase K, equal to the first, was added and incubated at 55°C for 3 hours. LPS samples were stored at -20°C until required.

2.26.2 Electrophoresis of lipopolysaccharide

Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (TSDS PAGE) was used to obtain high resolution of LPS. Gels were assembled as a two-phase gel with a 16.5% resolving component overlaid with a 4% stacking component. Electrophoresis was performed as previously described for SDS-PAGE. The acrylamide concentrations of the two phases was 49.5% *T*, 6% *C* and 49.5% *T*, 3% *C* respectively, where *T* represents the total acrylamide percentage (acrylamide and bisacrylamide), and *C* is the percentage of bisacrylamide (cross linker). All solutions were prepared with high quality milli Q water (Millipore). This system utilises two specific buffers for electrophoresis, ie. anode buffer (0.2 M Tris, pH8.9) and cathode buffer (0.1 M Tris, 0.1 M tricine, 0.1% SDS, pH 8.25). Electrophoresis was performed with the BioRad Mini-Protean II gel apparatus (BioRad), with the glass plates being thoroughly washed with 20% Decon 90 and rinsed well with milli Q water. Prior to loading, the LPS samples, together with loading dye, were boiled for 5 minutes. Samples were loaded under cathode buffer and electrophoresed for 5 h or until 1 hour after the dye front had reached the end of the gel.

LPS was visualized by oxidative silver staining (Tsai and Frasch, 1982). Following electrophoresis the gel was fixed overnight in a 40% ethanol, 5% acetic acid solution in a thoroughly washed and rinsed glass dish. The fixing solution was replaced with 0.7% periodic acid in 40% ethanol, 5% acetic acid and allowed to oxidize for 7-10 minutes. The gel was washed three times for 15 minutes with 200 ml milli Q water. After washing, the gel was stained with fresh silver staining reagent, containing 2 ml concentrated ammonium hydroxide, 28 ml of 0.1 M NaOH, 5 ml of 20% (w/v) silver nitrate, and milli Q water to 150

ml. The staining solution was left on for 10 minutes before being removed. The gel was then washed in milli Q water three times for 10 minutes. Once washed the stained gel was developed by the addition of 200 ml of developer, containing 10 mg citric acid and 100 µl of 37% formaldehyde. As the stain neared the desired intensity, the development was terminated by replacing the development solution with water or a mild acidic solution.

2.27 Immunization of mice with recombinant NMB0345 protein and bacterial challenge

2.27.1 Protection model and immunization schedule

Groups of ten six week-old female adult mice (BALB/c, Harlan) were vaccinated on days 0 and 21 with one of the following.

Immunizations:

1. Recombinant NMB0345 protein eluted in elution buffer (Clontech) was diluted in sterile PBS to a final concentration of 25 µg per 100 µl. The diluted protein was combined in a 1:1 ratio with Freunds incomplete adjuvant (Sigma, U.K.) and emulsified by passing through an 18 gauge needle (Merck) using a glass Luer-lock syringe (Sigma, U.K.). Mice were each given a total of 200 µl of the final preparation subcutaneously.

2. A 1:1 ratio of elution buffer (diluted in sterile PBS) and Freunds incomplete adjuvant (Sigma, U.K.) was emulsified as described above. Mice were each given a total of 200 µl of the final preparation subcutaneously.

3. *N. meningitidis* attenuated strain YH102 (MC58Δ*siaD*Δ*rfaF*) (Li *et al.*, 2004). Strain YH102 was grown overnight on BHI agar. Bacteria were prepared for 2 x 10^6 CFU/0.5 ml supplemented with 0.5% (wt/vol) iron dextran (Sigma, U.K.). Mice were incoculated intraperitoneally (I.P.) with 0.5 ml per mouse.

2.27.2 Live bacterial challenge

Challenge was performed on day 35 (two weeks following the second immunization). *N. meningitidis* strain MC58 was grown overnight on a solid medium and then harvested into sterile PBS. The number of CFU was estimated by measuring the OD of a lysate of the suspension prepared in lysis buffer (1% SDS, 0.1% NaOH) at 260 nm. The inoculum was prepared for 1 x 10^6 CFU/0.5 ml in BHI medium supplemented with 0.5% (wt/vol) iron dextran (Sigma, U.K.). Mice were each inoculated with 0.5 ml I.P. Animals were observed at least twice daily for 72 hours following inoculation.

2.28 Immunologic Assays

2.28.1 Serum Bactericidal Assay (SBA)

Serum bactericidal assays were performed using either murine or rabbit polyclonal sera collected from animals immunized with purified recombinant NMB0345 protein following the immunization protocol described. SBAs were performed according to the method described by Maslanka *et al.*, 1997, with the exception that the number of viable bacteria at the start and at the end of the incubation was determined by plating to BHI agar rather than GC agar. All assays were performed in duplicate (Maslanka *et al.*, 1997).

2.28.2 Whole cell Enzyme Linked Immunosorbent Assay (ELISA)

Bacterial suspensions were heat-killed by incubating at 56°C for 60 minutes. A flat-bottomed ELISA plate (Maxisorb; Nunc™) was coated with 100 µl of the heat-treated bacterial suspension (OD 1.0 measured at 540 nm) in carbonate bicarbonate coating buffer (Sigma, U.K.) either overnight at 4°C or at for 1 hour at 37°C. The wells were washed three times with 200 µl of PBS-Tween 20 (0.1%) with 30 seconds to 1 minute between each wash, then incubated with dilutions of sera for 2 hours at room temperature; pre-immune sera were used as negative controls. Following this incubation, the wells were washed three times with 200 µl of PBS-Tween 20 (0.1%). 100 µl horseradish peroxidase conjugated goat anti-mouse antibodies (DAKO) diluted 1:1000 in 1% goat serum in PBS-Tween 20 was added to the wells and the plate incubated for 2 hours at room temperature. Following this incubation, the wells were washed three times with 200 µl of PBS-Tween 20 (0.1%). The substrate, *o*-phenylenediamine (Sigma, U.K.) (0.4 mg/ml in 0.1 M citric acid/phosphate

buffer, pH 5.0) was then added to the wells. The reaction was stopped with 100 µl of 2 M H_2SO_4 or 3 N HCl. The absorbance was read at 490 nm or 492 nm.

2.29 Raising antibody against recombinant NMB0345 protein in rabbit

Purified recombinant NMB0345 protein was provided at a concentration of 1 mg/ml in PBS to CovalAb, U.K. A total of 200 µg of protein was given to two female rabbits (New Zealand) over four injections.

Immunization Protocol:

Day 0	Pre-bleed	5 ml taken
	1st Injection	1 ml antigen + 1 ml Freunds complete adjuvant
Day 21	2nd injection	1 ml antigen + 1ml Freunds incomplete adjuvant
Day 42	3rd injection	1 ml antigen
Day 54	Test bleed	5-20 ml taken
Day 63	4th injection	1 ml antigen
Day 74	Test bleed	5-20 ml taken
Day 88	Final bleed	

2.30 Whole Blood Assay (WBA)

Bacterial strains were grown on BHI agar at 37°C with 5 % CO_2. After 16 hours' growth, bacteria were sub-cultured onto BHI agar and allowed to grow for 4 hours at 37°C with 5% CO_2. Bacteria were collected using a sterile swab and re-suspended in 2 ml of sterile RPMI (without phenol red or L-glutamine) (Invitrogen). A 1:10 dilution of this suspension was made in RPMI and the OD of the suspension measured at 600 nm. Suspensions of each strain were made up to an OD of 1.0. 10 µl of this suspension is equivalent to approximately 10^8 CFU. Dilutions of 10^{-5}, 10^{-6}, and 10^{-7} of each bacterial suspension were made in RPMI in a 96 well microtitre plate and five 10 µl spots from each dilution plated to BHI agar for enumeration of bacteria in the inoculum.

Blood was taken by venous puncture and heparin (Leo Lab) added at 10 units per ml blood. Blood was taken no more than three hours prior to use and stored at room temperature. For each strain being tested, 1 ml blood was placed into a 7 ml tube. 10 µl of bacterial suspension was added to each tube and the tube inverted 5 times. 10 µl was taken from each tube and used to inoculate two wells of a 96 well microtitre plate containing 90 µl RPMI. Dilutions were made in RPMI in a 96 well microtitre plate. The tubes were placed into a sealed plastic bag with tissue placed inside. The bag was placed on a rocker (10 rev/min) and incubated at 37°C, 5 % CO_2. 10 µl of blood was taken from each tube following 45 minutes and 90 minutes incubation and 1:10, 1:100, 1:1000, 1:10,000, $1:10^5$ and $1:10^6$ dilutions were made in a 96 well plate using a multi-channel pipette. Five 10 µl drops from each dilution for each strain were plated to BHI agar. Spots were allowed to

dry. The plates were then inverted and incubated at 37°C, 5 % CO_2 for enumeration of bacteria.

2.31 Serum-sensitivity assay

Bacterial strains were grown on BHI for 16 hours at 37°C, 5% CO_2. Each strain was sub-cultured on BHI agar and grown for four hours at 37°C, 5% CO_2. Bacteria were harvested from the plates into 400 µl of PBS in a 2 ml screw-cap tube and centrifuged at 450 *g* for 5 minutes. The supernatant was then transferred to a new 2 ml tube and re-centrifuged at 15,700 *g* for 5 minutes. The supernatant was then discarded and the pellet resuspended in 400 µl PBS. The OD of a 1:50 diluted lysate of the suspension made in P2 buffer (Qiagen) was measured at 260 nm. Inocula were prepared to a concentration of 10^4 CFU/100 µl in Dulbecco's Modified Eagle's Medium (DMEM) glutaMAX™ (Invitrogen). The assay was performed using a 96 well plate. 125 l of bacterial suspension was placed in the first well of each row. 100 µl of normal human serum or heat-inactivated serum (heated to 56°C for 45 minutes prior to use in the assay) was placed into the first well of each row of the 96 well microtitre plate. The following dilutions of serum (1:2, 1:4, 1:8, 1:16, 1:32, 1:64) were made in DMEM glutaMAX™ (Invitrogen) using a multi-channel pipette. 125 µl of bacterial suspension was placed in the first well of each row. 100 µl of each suspension was placed into each of the remaining wells. The wells were mixed by pipetting 10 times using a multi-channel pipette, using new tips to mix each row. Following incubation of the plate at for 1 hour, five 10 µl drops from each of the following dilutions of serum, (1:2, 1:4, and

1:8), were plated onto BHI agar. The spots were allowed to dry. The plates were then inverted and incubated at 37°C, 5% CO_2 overnight and the bacteria enumerated.

2.32 Microscopy of *N. meningitidis* strains

2.32.1 Immunofluorescence

Chang epithelial cells were grown to monolayers of 10^5 cells on glass coverslips (BDH) using the method described for the adhesion or invasion assays described above except that following incubation with the bacteria and washing with RPMI (adhesion assay) or incubation with gentamicin and washing with RPMI (invasion assay), cells and bacteria were fixed onto coverslips by the addition of 3% paraformaldehyde (PFA) for 15 minutes. The PFA was then removed and the coverslips washed twice with PBS. 1 ml of fresh PBS and 10 µl of 1M $(NH_4)_2SO_4$ were then added. Immunostaining was performed using an murine monoclonal anti-PorA P1.15 antibody (NIBSC, U.K.) as the primary antibody at a 1:150 to 1:200 dilution and donkey anti-mouse Texas Red conjugated antibody (Calbiochem) as the secondary antibody at a 1:200 dilution. Antibody dilutions were made in 0.1% saponin in PBS containing 10% horse serum. The coverslips were then washed twice in PBS. 40 µl drops of diluted antibody were placed onto parafilm (Merck). Coverslips were placed bacteria-side down onto the drops and incubated for 30 minutes. Following incubation with the final antibody, coverslips were washed twice in PBS and once in dH_20. Coverslips were then mounted onto glass slides (BDH) using Aqua polymount (Polyscience) and allowed to dry. Images were obtained using an Olympus

BX150 microscope and were taken at x 40 and x 100 objective. Slides were stored at room temperature.

2.32.2 Transmission electron microscopy (TEM)

Electron microscopy was performed by David Ferguson, John Radcliffe Hospital, Oxford. *N. meningitidis* strains were grown on BHI agar for 16 hours at 37°C with 5% CO_2. Bacteria were fixed in 4% glutaraldehyde and 0.1 M phosphate buffer. Samples were post-fixed in osmium tetraoxide in 0.1 M phosphate buffer, then dehydrated in ethanol. Samples were then treated with propane oxide and embedded in Spur's epoxy resin. Thin sections of samples were stained with uranyl acetate and lead citrate and images taken using a JEOL 1200 EX transmission electron microscope.

2.33 Peptidyl Prolyl *cis-trans* Isomerase (PPIase) assay

Recombinant NMB0345 protein purified by using a Talon™ metal affinity resin (Clontech) as described above, was desalted and exchanged into HEPES buffer pH 7.8 (Sigma, U.K.) using a Hi-trap desalting column (Pharmacia). The protein was then concentrated to 250 mg/ml using a Centricon column. PPIase activity of the protein was determined using a coupled assay in which the PPIase was combined with α–chymotrypsin and substrate (either the synthetic short peptide N-succinyl-Ala-Ala-Pro-Phe-p-nitroanalide or N-succinyl-Ala-Phe-Pro-Phe-p-nitroanalide (Bachem). 50 µl of PPIase (0.05 µg/ml) was added to 50 µl of 6 mg/ml chymotrypsin (Sigma, U.K.) dissolved in 0.05 M HEPES pH 7.8 and 1850 µl of

0.05 M HEPES pH 7.8 pre-equilibrated at 10°C, in a 3 ml cuvette (Elkay) and the mixture equilibrated at 10°C for 3 minutes. 50 µl of 2 mg/ml substrate in 0.05 M HEPES pH 7.8 was added and mixed. OD readings at 390 nm were taken every second over a period of three minutes using a spectrophotometer (Shimadzu UV-1601) linked to UVProbe software. Controls included reactions run in the absence of substrate and reactions run in the absence of PPIase. All experiments were performed in triplicate.

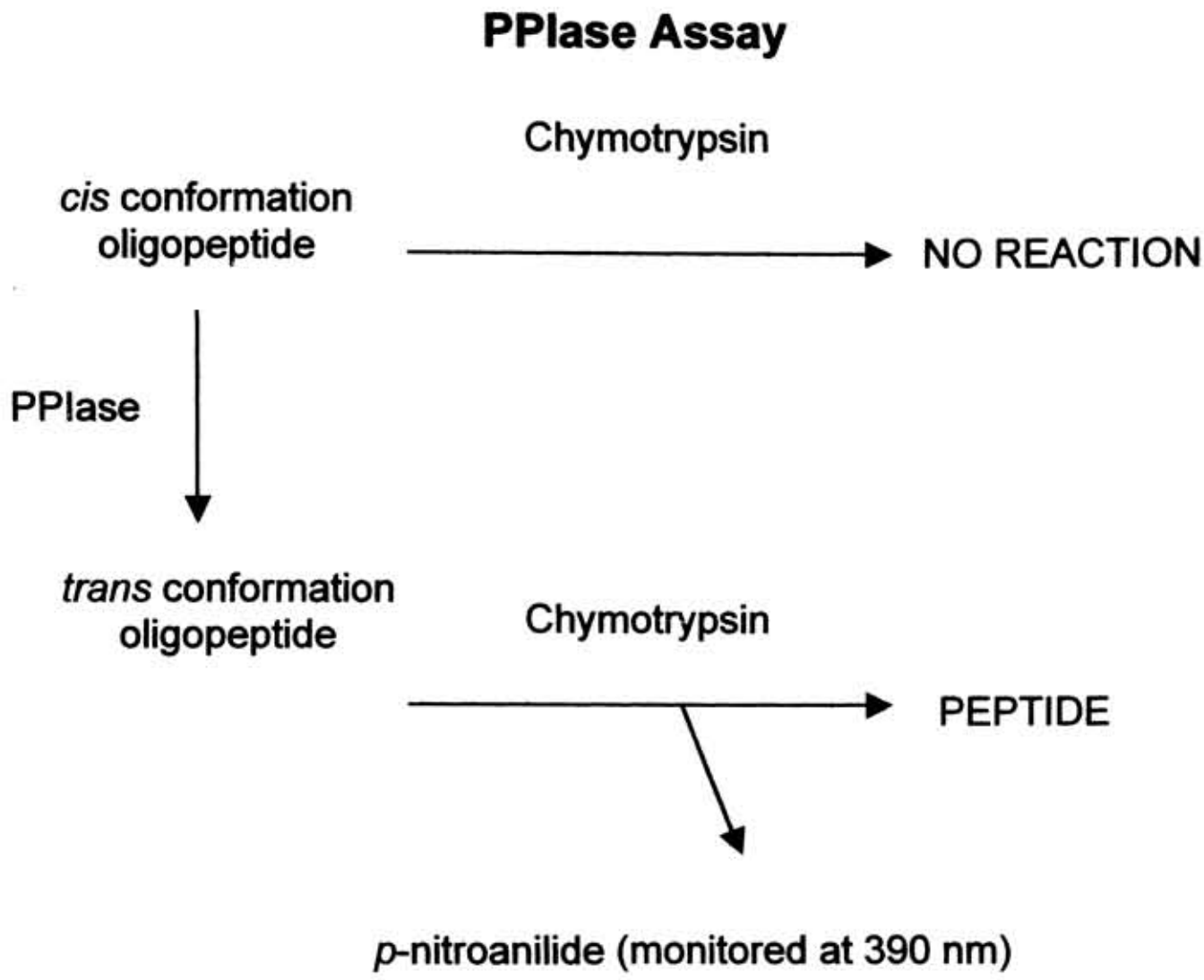


Fig. 2.1 Schematic diagram of the PPIase assay

2.33.1 Inhibition studies

The method described above was repeated in the presence of FK506 (Calbiochem) (final concentration 500 nM in HEPES pH 7.8) an inhibitor of PPIase activity in reactions including 0.025 μg/ml recombinant NMB0345 protein.

2.34 Mass spectrometric analysis

Mass spectrometry was performed by Paul Hitchen, Department of Biochemistry, Imperial College.

2.34.1 Enzymatic in gel digestion of proteins and removal of stain

Sample bands of interest were carefully excised from the gel then transferred to clean 0.5 ml tubes (Eppendorf). 500 μl of acetonitrile/0.1M ammonium bicarbonate (1:1 (v/v)) solution was then added to the gel pieces for 5 minutes to remove the stain. Gel pieces were then dried using a SpeedVac (Savant). 40 μl of trypsin (1μg/ml in 50mM NH_4HCO_3, pH 8.4) was added to the gel pieces. Sufficient (60 μl) 0.1 M ammonium bicarbonate (1:1 (v/v)) was added to each of the samples, which were then incubated at 37°C overnight.

2.34.2 Elution of peptides from the gel pieces

Supernatant from the tryptic digests were removed to clean 0.5 ml tubes and 50 μl of 0.1% trifluoroacetic acid (TFA) was added to the gel pieces, which were incubated for 1hour at 37°C to halt the digestion. The 0.1% TFA was pooled with the corresponding supernatant, then 200 μl of acetonitrile/0.1% TFA (6:4 (v/v)) solution was added to the gel pieces which

were incubated for a further 1hour at 37°C. This step was repeated twice and the supernatants pooled. The samples were reduced in volume (to approximately 60 µl) on a SpeedVac (Savant).

2.34.3 MicroTrap purification of tryptic peptides

Samples were loaded directly onto a C-18 MicroTrap (Jones Chromatography, Hengoel) peptide cartridge and then eluted manually using a 25 µl glass syringe. The column is first equilibrated using 10 x 20 µl of methanol, 10 x 20 µl 100% acetonitrile, and finally 10 x 20 µl of 0.1% TFA. Approximately 20 µl of sample was loaded. The elution was performed in three steps: 10 x 20 µl of 0.1% TFA to wash out any salt contamination, followed by 20 µl of acetonitrile/0.1%TFA (3:7 (v/v)) and then 20 µl of acetonitrile/0.1%TFA (6:4 (v/v)), collected in separate Eppendorf tubes. The column was re-equilibrated between peptide purifications. MicroTrap-purified peptides were then analysed by mass spectrometry.

2.34.4 Electrospray mass spectrometry

Sample aliquots of 1-2 µl were loaded directly into the Q-ToF MS (Micromass, UK) via a nanoES spray capillary needle (MDS Proteomics). The column and collision gases used were nitrogen and argon respectively. A potential of 1.5kV was applied to the nanoflow tip in the ion source. Collision gas pressure was maintained at 10^{-4} mbar and collision energies typically did not exceed 60eV. Primary and secondary ion spectra were collected in the positive mode. Data were acquired and processed using Masslynx software (Micromass, UK). The instrument was pre-calibrated using a 1 *p*mol/μl solution of [Glu^{1}]-fibrinopeptide B in acetonitrile/5% aqueous acetic acid (1:3 (v/v)).

3 Genetic and phenotypic analysis of the NMB0342-NMB0348 locus

An STM screen of a library of 2,850 transposon mutants of the serogroup B isolate C311$^+$ identified 73 genes in *N. meningitidis* that are required for bacteraemic infection in an infant rat model (Sun *et al.*, 2000). Nucleotide sequences flanking the transposon insertion sites in C311$^+$ were searched against the MC58 genome sequence and identified a number of genes encoding known virulence determinants of *N. meningitidis*, such as capsular polysaccharide and LPS. However, a large proportion of the genes identified have not previously been implicated in *N. meningitidis* pathogenesis, including NMB0342 and NMB0345 (Sun *et al.*, 2000), and it is not known whether any of these genes play roles at other stages of meningococcal infection such as colonization. As the NMB0342 and NMB0345 genes each have homologues in other human pathogens, the *ispA* gene of *S. flexneri* and *cbf* of *C. jejuni* respectively, and are located within a potential operon of seven genes, the role of this locus (NMB0342-NMB0348) in meningococcal pathogenesis was investigated.

To begin to understand the potential function of some of these genes during the disease process, database searches were performed using sequences obtained from the MC58 genome sequence. The sequences of both NMB0342 and NMB0345 are highly conserved (identities at the nucleotide level of 92% and 100%, respectively) between isolate C311$^+$ and the MC58 genome sequence, consistent with the results from the analysis of other STM mutants. MC58 and C311$^+$ belong to the same electrophoretic type (ET-5). Also included in these searches were the sequence of adjacent ORFs,

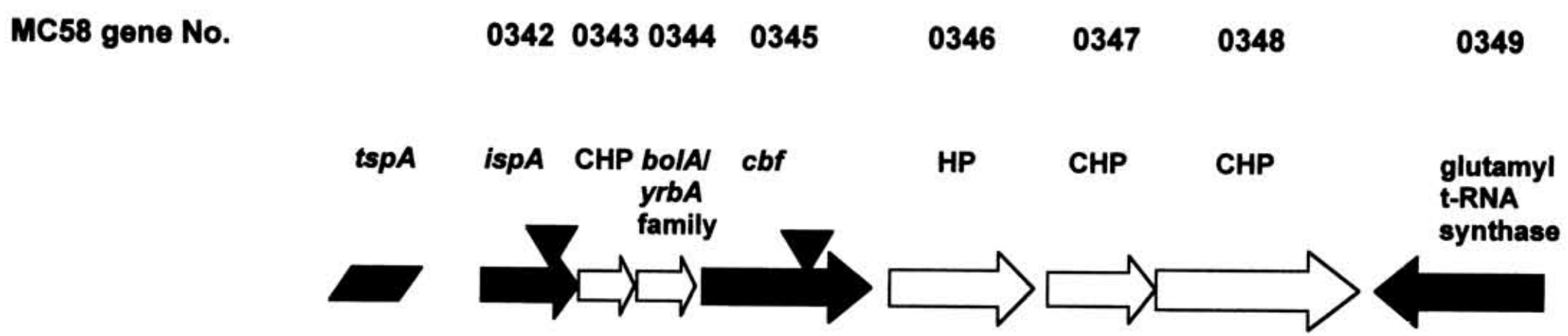
NMB0343, NMB0344, NMB0346, NMB0347, and NMB0348 as the contiguous arrangement of these genes suggested that they may form an operon and therefore may play a role in a related function relevant to the pathogenesis of the bacterium (Fig. 3.1).

Many bacterial pathogens that cause disease in humans employ similar mechanisms to overcome host defences and establish infection (Finlay and Falkow, 1997). An example of such a mechanism is the type III secretion system (TTSS) which is found in numerous gram-negative bacteria including *Yersinia enterocolitica* (Haller *et al.*, 2000), *Salmonella typhimurium* (Darwin and Miller, 2001) and *S. flexneri* (Blocker *et al.*, 1999). Knowledge of the function of genes conserved in other pathogenic microorganisms may provide clues to the role of homologues in *N. meningitidis*. Therefore, the conservation of the genes of the NMB0342-NMB0348 locus within *Neisseria* spp. and other bacterial pathogens was examined. In addition, the requirement for each of the genes of the locus during systemic infection was investigated.

3.1 Database searches of pathogenic bacteria

A search of the genome sequences from a range of Gram-negative and Gram-positive bacterial pathogens was performed using the translated sequences of the individual ORFs from the NMB0342-NMB0345 locus and the BLASTP algorithm (Altschul *et al.*, 1997). Initial analysis demonstrated that the entire locus is conserved in the available genome sequences of *N. meningitidis* serogroup A strain Z2491, serogroup B strain MC58, and serogroup C strain FAM18, and in *N. gonorrhoeae* strain FA1090 (Table 3.1).

Fig. 3.1 The NMB0342-NMB0348 locus



The NMB0342-NMB0348 locus showing the relative positions of the Tn*10* insertion sites of the NMB0342 and NMB0345 mutants identified in an STM analysis of the *N. meningitidis* serogroup B isolate, C311^{+}. The NMB0342 and NMB0345 genes are shown in blue.

An identity of 20% or higher to MC58 sequences was used as the cut-off value for inclusion as a homologue. The genetic organization of the complete locus was not found to be conserved in any other bacteria surveyed. However, homologues of each of the genes of the NMB0342-NMB0348 locus are present in the genomes of *E. coli* K12, *Salmonella enterica* serovar Typhi, *Pseudomonas aeruginosa*, and *S. flexneri*. The organization of the homologues of NMB0342 and NMB0343 in *P. aeruginosa,* PA3201 and PA3202 is identical to that of *N. meningitidis*. Like *N. meningitidis*, *H. influenzae* also inhabits the nasopharynx and can cause septicaemia and bacterial meningitis (Moxon, 1992). The homologues of NMB0342 and NMB0343 in *H. influenzae* are separated by a single ORF encoding a hypothetical protein, HI0827. ORF HI0827 is 462 bp in length and has 57% identity to the MC58 gene NMB0925, a putative acyl-CoA thioester hydrolase. A similar organization is found in *Vibrio cholerae*, the causative agent of cholera. The extra ORF in *V. cholerae*, VC1701 has 62% identity to NMB0925. However, no NMB0345 or NMB0346 homologues are present in either of *H. influenzae* or *V. cholerae*.

Table 3.1 **Results of searches using translated MC58 genome sequences of ORFs of the NMB0342-NMB0348 locus and the BLASTP or TBLASTN algorithms against the genome sequences of a range of pathogenic bacterial species**

Neisseria meningitidis **serogroup B MC58**	**NMB0342**	**NMB0343**	**NMB0344**	**NMB0345**	**NMB0346**	**NMB0347**	**NMB0348**
Neisseria meningitidis **serogroup A Z2491**	**NMA2145** 91% Identity	**NMA2144** 96% Identity	**NMA2143** 96% Identity	**NMA2142** 95% Identity	**NMA2141** 97% Identity	**NMA2140** 93% Identity	**NMA2139** 94% Identity
Neisseria meningitidis **serogroup C FAM18**	**Coordinates: 1861227-1860700** 91% Identity	**Coordinates: 1860682-1860407** 96% Identity	**Coordinates: 1860404-1860129** 100% Identity	**Coordinates: 1860077-1859214** 95% Identity	**Coordinates: 1859071-1858385** 97% Identity	**Coordinates: 1858308-1857820** 100% Identity	**Coordinates: 1857786-1856779** 96% Identity
Neisseria gonorrhoeae **FA1090**	**Coordinates: 1614082-1613555** 86% Identity	**Coordinates: 1613537-1613262** 93% Identity	**Coordinates: 1613259-1612984** 98% Identity	**Coordinates: 1612932-1612069** 94% Identity	**Coordinates: 1611927-1611241** 95% Identity	**Coordinates: 1611164-1610676** 99% Identity	**Coordinates: 1582884-1581907** 93% Identity
E.coli **K12**	***yclB*** 33% Identity	***yclI*** 52% Identity	***bolA*** 35% Identity	***ybaU*** 22% Identity	***surA*** 20% Identity	***yihZ*** 44% Identity	***yjbN*** 54% Identity

Table 3.1 **Results of searches using translated MC58 genome sequences of ORFs of the NMB0342-NMB0348 locus and the BLASTP or TBLASTN algorithms against the genome sequences of a range of pathogenic bacterial species**

***Salmonella enterica subsp. enterica serovar Typhi* CT18**	***yclB*** 32% Identity	**STY1313** 48% Identity	***bolA*** 37% Identity	***ppiD*** 22% Identity	***surA*** 20% Identity	***yihZ*** 46% Identity	***yjbN*** 50% Identity
***Pseudomonas aeruginosa* PAO1**	**PA3201** 30% Identity	**PA3202** 54% Identity	***bolA*** **PA0857** 35% Identity	**PA0699** 21% Identity	***surA*** 26% Identity	**PA5079** 48% Identity	**PA2795** 51% Identity
***Vibrio cholerae* El-Tor N16961**	***yclB*** **VC1700** 33% Identity	***ycII*** **VC1702** 51% Identity	**VC2296** 33% Identity	x	x	**VC2741** 46% Identity	**VC0379** 50% Identity
***Shigella flexneri* 2a str. 301**	*ispA* SF1257 33% Identity	***ycII*** **SF1254** 52% identity	***bolA*** **SF0379** 35% identity	***ybaU*** SF0386 22% identity	***surA*** SF0050 20% identity	***dtd*** **SF3959** 44%identity	***yjbN*** **SF4156** 54%identity
***Yersinia pestis* C092**	***ispZ*** **YPO2196** 33% Identity	**YPO2192** 51% Identity	***bolA*** 31% Identity	x	x	**YPO0029** 44% Identity	**YPO0316** 51% Identity
***Rickettsia prowazekii* Madrid E**	**RP392** 23% Identity	x	**RP245** 34% Identity	**RP576** 22% Identity	x	x	**RP011** 28% Identity

Table 3.1 **Results of searches using translated MC58 genome sequences of ORFs of the NMB0342-NMB0348 locus and the BLASTP or TBLASTN algorithms against the genome sequences of a range of pathogenic bacterial species**

***Haemophilus influenzae* Rd KW20**	**HI0826** 28% Identity	**HI0828** 51% Identity	**HI0163** 29% Identity	x	x	**HI0670** 45% Identity	**HI0634** 54% Identity
***Helicobacter. pylori* J99**	x	x	x	**jhp0161** 23% Identity	**jhp0161** 21% Identity	x	**jhp0664** 23% Identity
Bacillus subtilis	x	x	x	***yacD*** 21% Identity	***prsA*** 27% Identity	***yrvI*** 55% Identity	***yfjN*** 26% Identity
***Campylobacter jejuni* NCTC11168**	x	x	x	*peb4/cbf2* Cj0596 24% Identity	x	x	**Cj0123c** 22% Identity
***Staphylococcus aureus* Mu50**	x	x	x	x	**SA1573** 22% Identity	**SA1633** 44% Identity	**SAV0089** 25% Identity
***Listeria monocytogenes* EGD-e**	x	x	x	x	x	**lmo1522** 48%	**lmo0227** 24% Identity
***Streptococcus pneumoniae* R6**	x	x	x	x	x	**Spr1486** 40% Identity	**Spr1994** 24% Identity
***Mycobacterium tuberculosis* H37Rv**	x	x	x	x	x	**Rv1897c** 43% Identity	**Rv0823c** 28% Identity

Homologues of the first three genes of the locus, NMB0342, NMB0343, and NMB0344 are absent from the genomes of *Helicobacter pylori*, *Bacillus subtilis*, and *Campylobacter jejuni*. In *Listeria monocytogenes*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis*, only the last two genes of the locus, NMB0347 and NMB0348 have obvious homologues. Homologues of NMB0348 were found in all of the bacterial species included in this analysis. Genes of the locus are the least conserved in *C. jejuni*; only homologues of NMB0345 and NMB0348 are present in this genome. Genes that are recognizable homologues of NMB0345 are present in fewer of the bacterial genomes queried than are NMB0342 homologues.

3.2 Conservation of the NMB0342-NMB0348 locus in *Neisseria* spp.

3.2.1 PCR and Southern Analysis

Genes that are specifically required for virulence are likely to be more conserved in pathogenic bacteria than commensal species. To test this hypothesis in regard to the NMB0342-NMB0348 locus, PCR and Southern analysis was carried out on a collection of 48 strains of pathogenic and non-pathogenic *Neisseria* spp. (Barrett and Sneath, 1994) (See Appendix A for full list of strains). The strains were selected to encompass the known genetic diversity of *Neisseria* spp. based on MLST (Maiden *et al.*, 1998) and MLEE (Pizza *et al.*, 2000) analyses. The pathogenic strains included three serogroup A strains, 16 representative disease-associated serogroup B strains, one strain each from serogroups W^{135}, X, Y, and Z, and two strains of *N. gonorrhoeae*.

Initially, the conservation of the NMB0342-NMB0348 locus among *Neisseria* spp. was examined by PCR amplification of the individual ORFs, NMB0342, NMB0345, and NMB0348. Oligonucleotide primers based on the MC58 genome sequence were designed to anneal within each ORF. (The sequence of all oligonucleotide primers used in this study is given in Appendix B). NMB0342 homologues were amplified with primers NG197 and NG198; primers NG216 and NG217 were used for amplification of NMB0345, and NMB0348 was amplified using primers NG377 and NG378. The expected sizes of PCR products amplified from MC58 genomic DNA are as follows: NMB0342, 526 bp, NMB0345, 864 bp, and NMB0348, 1008 bp. The results of PCR analysis are given in the Appendix C (Fig. A1.1-A1.3). PCR analysis of strains 1-48 was performed to determine the presence of NMB0342 and NMB0345 sequences (Table 3.2 and 3.3). PCR amplification of NMB0348 was only attempted for strains 1-16 due to difficulties in obtaining PCR products for this gene (Table 3.2). PCR products for NMB0348 were successfully amplified from all *N. meningitidis* strains with the exception of the serogroup A strain F6124 and serogroup B strains 2996 and B16/B6.

For Southern analysis, genomic DNA from each of the 48 representative *Neisseria* strains was digested with *Cla*I and Southern blots were hybridized with PCR amplified fragments of the NMB0342, NMB0345, or NMB0348 genes as probes (Appendix D). The expected sizes of bands for strain MC58 based on the published MC58 genome sequence are as follows: NMB0342, 13.5 kb, NMB0345, 13.5 kb and 5.1 kb, and NMB0348, 3.6 kb.

Table 3.2 **PCR and Southern analysis of *N. meningitidis* isolates for the presence of NMB0342, NMB0345, and NMB0348 sequences**

Reference				NMB0342		NMB0345		NMB0348	
No.	Serogroup	Strain	MLEE Assignment	PCR	Southern (kb)	PCR	Southern (kb)	PCR	Southern (kb)
1	A	Z2491	subgroups IV-1, IV-2	+	10.2	+	10.2	+	3.6
2	A	F6124		+	10.2	+	10.2	-	3.6
3	A	205900	subgroup IV-1	+	10.2	+	10.2	+	3.6
4	C	90/18311	ET-37	+	10.2	+	10.3	+	3.6
5	W-135	A22	other	+	10.2	+	10.2	+	3.6
6	X	E26	other	+	10.2	+	1.9	+	4.1
7	Y	860800	other	+	7.0	+	1.9	+	5.1
8	Z	E32	other	+	10.2	+	1.9	+	3.6
9	B	2996		+	10.2	+	1.9	-	3.6
10	B	528		+	10.2	+	1.9	+	3.6
11	B	BZ169	ET-5 complex	+	10.2	+	1.9	+	4.2
12	B	BZ83	ET-5 complex	+	10.2	+	1.9	+	4.2
13	B	H44/76	ET-5 complex	+	10.2	+	1.9	+	4.2
14	B	MC58[+]	ET-5 complex	+	10.2	+	1.9	+	4.2
15	B	C311[+]		+	10.2	+	1.9	+	4.2
16	B	B16/B6		+	10.3	+	10.2	-	3.8
17	B	1000	other	+	10.4	+	1.7		3.9
18	B	297-0	other	+	10.4	+	1.7		3.9
19	B	BZ 133		+	7.0	+	1.7		3.9
20	B	BZ 147	other	+	10.4	+	1.7		3.9
21	B	BZ 198	Lineage 3	+	7.0 10.4	+	1.7		4.7
22	B	BZ 232	other	+	10.4	+	1.7		3.9
23	B	NG 3/88	other	+	10.4	+	1.7		4.7
24	B	NG 6/88	other	+	10.4	+	1.7		4.7
25	B	NG E28	other	+	10.4	+	1.7		3.9
26	B	NG E31	other	+	10.4	+	1.7		3.9
27	B	NG F26	other	+	10.4	+	1.7		4.7
28	B	NG H15	other	+	10.4	-	1.7		3.8
29	B	NG H36	other	+	10.4	+	1.7		3.9
30	B	NG H38	other	+	10.4	+	1.7		3.9
31	B	NGP165	ET-37	+	10.4	+	11.0		3.9
32	B	SWZ107	other	+	10.4	+	1.65		4.7

Table 3.2 **PCR and Southern analysis of *N. meningitidis* isolates for the presence of NMB0342, NMB0345, and NMB0348 sequences**

The results of PCR and Southern analyses of 32 strains of *N. meningitidis* including 24 serogroup B isolates (No.'s 9-32) for the presence of the NMB0342, NMB0345, and NMB0348 sequences are summarized in the table above. A '+' indicates that a product of the expected size was detected. The sizes of bands detected by Southern analysis are given in kb.

Table 3.3 **PCR and Southern analysis of *N. gonorrhoeae*, commensal *Neisseria* spp., and *E. coli* for the presence of NMB0342, NMB0345, and NMB0348 sequences**

			Results of PCR and Southern Analysis				
			NMB0342		NMB0345		NMB0348
No.	Species	Strain	PCR	kb	PCR	kb	kb
33	*N. gonorrhoeae*	FA1090	+	10.2 3.5	+	11.6	10.3 **4.4**
34	*N. gonorrhoeae*	MS11	+	10.2 3.5	+	11.6	10.3 **4.4**
35	*N. lactamica*	L13	-	6.9	+	10.1	8.5
36	*N. lactamica*	L18	+	6.9	+	10.1	8.5
37	*N. polysaccharea*	P1	-	8.0	+	10.7	8.5
38	*N. polysaccharea*	P4	-	9.0	+	11.0	8.5
39	*N. mucosa*	M7	+	8.0 3.5	+	10.6	8.5
40	*N. cinerea*	F1	+	**4.4**	-	9.5	8.5 4.4
41	*N. cinerea*	F3	+	**4.4**	+	9.5	10.1
42	*N. elongata*	I1	-	-	-	-	**7.45**
43	*N. elongata*	I3	-	-	+	-	7.5
44	*N. sicca*	Q29	-	-	-	-	5.0
45	*N. perflava*	U15	-	-	-	-	-
46	*N. subflava*	U37	-	-	+	-	-
47	*N. animalis*		-	-	-	-	-
48	*E. coli*	DH5α	-	-	-	-	-

Table 3.3 **Southern analysis of *N. gonorrhoeae*, commensal *Neisseria* spp., and *E. coli* for the presence of NMB0342, NMB0345, and NMB0348 sequences**

The results of PCR and Southern analyses of 16 strains including *N. gonorrhoeae* (No. 33-34), commensal *Neisseria* spp. (No. 35-47), and *E. coli* (No. 48). '+' indicates that a product of the expected size was detected. The sizes of bands detected by Southern analysis are given in kb.

The combined results of PCR and Southern analyses demonstrated that the NMB0342, NMB0345, and NMB0348 sequences are present in all isolates of pathogenic *Neisseria* spp. tested including *N. meningitidis* serogroups A, B, C, W, X, Y, and Z (Appendices C & D). In a number of instances, PCR failed to detect the presence of genes that were shown to be present by Southern analysis. For example, NMB0348 was not amplified from strains F6124 or B16/B6 (Table 3.2), but was detected by Southern hybridization. Although a high correlation was observed between the results of PCR and Southern analysis, the value of performing Southern analysis of strains for which genome sequence is unavailable is emphasized by the presence of hybridizing bands in these isolates.

The sizes of the hybridizing bands observed by Southern analysis using probes for NMB0342, NMB0345, and NMB0348 were largely conserved across the serogroup A, C, and W isolates tested. The size of the NMB0342 band was very similar (~10 kb) across strains 1-32 (serogroups A, C, W, X, Z, B), with the exception of the serogroup Y strain 860800 and serogroup B strains BZ133 and BZ198 which gave bands of 7.0 kb (Table 3.2).

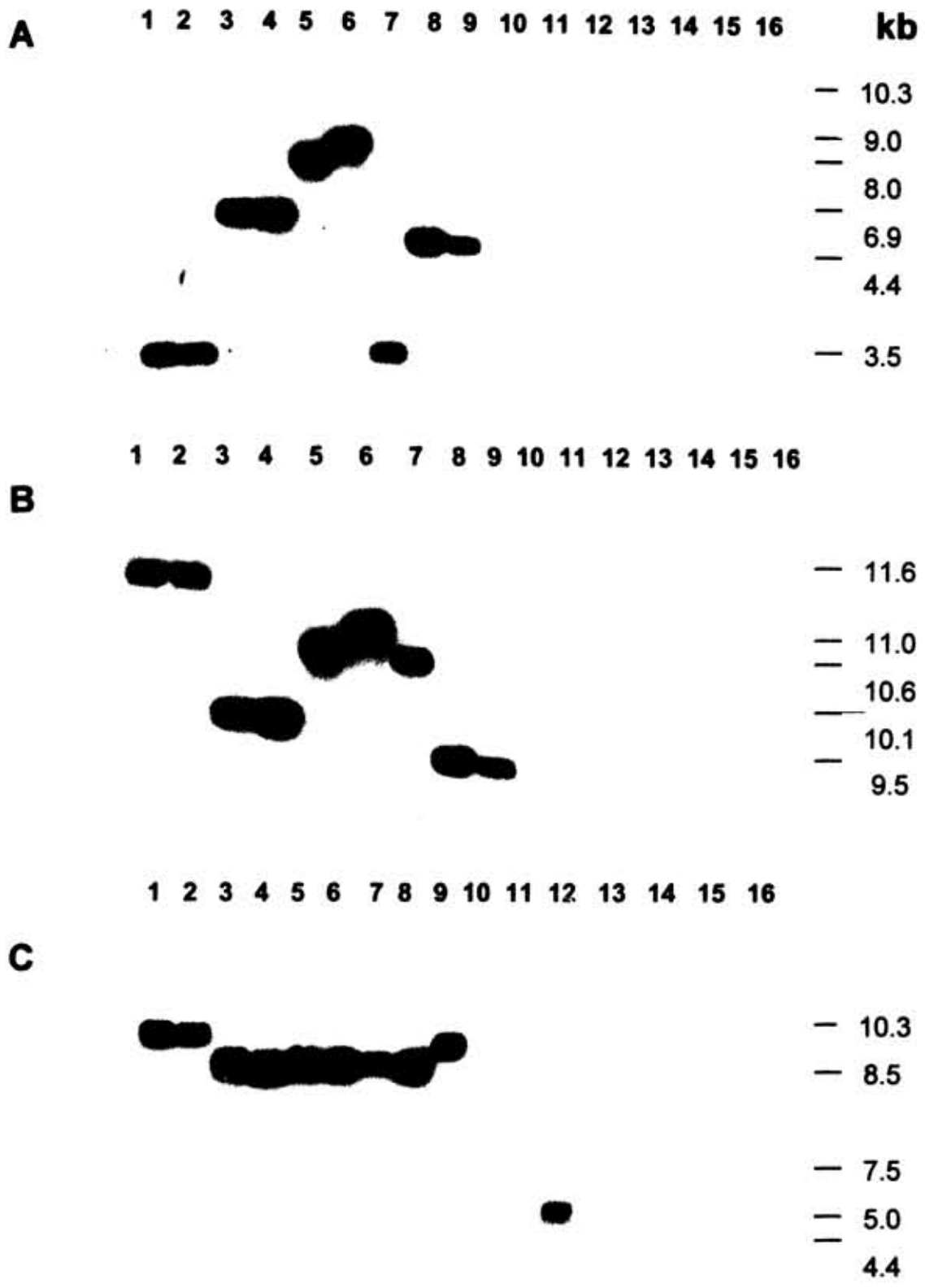
All of the serogroup B strains probed with NMB0345 gave hybridizing bands of 1.6-1.9 kb except for B16/B6 and NGP165. These strains gave bands of ~10.2 kb and ~11.0 kb, respectively, sizes similar to those obtained for the serogroup A, C, and W isolates representing a distinct difference between the serogroup B strains and the serogroup A, C, and W135 isolates tested. Apart from serogroup B isolates, the 1.6-1.9 kb band size was also observed in serogroup X, Y, and Z isolates.

Greater variation was observed in band sizes obtained across strains probed with the NMB0348 sequence suggesting that the NMB0348 sequence may be less conserved across *Neisseria* spp. than the NMB0342 and NMB0345 sequences. PCR products of the predicted size for NMB0342 and NMB0345 were amplified from both *N. gonorrhoeae* strains.

3.2.2 Analysis of non-pathogenic *Neisseria* spp.

As PCR analysis gave false-negative results for some pathogenic *Neisseria* strains, conclusions from the analysis of non-pathogenic species were based mainly on the results of Southern hybridization (Table 3.3). Southern analysis of the non-pathogenic *Neisseria* species allows them to be divided into three distinct groups (Fig. 3.2). NMB0342, NMB0345, and NMB0348 are all present in *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria mucosa*, and *Neisseria cinerea*. In contrast, none of these sequences were found in *Neisseria perflava*, *Neisseria subflava*, and *Neisseria animalis*. In *N. lactamica* and *N. cinerea*, the band sizes obtained for NMB0342, NMB0345, and NMB0348 were similar or identical for the two strains tested for each species.

Fig. 3.2 Southern analysis of *Neisseria* spp. for the presence of NMB0342, NMB0345, and NMB0348 sequences



Key:

No.	Species	Strain
1	*N. gonorrhoeae*	FA1090
2	*N. gonorrhoeae*	MS11
3	*N. lactamica*	L13
4	*N. lactamica*	L18
5	*N. polysaccharea*	P1
6	*N. polysaccharea*	P4
7	*N. mucosa*	M7
8	*N. cinerea*	F1
9	*N. cinerea*	F3
10	*N. elongata*	I1
11	*N. elongata*	I3
12	*N. sicca*	Q29
13	*N. perflava*	U15
14	*N. subflava*	U37
15	*N. animalis*	
16	*E. coli* DH5α	

Southern analysis to examine the conservation of the NMB0342 (Panel A), NMB0345 (Panel B), and NMB0348 (Panel C) sequences in *N. gonorrhoeae* (lanes 1-2), commensal *Neisseria* spp. (lanes 3-15), and *E. coli* (lane16). Chromosomal DNA from each strain listed in the key are probed with NMB0342 (Panel A), NMB0345 (Panel B), and NMB0348 (Panel C) sequences.

Fig. 3.2 **Southern analysis of *Neisseria* spp. for the presence of NMB0342, NMB0345, and NMB0348 sequences**

Southern analysis to examine the conservation of the NMB0342 (Panel A), NMB0345 (Panel B), and NMB0348 (Panel C) sequences in *N. gonorrhoeae* (lanes 1-2), commensal *Neisseria* spp. (lanes 3-15), and *E. coli* (lane16). Chromosomal DNA from each strain listed in the key are probed with NMB0342 (Panel A), NMB0345 (Panel B), and NMB0348 (Panel C) sequences.

NMB0342 and NMB0345 homologues were present in all strains in which the NMB0348 sequence is present with the exception of *N. elongata* I3 and I1, and *Neisseria sicca* Q29. Faint bands (~7.5 kb) were observed in *N. elongata* I3 and I1 and a band of ~5.0 kb was observed in *N. sicca* Q29 when probed with NMB0348.

3.3 Construction of mutants with insertions in genes of the NMB0342-NMB0348 locus

To establish whether genes present in the NMB0342-NMB0348 locus are involved in *N. meningitidis* pathogenesis, mutations were introduced into NMB0343, NMB0344, NMB0346, NMB0347, and NMB0348 (Fig. 3.3). Initially, the gene of interest was amplified from C311^{+} genomic DNA with 0.3-2.0 kb of flanking sequence. The location of the primers used is shown in Fig. 3.4 and the sizes of fragments amplified are given in Table 3.4. The PCR product amplified with oligonucleotides NG240 and NG241 contains both the NMB0343 and NMB0344 genes and therefore it was not necessary to generate separate PCR products for these genes. PCR amplification products were ligated into pCR®2.1 TOPO® (Fig. 3.3).

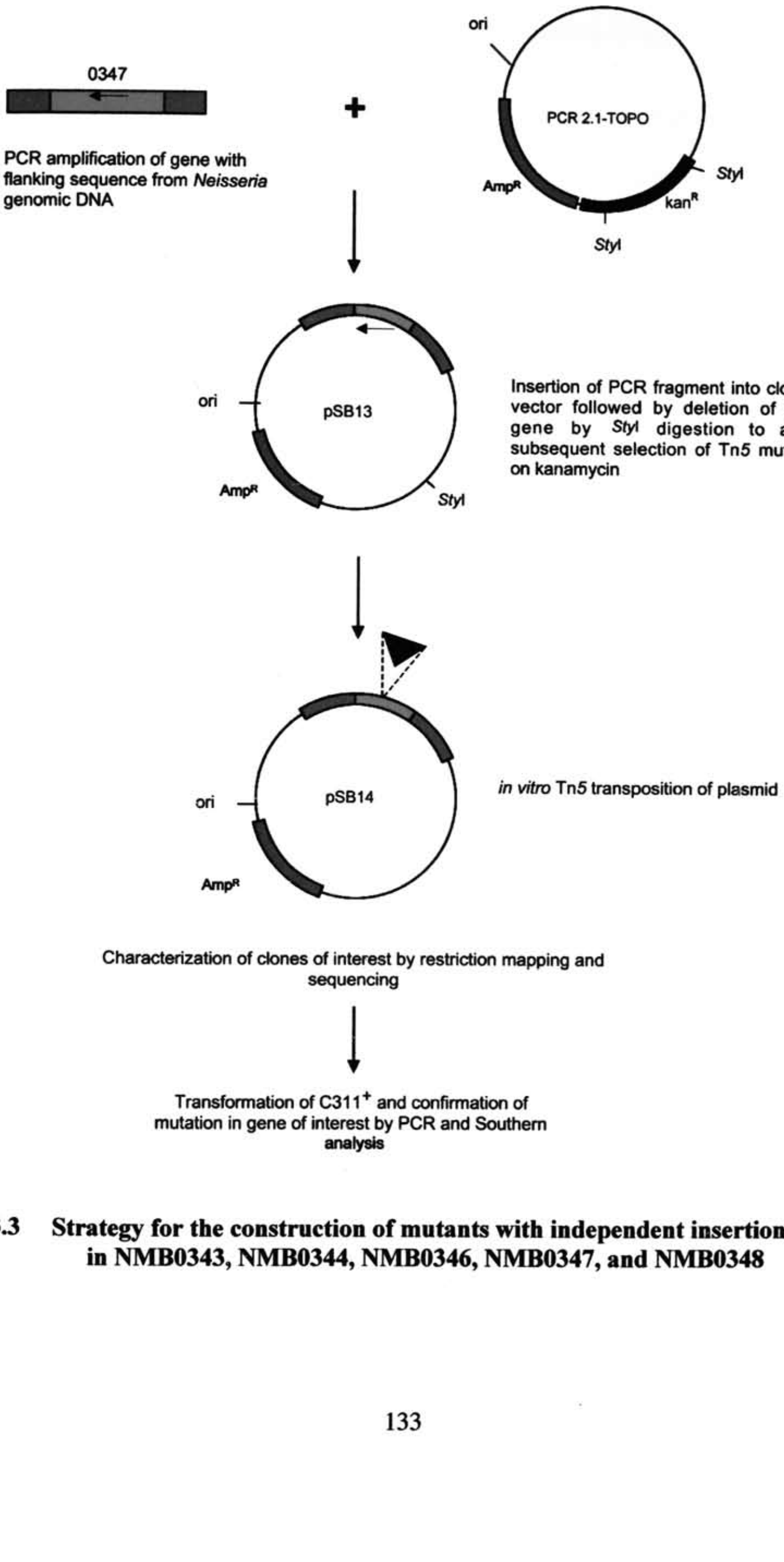


Fig. 3.3 Strategy for the construction of mutants with independent insertions in NMB0343, NMB0344, NMB0346, NMB0347, and NMB0348

Fig. 3.3 **Strategy for the construction of mutants with transposon insertions in NMB0343, NMB0344, NMB0346, NMB0347, and NMB0348**

Mutants with insertions in NMB0343, NMB0344, NMB0346, NMB0347, and NMB0348 were constructed using a strategy involving the amplification of the gene of interest and ligation of the PCR product into a cloning vector (pCR®2.1 TOPO®). The kan^R gene was deleted by *StyI* or *SphI* digestion and self-ligation of the vector. The resulting plasmid was subjected to *in vitro* EZ::TN™<KAN-2> transposition and the position of the transposon insertion mapped by restriction digest and nucleotide sequencing using transposon-specific oligonucleotide primers. Plasmids with EZ::TN™<KAN-2> insertions in the gene of interest were used to transform the serogroup B strain $C311^+$. The origin of replication is indicated as 'ori'. An arrow is used to indicate the orientation of the NMB0347 gene (direction of transcription).

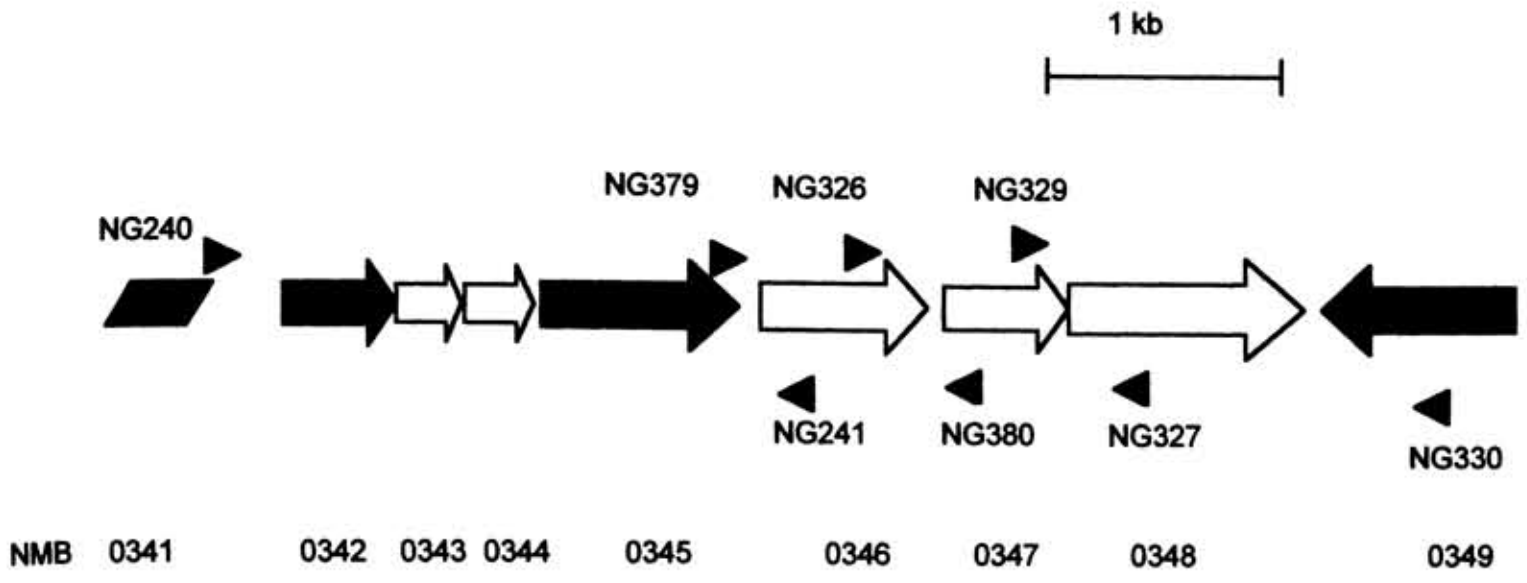


Fig. 3.4 Location of primers used to amplify fragments of the NMB0342-NMB0348 locus

Oligonucleotide primers (represented by small black arrows) were designed according to the published MC58 genome sequence. These primers were used to amplify fragments of the NMB0342-NMB0348 locus for ligation into pCR®2.1TOPO®. NMB0342 and NMB0345 are shown in blue.

Fragment Amplified	Primers	Length of Fragment (bp)
NMB0343 + ~1 kb on either side of gene (containing NMB0344)	NG240/NG241	2,258
NMB0346 + ~150bp on either side of gene	NG379/NG380	1,084
NMB0347 + ~0.5 kb on either side of gene	NG326/NG327	1,489
NMB0348 + ~0.5 kb on either side of gene	NG329/NG330	2,008

Table 3.4 PCR fragments amplified for mutagenesis of genes and for the construction of *N. meningitidis* mutants

ORFs of the NMB0342-NMB0345 locus were PCR amplified with 0.3-2.0 kb of flanking sequence using oligonucleotide primers designed according to the published MC58 genome sequence.

Fig. 3.5 **Restriction mapping to confirm presence and orientation of NMB0346 insert in pCR 2.1 TOPO and mapping of plasmid DNA isolated from transformants following *in vitro* Tn*5* transposition of pSB24**

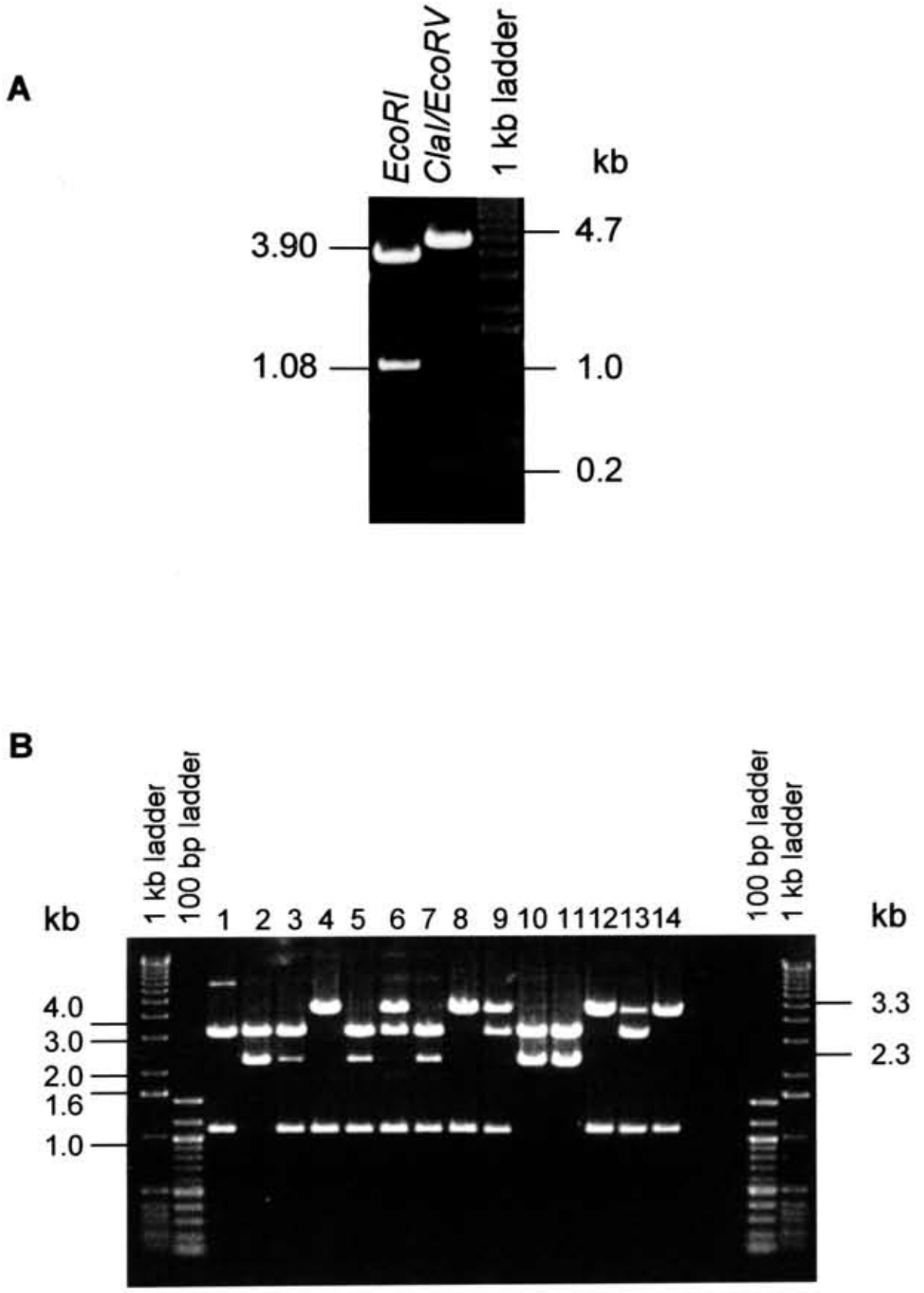


Fig. 3.5 **Restriction mapping to confirm presence and orientation of NMB0346 insert in pCR 2.1 TOPO and mapping of plasmid DNA isolated from transformants following *in vitro* EZ::TN™<KAN-2> transposition of pSB24**

*Eco*RI digestion of pSB11 gave bands of the expected size; 3.902 kb (pCR®2.1 TOPO®), 1.084 kb (NMB0346 insert). *Cla*I/*Eco*RV digestion of pSB11gave bands of 4.7 kb, 1.0 kb, and 0.2 kb (Panel A). Plasmid DNA from 14 transformants (Panel B, lanes 1-14) resulting from EZ::TN™<KAN-2> transposition of pSB24 was digested with *Eco*RI to check for the presence of EZ::TN™<KAN-2> in the insert. Plasmids from three transformants (Panel B, lanes 2, 10, and 11) were found to have a EZ::TN™<KAN-2> insertion in the insert. These plasmids gave bands of 3.3 kb (pCR ®2.1TOPO® with kan^R deleted) and 2.3 kb (NMB0346 insert (1.1 kb + EZ::TN™<KAN-2> (1.2 kb)), indicated.

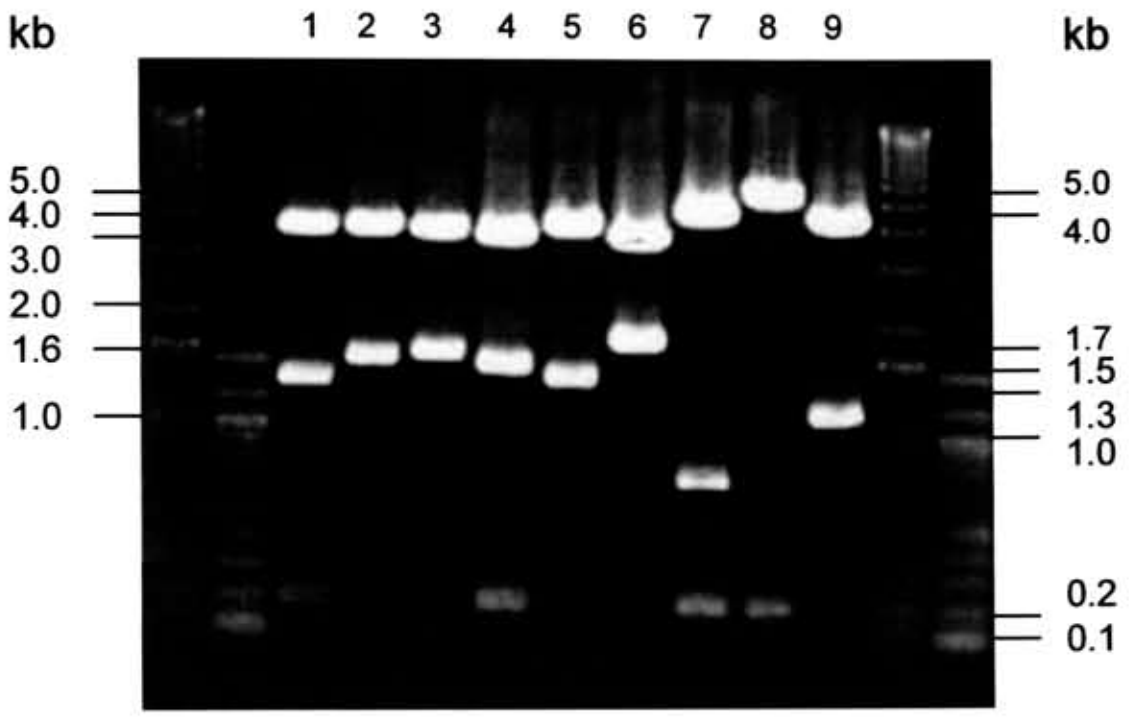
Plasmid DNA isolated from transformants was checked for the presence of an insert of the correct size by performing an *Eco*RI digest. *Eco*RI recognition sites flank the TA cloning site of pCR®2.1 TOPO® and are not present within the sequence of any of the products amplified. The construction of the NMB0346 mutant is described here in detail. The same method was used for the construction of mutants with transposon insertions in NMB0343, NMB0344, NMB0347, and NMB0348. pSB11 consists of pCR®2.1 TOPO® harbouring the NMB0346 insert and digestion of this plasmid with *Eco*RI gave bands of the expected size; 3.9 kb (vector) and 1.08 kb (NMB0346 insert). (Fig. 3.5, Panel A). Orientation of the insert in pSB11 was mapped by digestion of pSB11 with *Cla*I/*Eco*RV (Fig. 3.5, Panel A) giving bands of 4.74 kb and 0.24 kb. *Cla*I recognises a single site within the insert of pSB11 and does not cut in the vector. *Eco*RV cuts once in the vector and does not cut in the insert.

The kanamycin resistance (kan^R) gene of pCR®2.1 TOPO® was deleted from pSB11 by restriction digestion with *Sty*I, then self-ligation and transformation into *E. coli*. Transformants were selected on carb (Fig. 3.3). The kan^R gene was deleted to allow the subsequent selection of transformants following *in vitro* transposon mutagenesis using a Tn*5*-derivative that contains a kan^R gene (EZ::TN™<KAN-2). Transformants were replica plated to LB kan and LB carb to confirm loss of the kan^R cassette. The resulting plasmid, pSB24, was then used as a target for *in vitro* EZ::TN™<KAN-2> transposition. The products of the transposition reaction were used to transform *E. coli* to kanamycin resistance.

Plasmids isolated following *in vitro* transposition of pSB24 were digested with *Eco*RI to identify those in which the transposon had inserted into the desired ORF (Fig. 3.5, Panel B). Transformants 2, 10, and 11 contained plasmids that gave bands of the expected size; 3.3 kb (pCR ®2.1TOPO® with kan^R deleted) and 2.3 kb (NMB0346 insert (1.1 kb + EZ::TN™<KAN-2> (1.2 kb)). DNA was digested with each of *Cla*I, *Hind*III, and *Nru*I/*Eco*RV to map the position of the transposon in the inserts of plasmids isolated from transformants 2, 10, and 11; the transposon was found to be located within the NMB0346 sequence in transformant 2 (Fig. 3.6, Panels A and B). *Cla*I cuts once in the insert and twice in the transposon, but does not cut the vector. *Nru*I cuts once in the transposon, but does not cut within the insert or vector. *Eco*RV cuts once in the vector. Only *Hind*III cuts once in the transposon and once within the vector, but does not cut in the insert. The expected size of plasmids containing the NMB0346 insert is 4.362 kb (3.278 kb (vector with kan^R cassette deleted), 1.084 kb (NMB0346 insert).

Fig. 3.6 Restriction mapping of plasmid DNA isolated from transformants 2, 10, and 11 to determine the position of Tn*5* in the insert

A



Loading order:

1 *Cla*I transformant 2
2 *Hind*III transformant 2
3 *Nru*I/*EcoRV* transformant 2
4 *Cla*I transformant 10
5 *Hind*III transformant 10
6 *Nru*I/*EcoRV* transformant 10
7 *Cla*I transformant 11
8 *Hind*III transformant 11
9 *Nru*I/*EcoRV* transformant 11

B

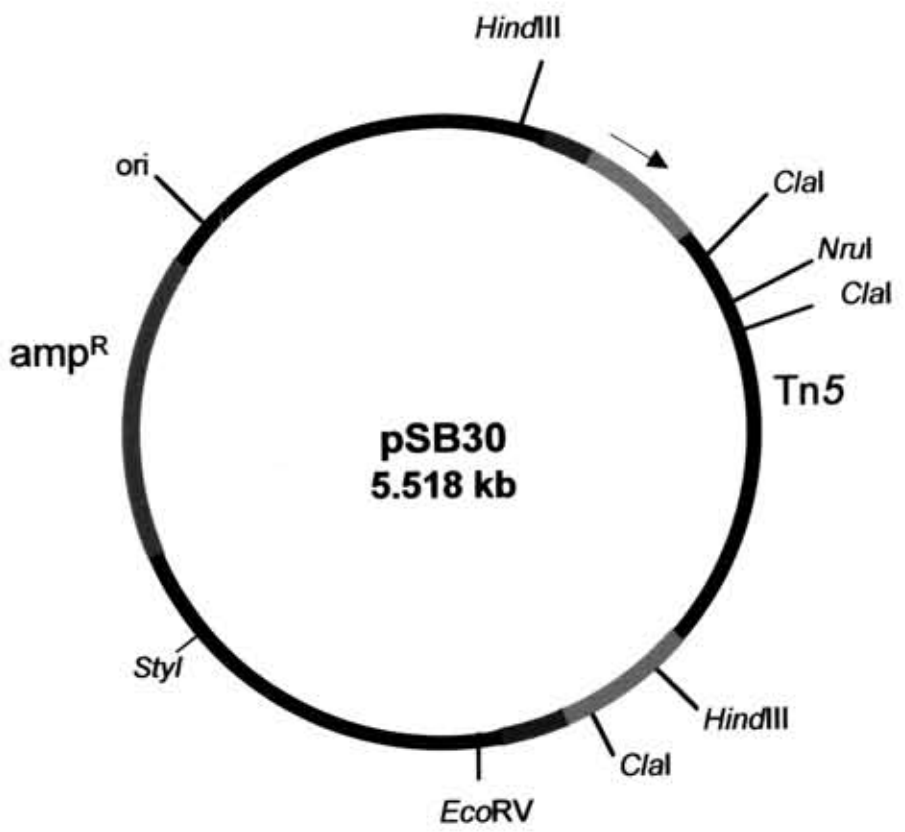


Fig. 3.6 Restriction mapping of plasmid DNA isolated from transformants 2, 10, and 11 to determine the position of EZ::TN™<KAN-2> in the insert

Plasmid DNA isolated from transformants 2, 10, and 11 following *in vitro* EZ::TN™<KAN-2> transposition of pSB24 was digested with either *Cla*I (lanes 1, 4, and 7), *Hind*III (lanes 2, 5, and 8), or *Nru*I/*Eco*RV (lanes 3, 6, and 9) to map the position of the EZ::TN™<KAN-2> insertion in the insert (Panel A). The band sizes obtained from each of these digests are shown. The band sizes obtained by *Cla*I, *Hind*III, and *Nru*I/*Eco*RV digests of plasmid DNA isolated from transformant 2 confirmed that the transposon has inserted into the NMB0346 sequence in this plasmid. The transposon insertion in pSB30 (pSB24 with EZ::TN™<KAN-2> insertion in NMB0346) is represented in black (Panel B). The NMB0346 ORF is represented in red with intergenic sequence represented in blue. The ampicillin resistance gene is shown in green. The position of *Cla*I, *Hind*III, *Nru*I, and *Eco*RV restriction sites are indicated. The origin of replication is indicated as 'ori'. An arrow is used to indicate the orientation of the NMB0346 gene (direction of transcription).

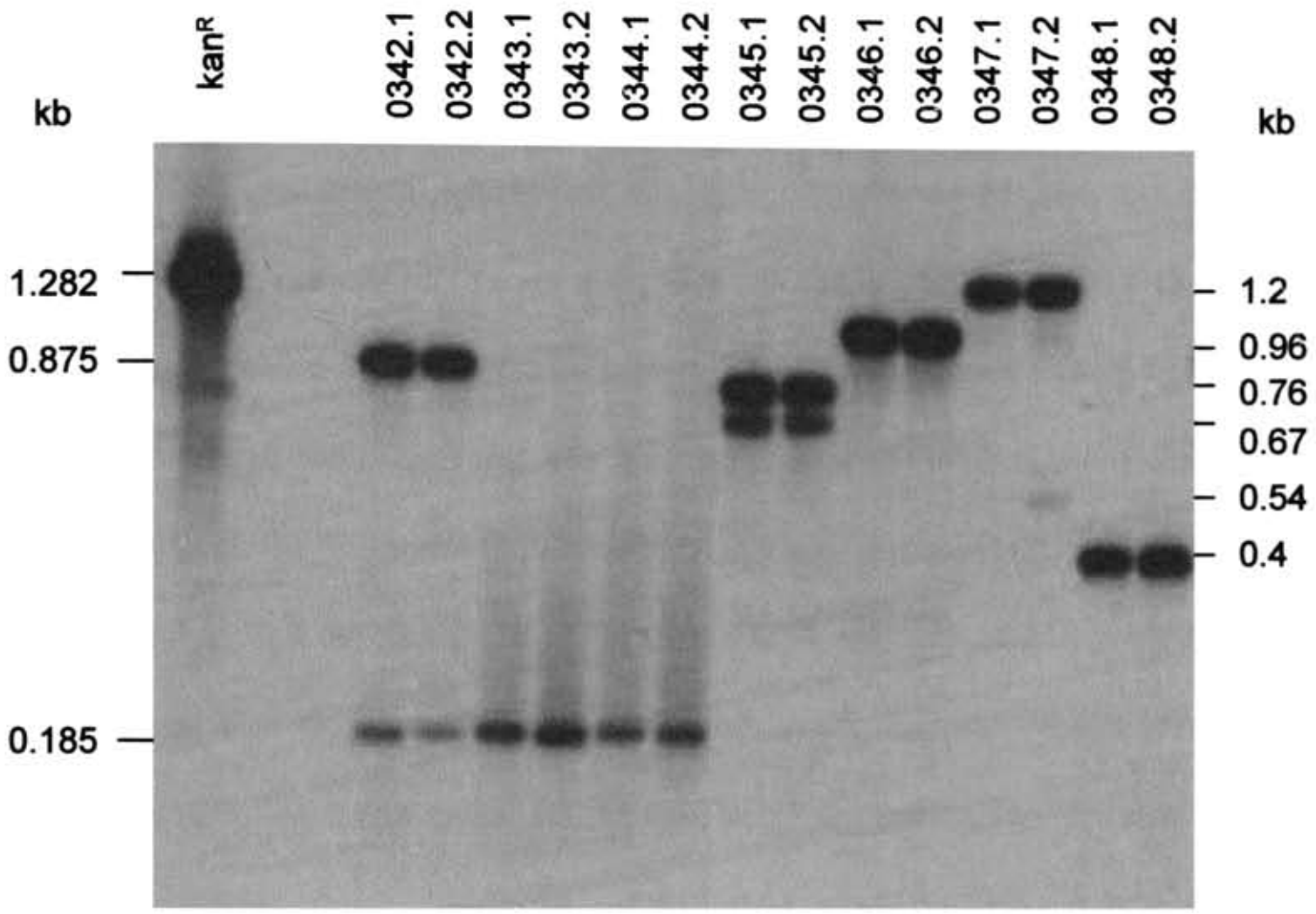
The expected size of plasmids with a EZ::TN™<KAN-2> insertion within the insert of pSB24 is 5.6 kb (3.3 kb (vector), 1.1 kb insert + EZ::TN™<KAN-2> (1.2 kb)). The precise position of the transposon insertion was determined by nucleotide sequencing using transposon-specific primers provided by the EZ::TN™<KAN-2> Insertion Kit; KAN-2 FP-1 forward primer and KAN-2 RP-1 reverse primer (Table 3.5). The EZ::TN™<KAN-2> insertion site in transformant 2 was found to be within the NMB0346 sequence (497 bp from 5′ end of the gene). The length of NMB0346 is 756 bp. The EZ::TN™<KAN-2> insertion site in transformant 10 was found to be within the NMB0347 sequence present in the insert. The EZ::TN™<KAN-2> insertion site in transformant 11 mapped to the intergenic region present at the 5′ end of NMB0346 sequence (20 bp from the 5′ end of the insert). Therefore, plasmid DNA from transformant 2, designated as pSB30, was used to transform strain C311^{+} to kan resistance. Diagrams of each of the plasmids cloned for construction of mutants are given in Appendix E. Southern analysis of genomic DNA isolated from *N. meningitidis* transformants hybridized against a kan^{R} cassette probe was performed to confirm that the desired mutations had integrated into the chromosome (Fig. 3.7). As genomic DNA from each strain was digested with *Cla*I for Southern analysis, the detection of two bands in the NMB0342 and NMB0345 mutants can be explained by the presence of a *Cla*I site within the kan^{R} cassette of Tn*10*. Two independent colonies were taken from each transformation and these were designated by the gene number (gene with transposon insertion) and colony number (eg. NMB0346.1 and NMB0346.2). Each of the alternative plasmid constructs listed in table 3.5 was used to transform strain C311^{+} and mutations were confirmed using a similar strategy to that described above for NMB0346.

Construction and mapping of the pJan3col23 and pJan3col2 plasmids was performed by Janine van Nes.

Plasmid	EZ::TN™<KAN-2> insertion in NMB locus	Length of Gene	EZ::TN™<KAN-2> insertion site (Distance from 5′ end of gene)	EZ::TN™<KAN-2> insertion site (Coordinate in MC58 genome)
pJan3col23	NMB0343	276 bp	257 bp	353422
pJan3col2	NMB0344	276 bp	154 bp	353597
pSB30	NMB0346	756bp	355 bp	355063
pSB14	NMB0347	489 bp	140 bp	355679
pSB20	NMB0348	1008 bp	155 bp	356216

Table 3.5 Position of EZ::TN™<KAN-2> in plasmids generated for the construction of *N. meningitidis* mutants

Fig. 3.7 Southern analysis of mutants constructed with independent insertions in genes of the NMB0342-NMB0348 locus



Genomic DNA was isolated from two independent colonies of each of the mutants constructed and digested with *ClaI* for Southern analysis. The blot was probed with a kan^R cassette excised from $pUC4kan^R$. Bands of the same size were detected for both colonies for each mutant. The kan^R probe (1.282 kb) was run in lane 1.

3.4 Requirement for genes of the NMB0342-NMB0348 locus by *N. meningitidis* for the establishment of septicaemic disease

To establish whether genes of the NMB0342-NMB0348 locus are required for bloodstream infection, the competitive index (C.I.) was estimated for mutants with transposon insertions in individual ORFs of the locus in the infant rat model of infection. A 1:1 ratio of wild-type to mutant bacteria was given to animals by IP inoculation and the ratio of strains in the bloodstream determined at a time of between 20-24 hours post-infection. This method was chosen for investigation as the influence of host-to-host variation is reduced in mixed inoculum experiments. The C.I. values are shown in (Fig. 3.8). The NMB0342 and NMB0345 mutants are markedly attenuated (C.I.s < 0.001), confirming the results of the STM screen in which these genes were originally identified. C.I. values determined for the NMB0343 and NMB0344 mutants (0.215 and 0.250 respectively) indicate that these mutants are more attenuated than the NMB0347 and NMB0348 mutants (C.I.s of 0.603 and 0.340 respectively) suggesting that the first four genes of the locus (NMB0342, NMB0343, NMB0344, and NMB0345) are more important for the establishment of bacteraemic infection than the last three genes of the locus (NMB0346 (C.I. of 1.06), NMB0347, and NMB0348).

A number of *N. meningitidis* genes encoding virulence determinants such as LPS and capsule undergo phase-variation (Hammerschmidt *et al.*, 1996) (de Vries *et al.*, 1996). Therefore, it is possible that a mutant is attenuated as a result of phase-variation of other surface-expressed molecules and not as a direct result of the transposon insertion. All

Fig. 3.8 C.I. values of mutants with insertions in genes of the NMB0342-NMB0348 locus

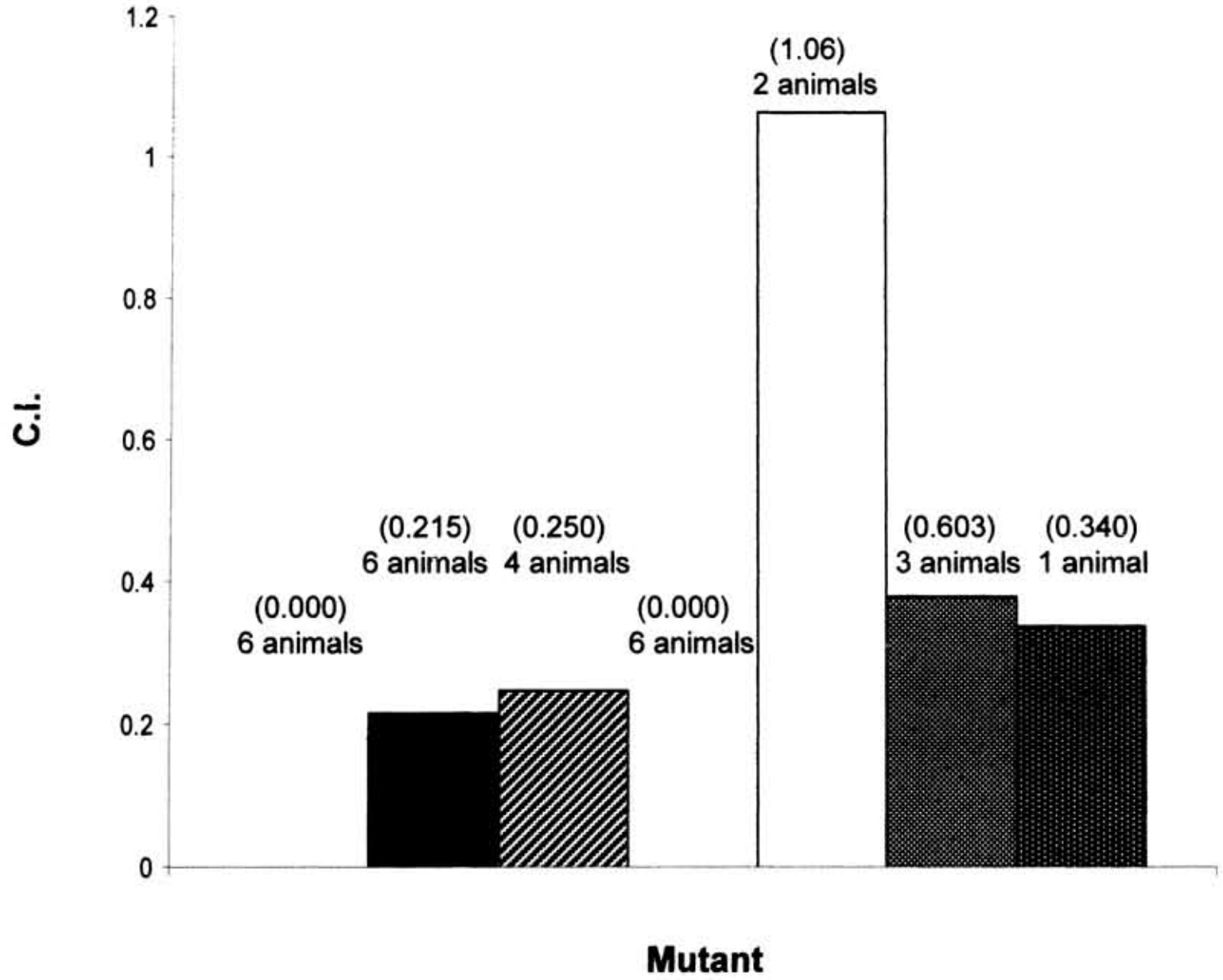


Fig. 3.8 **C.I. values of mutants with insertions in genes of the NMB0342-NMB0348 locus**

Groups of five day-old infant rats were inoculated intraperitoneally with a 1:1 ratio of mutant to wild-type bacteria at a total dose of 2 x 10^6 CFU. At 21 hours post-inoculation, blood was taken via a tail vein bleed and dilutions were plated to media with and without kanamycin. The C.I. of a mutant is calculated as the ratio of mutant to wild-type in the blood divided by the ratio of mutant to wild-type in the inoculum. The mean C.I. for each mutant is given in brackets with the number of animals used for calculation of the C.I. (surviving animals) shown below.

C.I.'s were determined using at least two independent transformants for each gene, providing further evidence that the attenuated phenotypes observed are due to mutations in the gene of interest. The C.I. values given are derived from the average of data obtained from experiments using these two independent transformants.

3.5 Complementation analysis of attenuated mutants

Examination of the C.I.s of individual mutants indicates that loss of NMB0345 function is sufficient to attenuate *N. meningitidis*. As the NMB0343 and NMB0344 mutants have C.I.s of 0.215 and 0.250 respectively, it is possible that the attenuation of the NMB0342 mutants may result from polar effects on downstream genes. Therefore, the NMB0342 mutant was complemented. The relative lack of attenuation of the NMB0343 mutant indicates that it is likely that loss of the NMB0342 product function, rather than an effect on downstream gene expression, is responsible for the inability of the NMB0342 mutant to cause bacteraemia, although it is possible that the NMB0342 may have a partial polar effect on NMB0343 and NMB0344. As the C.I. of the NMB0346 mutant is 1.06 (Fig. 3.8), it is unlikely that polar effects result from the transposon insertion in the NMB0345 mutant. Therefore, it was not necessary to perform complementation analysis of the NMB0345 mutant.

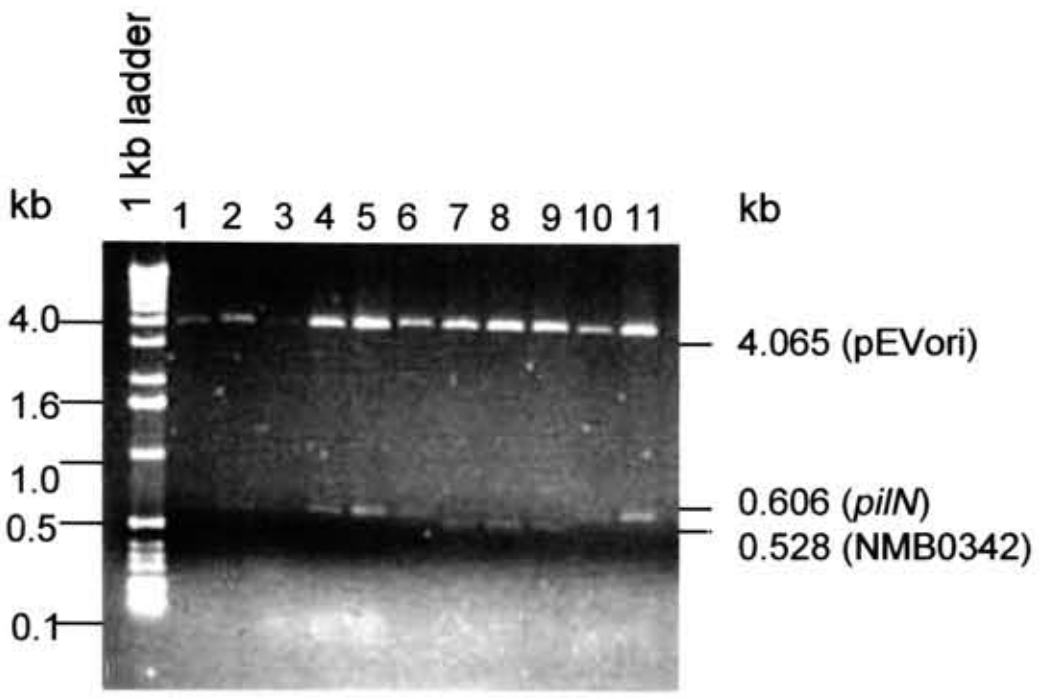
To confirm whether the transposon insertion in the NMB0342 gene is the cause of attenuation of the bacterium in the infant rat model, complementation analysis, where wild-type copies of the target gene are supplied *in trans*, was performed. The NMB0342

ORF (528 bp) was amplified from C311^{+} genomic DNA, incorporating a *Mlu*I site at the 5′ end and a *Bam*HI site at the 3′ end of the gene. The PCR product was ligated into pCR®2.1 TOPO® to generate pSB2. The plasmid was mapped and confirmed by restriction digestion.

Plasmid pEV*pilN*ori (4.671 kb) was constructed by Yao-hui Sun for complementation of *N. meningitidis* mutants (Winzer *et al.*, 2002) and contains a pACYC origin of replication, an erythromycin resistance gene, and a FLAG epitope. The *pilN* gene of *N. meningitidis* is ligated between the *Mlu*I and *Bam*HI sites of the vector. pEV*pilN*ori also contains the *N. meningitidis* genes, NMB0102 and NMB0103, allowing delivery of a single copy of the gene of interest *in trans* into the intergenic region located between NMB0102 and NMB0103 in the *N. meningitidis* chromosome.

An *Mlu*I/*Bam*HI fragment of NMB0342 isolated by digestion of pSB2 was ligated into the corresponding sites of pEV*pilN*ori lacking *pilN*, here designated as pEVori. The resulting complementation plasmid, pSB27 (4.593 kb, NMB0342 ligated in pEVori) and pEV*pilN*ori were mapped by restriction digestion using *Mlu*I/*Bam*HI, *Mlu*I/*Hinc*II, and *Cla*I. *Hinc*II and *Cla*I recognition sites are not present in *pilN*. *Mlu*I/*Bam*HI digests of pEV*pilN*ori, pSB27 gave bands of the expected sizes; pEV*pilN*ori (4.065 kb, 0.606 kb (*pilN*)) and pSB27 (4.065 kb, 0.528 kb (NMB0342 insert) (Fig. 3.9). *Mlu*I/*Hinc*II digests of pSB27 and pEV*pilN*ori also gave bands corresponding to the expected sizes; pSB27 (3.089 kb, 1.003 kb, and 0.436 kb) (Fig. 3.10, Panel A), pEV*pilN*ori (4.065 kb (pEVori), 0.6 kb (*pilN*)). *Hinc*II cuts once in NMB0342 and once in pEVori. *Cla*I digest

Fig. 3.9 **Restriction mapping of plasmids isolated following transformation of *E. coli* with ligations of NMB0342 or NMB0345 into pEVori**



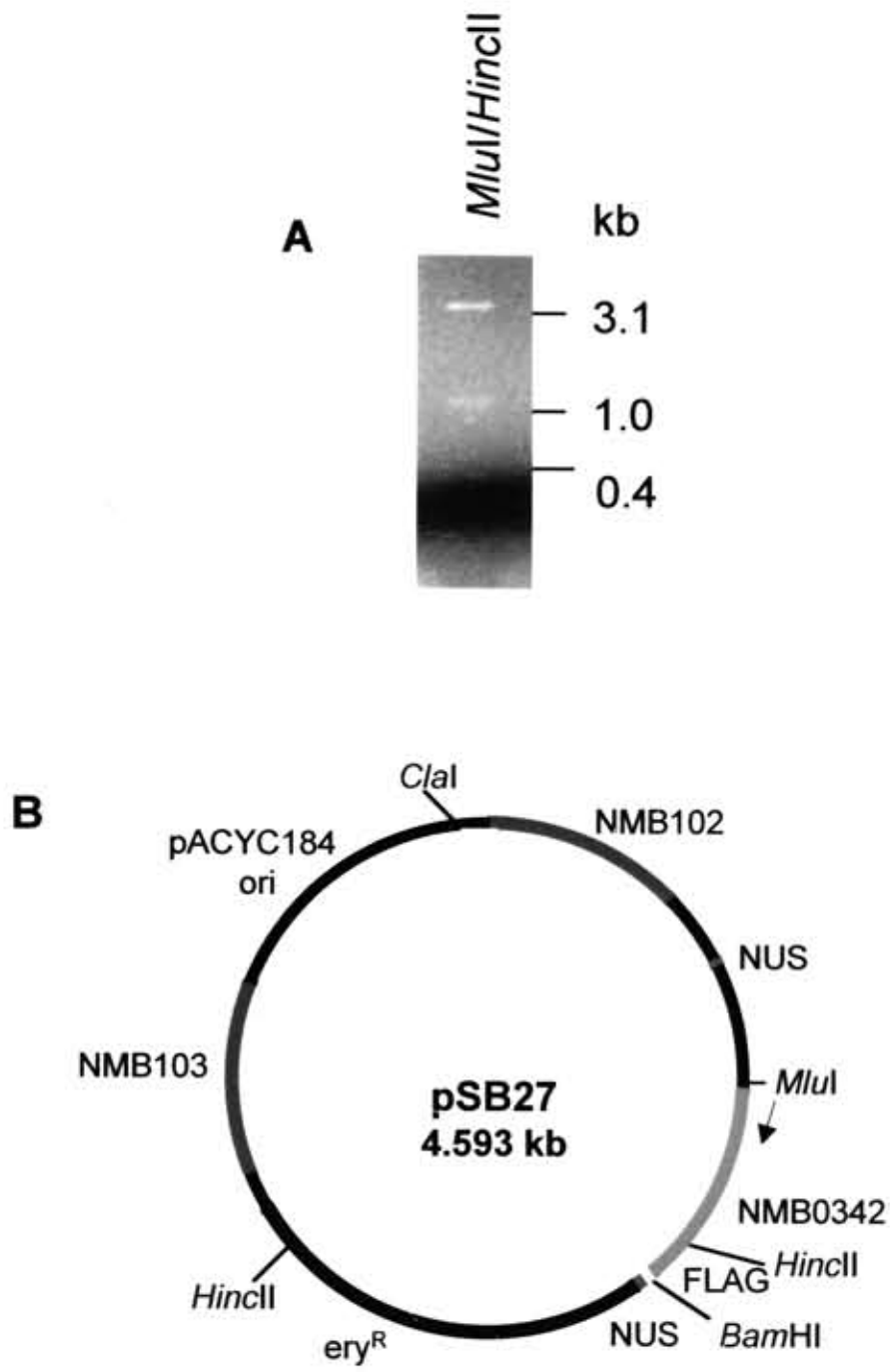
Plasmid DNA isolated from transformants 1-11 was digested with *Mlu*I and *Bam*HI to check for the presence of insert. Plasmids isolated from transformants 7, 8, and 9 have the NMB0342 insert (0.528 kb) in pEVori (4.065 kb). Plasmids isolated from transformants 1-6, 10, and 11 have *pilN* (0.606 kb) inserted in pEVori.

of pEV*pilN*ori gave bands of the expected size; (4.670 kb). *Cla*I cuts once once in pEVori (data not shown). The resulting complementation plasmid, pSB27, (Fig. 3.10, Panel B), was used to transform the NMB0342 mutant. Southern analysis of genomic DNA from each of the transformants hybridized with kan^R, ery^R, or NMB0342 probes was performed to confirm that the desired mutations had integrated into the chromosome by homologous recombination.

The results of Southern analysis of the complemented NMB0342 mutants are shown in Fig. 3.11. Hybridization with the NMB0342 probe detected a single copy of NMB0342 in $C311^+$ and in the NMB0342 mutants, 0342.1/$C311^+$ and 0342.2/$C311^+$ and two copies of NMB0342 in the NMB0342 complemented mutants (0342.1 pEVori and 0342.2 pEVori). The expected band size for $C311^+$ DNA cut with *Cla*I, then probed with NMB0342 is ~13.4 kb. The expected band sizes for NMB0342 mutants probed with NMB0342 are 12.6 kb and 2.0 kb. The detection of two bands is due to the presence of a *Cla*I site in Tn*10*.

Hybridization with the ery^R probe detected a single band (representing the ectopically-integrated copy of NMB0342) in each of the complemented NMB0342 mutants. Hybridization with the kan^R probe detected bands in all four NMB0342 mutants. The presence of two bands in NMB0342 mutants is due to the presence of a *Cla*I site in Tn*10*. No band was detected in the wild-type strain, $C311^+$. Expected band sizes for NMB0342 complemented mutants probed with NMB0342 are 12.6 kb, 2.0 kb, and an additional band of 11.5 kb which corresponds to the *Cla*I fragment containing the ectopically

Fig. 3.10 Restriction mapping of plasmid pSB27



*Mlu*I/*Hinc*II digestion of pSB27 generated bands of the expected size; 3.089 kb, 1.0 kb, and 0.4 kb (Panel A). Plasmid pSB27 (Panel B) was used to complement the NMB0342 mutant. Fragments of NMB0102 and NMB0103 are shown in blue. NMB0342 is represented in red. *Neisseria* uptake sequences (NUS) are represented in green. The erythromycin resistance gene (ery^R) is represented in grey. The FLAG epitope is shown in yellow. The pACYC184 origin of replication is represented in black. Restriction sites used for mapping are also shown. An arrow is used to show the orientation of the NMB0342 gene (direction of transcription).

Fig. 3.11 Southern analysis of complemented NMB0342 mutants

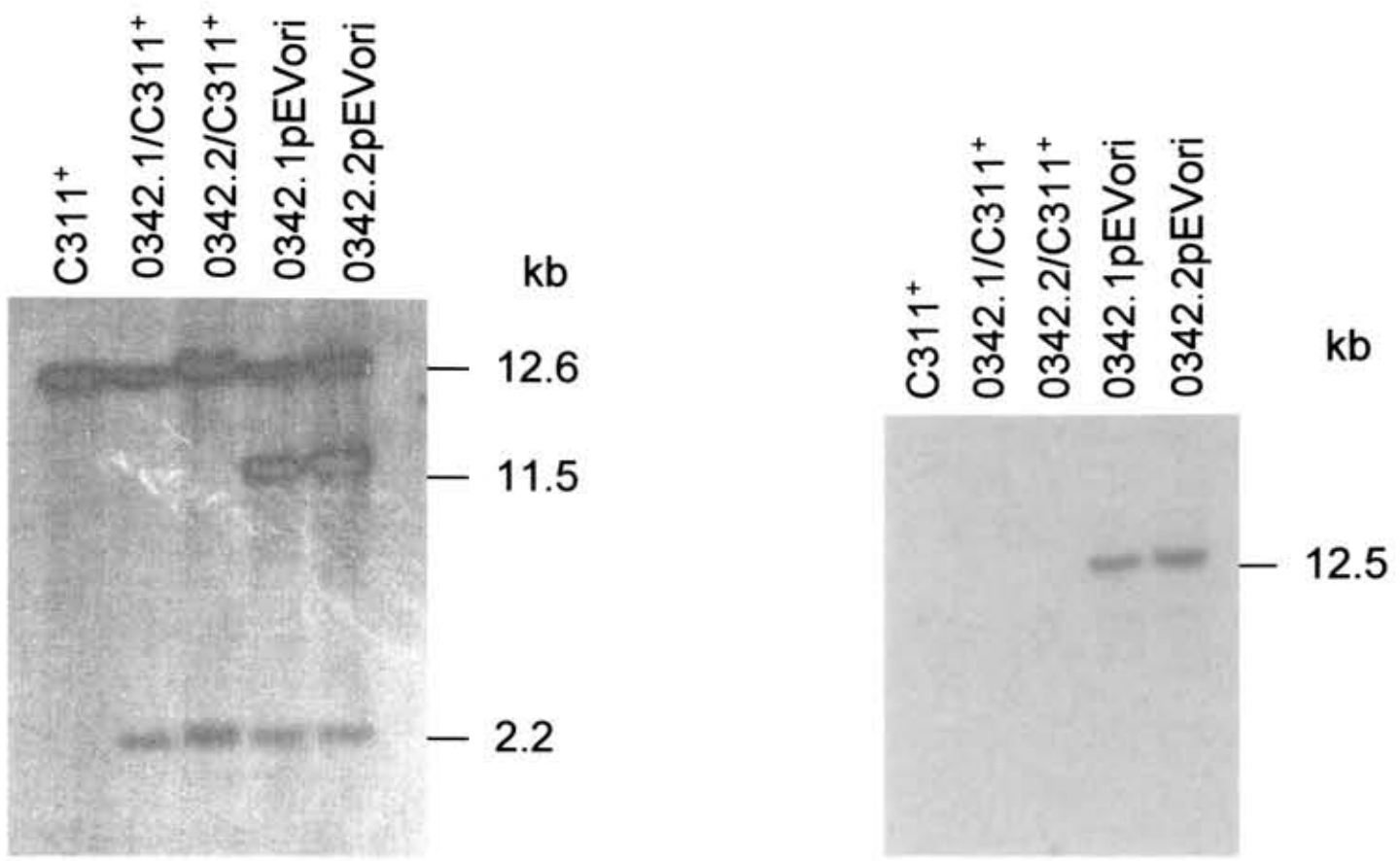


Fig. 3.11 **Southern analysis of complemented NMB0342 mutants**

Genomic DNA isolated from C311^{+}, two independent colonies of the NMB0342 mutant (0342.1/C311^{+} and 0342.2/C311^{+}), and two independent colonies of the NMB0342 complemented mutant (0342.1 pEVori and 0342.2 pEVori) was digested with *Cla*I. Southern blots were hybridized against either NMB0342 (Panel A), ery^{R} (Panel B), or kan^{R} (Panel C) genes.

integrated copy of NMB0342. The expected band sizes for the NMB0342 complemented mutants probed with ery^R and kan^R are ~12.7 kb and ~ 0.9 kb respectively.

3.6 Competitive index of the complemented NMB0342 mutant

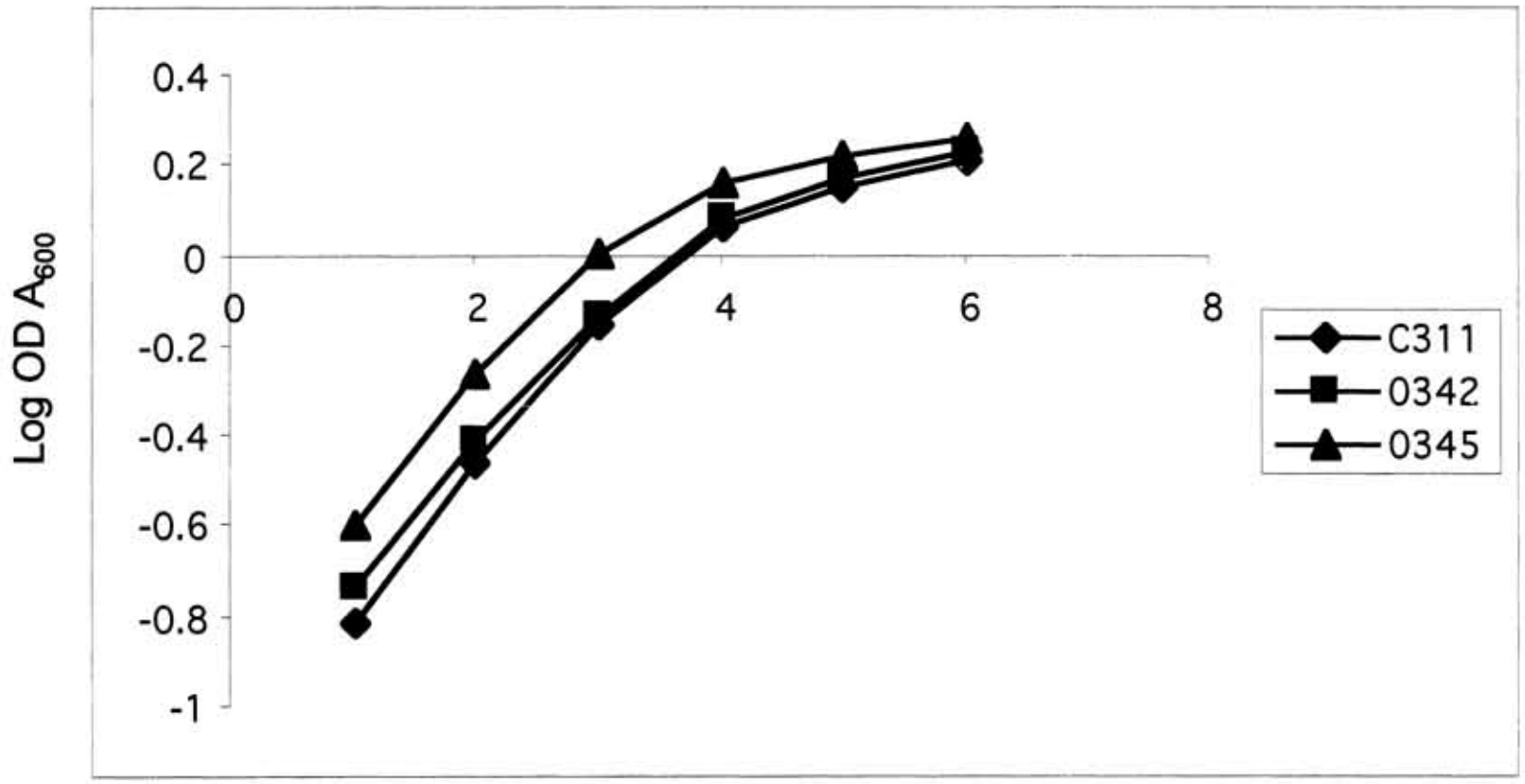
C.I.s were determined using at least two independent complemented NMB0342 mutants in order to minimize the potential influence of phase variation on observed phenotypes. The complemented NMB0342 mutant gave a C.I. of 0.918 when compared with the wild-type bacterium, $C311^+$. This value was determined by taking an average of the C.I.s determined from the infection of two animals. As the C.I. of the original NMB0342 mutant was <0.001, complementation of the NMB0342 mutant with a single wild-type copy of NMB0342 *in trans*, almost completely restored the virulence of the bacterium and the phenotype resulting from mutation of the NMB0342 gene does not result from polar effects of the transposon insertion on downstream genes.

3.7 *In vitro* growth of the NMB0342 and NMB0345 mutants

The NMB0342 and NMB0345 mutants are markedly attenuated in the infant rat model of meningococcal septicaemia (Sun *et al.*, 2000). It is possible that the attenuation observed *in vivo* is due to a growth defect. Therefore, the *in vitro* growth of these mutants was measured. 10 ml of proteose peptone broth supplemented with 2% vitox (Oxoid) was inoculated with 10^9 CFU of $C311^+$, the NMB0342 mutant, or the NMB0345 mutant, and incubated for six hours at 37°C with 5% CO_2 at 10 rev/minute. The OD of the cultures

was read at 600 nm at 60 minute intervals. Three independent experiments showed that the growth of NMB0342 and NMB0345 mutants was similar to that of the wild type strain, C311^{+}. Therefore, these mutants do not display a detectable growth defect *in vitro* (Fig. 3.12).

Fig. 3.12 ***in vitro*** **growth of the NMB0342 and NMB0345 mutants**



The *in vitro* growth of the NMB0342 and NMB0345 mutants was analysed by measuring the OD_{600} of three independent flasks containing 10 ml of BHI broth inoculated with 10^9 CFU of either $C311^+$, the NMB0342 mutant, or the NMB0345 mutant. The OD_{600} was measured hourly for six hours.

Discussion

The NMB0342 and NMB0345 genes of *N. meningitidis* are located 622 bp apart within a potential operon of seven genes (NMB0342-NMB0348). The proximity of these genes and the presence of homologues in known human pathogens such as *S. flexneri* and *C. jejuni* suggested the possibility that these genes may be functionally linked. As the complete genome sequences of many bacterial pathogens are now available and can be accessed using on-line genome databases, searches were performed using published MC58 genome sequence of each of the genes of the NMB0342-NMB0348 locus against 16 complete and two incomplete bacterial genomes (*N. meningitidis* serogroup C strain FAM18 and *N. gonorrhoeae* strain FA1090) using NCBI databases and the BLASTP and TBLASTN algorithms. The results of these searches demonstrate that homologues of NMB0342 and NMB0343 exist and are linked in a range of Gram-negative bacteria. The genomes queried include the Gram-negative bacterium *H. influenzae* (Rosenstein and Perkins, 2000) and Gram-positive bacteria, *S. pneumoniae* (Koedel *et al.*, 2002), and *Listeria monocytogenes* (Doganay, 2003), which like *N. meningitidis* can all cause meningitis and septicaemia. However, in some species, these homologues are separated by an intervening gene(s) (Table 3.1). In both *E. coli* K12 and *Y. pestis*, one of the intervening genes is *tonB*. The TonB protein, in association with ExbB and ExbD, is involved in energy transduction across the cell membrane for the transport of both iron from host iron-binding proteins, and vitamin B12 (Postle, 1990). However, this differs from the genetic organization in serogroup B *N. meningitidis* strain MC58 where the *tonB* gene designated as ORF NMB1730 is not linked to the NMB0342 and NMB0345 genes.

Although homologues of NMB0344 (*bolA/yrbA*) are present in most bacteria, these homologues do not appear to be linked to the NMB0342 and NMB0343 homologues in other species when they are found (Table 3.1). The linkage of NMB0342 and NMB0343 homologues in a range of Gram-negative bacteria suggests that their functions may be related and that the expression of these genes may be co-regulated. The requirement for the *S. flexneri* homologue of NMB0342, *ispA* for effective intracellular septation (Hong et al., 1998) (Mac Siomoin *et al.*, 1996) and the implication that the *E. coli* homologue of NMB0343, *bolA*, is involved in septum formation mediated by *ftsZ*, a gene which plays a central role in cell division, suggests that these genes have related functions in bacterial cell division (Aldea *et al.*, 1990) (de Boer *et al.*, 1992).

The first three genes of the NMB0342-NMB0348 locus are absent from the genomes of all Gram-positive bacteria surveyed (*S. aureus*, *S. pneumoniae*, *L. monocytogenes*, and *B. subtilis*). In *S. aureus*, only homologues of the last three genes of the locus are present, while in *L. monocytogenes* and *S. pneumoniae*, only the NMB0347 and NMB0348 sequences are present. The only gene of the NMB0342-NMB0348 locus to have homologues in all bacterial species surveyed is NMB0348. Results of searches performed using the BLASTP algorithm demonstrated the presence of multiple NMB0348 homologues within many of the bacterial genomes surveyed (data not shown). However, the function of these proteins is unknown. The NMB0345 and NMB0346 sequences share homology with each other, but are the least conserved in other bacterial species based on the cut-off values used for searches of 20% identity or higher.

Examination of available *Neisseria* genome sequences demonstrates that the NMB0342-NMB0348 locus is conserved in *N. meningitidis* strains Z2491 (serogroup A), MC58 (serogroup B), FAM18 (serogroup C) and *N. gonorrhoeae* strain FA1090. This observation was extended by performing PCR and Southern analysis of the locus in a range of isolates.

Following a survey of the conservation of genes of the NMB0342-NMB0348 locus in a range of pathogenic and commensal *Neisseria* spp., a high degree of concordance was observed between the results generated by PCR amplification and results of Southern analysis for the majority of isolates tested. However, in some instances, there are discrepancies between the results of PCR and Southern analyses. This is apparent in the pathogenic *Neisseria* spp. such as serogroup A isolate F6124, and serogroup B isolates, 2996 and B16/B6, which are negative by PCR, but positive by Southern for the presence of NMB0348. These discrepancies are also seen in non-pathogenic *Neisseria* spp. such as *N. lactamica*, *N. polysaccharea*, and *N. cinerea* indicating that PCR analysis gives rise to false-negative results in some strains. This is likely to result from differences in nucleotide sequence between strains; all oligonucleotides for PCR were designed based on the published MC58 genome sequence (Tettelin *et al.*, 2000).

NMB0342, NMB0345, and NMB0348 sequences are present in all strains of *N. meningitidis* tested regardless of serogroup, but are not conserved in non-pathogenic *Neisseria* spp. Homologues of NMB0342, NMB0345, and NMB0348 are present in *N. gonorrhoeae*, *N. lactamica*, *N. polysaccharea*, *N. mucosa*, and *N. cinerea*, consistent with the observation that with the exception of *N. cinerea*, these species together with *N.*

meningitidis, form a single genospecies on the basis of their nucleotide relatedness (Guibourdenche *et al.*, 1986). However, a study by Welch *et al.*, 2002 has shown that although genes present in related bacterial species may share a high level of identity, overall gene content can differ extensively (Welch *et al.*, 2002).

The identification of two mutants attenuated for septicaemic disease with transposon insertions in NMB0342 and NMB0345, the close proximity of these genes in the *N. meningitidis* genome, and the conservation of the genetic organization of the NMB0342-NMB0348 locus in published serogroup A, B, and C genome sequences suggested that the genes of the NMB0342-NMB0348 locus may play an important role in *N. meningitidis* pathogenesis. Therefore, a series of mutants with transposon insertions in each of the predicted ORFs of the locus were constructed to allow an assessment of the requirement for these genes during pathogenesis.

Cloning of a PCR product consisting of the NMB0346 sequence with 1 kb of flanking sequence on either side of the gene was initially unsuccessful, with no transformants obtained. New primers were subsequently designed in order to amplify a shorter fragment consisting of the NMB0346 sequence with approximately 150 bp of flanking sequence on either side of the gene. This fragment was successfully cloned into the pCR® 2.1 TOPO® cloning vector. As the insert contained the complete NMB0345 ORF, the failure to obtain transformants using the original insert may have been due to toxicity of the NMB0345 gene product in *E. coli*.

The method of *in vitro* EZ::TN™<KAN-2> transposition used in this study for the construction of insertional mutants has not been used previously for the study of *N. meningitidis*. The availability of the complete genome sequence of the serogroup B isolate MC58 allowed the design of primers for the amplification by PCR of individual genes. PCR products were amplified from genomic DNA isolated from strain C311^{+}. PCR products were subsequently cloned into pCR®2.1 TOPO® and the resulting plasmids were used as targets for EZ::TN™<KAN-2> transposition following deletion of the kan^{R} cassette present in the vector. This permitted selection of transformants on the basis of kanamycin resistance encoded by the transposon. Mutations were introduced into the *Neisseria* genome by homologous recombination. This methodology has proven to be straightforward as it avoids the requirement for suitable restriction sites for cloning in the gene of interest, or for inverse PCR.

The C.I. values obtained for the NMB0342-NMB0348 locus mutants indicate that the transposon insertion in NMB0342, which results in a C.I. of <0.001, is unlikely to be strongly polar as the C.I.s determined for mutants with insertions in the genes immediately downstream are 0.215 and 0.250 for each mutant respectively. However to exclude the possibility of polar effects contributing to the attenuation of the NMB0342 mutant, complementation analysis was performed. A wild-type copy of the NMB0342 gene, ligated into the pEV*pilN*ori vector for complementation, was introduced into the corresponding NMB0342 mutant. This vector allows ectopic integration of a single copy of the wild-type gene into the *Neisseria* chromosome. Ectopic integration allows the stable expression of genes in single copy and avoids artefacts associated with trans

complementation by genes maintained on plasmids (Haldimann and Wanner, 2001). Complementation of the NMB0342 mutant restored the virulence of the bacterium demonstrating the requirement for NMB0342 for survival in the bloodstream. A transposon insertion in NMB0345 results in a C.I. of <0.001. The C.I. value determined for the NMB0346 mutant (1.06) suggests that this mutation does not have polar effects on downstream genes. The C.I.s determined for mutants with insertions in NMB0347 (0.603) and NMB0348 (0.340) indicate that these mutants are only partially attenuated and therefore the attenuation of a mutant with a transposon insertion in NMB0345 is unlikely to have resulted from polar effects on either NMB0347 or NMB0348.

All C.I.s were determined by harvesting blood (output) at approximately 21 hours post-infection and dividing the ratio of mutant to wild-type bacteria in the output by the ratio of mutant to wild-type in the inoculum. This time-point was the same used for studies with the original mutants. 4E3 (transposon insertion in NMB0342) and 25E5 (transposon insertion in NMB0345) were identified in the STM screen of a library of *N. meningitidis* mutants. Blood was harvested at 20-24 hrs post-infection. However, it is possible that C.I.s determined at earlier or later time-points will give differing C.I. values and may reflect on the particular stages of septicaemic disease.

Current studies have demonstrated the requirement for the NMB0342 and NMB0345 genes by *N. meningitidis* for septicaemic disease, but do not strongly implicate the other genes of the NMB0342-NMB0348 locus at this stage of pathogenesis. However, it is possible that these genes are involved at other steps of *N. meningitidis* pathogenesis. In

addition, the linkage of these genes to NMB0342 and NMB0345 and to homologues of NMB0342 and NMB0345 in a range of pathogenic *Neisseria* spp. suggests that they may have functions related to the virulence of the organism. Strains with independent insertions in the genes of the NMB0342-NMB0348 locus will allow further phenotypic analysis in a range of model systems. Furthermore, the conservation of the NMB0342 and NMB0345 genes in pathogenic and non-pathogenic *Neisseria* spp. and the requirement for these genes in the establishment of meningococcal septicaemia indicate that the proteins encoded by these genes may have potential as vaccine candidates.

4 Phenotypic characterization of the NMB0342 and NMB0345 mutants

Genetic analysis of the NMB0342-NMB0348 locus described in the preceding chapter demonstrated that two genes of the locus, NMB0342 and NMB0345 are required for establishing bacteraemic infection in the infant rat model following IP inoculation. In order to cause bacteraemia, pathogenic bacteria utilize numerous virulence factors required for vital functions including the avoidance of host immunity, acquisition of nutrients, and for some bacteria, tropism to distinct cell types and production of host damage (Cornelissen and Sparling, 1994) (Kaufmann, 1993) (Finlay and Falkow, 1989). The aim of this chapter is to define the molecular mechanisms by which the NMB0342 and NMB0345 genes contribute to the pathogenesis of meningococcal infection.

The pathogenesis of meningococcal disease is complex, involving colonization of the nasopharynx, passage through cells lining the nasopharyngeal mucosa and blood vessels, and survival in the bloodstream resulting in sepsis and/or meningitis if the bacteria are able to invade the central nervous system (CNS). Survival and replication of *N. meningitidis* in the bloodstream is a pre-requisite for the establishment of severe meningococcal disease (van Deuren *et al.*, 2000). At each of the known stages of pathogenesis, the bacterium must overcome a variety of host defence mechanisms requiring the concerted expression of multiple bacterial virulence factors, many of which are known to undergo high frequency phase and antigenic variation (Hammerschmidt *et al.*, 1996) (de Vries *et al.*, 1996). Furthermore, many meningococcal virulence determinants have roles during multiple steps of pathogenesis (Hammerschmidt *et al.*,

1996) (Jennings *et al.*, 1995) (Sarkari *et al.*, 1994) (de Vries *et al.*, 1996). Therefore, the requirement for the NMB0342 and NMB0345 genes in two key stages of meningococcal pathogenesis, colonization and bloodstream infection, was investigated.

In the preceding chapter, the infant rat model of meningococcal septicaemia was used to assess the ability of *N. meningitidis* to survive and replicate in the bloodstream. However, as *N. meningitidis* is a commensal and pathogen of humans only, experiments were initially performed to validate the results from the infant rat model in assays using human blood and serum. In order to determine whether the NMB0342 and NMB0345 genes are required at other stages of pathogenesis, the ability of the NMB0342 and NMB0345 mutants to adhere to cultured human epithelial cells was also examined.

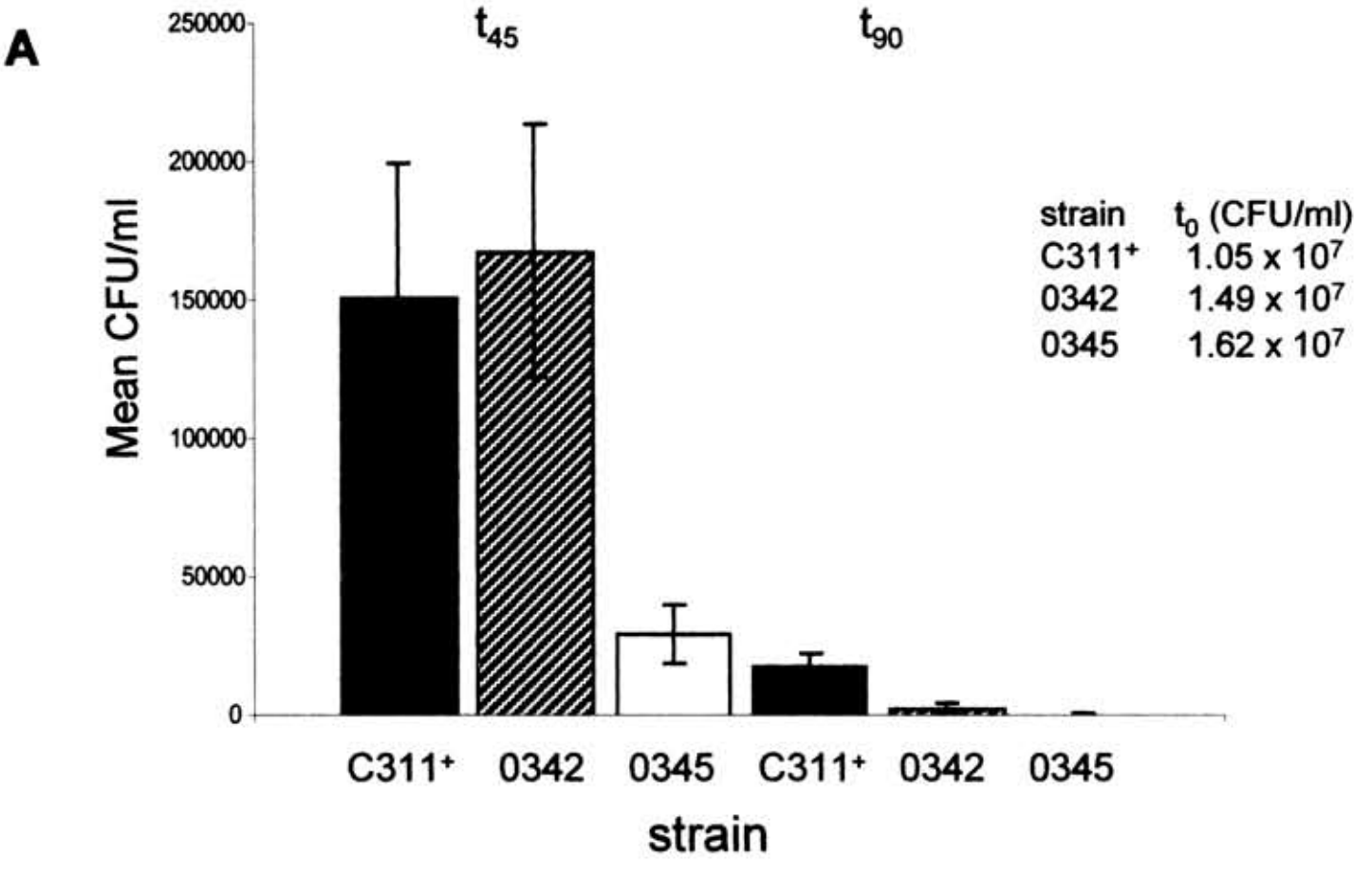
4.1 Survival of the NMB0342 and NMB0345 mutants in whole blood

The NMB0342 and NMB0345 mutants were initially examined in an *ex vivo* human whole blood model of infection (Ison *et al.*, 1995; Ison *et al.*, 1999). The primary advantage of this model is that it closely resembles the environment of the systemic circulation using human whole blood and comprises phagocytic cells such as polymorphonuclear leucocytes and monocytes as well as antibodies and factors of the complement pathway.

For each strain tested, ($C311^+$, NMB0342 mutant, and NMB0345 mutant), 5 ml of heparinized human whole blood was inoculated with 10^8 CFU of the respective strain and

incubated at 37°C. In the first experiment (Fig. 4.1 Panel A), the NMB0342 mutant was recovered at similar levels to the wild-type following 45 minutes incubation (t_{45}). However, in a second experiment using blood collected from the same donor, (Fig. 4.1 Panel B), the NMB0342 mutant was recovered at only 32.7% of wild-type levels by t_{45}. In an average of two experiments, the NMB0345 mutant was recovered at approximately 40% of wild-type levels at t_{45}. At t_{90}, both mutants were recovered at reduced numbers compared to the wild-type; 2.8% and 11.6% of wild-type for the NMB0342 and NMB0345 mutants respectively, in an average of two experiments (Fig 4.1, Panels A and B). As the number of bacteria at t_0 for the three strains was similar for both experiments, the reduction in CFU observed reflects the decreased survival of the mutants over the incubation period (Fig. 4.1, Panels A and B). These experiments demonstrate that after both 45 and 90 minutes incubation in whole blood, the NMB0342 and NMB0345 mutants are attenuated in whole blood compared to the wild-type bacterium, C311$^+$. The rapid reduction in the numbers of the mutants compared with the wild-type suggests that the mutants do not have a growth defect, but are sensitive to killing. The results of these assays confirm the attenuation of the NMB0342 and NMB0345 mutants observed in the infant rat model of infection.

Fig 4.1 **Survival of the NMB0342 and NMB0345 mutants in the human whole blood assay**



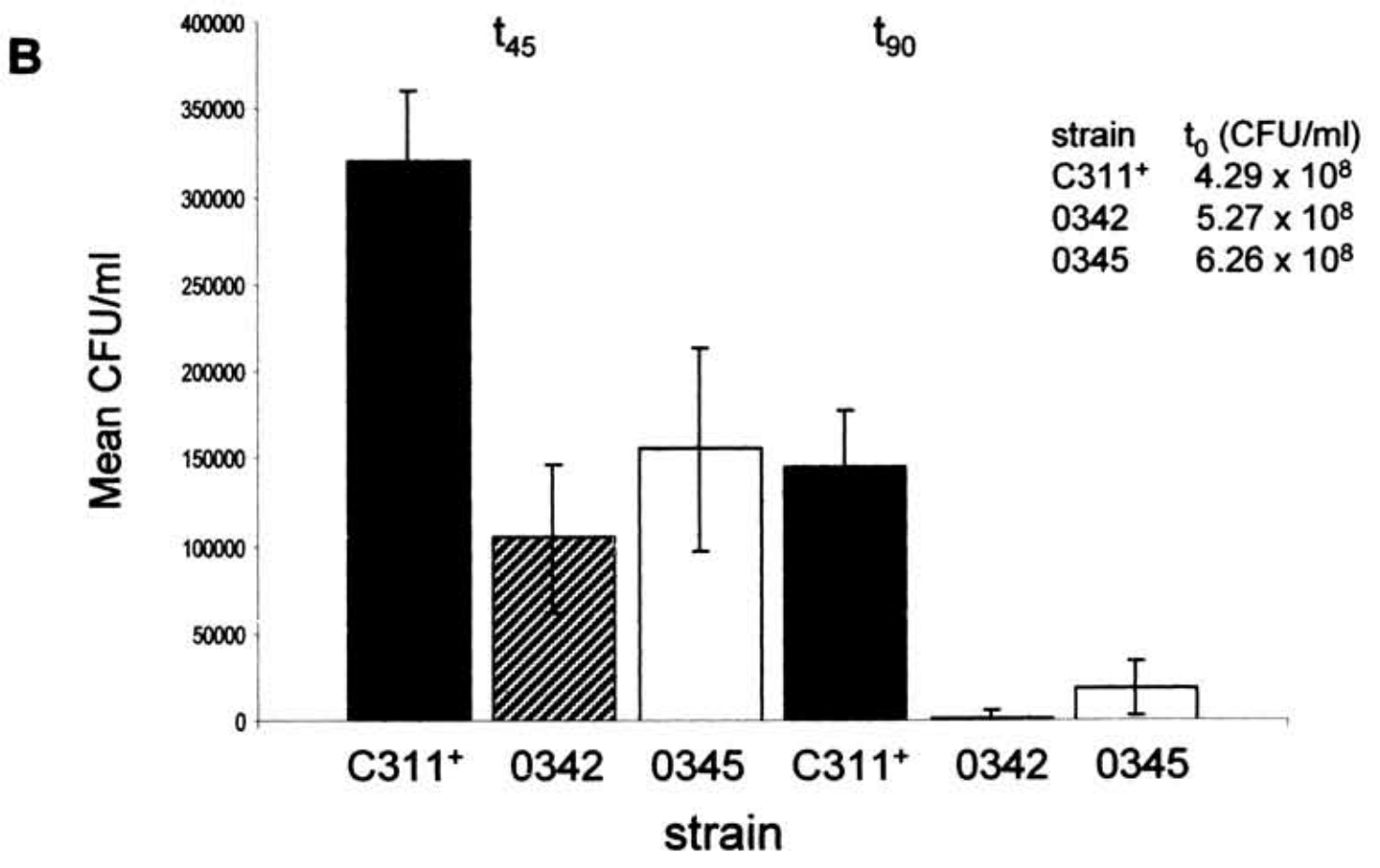


Fig. 4.1 **Survival of the NMB0342 and NMB0345 mutants in whole blood**

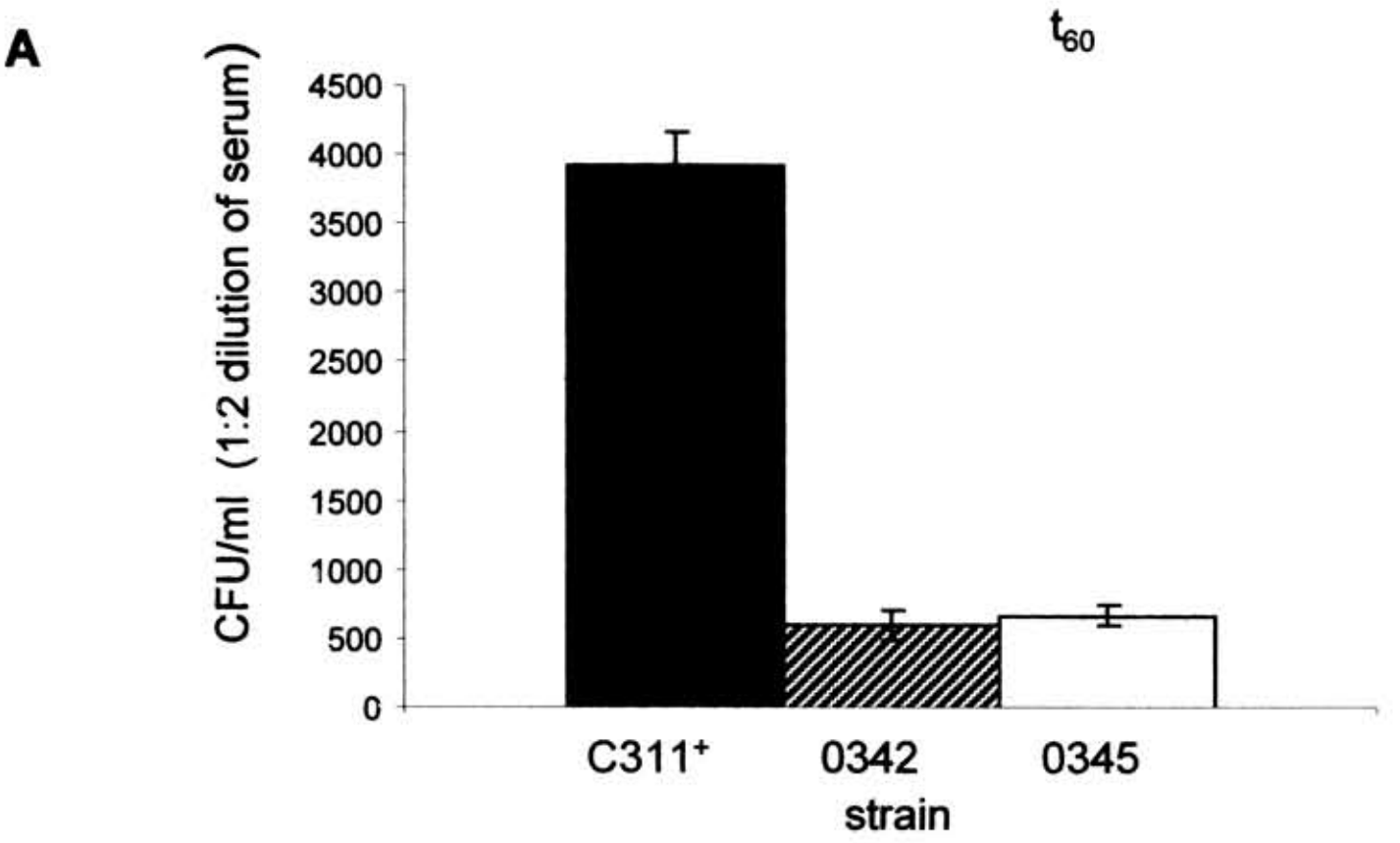
10^8 CFU of C311$^+$, the NMB0342 mutant, or the NMB0345 mutant were inoculated into 5 ml of heparinized human whole blood. Aliquots (10 μl) were plated to BHI agar after 45 minutes (t_{45}) and 90 minutes (t_{90}) incubation. The number of bacteria recovered at each time point is represented in CFU/ml. The results of two independent assays are shown in panels A and B. Error bars represent the standard deviation of the mean.

4.2 Serum-sensitivity of the NMB0342 and NMB0345 mutants

In human disease, the complement-mediated bactericidal activity of sera is a fundamental mechanism preventing the replication of *N. meningitidis* in the blood (van Deuren *et al.*, 2000). Following the entry of meningococci into the bloodstream, all three complement pathways (classical pathway, alternative pathway, and mannose-binding lectin (MBL) pathway) are activated (Emonts *et al.*, 2003). The increased incidence of recurrent meningococcal disease in patients with deficiencies of the complement pathway highlights the importance of this system for the clearance of meningococci (Figueroa *et al.*, 1993). Therefore, the requirement of the NMB0342 and NMB0345 genes for resistance to complement-mediated killing was investigated. The serum fraction of the blood contains both complement factors and antibodies while cellular components of the blood are absent. The survival of wild-type strain, C311^{+}, and the NMB0342 and NMB0345 mutants in normal human serum (NHS) was measured by performing serum-sensitivity assays using serum collected from blood obtained from the same donor used for the whole blood assays. Strains were tested in the following dilutions of serum (1:2, 1:4, 1:8, 1:16, 1:32, 1:64). Heat-inactivated NHS was used as a control. Two independent experiments demonstrated that in a 1:2 dilution of serum, the NMB0345 mutant is sensitive to serum-killing compared to the wild type, C311^{+} (Figure 4.2, Panels A and B). The first experiment (Fig. 4.2 Panel A) demonstrated that the NMB0345 mutant is more serum-sensitive than the wild-type as it was recovered at approximately 500 CFU/ml

compared to approximately 4 x 10^3 CFU/ml for the wild-type after 60 minutes' incubation. In this experiment, the NMB0342 mutant was also recovered at approximately 500 CFU/ml suggesting that this mutant may also be more serum-sensitive than the wild-type. However, a second experiment (Fig. 4.2 Panel B) showed that at 30 minutes incubation in a 1:2 dilution of human serum, the NMB0342 mutant was recovered at similar numbers to the wild-type (6 x 10^3 to 7 x 10^3 CFU/ml|), whereas the NMB0345 mutant was recovered at approximately 2 x 10^3 CFU/ml (Fig. 4.2 Panel B). No visible difference was observed between the number of wild-type and NMB0342 or NMB0345 mutant recovered following incubation in a 1:4 or higher dilution of serum.

Fig 4.2 **Serum-sensitivity of the NMB0342 and NMB0345 mutants**



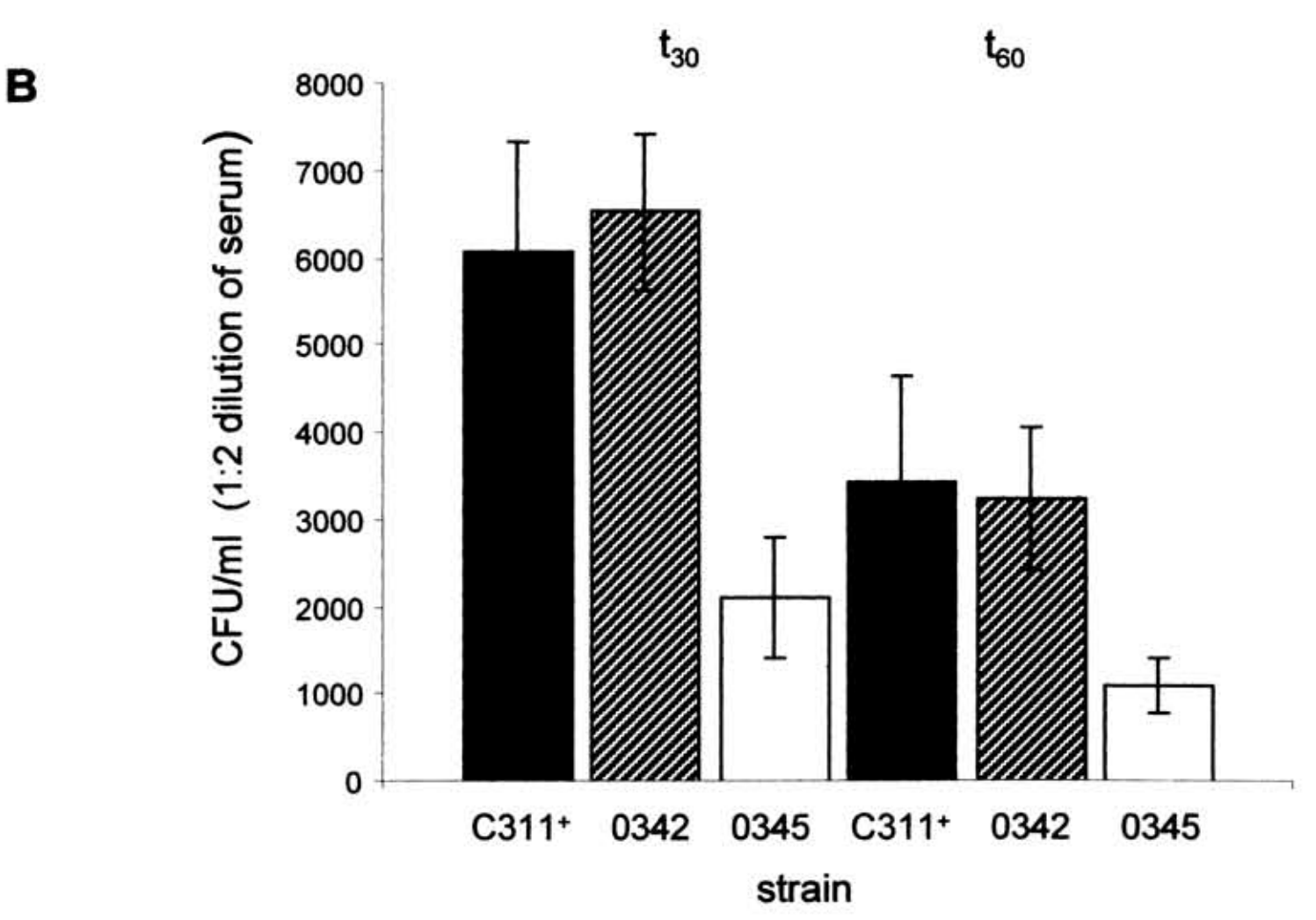


Fig. 4.2 Serum-sensitivity of the NMB0342 and NMB0345 mutants

10^4 CFU of C311$^+$, the NMB0342 mutant, or the NMB0345 mutant were inoculated into a 1:2 dilution of normal human serum in a 96-well microtitre plate. Aliquots (10 μl) from each well were plated to BHI agar after 30 (t_{30}) or 60 minutes (t_{60}) incubation. The number of bacteria recovered at each time point is represented in CFU/ml. No killing of the strains was observed using heat-treated NHS. The results of two independent assays are shown in panels A and B. Error bars represent the standard deviation of the mean.

A second experiment (Fig. 4.2 Panel B) showed that at 30 minutes incubation in a 1:2 dilution of human serum, the NMB0342 mutant was recovered at similar numbers to the wild-type (6×10^3 to 7×10^3 CFU/ml), whereas the NMB0345 mutant was recovered at approximately 2×10^3 CFU/ml (Fig. 4.2 Panel B). In this experiment, the NMB0342 mutant was also recovered at similar numbers to the wild-type after 60 minutes incubation. This is similar to the result obtained after 45 minutes' incubation in heparinized human whole blood at 45 minutes (Fig. 4.1, Panel A). Therefore, the reduction in mutant numbers compared to the wild-type strain, $C311^+$, observed in the whole blood assay was also observed in the serum-sensitivity assay in which cellular components of the blood are absent.

4.3 Survival of the NMB0342 and NMB0345 mutants in $C3^{-/-}$ C57BL/6 mice

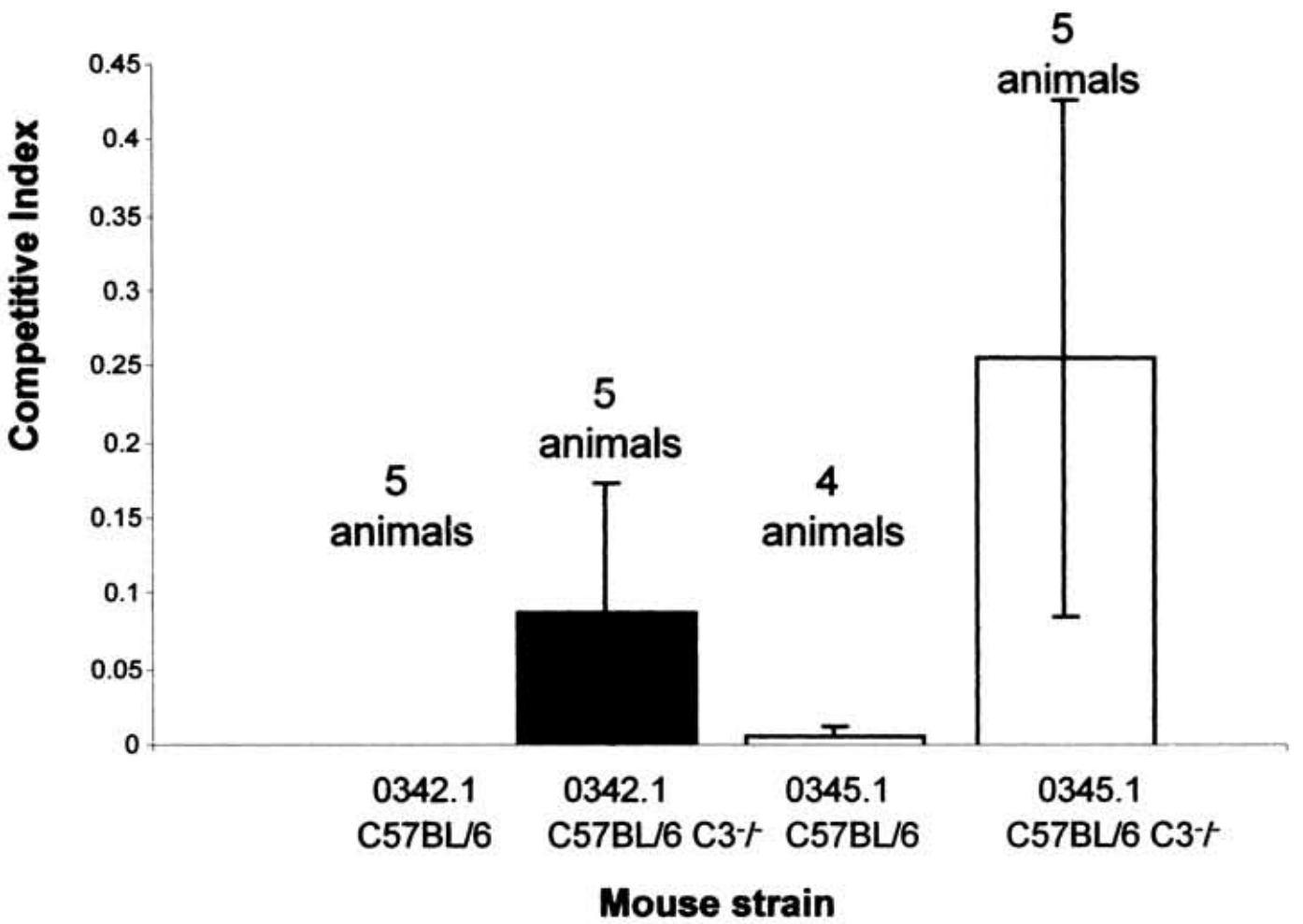
In vitro analyses (whole blood and serum-sensitivity assays) indicate that sensitivity to complement-mediated killing may be a potential mechanism underlying the attenuation of the NMB0342 and NMB0345 mutants in the infant rat. Therefore, experiments were performed to assess the involvement of the complement system on the behaviour of these mutants *in vivo* using a murine model of infection. Complement factor C3 is the point at which the classical, alternative, and MBL pathways of complement activation converge (Figueroa and Densen, 1991), and is therefore central to the complement pathway. As C3 is required for the activation of other components of the complement pathway prior to the assembly of a membrane attack complex, animals lacking factor C3 have no complement-mediated bactericidal activity (Wessels *et al.*, 1995).

Five wild-type (C57BL/6) and five C3-deficient (C57BL/6 C3$^{-/-}$) mice were inoculated with a 1:1 ratio of wild-type (C311^{+}) and mutant (either NMB0342 or NMB0345) bacteria. Blood was taken via a tail vein bleed at 21 hrs after inoculation and dilutions of this blood were plated to both BHI agar with or without kanamycin. Both mutants were highly attenuated in wild-type mice with C.I.s of less than 0.001 and 0.005 for the NMB0342 and NMB0345 mutants respectively (Fig 4.3). C.I.s for both mutants were higher in C3-deficient mice than in the wild-type mice; C.I. values of 0.087 and 0.257 for the NMB0342 and NMB0345 mutants respectively. The results indicate that while both mutants are sensitive to killing by complement, this enhanced sensitivity contributes more to the attenuation of the NMB0345 mutant than the NMB0342 mutant and is consistent with the results of the serum-sensitivity assay (Fig. 4.2, Panel B) in which numbers of the NMB0342 mutants recovered after both 30 minutes and 60 minutes incubation in normal human serum are similar to the number of wild-type strain C311^{+} recovered, while reduced numbers of the NMB0345 mutant compared to wild-type are recovered at both time points. This result suggests that the attenuation of the NMB0342 mutant in the infant rat model may be due to a combination of defects.

4.4 LPS profile of the NMB0342 and NMB0345 mutants

LPS is one of the main mediators of *N. meningitidis* serum-resistance and phase variation in LPS biosynthetic genes can result in a significant increase in the serum-sensitivity of

Fig. 4.3 Competitive index of NMB0342 and NMB0345 mutants in C57BL/6 and C57BL/6 C3-/- mice



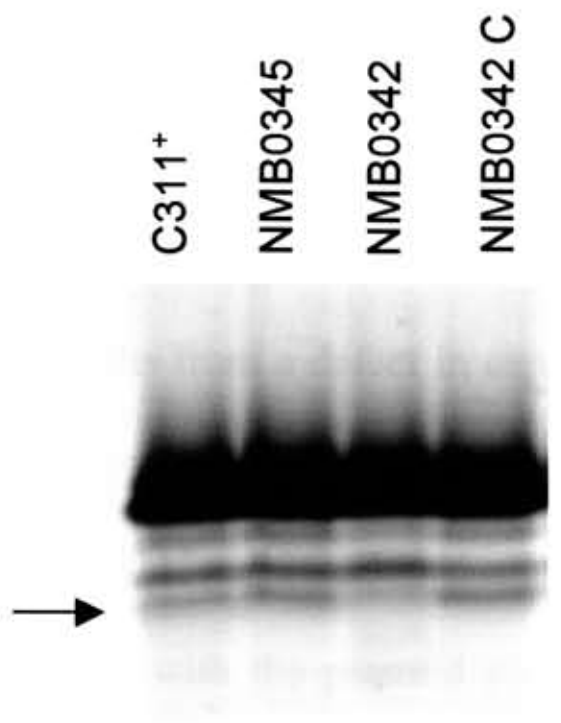
Five wild-type (C57BL/6) and five C3-deficient (C57BL/6 $C3^{-/-}$) mice were inoculated with a 1:1 ratio of $C311^{+}$ and either the NMB0342 or NMB0345 mutant at a dose of 5×10^4 CFU. The ratio of mutant to wild-type bacteria in the blood was determined at 21 hrs post-inoculation. The error bars represent the standard deviation of the mean.

N. meningitidis (Vogel and Frosch, 1999) (Kahler and Stephens, 1998) (Estabrook *et al.*, 1997) (Geoffroy *et al.*, 2003). In addition to the contribution of the polysialic acid serogroup B capsule to the serum resistance, the sialylation of LPS enhances the resistance of *N. meningitidis* to complement-mediated lysis and opsonophagocytosis by neutrophils (Estabrook *et al.*, 1997) (Kahler and Stephens, 1998) (Estabrook *et al.*, 1992). As the NMB0342 and NMB0345 mutants display reduced serum-resistance compared to the wild-type, C311^{+}, the LPS profile of each strain was compared with that of the wild-type. The LPS profiles of the mutant strains were found to be identical to the wild-type with the exception of a slight reduction in the intensity of a low molecular weight band in the NMB0342 mutant (Fig. 4.4). Lower molecular weight bands may represent truncated glyco-forms resulting from phase variation in LPS biosynthesis genes (Berrington *et al.*, 2002). LPS extractions and gel electrophoresis were performed by Nick West.

4.5 Capsulation of the NMB0342 and NMB0345 mutants

The polysaccharide capsule of *N. meningitidis* is required for serum-resistance (Kahler *et al.*, 1998; Kahler and Stephens, 1998). Phase variation events resulting in differences in capsulation can have a significant effect on the virulence of the bacterium (Hammerschmidt *et al.*, 1996) (Frosch *et al.*, 1989) (Kahler *et al*, 1998) (Mackinnon *et al.*, 1993). As both the NMB0342 and NMB0345 mutants are highly sensitive to serum killing, the capsulation status of the NMB0342 and NMB0345 mutants was established by performing a whole cell ELISA using a murine monoclonal *N. meningitidis* serogroup B anti-capsular antibody (Murex).

Fig. 4.4 LPS profile of the NMB0342 and NMB0345 mutants



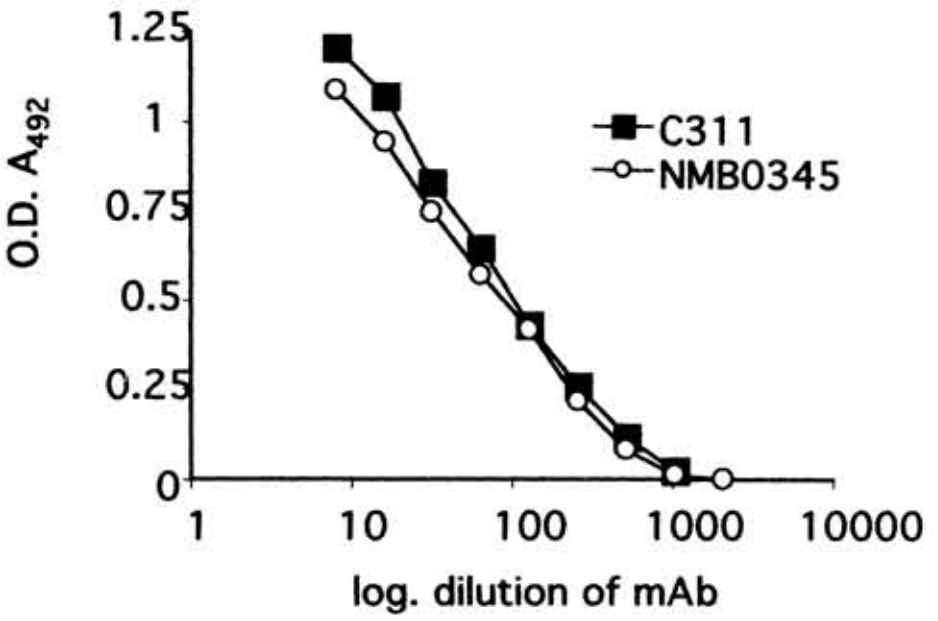
LPS prepared from wild-type strain C311$^+$, the NMB0342 and NMB0345 mutants, and the complemented (C) NMB0342 mutant was run on a tricine gel and silver-stained. In the NMB0342 mutant, a minor band (indicated by an arrow) is slightly reduced in its intensity compared to the wild-type.

The results of the ELISA indicate that capsulation of the NMB0342 (data not shown) and NMB0345 mutants is similar to that of the wild-type strain, $C311^+$ (Fig. 4.5).

4.6 Serum-sensitivity of the NMB0345 mutant in a capsule-deficient background

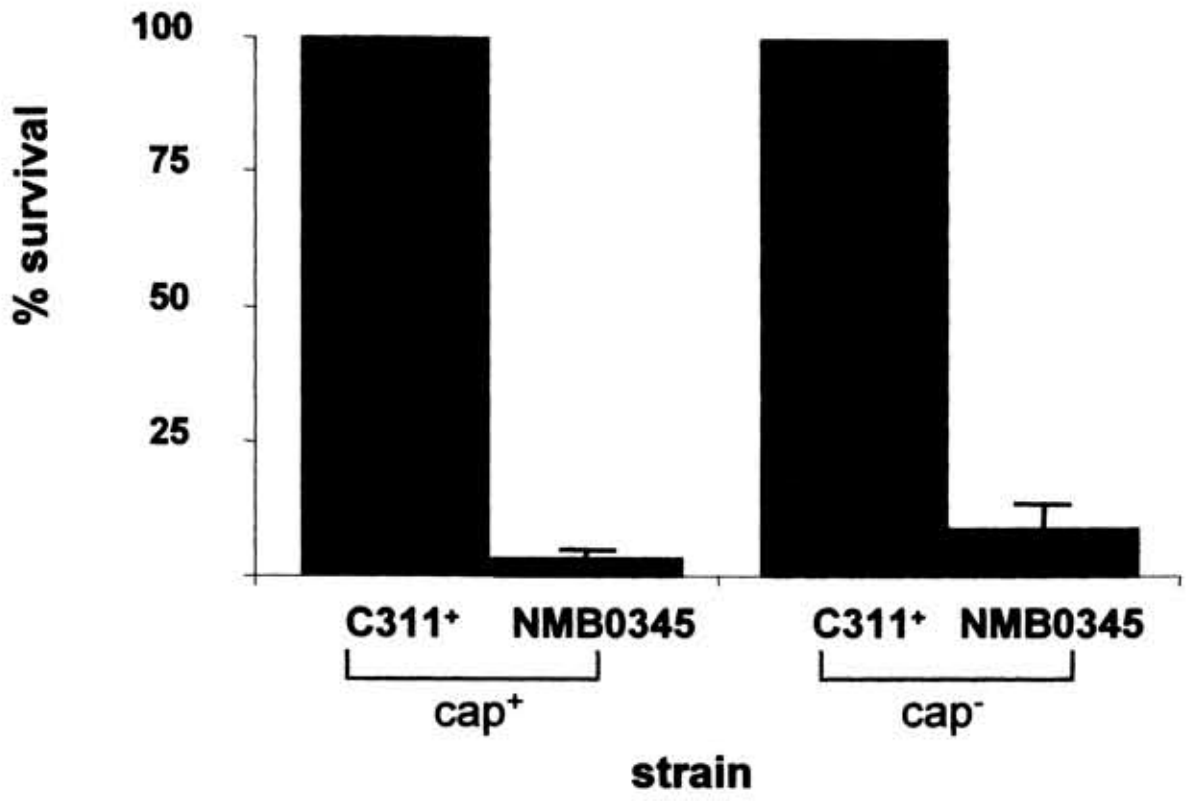
In order to determine whether the reduced survival of the NMB0345 mutant in whole blood and normal human serum results from a defect in capsule biosynthesis that is not detected by whole cell ELISA, capsule-negative $C311^+$ and NMB0345 strains were constructed. The capsule-deficient $C311^+$ and NMB0345 mutant strains were constructed by transforming the respective strain with the plasmid pIP10 (Virji *et al*, Mol Micro 1995). pIP10 consists of pUC18 harbouring a copy of the *siaD* gene containing a deletion and the *ermC* gene which encodes erythromycin resistance. Transformants were selected on BHI media containing erythromycin. The survival of these strains in normal human serum was analyzed. Results of this experiment demonstrate that the difference in survival between the non-encapsulated $C311^+$ and NMB0345 strains is similar to the difference in survival between the capsulated strains (Fig. 4.6). The number of CFU of non-encapsulated NMB0345 mutant is approximately 10% of the number of CFU of wild-type strain $C311^+$ recovered. Therefore, the decreased survival of the NMB0345 mutant in normal human serum compared to the wild-type strain is unlikely to be due to alterations in capsule expression.

Fig. 4.5 Analysis of the capsulation of the NMB0345 mutant by ELISA



Capsulation of wild-type strain C311^{+} and the NMB0345 mutant was analyzed by performing a whole cell ELISA using a murine monoclonal *N. meningitidis* serogroup B anti-capsular antibody (Murex).

Fig. 4.6 Effect of capsulation on the serum-sensitivity of the NMB0345 mutant



10^4 CFU of capsulated or non-encapsulated strains of either wild-type, C311$^+$ or NMB0345 mutant were inoculated into a 1:2 dilution of normal human serum in a 96-well microtitre plate. Aliquots (10 μl) from each well were plated to BHI agar after 60 minutes (t_{60}) incubation. Survival of the strains is represented as a percentage of wild-type recovered. No killing of the strains was observed using heat-treated NHS.

4.7 Adherence of the NMB0342 and NMB0345 mutants to epithelial cells

Results obtained from the infant rat model of infection, whole blood assays, and serum-sensitivity assays, confirm that the NMB0342 and NMB0345 genes are required for bloodstream infection. In order to establish whether the NMB0342 and NMB0345 genes are involved at another stage of pathogenesis, the ability of the NMB0342 and NMB0345 mutants to adhere to cultured human epithelial cells was investigated.

Chang epithelial cells (conjunctival cell line), which are considered to be derived from respiratory tissue, have been used extensively to study the interaction of *N. meningitidis* with epithelial cells (Virji *et al.*, 1992; Virji *et al.*, 1993; Virji *et al.*, 1995). Monolayers of approximately 10^5 Chang cells were grown in wells of a 24-well microtitre plate and infected with approximately 10^8 CFU of either strain C311$^+$, the NMB0342 mutant, or the NMB0345 mutant. Bacterial association with epithelial cells was determined by washing to remove non-adherent bacteria, followed by lysis of the epithelial cells with saponin and plating for enumeration of viable bacteria. In three experiments, NMB0342 was recovered at similar levels to the wild-type (Fig. 4.7, Panels A, B, and D), while in a single experiment, the NMB0342 mutant was recovered at only 43% of wild-type (Fig. 4.7, Panel C). Four independent experiments demonstrated that the NMB0345 mutant has reduced adherence to Chang epithelial cells compared to the wild-type strain (Fig. 4.7). Overall, the NMB0345 mutant was recovered at 34.2% of wild-type (standard deviation 11.5) in an average of four experiments.

In order to ensure that the reduction in numbers of mutant bacteria recovered compared to the wild-type bacteria is not due to decreased survival in 1% saponin, the sensitivity of the three strains to saponin was tested. 1×10^5 CFU of each strain was inoculated into 1% saponin in PBS in a 96-well microtitre plate in triplicate. Following 30 minutes' and 60 minutes' incubation dilutions were plated to BHI agar for enumeration. No difference in survival was seen for either mutant compared to the wild-type (Appendix F).

Fig. 4.7 **Adherence of the NMB0342 and NMB0345 mutants to cultured Chang epithelial cells**

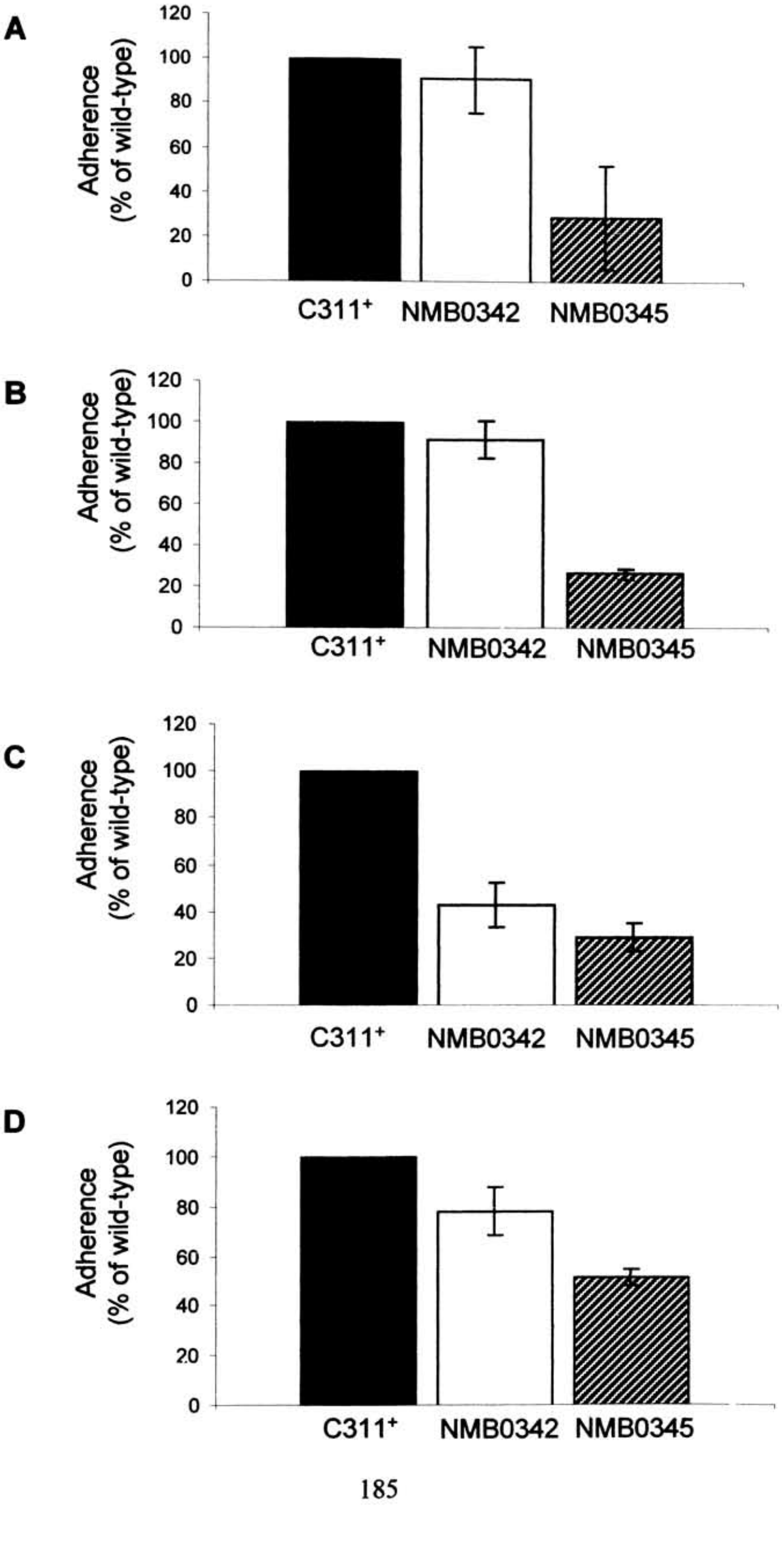


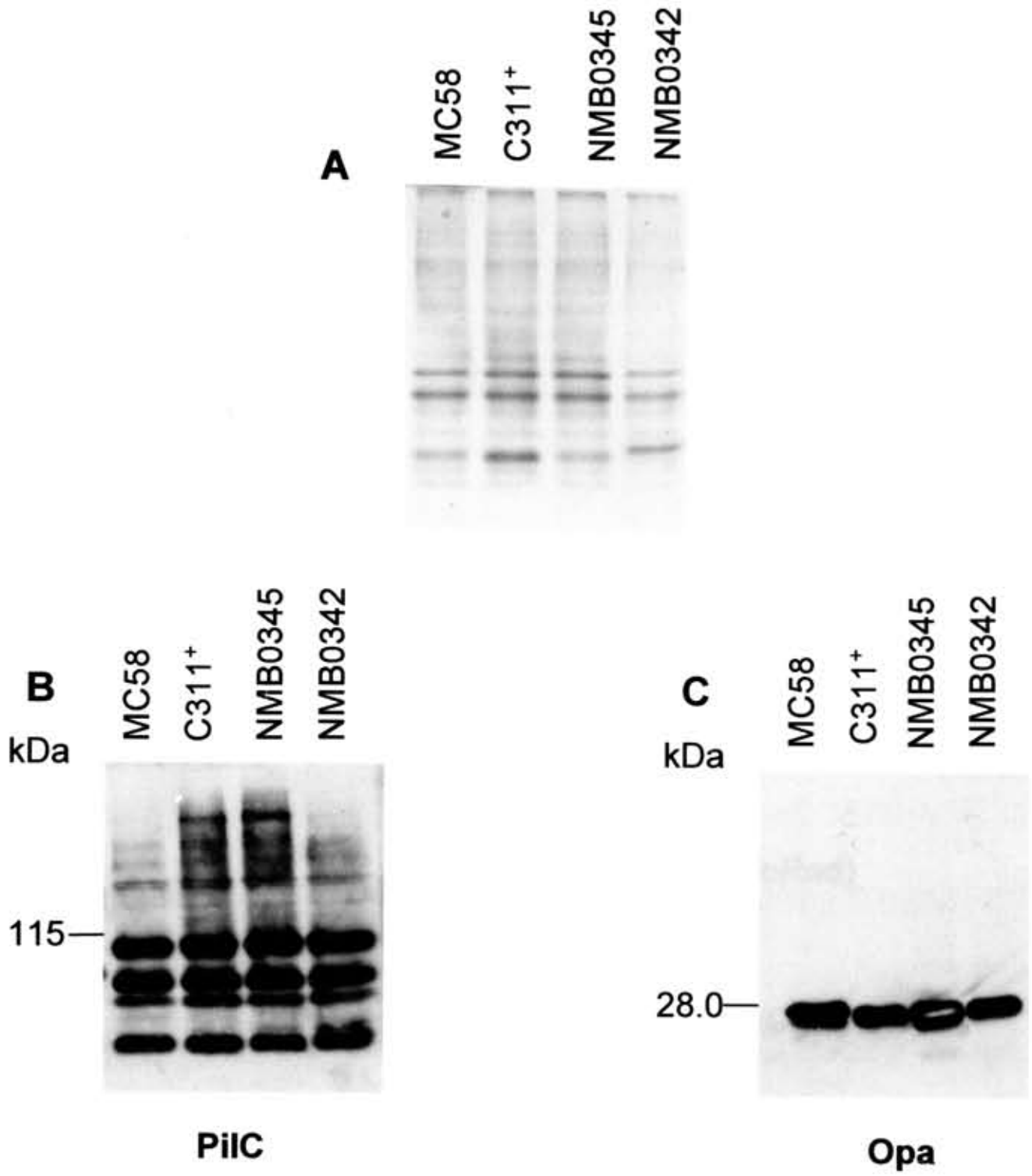
Fig. 4.7 **Adherence of the NMB0342 and NMB0345 mutants to cultured Chang epithelial cells**

10^8 CFU of either C311$^+$, the NMB0342 mutant, or the NMB0345 mutant were used to infect monolayers of approximately 10^5 Chang cells grown in a 24 well microtitre plate. Each strain was used to infect three wells. Following incubation for three hours at 37°C with 5% CO_2, the bacterial suspension was removed from each well and the wells washed three times. Chang cells were lysed with 1% saponin in PBS and five 10 µl aliquots from each well were plated to BHI agar. The number of bacteria recovered for each mutant is given as % of wild-type (C311$^+$), with wild-type represented as 100%. The results of four independent assays are shown in panels A, B, C, and D. Error bars represent the standard deviation.

4.8 Expression of major adhesins by the NMB0342 and NMB0345 mutants

To establish whether the reduction in adherence to epithelial cells by the NMB0342 and NMB0345 mutants can be attributed to alterations in the expression of known adhesins such as pili and Opa proteins, Western blot analysis was performed to compare the expression of these adhesins in each of the mutants with the wild-type strain, C311^{+} (Fig. 4.8). Using murine monoclonal antibodies (Opa, PilC, SM1), the banding patterns of the antibodies to the mutants were identical to that of the wild-type for each of the proteins tested with the exception of the banding pattern of the NMB0342 mutant detected by the SM1 antibody which differed from that of the wild-type. The original STM mutants, derived from single colonies, were used for testing all adhesins. Therefore, the difference observed in the banding pattern of the NMB0342 mutant detected by the SM1 antibody may be due to a phase variation event in this colony. In order to address this possibility, genomic DNA was isolated from this mutant and used to transform the wild-type strain C311^{+} and transformants selected on kanamycin. Stocks grown from three independent colonies of NMB0342 and four independent colonies of NMB0345 taken from these transformations were subsequently tested with the SM1 antibody. The banding pattern obtained for each of these colonies was found to be identical to that of the wild-type (Fig. 4.9). Transformants NMB0342.1 and NMB0345.1 tested with the SM1 antibody (Fig. 4.9) were the strains used in adhesion assays. It is therefore unlikely that the reduced adherence of the mutants is due to a mutation or phase variation in the genes encoding these adhesins.

Fig. 4.8 Expression of major adhesins by the NMB0342 and NMB0345 mutants



Whole cell lysates of MC58, C311$^+$, and the NMB0342 and NMB0345 mutants were analyzed by SDS-PAGE. The polyacrylamide gel was stained with Coomassie Blue (Panel A). Western blots of the same strains were prepared in order to analyze the expression of PilC and Opa. Pilus expression by the NMB0342 and NMB0345 mutants was analyzed using a murine monoclonal antibody raised against PilC (Panel B). Expression of Opa was analyzed using a murine monoclonal antibody raised against the Opa protein (Panel C).

Fig. 4.9 SM1 detection of Class I pilus expression by the NMB0342 and NMB0345 mutants



Loading order:

1 Pre-stained marker (BioRad)
2 C311^{+}
3 NMB0342.1
4 NMB0342.2
5 NMB0342.3
6 NMB0345.1
7 NMB0345.2
8 NMB0345.3
9 NMB0345.4

Whole cell lysates were prepared from strain C311^{+}, three independent colonies of the NMB0342 mutant, and four independent colonies of the NMB0345 mutant and were analysed by SDS-PAGE. Western blot analysis of the strains detected the expression of Class I pili using murine monoclonal antibody, SM1.

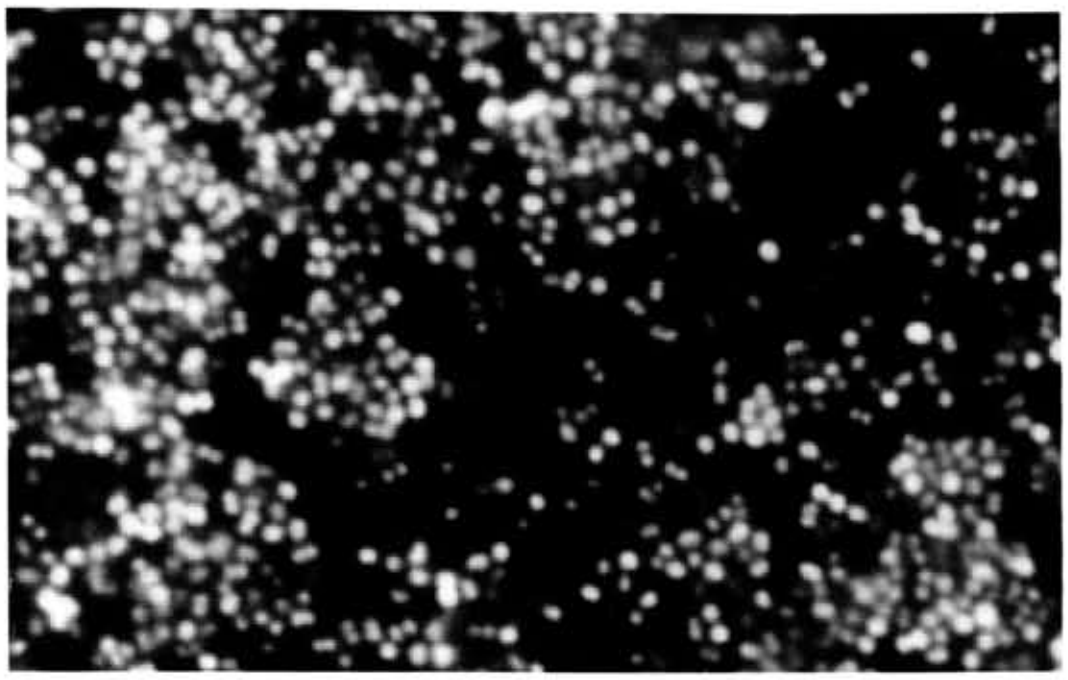
Invasion assays were also performed using the same transformants used for the adhesion assays. However, no colonies were recovered following lysis of the Chang cells with saponin. Subsequent comparison of the C311^{+} and MC58 strains revealed that while both strains were able to adhere to Chang cells, only strain MC58 was able to invade under the experimental conditions used.

4.9 Immunofluorescence and electron microscopy of the NMB0342 and NMB0345 mutants

NMB0342 is a homologue of the *S. flexneri* gene, *ispA*. Using immunoflurorescence microscopy, an *S. flexneri ispA* transposon mutant was shown to be defective in septation, forming long filamentous bacteria within epithelial cells (Mac Siomoin *et al.*, 1996) (Hong *et al.*, 1998). Adhesion assays demonstrated that the NMB0345 mutant is defective in the level of adherence to epithelial cells. Therefore, in order to further characterize these mutants, immunofluorescence and electron microscopy were used to compare the morphology of the NMB0342 and NMB0345 mutants with the wild-type strain, C311^{+} (Fig. 4.10). Strain C311^{+} and the NMB0342 and NMB0345 mutants were grown overnight on BHI agar and fixed in paraformaldehyde, then immunostained using an anti-PorA antibody as the primary antibody and Texas Red-conjugated secondary antibody. Images were taken using the x100 objective (Fig. 4.10c). No differences were seen in the size or morphology of cells of either mutant compared to the wild-type. The NMB0342 mutant bacteria were further analyzed by electron microscopy (Fig. 4.11). However, no differences were detected in the size or morphology of the NMB0342 mutant cells when compared to the wild-type.

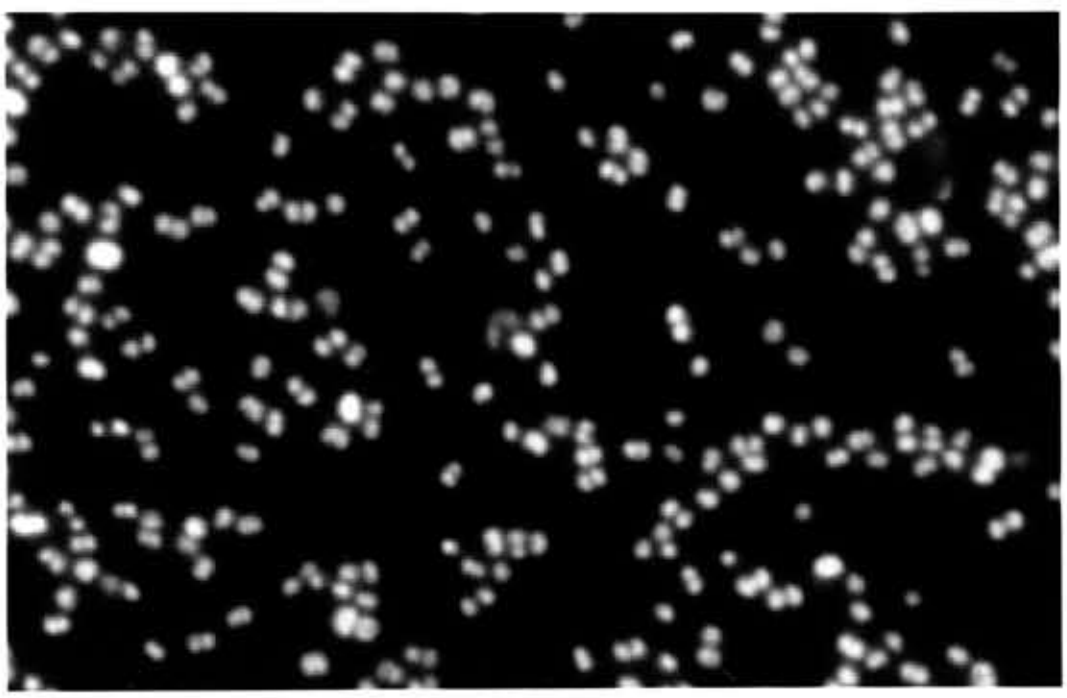
Fig. 4.10 a **Immunofluorescence microscopy of the NMB0342 and NMB0345 mutants**

i



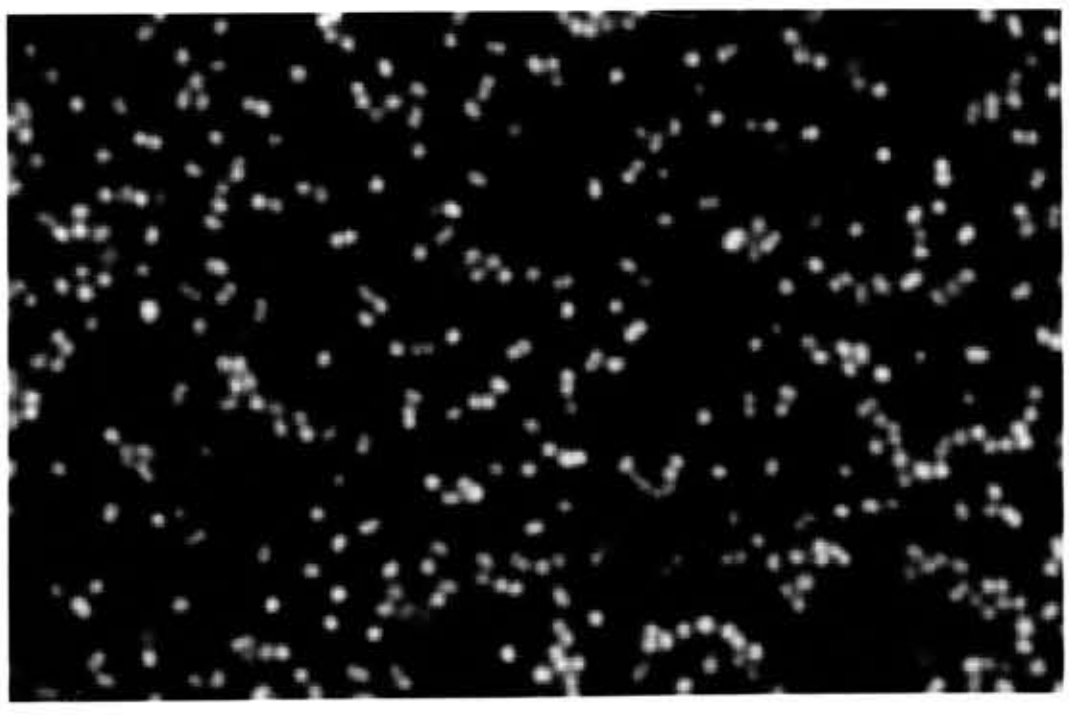
C311⁺ DAPI

ii



NMB0342 DAPI

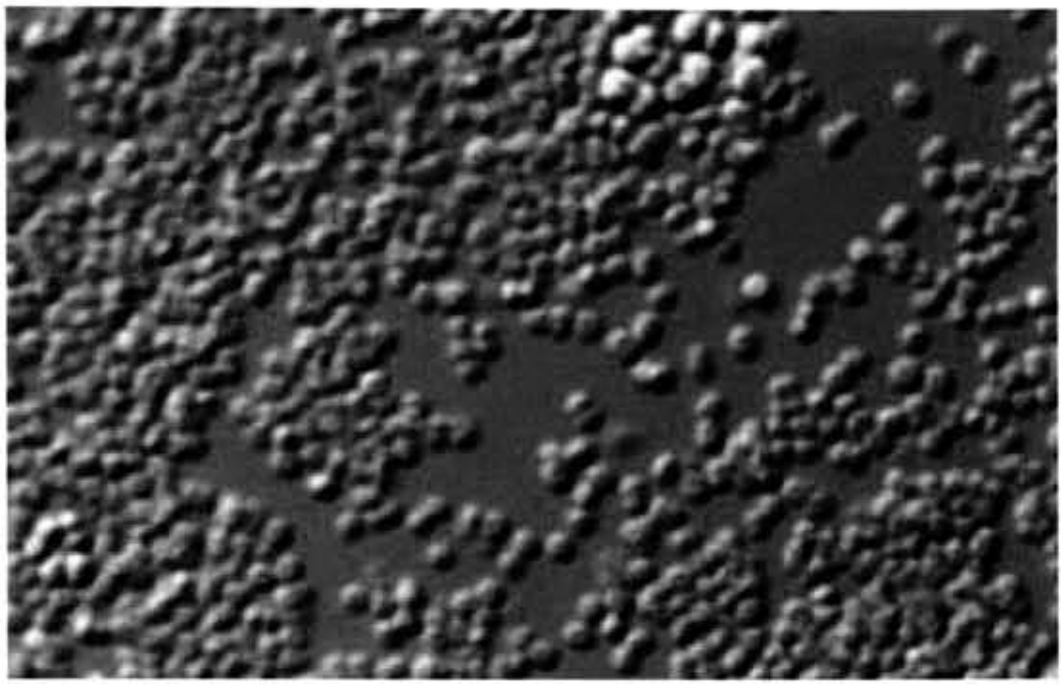
iii



NMB0345 DAPI

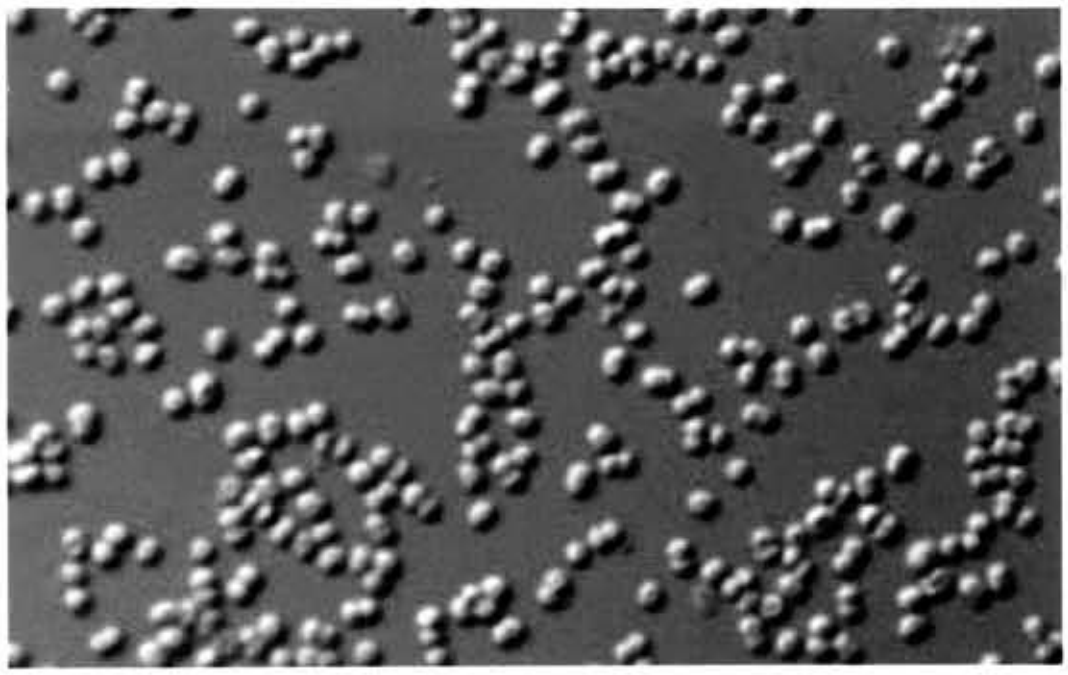
Fig. 4.10b **Immunofluorescence microscopy of the NMB0342 and NMB0345 mutants**

i



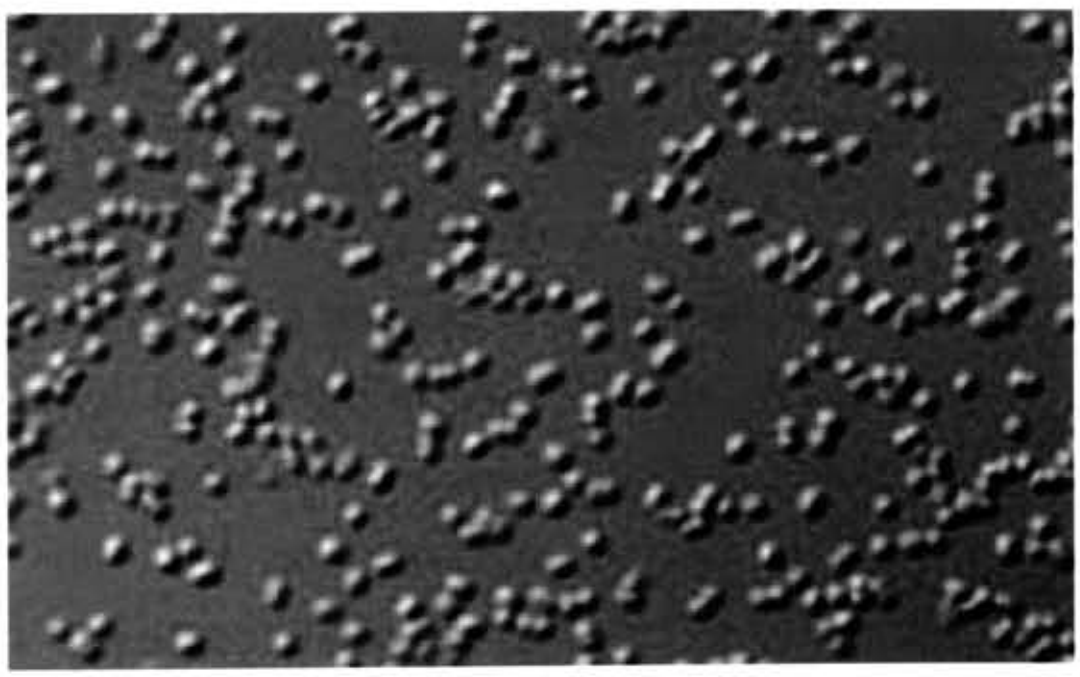
C311⁺ DIC

ii



0342 DIC

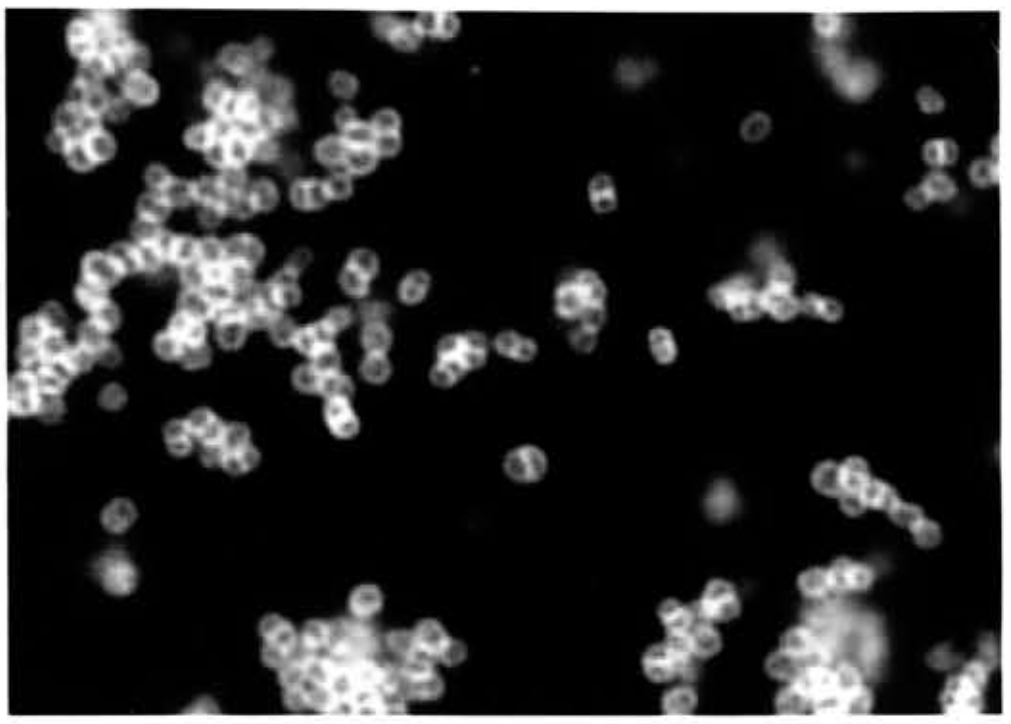
iii



0345 DIC

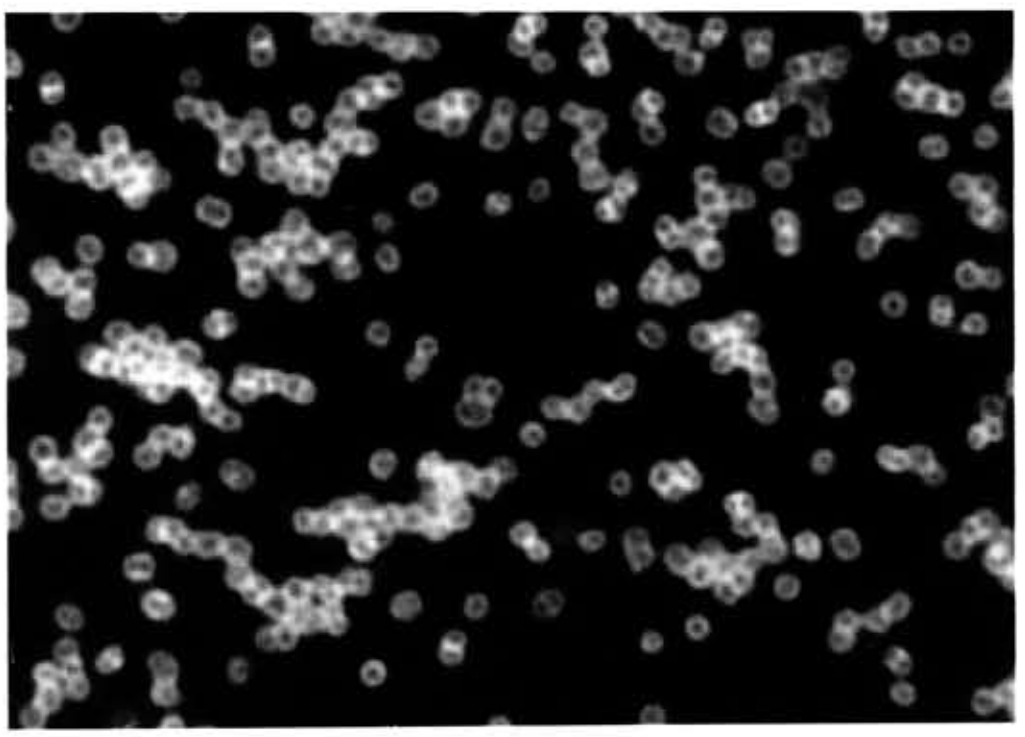
Fig. 4.10c **Immunofluorescence microscopy of the NMB0342 and NMB0345 mutants**

i



0342 PorA

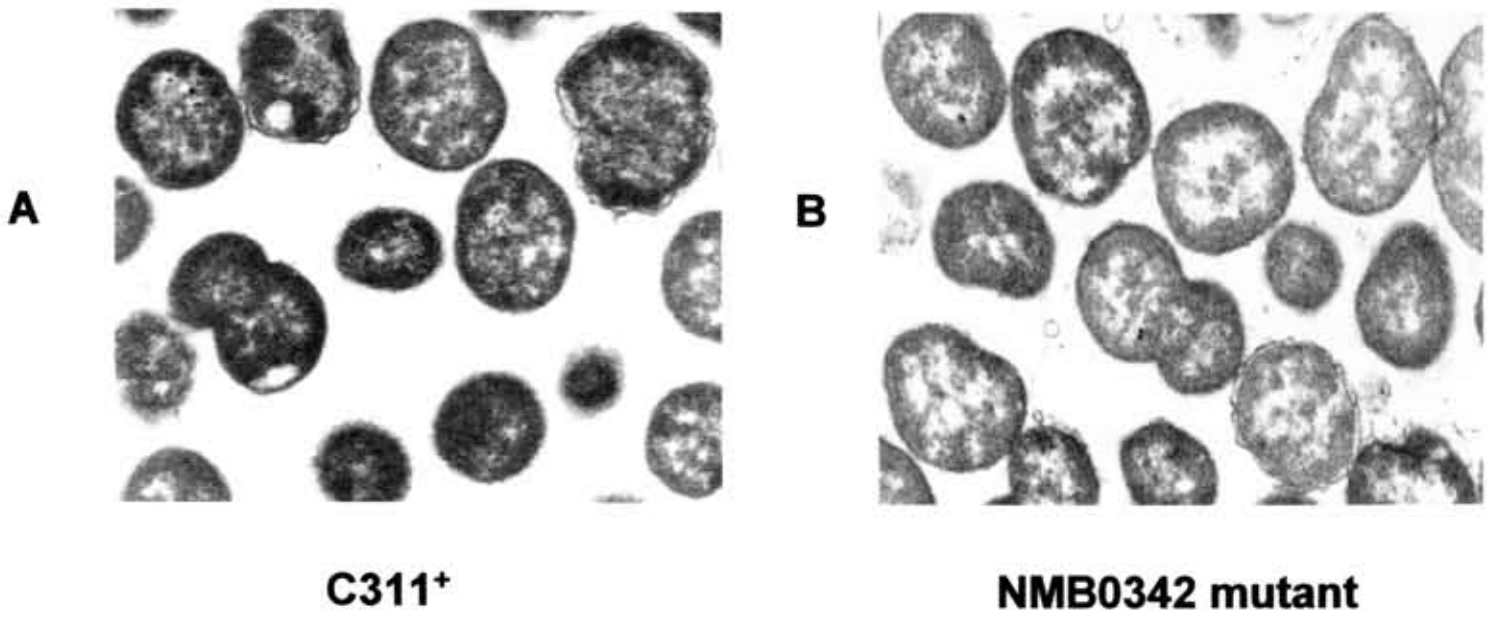
ii



0345 PorA

The wild-type strain, C311^{+}, the NMB0342 mutant, and the NMB0345 mutant were analyzed by immunofluorescence staining using DAPI (a), DIC (b), and an anti-PorA antibody (c) at a magnification of x100.

Fig. 4.11 Electron micrographs of wild-type strain C311$^+$ and the NMB0342 mutant



The wild-type strain, C311$^+$, and the NMB0342 mutant are shown at a magnification of 12,000x (Panels A and B) and 30,000x (Panels C and D). All electron microscopy was performed by David Ferguson, John Radcliffe Hospital, Oxford.

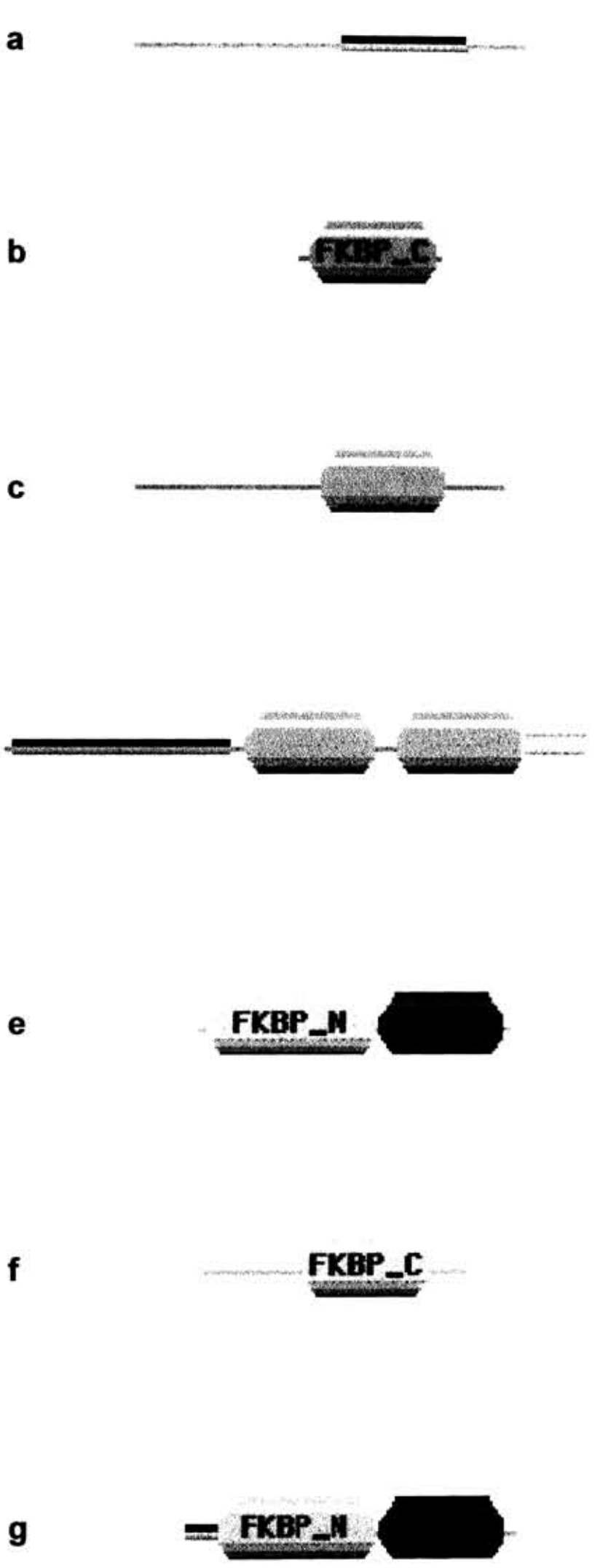
4.10 Biochemical function of the NMB0345 protein

The NMB0345 gene product is predicted to be a peptidyl prolyl *cis-trans* isomerase (PPIase) (Fig. 4.12a). PPIases are highly conserved proteins found in a wide range of prokaryotic and eukaryotic organisms (Kay, 1996). These enzymes are involved in protein folding and accelerate the *cis-trans* isomerization of proline peptide bonds (Xaa-Pro) where Xaa represents any natural amino acid (Fischer and Bang, 1985) (Lang *et al.*, 1987) (Bachinger, 1987). These enzymes have a conserved core region with additional N- and C- terminal extensions with sequence motifs of mainly unknown function. Three structurally distinct families of PPIases have been identified; i) cyclophilins which bind cyclosporin A (CsA), ii) FKBPs (FK506-binding protein) (11.8-158 kDa) which bind the drug FK506, and iii) parvulins (10 kDa). Cyclosporin A and FK506 are potent immunosuppressive agents that are highly effective in preventing organ transplant rejection in humans (Fischer *et al.*, 1989) (Schreiber, 1991), (Galat, 1993) (Rahfeld *et al.*, 1994).

A number of PPIases found in pathogenic microorganisms have been shown to be involved in virulence. These include the SurA protein of *Salmonella typhimurium*, *Lp*Mip of *Legionella pneumophila*, *Tc*Mip of *Trypanosoma cruzi*, and the Mip-like protein of *Chlamydia trachomatis*, (Lazar and Kolter, 1996) (Webb *et al.*, 2001) (Behrens *et al.*, 2001) (Fischer *et al.*, 1992) (Moro *et al.*, 1995). Although NMB0345 does not have a high level of sequence identity to these proteins, analysis of the published amino acid sequences of these proteins using the Pfam database (Protein families database of

alignments and HMMs, http://www.sanger.ac.uk/Software/Pfam) reveals that they all contain conserved PPIase domains (Fig. 4.12).

Fig. 4.12 **Location of conserved domains present in PPIases of pathogenic microorganisms**



NMB0345
288 residues
PPIase domain: 154-244

NMB0027
107 residues
FKBP C domain: 10-103

Cbf
273 residues
Rotamase domain: 138-228

SurA
428 residues
Rotamase domains:
178-272, 289-382

***Lp*Mip**
233 residues
FKBP N domain: 10-132
FKBP C domain: 136-227

***Tc*Mip**
196 residues
FKBP domain: 76-168

***Ct*Mip-like protein**
243 residues
FKBP N domain: 25-140
FKBP C domain: 142-232

Fig. 4.12 **Location of conserved domains present in PPIases of pathogenic microorganisms**

Published genome sequences of the NMB0345 (a) and NMB0027 (b), proteins of *N. meningitidis*, Cbf of *Campylobacter jejuni* (c), SurA of *Salmonella typhimurium* (d), LpMip of *Legionella pneumophila* (e), TcMip of *Trypanosoma cruzi* (f), and the Mip-like protein of *Chlamydia trachomatis* (g) were analyzed using the Pfam database (Protein families database of alignments and HMMs) at http://www.sanger.ac.uk/Software/Pfam/search.shtml and revealed the presence of conserved PPIase domains. Amino acid sequences were obtained from http://www.ncbi.nlm.nih.gov/ entrez/query.fcgi?db=Protein.

The Pfam database is a large collection of protein families and domains containing multiple alignments of protein families (Bateman *et al.*, 2004). NMB0346, which has 20% identity to NMB0345, is also predicted to be a PPIase. The NMB0027 gene, which has similarity to a human FKBP, has previously been cloned from *N. meningitidis* serogroup B strain M986 (Perry *et al.*, 1988) and the gene product expressed as a fusion protein in *E. coli* (Fig. 4.12b). The recombinant protein is an active PPIase that is inhibited by FK506 (Sampson and Gotschlich, 1992). However, no involvement in virulence has been reported for this protein.

Recombinant NMB0345 protein was assayed for PPIase activity *in vitro* using a method originally developed by Fischer and colleagues which relies on the ability of α–chymotrypsin to release p-nitroaniline from synthetic oligopeptide substrates only when the bond present on the N-terminal side of the proline residue is in the *trans* conformation (Fischer *et al.*, 1989). The release of *p*-nitroaniline is monitored at 390 nm and PPIase activity is measured as a change in absorbance per second (Abs/s) (Fig. 4.9.5). By taking into account the extinction coefficient of the starting oligopeptide substrate, this value is converted to μM per second (μM/s) representing turnover of substrate.

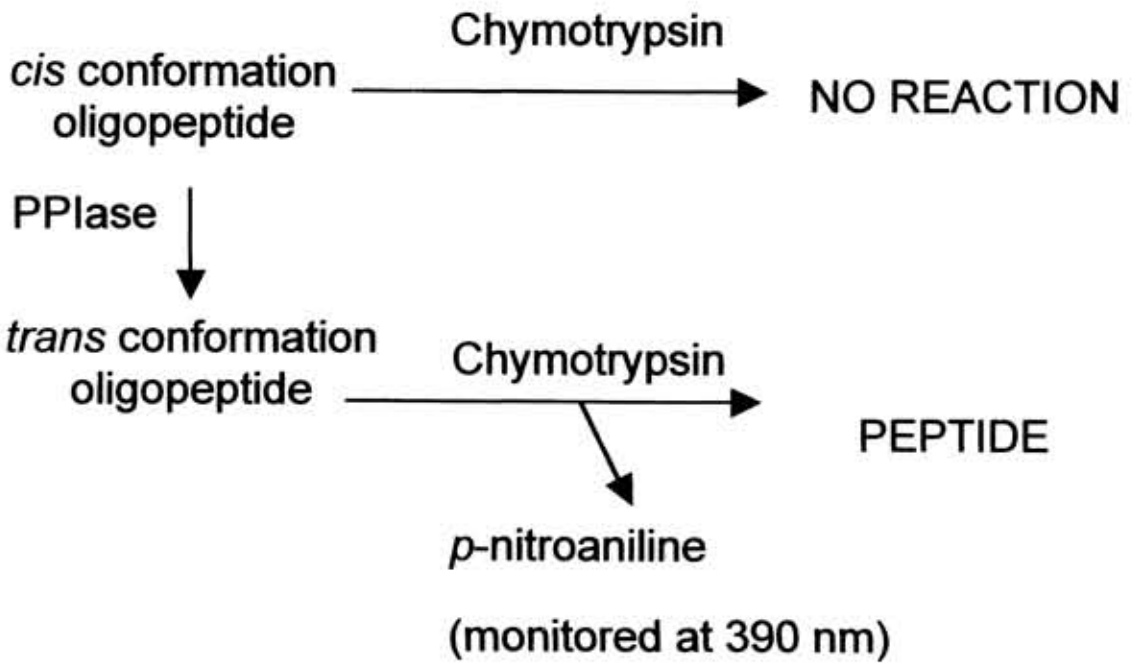


Fig. 4.13 Schematic diagram of the PPIase assay

In order to test the substrate specificity of the enzyme, assays were performed using two different model synthetic oligopeptide substrates. These substrates differ only in the amino acid preceding the proline residue (Xaa-Pro); Xaa representing either alanine or phenylalanine in the two substrates (Fig. 4.9.5). PPIases accelerate the isomerization of the Xaa-Pro bond from *cis* to *trans* conformation (Fischer *et al.*, 1989). FKBP-type PPIases usually have a strong preference for substrates with a large hydrophobic residue preceding the proline residue (Harrison and Stein, 1990). Therefore, the most commonly used substrates incorporate either a phenylalanine or alanine residue preceding the proline residue (Kay, 1996).

N-succinyl-Ala-**Phe**-Pro-Phe-*p*-nitroaniline

N-succinyl-Ala-**Ala**-Pro-Phe-*p*-nitroaniline

Fig. 4.9.5 Oligopeptide substrates used for analysis of NMB0345 PPIase activity

Purification of recombinant NMB0345 protein is described in the following chapter. No PPIase activity for the NMB0345 protein was observed using the 'Phe' substrate (Table 4.1a). PPIase activity was observed with the 'Ala' substrate when compared to the control reactions in which no NMB0345 protein was added (Table 4.1b). This activity was inhibited by the addition of FK506 to the assay to a final concentration of 500 nM and gave values similar to those obtained for reactions performed in the absence of NMB0345. The PPIase activity of FKBPs (FK506-binding proteins) is known to be inhibited by FK506 (Siekierka *et al.*, 1990) (Tropschug *et al.*, 1990).

	Reaction	μM/s
1	phenylalanine substrate	0.82
2	phenylalanine substrate + NMB0345	0.63

Table 4.1a **PPIase assay of recombinant NMB0345 protein using oligopeptide substrate N-succinyl-Ala-Phe-Pro-Phe-*p*-nitroaniline**

Two reactions were performed using the phenylalanine substrate N-succinyl-Ala-Phe-Pro-Phe-*p*-nitroaniline. 1) phenylalanine substrate + chymotrypsin in the absence of NMB0345 protein. 2) phenylalanine substrate + NMB0345 protein + chymotrypsin. Each reaction was performed in triplicate.

	Reaction	μM/s
1	alanine substrate	1.36
2	alanine substrate + NMB0345	1.79
3	alanine substrate + NMB0345 + FK506	1.66
4	alanine substrate + FK506	1.25

Table 4.1b **PPIase assay of recombinant NMB0345 protein using oligopeptide substrate N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline**

Four types of reactions were performed using the alanine substrate N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline. Each reaction was performed in triplicate. 1) Alanine substrate + chymotrypsin in the absence of NMB0345 protein. 2) Alanine substrate + NMB0345 + chymotrypsin. 3) Alanine substrate + NMB0345 protein + the inhibitor FK506. 4) Alanine substrate + FK506.

Discussion

Analyses of the survival of the NMB0342 and NMB0345 mutants in whole blood, serum, and C3-deficient mice confirm that the attenuation of the NMB0345 mutant in the infant rat model of septicaemia is a largely a result of a lowered resistance to complement-mediated killing. As only a slight increase in the C.I. of the NMB0342 mutant is observed in C3-deficient mice compared to wild-type mice, it is likely that the attenuation of this mutant in the infant rat model results from a defect other than increased sensitivity to complement-mediated killing. The NMB0342 and NMB0345 gene products have not previously been recognized as being factors required for meningococcal serum resistance. So far, capsule and LPS expression have been shown to be the major mediators of serum resistance of *N. meningitidis* (Geoffroy *et al.*, 2003) (Frosch *et al.*, 1989). In a recent STM screen of a library of serogroup A *N. meningitidis* transposon mutants in human serum, all mutants identified were found to be defective in either capsule or LPS biosynthesis (Geoffroy *et al.*, 2003). Previous studies have demonstrated the importance of the capsule and LPS as the main virulence determinants required for meningococcal serum resistance (Mackinnon *et al.*, 1993) (Kahler *et al.*, 1998). The role of the serogroup B capsule and sialylation of LPS in inhibiting the bactericidal activity of normal human serum has been demonstrated by the increased sensitivity of capsule-deficient strains with mutations in genes required for capsule biosynthesis compared to wild-type in a 1:2 dilution of normal human serum (Kahler *et al.*, 1998).

The increased serum-sensitivity of the NMB0342 and NMB0345 mutants compared to the wild-type bacterium highlights a limitation of the STM methodology in that a sub-set of mutants, (aside from those with transposon insertion in essential genes) is excluded by the conditions of the assays described by Geoffroy and colleagues (Geoffroy *et al.*, 2003). These mutants may only be identified through focused studies on single mutants using physiologically relevant serum concentrations including neat or 1:2 dilutions of serum. Geoffroy and colleagues used a single defined concentration of serum (25%) in their assays to screen pools of tagged mutants. Mutants absent from output pools following five hours incubation were defined as being serum-sensitive. However, the lowered survival of the NMB0342 and NMB0345 mutants was only detected using a 1:2 dilution of serum. The results of this study indicate that individual analysis of mutants combined with the use of a serial dilution of serum including high concentrations of serum (neat or 50% serum) and plating at more than one time point may lead to the identification of further virulence determinants critical for meningococcal serum resistance. The use of more dilute concentrations of serum in assays is likely to have led to the classification of the NMB0345 mutant and possibly the NMB0342 mutant as 'serum-resistant'. However, the attenuation of these mutants in the infant rat clearly demonstrates that under *in vivo* conditions, this level of serum-sensitivity is significant.

The source of whole blood or serum can contribute to the level of bactericidal activity observed in *in vitro* assays as individuals vary in whether or not their blood has bactericidal activity against *N. meningitidis*. For the purpose of these experiments, a donor with bactericidal activity was selected in order to allow the relative survival of

mutant strains compared to that of the wild-type strain, C311$^+$, to be examined. For the whole blood assay, blood is collected just prior to use in experiments. Both experiments were performed within two hours of collection of blood. Although blood collected from the same donor was used in replicate experiments, blood collected on different days was found to have differing levels of bactericidal activity. For example, while reduced numbers of NMB0345 mutant bacteria were recovered compared to the wild-type strain at t_{45} in two separate experiments, reduced numbers of NMB0342 mutant were recovered at t_{45} in one experiment whereas in another experiment, this mutant was recovered at similar levels to the wild-type at t_{45}. The difference between the survival of the NMB0342 and NMB0345 mutants compared to the wild-type strain, C311$^+$ suggests that the NMB0345 mutant may be more susceptible to complement-mediated killing than the NMB0342 mutant. Differences between the bactericidal activity of blood collected from different donors have been observed in previous studies (Heist *et al.,* 1922). Therefore, a single donor was used in the present study. However, the use of pooled human serum may provide a more accurate reflection of the bactericidal activity of human whole blood.

To assess the contribution of susceptibility to complement-mediated killing to the attenuation of the NMB0342 and NMB0345 mutants *in vivo*, experiments were performed to compare the survival of the NMB0342 and NMB0345 mutants in wild-type and C3-deficient mice. The results of these experiments demonstrate that the NMB0342 and NMB0345 mutants are more sensitive to complement-mediated killing than the wild-type strain, C311$^+$, and indicate that the attenuation of the NMB0342 mutant is not wholly due to increased serum-sensitivity. As mutation of NMB0342 is likely to have

pleiotropic effects and the basis of the attenuation of the NMB0342 mutant is unclear, the NMB0345 mutant was selected for further study.

Examination of the capsulation and LPS profiles of the NMB0342 and NMB0345 mutants detected no defects in expression of capsule or LPS. Therefore, further examination of the possible functions of the NMB0342 and NMB0345 gene products is required to establish the basis of the serum-sensitivity of each of these mutants. Gram-negative bacteria possess a number of outer membrane proteins that are required for serum resistance. Examples of these proteins include the YadA protein of *Yersinia enterocolitica*, the DsrA protein of *H. ducreyi*, and the UspA1 and UspA2 proteins of *Moraxella catarrhalis* (Roggenkamp *et al.*, 1996) (Cole *et al.*, 2002) (Lafontaine *et al.*, 2000). YadA binds to complement factor H, leading to the degradation of C3b deposited on the cell surface preventing formation of the membrane attack complex (China *et al.*, 1993) (Pilz *et al.*, 1992). The mechanism by which the UspA1 and UspA2 proteins confer serum-resistance to *M. catarrhalis* is still under investigation. Interestingly, each of these proteins has also been shown to play an important role in adhesion.

A possible mechanism by which NMB0345 may confer serum resistance to the bacterium is through direct binding of either the NMB0345 gene product itself, or of a protein targeted as a substrate of NMB0345, to components of the complement pathway. A well-characterized protein that enhances the serum-resistance of bacteria by inactivating complement is the C5a peptidase of *S. pyogenes*. The C5a peptidase specifically degrades the complement factor C5a which is generated by proteolytic cleavage of C5

during complement activation (Webb *et al.*, 2001) (Chen and Cleary, 1990) (Cleary *et al.*, 1992). Inactivation of C5a peptidase significantly attenuates the bacterium and increases dissemination of the bacterium to the spleen in a murine model of infection (Ji *et al.*, 1996) (Husmann *et al.*, 1997) (Ji *et al.*, 1997). As the NMB0345 mutant is sensitive to complement-mediated lysis, proteases with functions similar to that of the C5a peptidase of *S. pyogenes* may be possible targets of the NMB0345 PPIase. Binding of the YadA adhesin of *Y. enterocolitica* to the serum complement-inhibiting factor H, is thought to be responsible for conferring serum resistance to the bacterium (China *et al.*, 1993). The Por1A outer membrane protein of *N. gonorrhoeae* has also been shown to inhibit complement activation by binding to Factor H, the key regulatory protein of the alternative pathway of complement, via the surface-exposed region of the fifth loop of the porin. A number of *N. gonorrhoeae* strains expressing Por1A can also suppress the classical complement pathway by binding to C4b-binding protein (C4 bp). Some serum-resistant gonococci strains expressing Por1B have also been shown to bind C4 bp (Ram *et al.*, 1999; Ram *et al.*, 2001) (Ram *et al.*, 1998a; Ram *et al.*, 1998b). A potential function of the NMB0345 gene product may be to alter the conformation of host proteins such as factors of the complement pathway thereby enhancing the serum resistance of the bacterium. The complement pathway consists of approximately 20 interacting soluble serum proteins that are activated by proteolytic cleavage leading to a cascade of complement activation (Medzhitov and Janeway, 2000). In order to be cleaved however, these proteins must be in the correct conformation (Neurath and Walsh, 1976). Therefore, a possible mechanism for prevention of complement activation may be through the modification and subsequent inactivation of complement factors by PPIases.

This study has shown that the NMB0345 mutant has reduced adherence to Chang epithelial cells. The expression of a large number of *N. meningitidis* genes, including those required for the expression of several known virulence determinants, is phase variable, mediated via reversible changes in the length of simple DNA repeats (Snyder *et al.*, 2001). Therefore, the expression of major adhesins of *N. meningitidis* known to undergo phase variation was examined. However, Western blot analysis may not be sufficiently sensitive to detect changes in the secondary and tertiary structure of adhesins. Furthermore, only a few adhesins (pili and Opa) were tested in this way. Therefore, the possibility remains that the reduced adherence of the NMB0345 mutant may be due to a phase variation event. In order to confirm the observed adhesion defect of the NMB0345 mutant, it will be necessary to either repeat the adhesion assays using multiple colonies of the NMB0345 mutant or to construct an independent NMB0345 mutant (remake the NMB0345 mutant by transforming strain C311^{+} with chromosomal DNA from the NMB0345 mutant) and evaluate its ability to adhere to Chang epithelial cells. The NMB0345 protein may play a role as an adhesin or be involved in the folding of a known or novel adhesin. A further possibility is that the NMB0345 protein may be required for binding to specific host cell extracellular matrix proteins such as vitronectin, which has been shown to be essential for the binding of *N. meningitidis* to epithelial cells (Unkmeir *et al.*, 2002).

PPIase activity has been demonstrated for proteins present in a number of pathogenic microorganisms including *S. typhimurium*, *L. pneumophila*, *T. cruzi*, and *C. trachomatis*. The SurA protein of *S. typhimurium* is a homologue of *E. coli* SurA (Fig. 4.12d). *E. coli*

surA or 'survival' gene was originally identified as a gene whose disruption leads to a reduction in stationary phase survival (Tormo *et al.*, 1990). An *S. typhimurium surA* mutant was found to be defective in its ability to adhere to and invade eukaryotic cells and was attenuated in a murine model of systemic infection. This strain has been proposed as an effective live oral vaccine (Sydenham *et al.*, 2000). NMB0345 and NMB0346 belong to same cluster of orthologous groups (COG) as the *surA* gene of *S. enterica* serovar Typhimurium C5 which encodes a PPIase (Lazar and Kolter, 1996). COGs are assembled by comparing protein sequences encoded in 43 complete genomes, representing 30 major phylogenetic lineages. Each COG consists of individual proteins or groups of paralogs from at least three lineages and therefore corresponds to an ancient conserved domain (http://www.ncbi.nlm.nih.gov/COG). The recent elucidation of the crystal structure of *E. coli* SurA has revealed that the protein has a bipartite structure and allowed identification of a core module implicated in membrane protein folding. This module contains a crevice that is thought to be involved in peptide-binding. The structure suggests a mechanism in which segments of polypeptide are sequentially or repetitively sequestered and released in the process of membrane protein-folding (Bitto and McKay, 2002).

*Lp*Mip (macrophage infectivity potentiator) is a 25 kDa protein present on the surface of the obligate intracellular bacterium, *L. pneumophila*, the causative agent of Legionnaire's disease (Helbig *et al.*, 2001) (Fig. 4.12e). Mip is an essential virulence factor required for efficient replication of *L. pneumophila* within human alveolar macrophages and protozoa, the natural host of *L. pneumophila* in aquatic environments (Cianciotto and

Fields, 1992). *Lp*Mip also contributes to the virulence of *L. pneumophila* in a guinea pig model of infection (Cianciotto *et al.*, 1990). The PPIase activity of *Lp*Mip has been demonstrated *in vitro*. This activity is effectively inhibited by FK506 and cyclosporin A (Fischer *et al.*, 1992) (Ludwig *et al.*, 1994). The recently determined crystal structure of *L. pneumophila* Mip has demonstrated that the protein exists as a dimer containing two PPIase active centers (Riboldi-Tunnicliffe *et al.*, 2001). The importance of the homodimeric structure and PPIase activity of *Lp*Mip in *L. pneumophila* virulence has been investigated by constructing *Lp*Mip mutants lacking the N-terminal half of the protein. Analyses of these mutants demonstrated that the N-terminal region and dimerization of the Mip protein are required for full virulence of *L. pneumophila* in the protozoan, *Acanthamoeba castellanii*, while PPIase activity was not found to be essential. In a guinea pig model of infection however, dimerization-deficient Mip mutants and mutants displaying very low levels of PPIase activity were found to be significantly attenuated (Kohler *et al.*, 2003). The results of these studies imply that *Lp*Mip may have different functions in eukaryotic cells and during animal infection. A recent study has demonstrated that the PPIase active site of *Lp*Mip is essential for efficient infection of eukaryotic cells. Monoclonal antibodies that bind specific epitopes of *Lp*Mip located within the active site of the enzyme were shown to successfully inhibit both *in vitro* PPIase activity and infection of *A. castellanii* (Helbig *et al.*, 2003).

*Tc*Mip is a virulence determinant of the protozoan, *T. cruzi*, the aetiological agent of Chagas' disease or American trypanosomiasis (Fig. 4.12f). The protein is thought to be involved in cell invasion. PPIase activity of *Tc*Mip and inhibition of PPIase activity by

the addition of FK506 has been demonstrated in *in vitro* PPIase assays. Elucidation of the crystal structure of *Tc*Mip has allowed comparison with the crystal structure of *Lp*Mip. Both *Lp*Mip and *Tc*Mip have a conserved PPIase domain and an exposed N-terminal helix (Pereira *et al.*, 2002) (Riboldi-Tunnicliffe *et al.*, 2001). The ability of *L. pneumophila* Mip to substitute effectively for *Tc*Mip in invasion assays suggests that these two proteins may have a common cellular target (Pereira *et al.*, 2002). Addition of recombinant *Tc*Mip to invasion assays has been shown to facilitate the infection of epithelial cells by *T. cruzi*. Furthermore, invasion was inhibited by the addition of FK506 or anti-*Tc*Mip antibodies raised in mice (Moro *et al.*, 1995). Pre-incubation of epithelial cells with recombinant *Tc*Mip (which was washed off prior to infection with *T. cruzi*) resulted in enhanced invasion by the parasites. This suggests that the target of *Tc*Mip activity may be located on the host cell membrane (Moro *et al.*, 1995).

Chlamydia trachomatis is an obligate intracellular bacterium that causes oculogenital infections. The 27 kDa Mip-like protein of *C. trachomatis* has sequence similarity to the Mip protein of *L. pneumophila* (Fig. 4.12g) and has been shown to have PPIase activity that is inhibited by FK506. Treatment of *C. trachomatis* with FK506 prior to infection of eukaryotic cells was found to reduce the infectivity of the organism suggesting that disruption of PPIase activity may block an early step(s) in the invasion process (Lundemose *et al.*, 1993).

A limitation of the PPIase assay is that in aqueous solution, the majority of oligopeptide substrate is in the *trans* conformation. However, a number of methods have been

developed to increase the proportion of *cis* isomer in the starting substrate and include dissolving the substrate in LiCl and trifluoroethanol (Kofron *et al.*, 1991). A further limitation is that the substrate concentrations required for maximal PPIase activity are higher than the solubility of the peptides (Kay, 1996). However, the difference in activity observed in reactions performed with and without recombinant NMB0345 protein in the presence of the N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline alanine substrate are comparable to the activities determined for other well-characterized PPIases such as *Lp*Mip and *Tc*Mip (Fischer *et al.*, 1992) (Moro *et al.*, 1995). Furthermore, the results of the assays performed in this study demonstrate that the PPIase activity of recombinant NMB0345 protein is inhibited by FK506, the immunosuppressive drug known to bind to and inhibit the activity of FKBP-type PPIases.

5 Assessment of recombinant NMB0345 protein as a vaccine candidate

Genetic characterization of the NMB0342–NMB0348 locus and analysis of mutants with insertions in genes of the locus has demonstrated that the NMB0342 and NMB0345 genes potentially play an important role in the pathogenesis of *N. meningitidis* infection. Both genes are required for the establishment of septicaemia in the infant rat as well as for survival in human whole blood and serum. In addition, the NMB0345 gene has an influence upon the adherence of the bacterium to Chang epithelial cells implicating a role in colonization. PCR and Southern analyses have demonstrated that both genes are conserved in all the pathogenic *Neisseria* spp. tested. Based on the contribution of these genes to virulence and their conservation in pathogenic isolates of *N. meningitidis*, the NMB0342 and NMB0345 gene products were considered as potential vaccine candidates.

This chapter describes the selection of NMB0345 for further study and evaluation of the NMB0345 gene product as a vaccine candidate. This protein was selected for the following reasons. First, Dense Alignment Surface (DAS) analysis, University of Stockholm, Sweden, http://www.sbc.su.se/~miklos/DAS/ (Cserzo *et al.*, 1997) predicted that that the NMB0345 protein has a single transmembrane sequence (the signal peptide sequence which is cleaved (Fig. 5.1, Panel A). Expression and purification of proteins with numerous transmembrane regions can be problematic due to their hydrophobic nature (Haris, 2001). NMB0342 has six transmembrane regions (Fig. 5.1, Panel B). Second, PSORT analysis, a program for the prediction of protein localization sites in

cells from amino acid sequence (http://psort.ims.u-tokyo.ac.jp/form.html) (Nakai and Kanehisa, 1991) indicated that NMB0345 encodes a potential lipoprotein. Two lipoproteins identified from the *N. meningitidis* serogroup B genome sequence, GNA33 and GNA1870, have been studied and shown to induce bactericidal antibodies in mice (Granoff *et al.*, 2001) (Masignani *et al.*, 2003). Lipidation is thought to enhance the immunogenicity of protective antigens (Baier *et al.*, 2000). Furthermore, comparison of the ability of lipidated and non-lipidated forms of the *Borrelia burgdorferi* protein OspA to induce a protective antibody response in mice indicate that the lipid attachment significantly enhances the immunogenicity of this protein (Erdile *et al.*, 1993). Finally, the *C. jejuni* homologue of NMB0345, Cbf, is a major antigenic peptide of this organism (Burucoa *et al.*, 1995).

Fig. 5.1 DAS™ analysis of NMB0342 and NMB0345 amino acid sequences

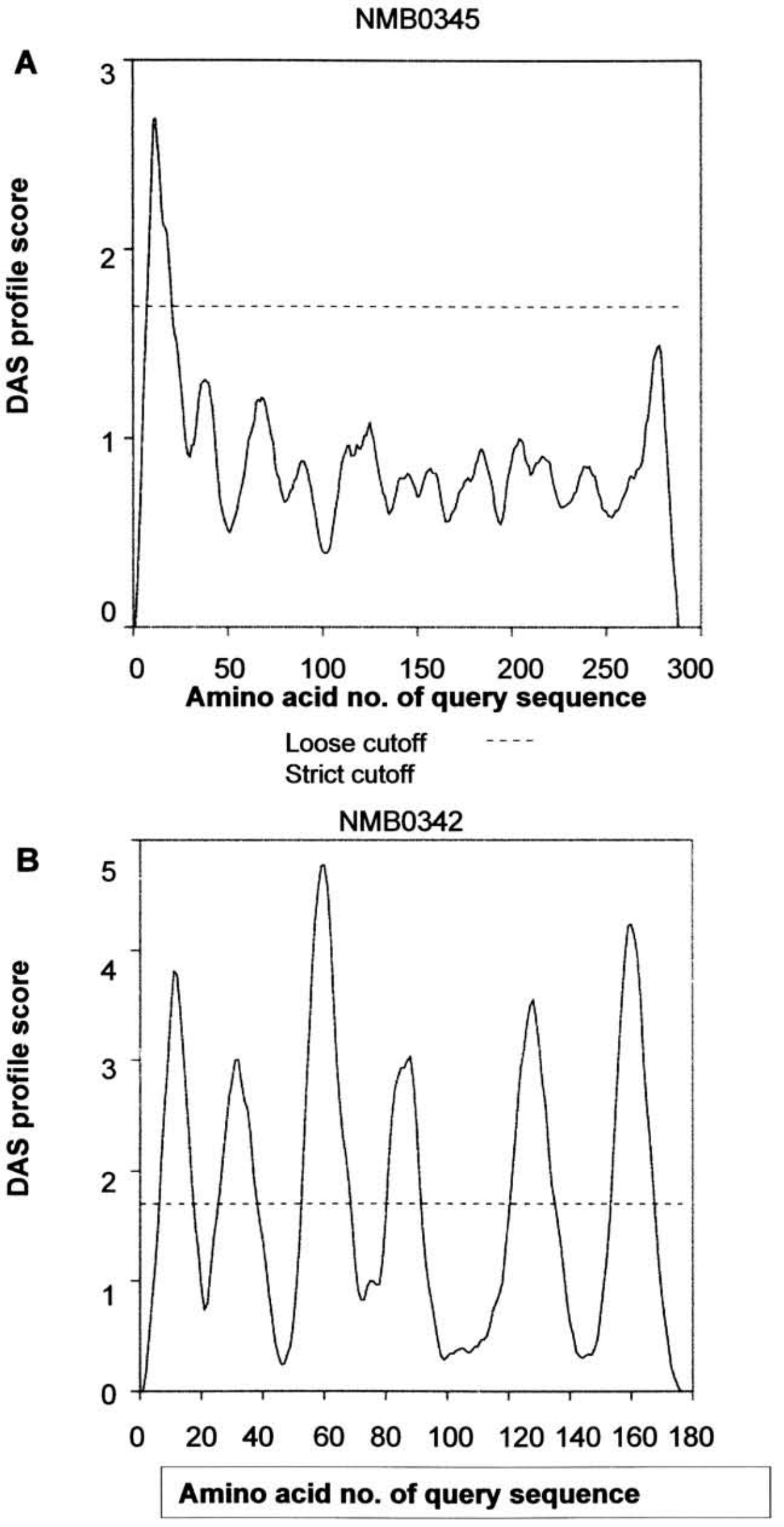


Fig. 5.1 'DAS' analysis of the NMB0342 and NMB0345 amino acid sequences

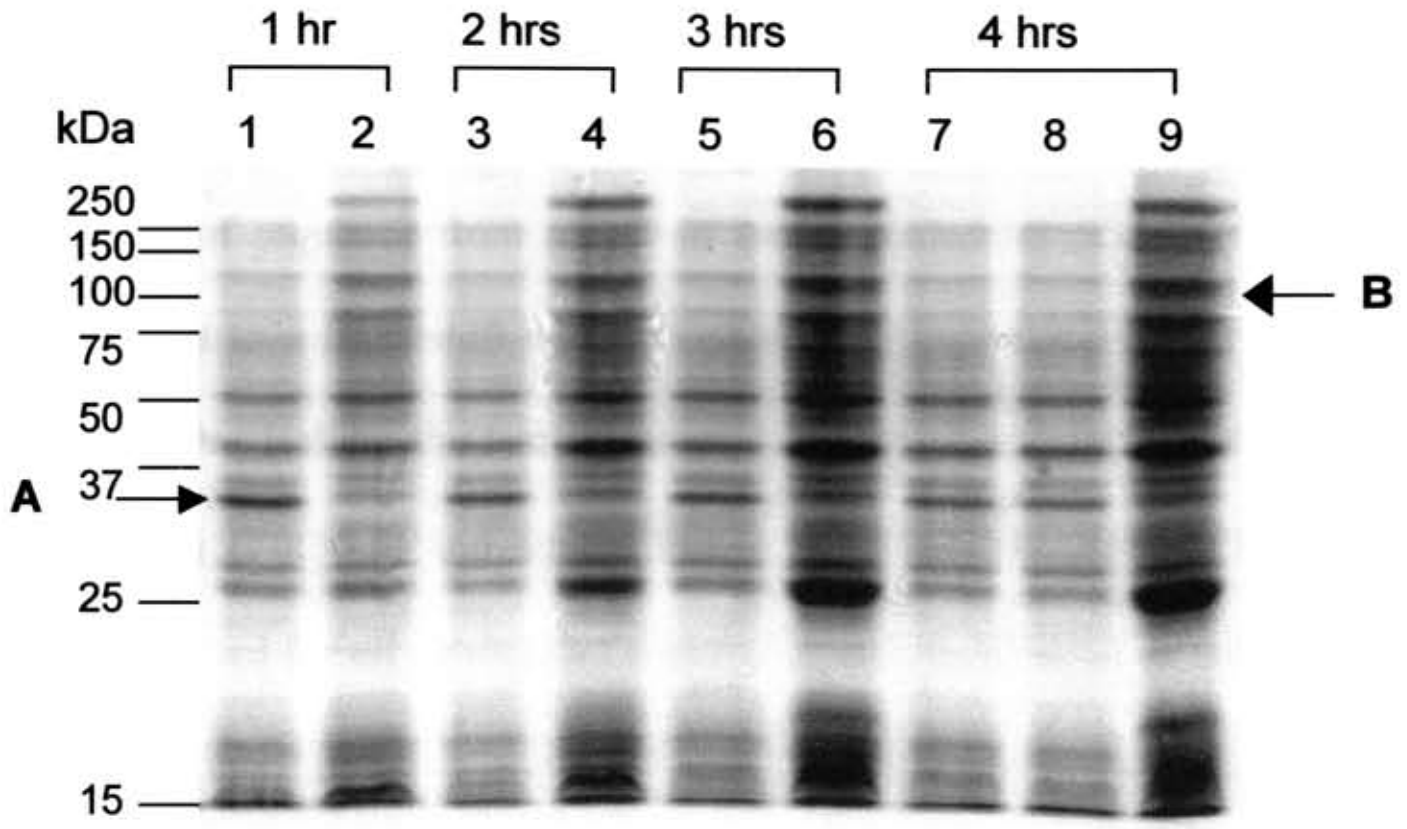
Predicted amino acid sequences of NMB0342 and NMB0345 were analyzed using the Dense Alignment Surface (DAS) server (http://www.sbc.su.se/~miklos/DAS/maindas.html). The NMB0342 protein is predicted to have six transmembrane segments (Panel B), while NMB0345 has a single transmembrane segment (Panel A). These curves are obtained by pairwise comparison of the proteins in a test set in an "each against the rest" fashion. The test set consists of 44 prokaryotic transmembrane proteins with known (experimentally determined) topologies (Czero *et al.*, 1997). Two cut-offs are indicated on the plots; the "strict" cutoff at 2.2 DAS score, and the "loose" cutoff at 1.7. The peak above 2.2 is informative in terms of the number of matching segments, while a peak above 1.7 gives the actual location of a transmembrane segment.

5.1 Cloning of NMB0345 and expression of the NMB0345 gene product in *E. coli*

To allow assessment of the NMB0345 protein as a vaccine candidate, the protein was expressed in *E. coli*. The entire open reading frame of NMB0345 was amplified by PCR from strain MC58 genomic DNA using oligonucleotide primers NG337 and NG338 that were designed according to the published MC58 genome sequence (Pizza *et al.*, 2000) MC58 genomic DNA was used in preference to strain C311^{+} as the complete sequence of the gene is known for this strain. BioXact™ polymerase was used as it has 3′-5′ exonuclease activity. This PCR product was ligated into the expression vector, pCR® T7/CT-TOPO®, which encodes a C-terminal 6xHis tag. (Please refer to Appendix G for details of the pCR® T7/CT-TOPO® vector). The resulting plasmid, pSB5, was transformed into *E. coli* DH5α competent cells. PCR amplification of the resulting transformants using primers NG337 and NG338 enabled identification of cells harboring recombinant plasmids. 10 ng of pSB5 was transformed into BL21 (DE3) pLysS *E.coli* for expression of the heterologous protein. An *E. coli* BL21 strain carrying the pLysS plasmid was used as pLysS expresses T7 lysozyme, an inhibitor of the T7 polymerase encoded by the pCR® T7/CT-TOPO® plasmid. 10 ng of plasmid PCR® T7/CT-lacZ, which encodes β–galactosidase, was also transformed into BL21 (DE3) pLysS cells as a positive control for expression. This plasmid encodes both an N-terminal and a C-terminal 6xHis tag. Each transformation was inoculated into separately into 50 ml LB according to the supplier's instructions. The OD_{600} of the overnight cultures were 0.78 and 1.15 for the *E. coli* pSB5 and *E. coli* pCR® T7/CT-LacZ cultures respectively. An aliquot of 0.5 ml of each of the overnight cultures was used to inoculate 10 ml of fresh media that was incubated for two hours at 37°C with shaking (250 rpm). However, the

OD_{600} of the *E. coli* pSB5 culture reached an OD_{600} of 0.01 after two hours compared to the OD of the *E. coli* pCR® T7/CT-LacZ culture, which reached an OD_{600} of 0.52. Therefore, the ligations were again transformed into BL21 (DE3) pLysS *E. coli* and an overnight culture of *E. coli* pSB5 and a fresh culture of *E. coli* pCR® T7/CT-LacZ (a dilution of the overnight culture) were induced for expression with IPTG (final concentration 1mM) and 0.5 ml aliquots of this culture collected at hourly intervals. 15 µl of each sample was analyzed by SDS-PAGE. A protein band of approximately 34 kDa was detected in the *E. coli* pSB5 (pLysS) samples at each of the time points (Fig. 5.2). The predicted size for the NMB0345 protein expressed by *E. coli* pSB5 (pLysS) is 36.2 kDa. A protein band of approximately 34 kDa was also observed in a sample from the uninduced *E. coli* pSB5 (pLysS) culture. This result was expected as it is known that *E. coli* produces low levels of T7 RNA polymerase even without induction with IPTG. However, this protein band was not present in the *E. coli* pCR® T7/CT-LacZ samples. A protein band of the predicted size for β-galactosidase (115 kDa) was present in the *E. coli* pCR® T7/CT-LacZ samples (Fig. 5.2). However, no band was detected in samples from *E. coli* pSB5 (pLysS) cultures by Western analysis using an anti-polyhistidine monoclonal antibody that recognizes the 6x His tag. Bands of 115 kDa (β-galactosidase) were detected by the antibody in the *E. coli* pCR® T7/CT-LacZ samples (data not shown). Nucleotide sequence analysis of the insert in pSB5 using primers NG337 and NG376 was performed and revealed the presence of a frameshift mutation at the 3′ end of the cloned NMB0345 insert leading to the loss of expression of the C-terminal His tag encoded by

Fig. 5.2 Expression of NMB0345 protein by *E. coli* pSB5



Loading order:

1 *E. coli* pSB5 (IPTG induced)
2 *E. coli* pCR® T7/CT-LacZ (IPTG induced)
3 *E. coli* pSB5 (IPTG induced)
4 *E. coli* pCR® T7/CT-LacZ (IPTG induced)
5 *E. coli* pSB5 (IPTG induced)
6 *E. coli* pCR® T7/CT-LacZ (IPTG induced)
7 *E. coli* pSB5 (IPTG induced)
8 *E. coli* pSB5 (uninduced)
9 *E. coli* pCR® T7/CT-LacZ (IPTG induced)

Samples were collected from *E. coli* pSB5 induced at 37°C for 1, 2, 3, and 4 hours and analyzed by SDS-PAGE. A band of approximately 34 kDa was observed in *E. coli* pSB5 samples (lanes 1, 3, 5, 7, and 8) and is indicated by an arrow marked 'A'. A band of similar intensity is seen in the uninduced sample (lane 8). This band was not seen in the control (pCR® T7/CT-lacZ) samples (lanes 2, 4, 6, and 9). A band of approximately 115 kDa (the predicted size of β–galactosidase) is seen only in the control samples. This band is indicated by an arrow marked 'B'.

the pCR® T7/CT-TOPO® vector. The frameshift mutation reduced the size of the expressed protein by approximately 3.3 kDa. The wild-type NMB0345 gene contains four adenine nucleotides at the 3′ end of the gene. The presence of an additional two (or more) adenine nucleotides (the exact number of adenine nucleotides was not clear from the chromatogram of sequence from pSB5) in the amplified NMB0345 PCR product present in pSB5 led to a frameshift mutation preventing expression of the 6xHIS tag. Therefore, a fragment lacking the last seven amino acids was amplified using primers NG337 and NG375 (Fig. 5.3) and ligated into pCR® T7/CT to generate the plasmid pSB10 which was used for all subsequent expression and purification of the NMB0345 protein.

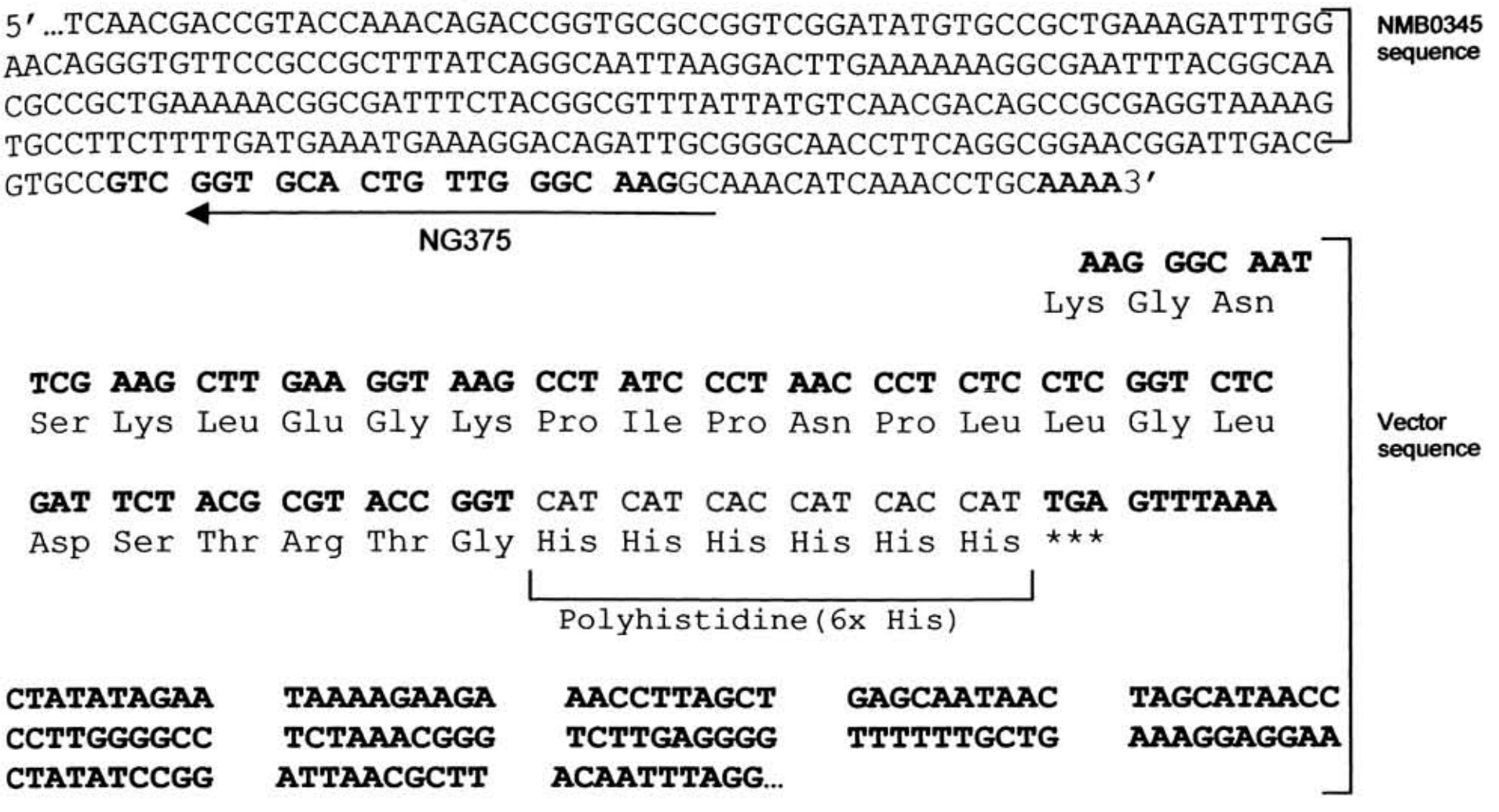
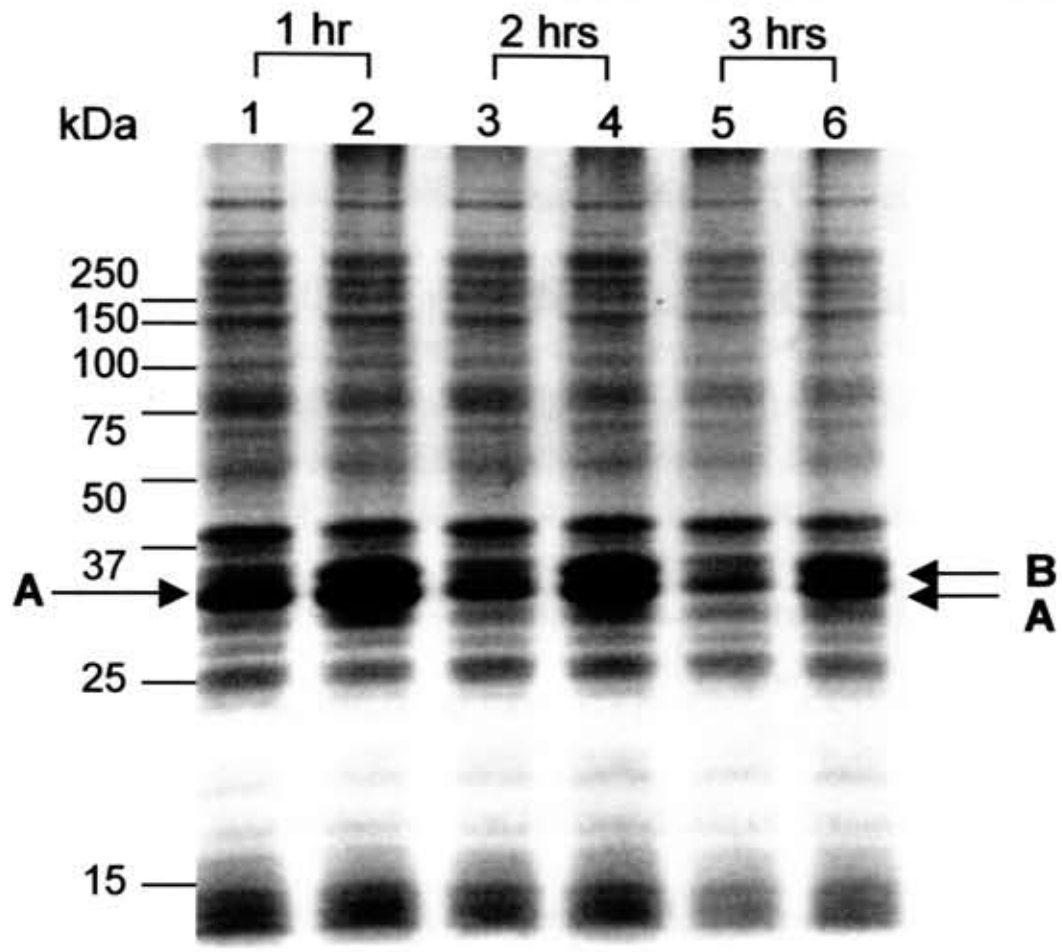


Fig. 5.3 Location of frameshift mutation in NMB0345 insert in pSB5

Nucleotides 569 - 864 of the published MC58 genome sequence of NMB0345 (864 bp) are shown with the location of the adenine nucleotides present at the 3′ end of the gene indicated in red. The sequence to which reverse primer NG375 (used for amplification of an NMB0345 PCR product lacking the last 21 nucleotides) anneals is shown in blue. pCR® T7/CT vector sequence is shown in bold with sequence encoding the 6x His tag shown in red.

This PCR product was ligated into pCR® T7/CT-TOPO® and transformed into *E. coli* DH5α. A recombinant plasmid, pSB10, was identified and transformed into BL21 (DE3) pLysS. The nucleotide sequence of the insert of pSB10 was confirmed by sequencing using oligonucleotide primers NG337 which anneals to the 5′ end of NMB0345 and NG376 which anneals downstream of the His tag encoded by the pCR® T7/CT-TOPO® vector. 10 ng of pCR® T7/CT-LacZ was used to transform BL21 (DE3) pLysS. Each transformation was separately inoculated in 50 ml LB and incubated at 37°C overnight. An aliquot of 0.5 ml of the *E. coli* pCR® T7/CT-LacZ (pLysS) overnight culture was used to inoculate 10 ml of LB, and this culture was split into two 5 ml cultures (IPTG-induced and uninduced), and grown to an OD_{600} of approximately 0.7. The overnight culture of *E. coli* pSB10 (pLysS) (OD_{600} of 0.865) was also split into two 5 ml cultures (IPTG-induced and uninduced). Aliquots were collected and analyzed by SDS-PAGE as before. A band of the predicted size (35.4 kDa) was detected by SDS-PAGE analysis that was not present in samples from the *E. coli* pCR® T7/CT-LacZ (pLysS) control (Fig. 5.4). The 35.4 kDa band appears to be less intense at three hours induction than after one and two hour's induction. This may be due to toxic effects of high levels of the NMB0345 protein in *E. coli*. Western analysis of the expression profiles demonstrated that the 35.4 kDa band reacted with a mouse anti-His monoclonal antibody (data not shown).

Fig. 5.4 Expression of NMB0345 protein by *E. coli* pSB10 (pLysS)



Loading order:

1 *E. coli* pSB10 uninduced
2 *E. coli* pSB10 induced
3 *E. coli* pSB10 uninduced
4 *E. coli* pSB10 induced
5 *E. coli* pSB10 uninduced
6 *E. coli* pSB10 induced

Samples were collected from *E. coli* pSB10 (pLysS) induced at 37°C for 1, 2, and 3 hours and analyzed by SDS-PAGE. A prominent band of approximately 34 kDa was observed in the uninduced samples (lanes 1, 3, and 5). This band is indicated by an arrow marked 'A'. The predicted size of NMB0345 amplified using primers NG337 and NG375 is 35.42 kDa. Prominent bands of approximately 34 and 36 kDa were observed in samples collected from cultures induced with IPTG (lanes 2, 4, and 6). These bands are indicated by arrows marked 'A' and 'B'.

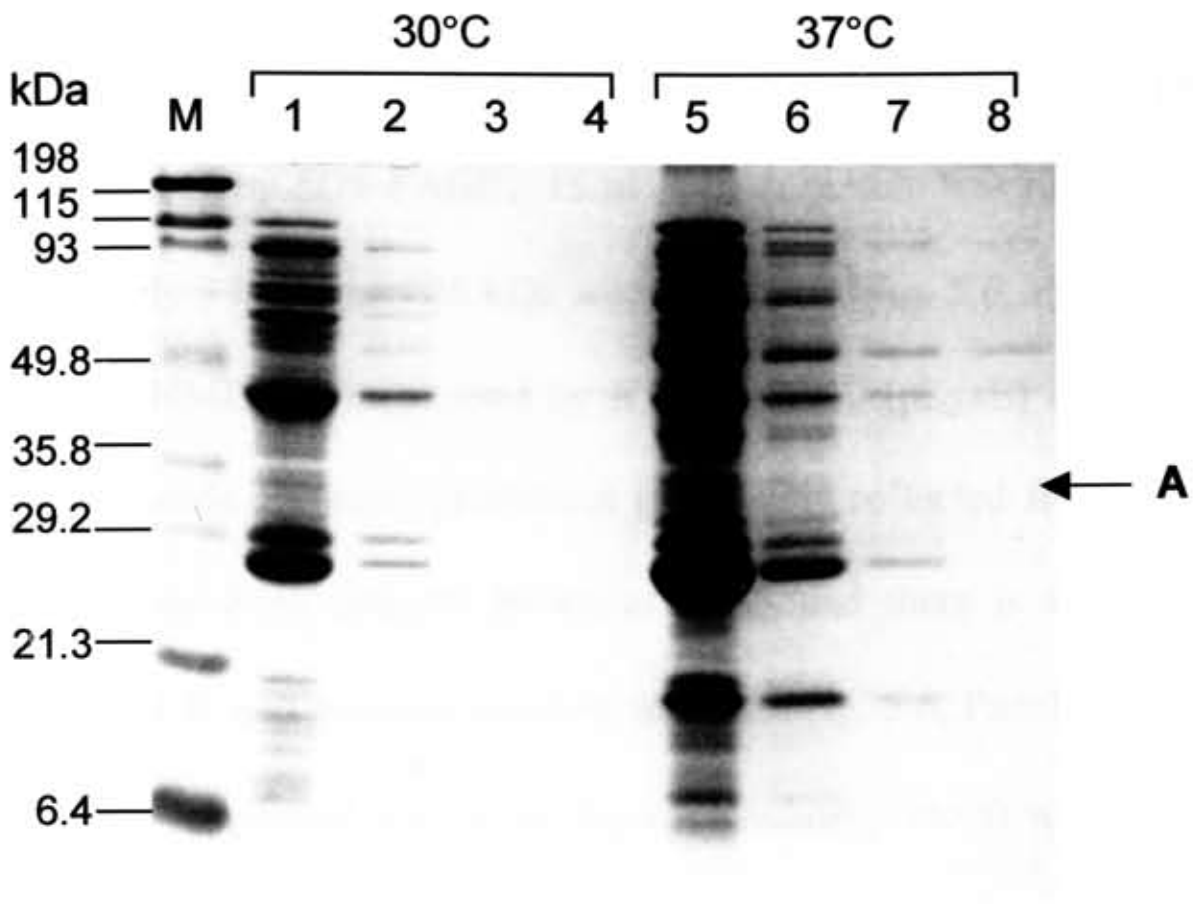
5.2 Purification of recombinant NMB0345 protein using a nickel column

Initially, purification of recombinant NMB0345 protein was attempted using a B-PER™ 6xHis Spin nickel column. Four ml of an overnight culture of pSB10 (pLysS) was used to inoculate 200 ml of LB. Expression was induced with IPTG once the culture reached an OD_{600} of approximately 0.6. Cultures were then incubated at either 30°C or 37°C for five hours (according to the manufacturer's instructions) after which the bacteria were harvested by centrifugation. Cell pellets were lysed by sonication and the proteins expressed were purified using a B-PER™ 6xHis Spin column. Aliquots from elutions collected from the column were analyzed by SDS-PAGE (Fig 5.5). However, no band of the expected size (35.4 kDa) was detected by SDS-PAGE or by Western analysis using an anti-polyhistidine monoclonal antibody indicating that expression of the protein was too low for purification. Therefore, the following steps were taken to optimize conditions for the expression and purification of the NMB0345 protein.

5.3 Optimization of conditions for expression and purification of recombinant NMB0345 protein

The low OD_{600} reached by bacteria containing the pSB5 and pSB10 plasmids compared to those harboring the control plasmid, pCR® T7/CT-*lacZ*, indicated that the NMB0345 gene product is toxic to *E. coli*. In order to reduce toxic effects, the BL21 (DE3) pLysE strain was used as the host strain for expression. The pLysE plasmid produces higher amounts of T7 lysozyme than pLysS and thereby reduces basal level expression of the gene of interest.

Fig. 5.5 Nickel column purification of recombinant NMB0345 protein expressed by *E. coli* pSB10 (pLysS) using a nickel column



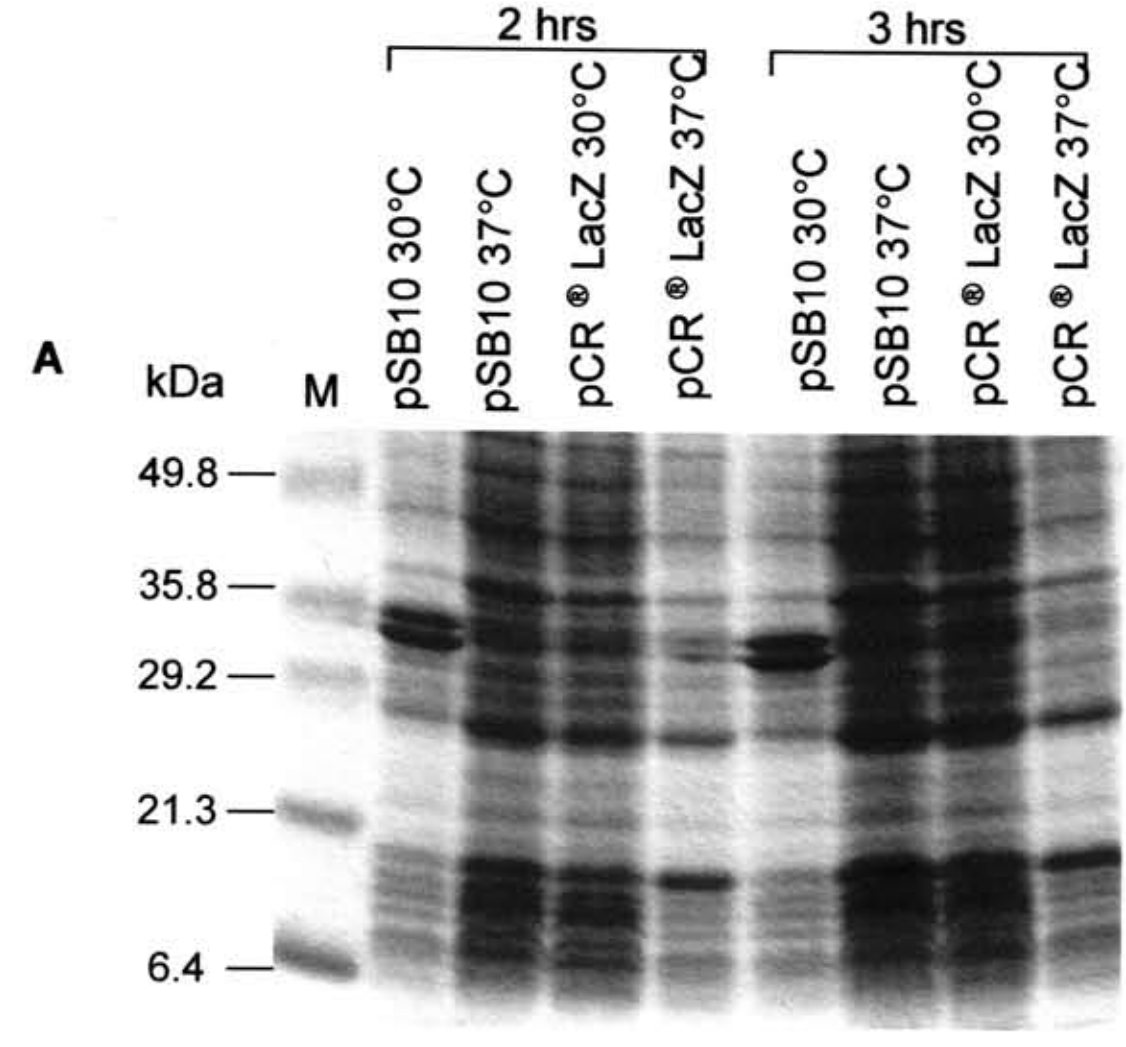
Fractions eluted:

M	Pre-stained marker (BioRad)
1	elution 1
2	elution 2
3	elution 3
4	elution 4
5	elution 1
6	elution 2
7	elution 3
8	elution 4

Cultures induced with IPTG for the expression of NMB0345 by *E. coli* pSB10 were incubated at either 30°C (lanes 1-4) or 37°C (lanes 5-8). Purification of NMB0345 protein was performed using a nickel purification column. Aliquots from each elution were analyzed by SDS-PAGE. No specific band of the predicted size for NMB0345 (approximately 35.4 kDa, indicated by an arrow labeled 'A') was observed.

A culture of *E. coli* pSB10 (pLysE) that had reached an OD_{600} of 0.39 was used as the host strain for expression. Expression of the NMB0345 protein in cultures of *E. coli* pSB10 (pLysE) incubated at 30°C and 37°C was compared (Fig 5.6, Panels A & B). Samples (0.5 ml) of these cultures were collected after induction over a five-hour period and the proteins were analyzed by SDS-PAGE. 15 µl of each sample was run on a 12% gel. Bands of approximately 34 kDa and 36 kDa were observed (Fig. 5.6, Panels A and B). The predicted size of NMB0345 expressed by *E. coli* pSB10 (pLysE) is 35.4 kDa. The 34 kDa and 36 kDa bands are more prominent in samples collected from cultures grown at 30°C than in those from cultures grown at 37°C, and there is a significant reduction in the background *E. coli* proteins resolved at 30°C (Fig. 5.6, Panels A and B). A protein band of 115 kDa (predicted size of the β-galactosidase protein) was observed in *E. coli* pCR® T7/CT-LacZ cultures (Fig. 5.6, Panel B). The 34 kDa and 36 kDa protein bands were of similar intensity at all time points (Fig. 5.6, Panels A and B). Western analysis using an anti-polyhistidine monoclonal antibody detected both the 34 and 36 kDa bands. However, the intensity of the 36 kDa band was much greater than that of the 34kDa band (Fig. 5.7). Bands were only detected in samples collected from cultures expressed at 30°C. The absence of bands detected by Western for samples collected from cultures expressed at 37°C is likely to be due to the low expression of recombinant NMB0345 protein at this temperature. In addition to the 115 kDa band (β-galactosidase) additional bands were detected in samples from cultures of *E. coli* pCR® T7/CT-LacZ and these are likely to represent products of proteolytic cleavage of the β-galactosidase protein. As expression was found to be optimal at 30°C, all subsequent culturing of *E. coli* pSB10 (pLysE) was performed at 30°C.

Fig. 5.6 **Comparison of expression profiles at 30°C and 37°C**



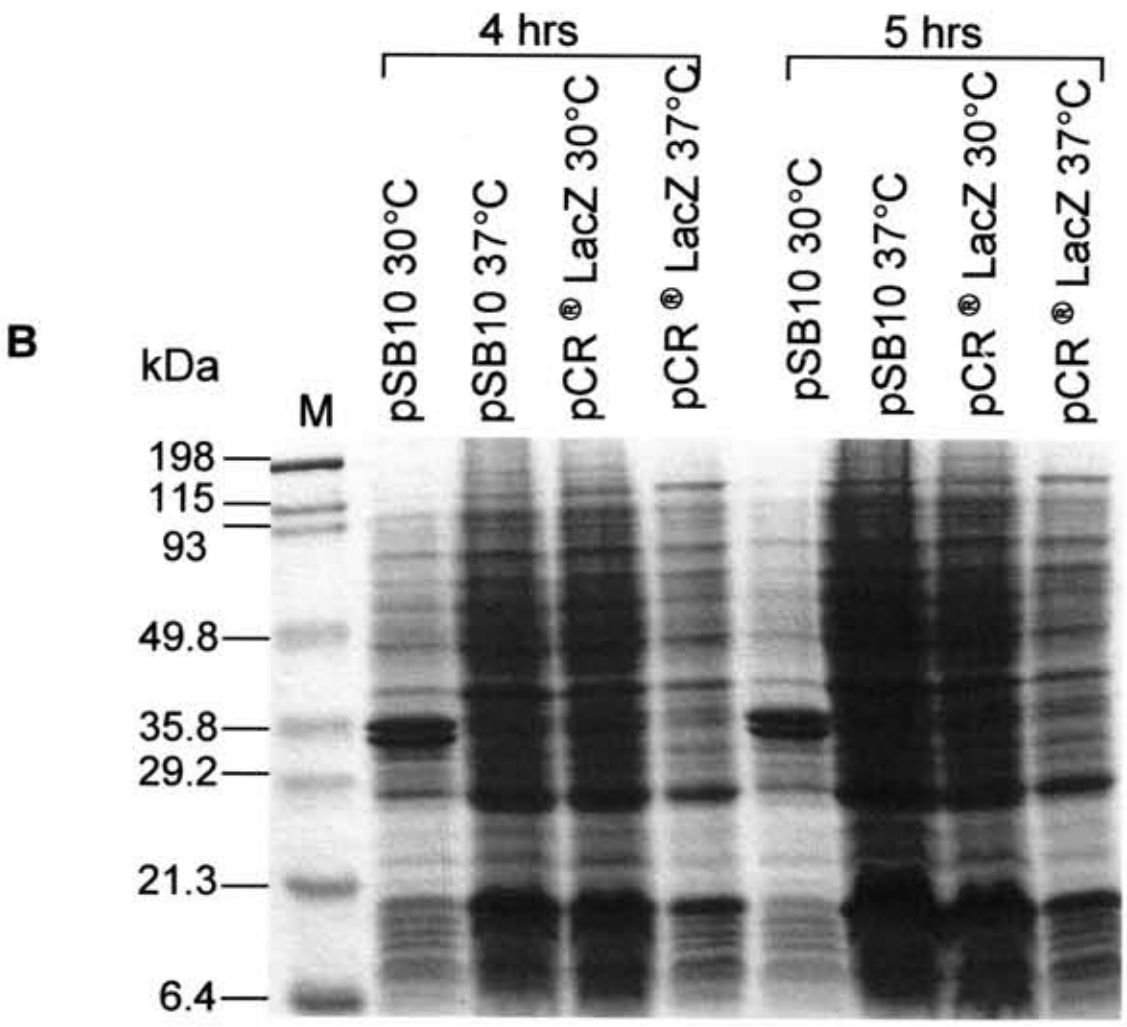


Fig. 5.6 **Comparison of expression profiles at 30°C and 37°C**

Cultures of *E. coli* pSB10 and pCR® T7/CT-LacZ were induced with IPTG and incubated at either 30°C or 37°C. Aliquots were collected at 2, 3, 4, and 5 hours and analyzed by SDS-PAGE (Panels A and B).

Fig. 5.7 Western blot analysis of NMB0345 expression at 30°C and 37°C

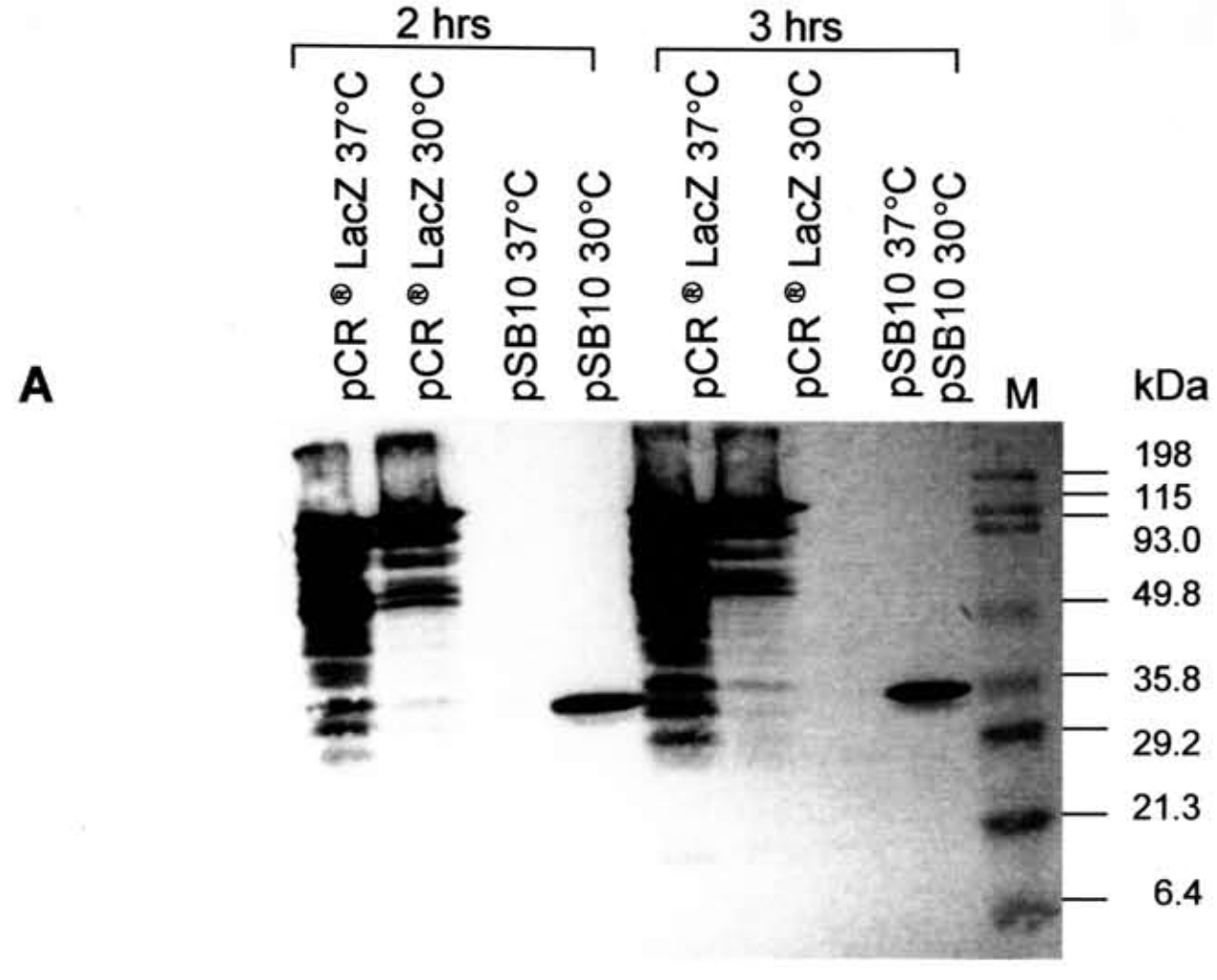


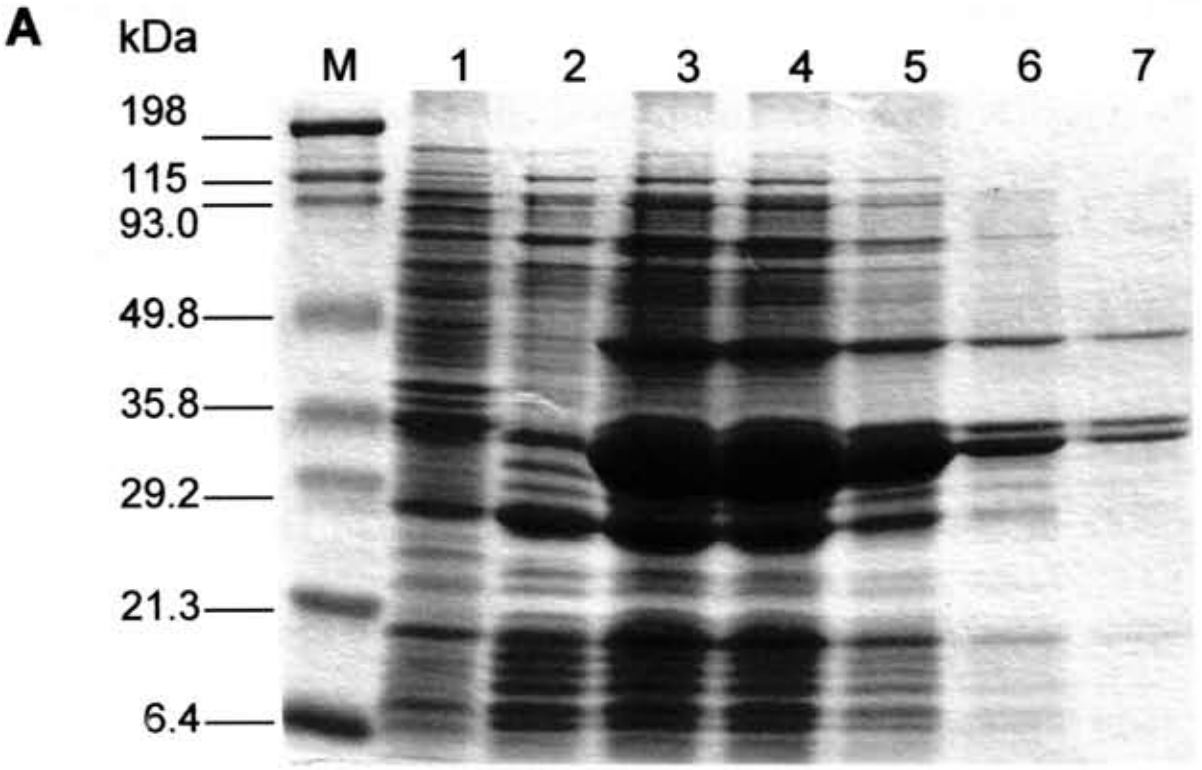
Fig. 5.7 **Western blot analysis of NMB0345 expression at 30°C and 37°C**

Western blot analysis of the samples collected from *E. coli* pSB10 expressed at 30°C and 37°C (Fig. 5.6) detected bands of approximately 36 kDa using a monoclonal anti-polyhistidine peroxidase conjugate antibody (Panels A and B).

5.4 Nickel column purification of recombinant NMB0345 protein using optimized conditions for expression

Initially, a B-PER™ 6x His Spin column (Pierce) was used for purification of NMB0345 protein expressed by *E. coli* pSB10 (pLysE). A culture of *E. coli* pSB10 (pLysE) was induced at on OD_{600} of 0.4-0.5 and expressed for 2.5 hours. Although two bands of approximately 34 kDa and 36 kDa were observed upon SDS-PAGE analysis (Fig. 5.8, Panel A) and were detected by Western analysis using an anti-polyhistidine monoclonal antibody (Fig. 5.8, Panel B), the level of background of *E. coli* proteins present in the eluate from the column was high. Therefore, purification was attempted using a Talon™ cobalt purification column (Clontech) which binds His-tagged proteins more specifically than nickel columns due to its requirement for the specific spatial positioning of histidine residues (Chaga *et al.*, 1999) (Hochuli *et al.*, 1987).

Fig. 5.8 Nickel column purification of recombinant NMB0345 protein expressed at 30°C



Loading Order:

M	pre-stained marker (BioRad)
1	wash 1
2	wash 2
3	elution 1
4	elution 2
5	elution 3
6	elution 4
7	elution 5

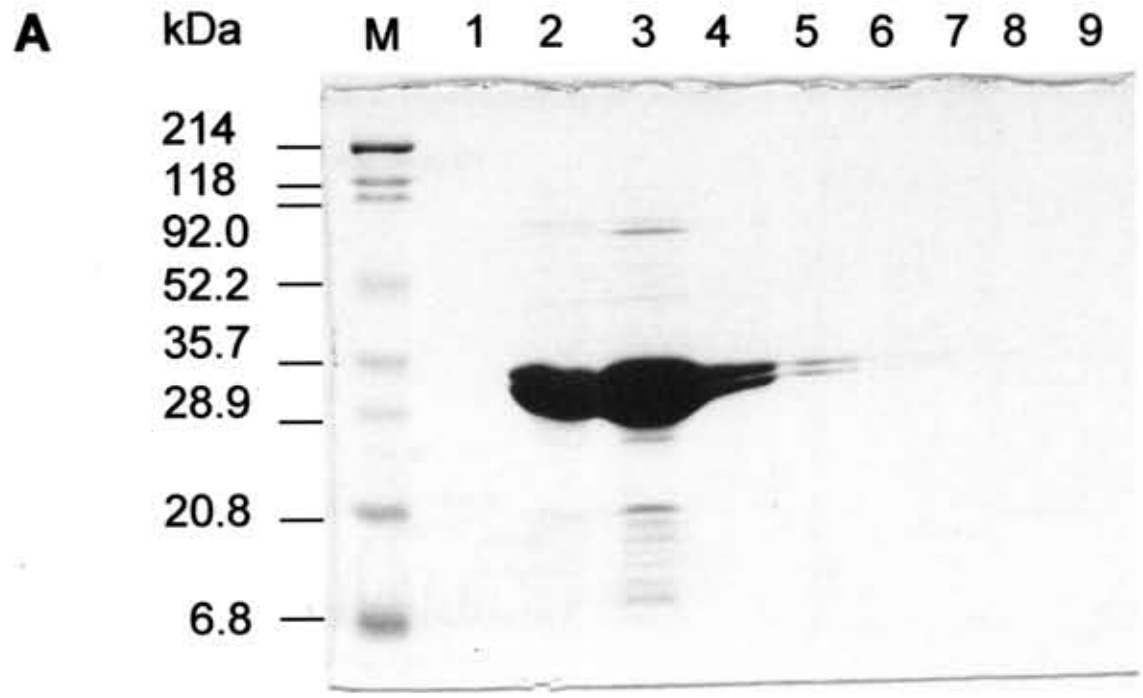
Fig. 5.8 Nickel column purification of recombinant NMB0345 protein expressed at 30°C

Purification of NMB0345 from *E. coli* pSB10 induced with IPTG at 30°C was performed using a nickel purification column. Aliquots of each of the washes and elutions collected were combined with 1 X SDS-PAGE sample buffer and run on a 12% SDS-PAGE gel. A high level of background was observed in the first three elutions (Panel A, lanes 3, 4, and 5). This background is significantly reduced in elutions 4 and 5 (Panel A, lanes 6 and 7). Western analysis of the same samples using an anti-polyhistidine monoclonal antibody detected two bands of approximately 34 kDa and 36 kDa in all five elutions. No other bands were detected (Panel B, lanes 3-7).

5.5 Cobalt column purification of the NMB0345 protein

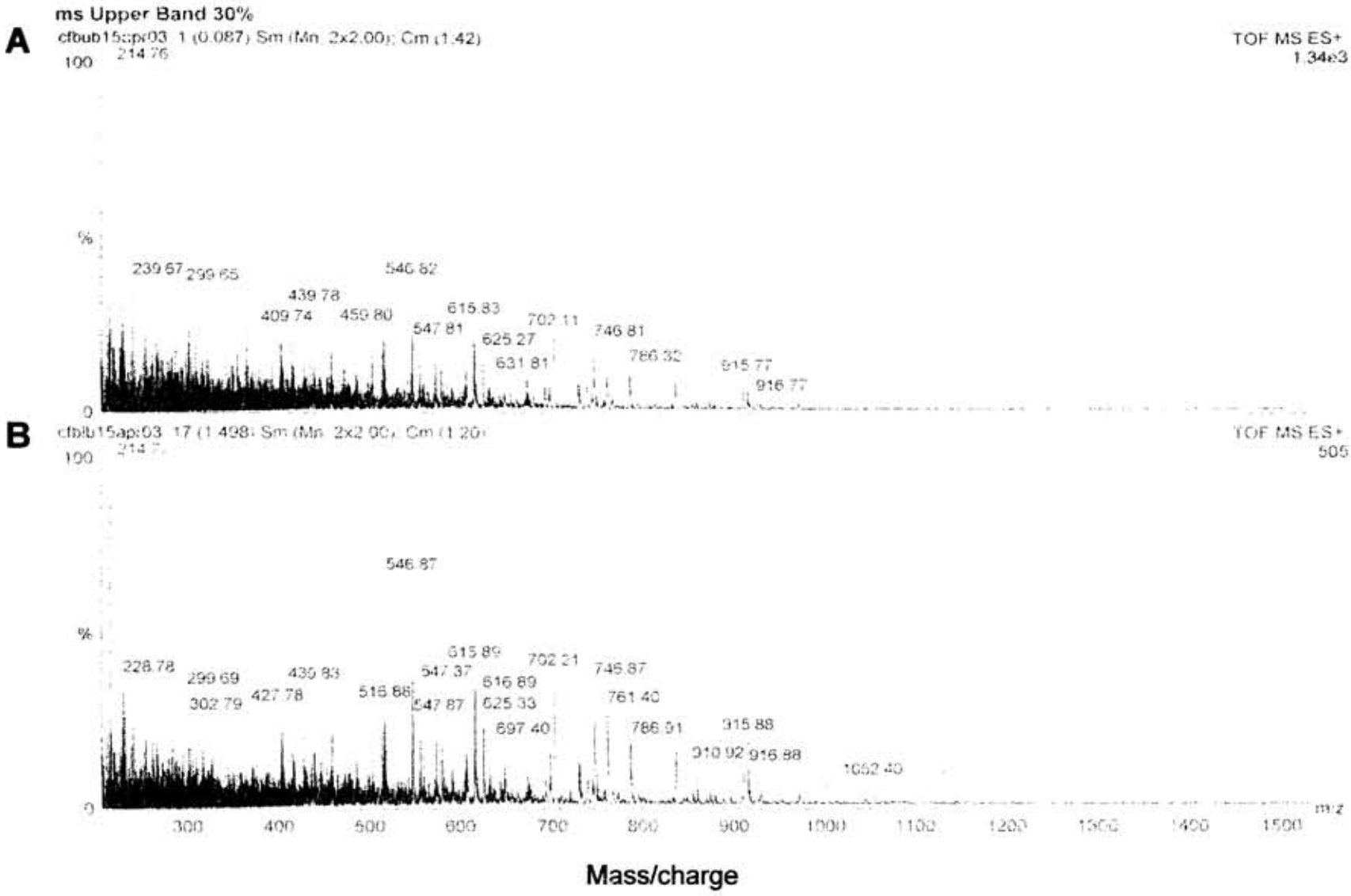
Purification using a Talon™ cobalt column (Clontech) eliminated most of the non-specific *E. coli* proteins. The two bands of 34 kDa and 36 kDa were observed (Fig. 5.9, Panel A). These bands were detected by Western analysis using a monoclonal anti-polyhistidine antibody. The lower molecular weight band was less intense than the higher molecular weight band (Fig 5.9, Panel B). The yield of recombinant NMB0345 protein purified from 125 ml of *E. coli* pSB10 (pLysE) culture was 1.5-2.0 mg. N-terminal sequencing (Protein and Nucleic Acid Chemistry Facility, University of Cambridge) of the 34 and 36 kDa bands excised from a 10% SDS-PAGE gel confirmed that the first 5 amino acids of the higher molecular weight band are identical to those from the published MC58 NMB0345 sequence. However, no sequence could be obtained for the lower band despite sequencing samples being prepared on two separate occasions. Therefore, the 34 kDa and 36 kDa protein bands were resolved by SDS-PAGE and subjected to spectrometric analyses to compare peptide maps resulting from individual tryptic digests. (Performed by Paul Hitchen). The results confirm that the spectra generated from the two bands are very similar. Comparison of the peptide maps of each band with the amino acid sequence of NMB0345 from the published MC58 genome confirms that both bands are NMB0345 (Pizza *et al.*, 2000) (Fig. 5.10).

Fig. 5.9 Cobalt column purification of recombinant NMB0345 protein



Purification of NMB0345 protein from an *E. coli* pSB10 (pLysE) culture induced with IPTG and expressed at 30°C was performed using a cobalt Talon purification column (Clontech). Aliquots of each elution were analyzed by SDS-PAGE (Panel A). The level of background observed in elutions 2 and 3 is significantly reduced in elutions 4-9. Western blot analysis of samples collected from the purification detected bands of approximately 34 kDa and 36 kDa in elutions (Panel B) using an anti-polyhistidine monoclonal antibody.

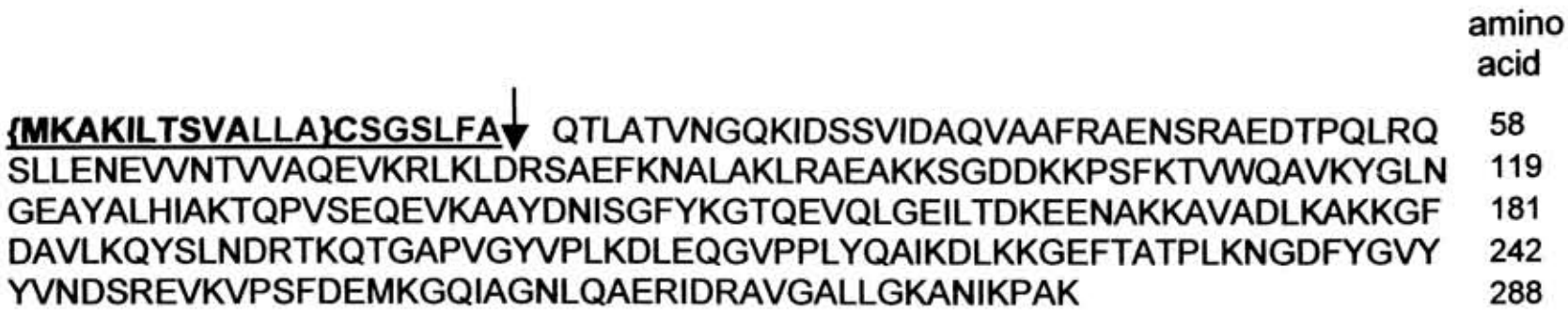
Fig. 5.10 Mass spectrometric analysis of the 34 kDa and 36 kDa protein bands isolated from SDS-PAGE following expression of *E. coli* pSB10 pLysE



Time of flight-mass spectrometric-electrospray analysis (TOF-MS-ES) of the 34 kDa (A) and 36 kDa (B) bands expressed by *E. coli* pSB10 (pLysE) gave very similar profiles for the two bands. Comparison of the peaks observed to expected peptide masses calculated from published protein sequence data for NMB0345 confirms that both bands are the NMB0345 protein. The x-axis represents mass (m) over charge (y). The y-axis is an arbitrary scale where the largest peak on the mass spectrometric spectra is assigned 100%. If a signal has a mass of 1000 and carries a single charge it appears as 1000. If it carries 2 charges it appears as 500 (1000÷2). Only the intact peptide appears as a doubly charged signal, while all the others (fragments) will be singly charged. Mass spectrometry was performed by Paul Hitchen.

PSORT analysis and the presence of a putative 'lipobox' within the N-terminal sequence of NMB0345 suggest that this protein may be lipidated. In *E. coli*, the conserved lipobox motif of lipoprotein precursors contains an invariable cysteine residue that is lipid modified by diacylglyceryl transferase (Lgt), prior to precursor cleavage by signal peptidase II (Sankaran and Wu, 1994). A hydrophobic signal peptide including the consensus motif for lipid conjugation, leucine-X-Y-cysteine [LVI][ASTVI][GAS][C] (Wu *et al.*, 1982), is present at the amino terminus of the NMB0345 protein sequence (Fig. 5.11). The spectrometric analyses of the 34 kDa and 36 kDa protein bands performed for this study would not have detected lipidation as the analysis of post-translational modification of proteins by mass spectrometry would require detailed peptide mapping of the protein.

Fig. 5.11 Location of a putative lipobox in the NMB0345 protein sequence



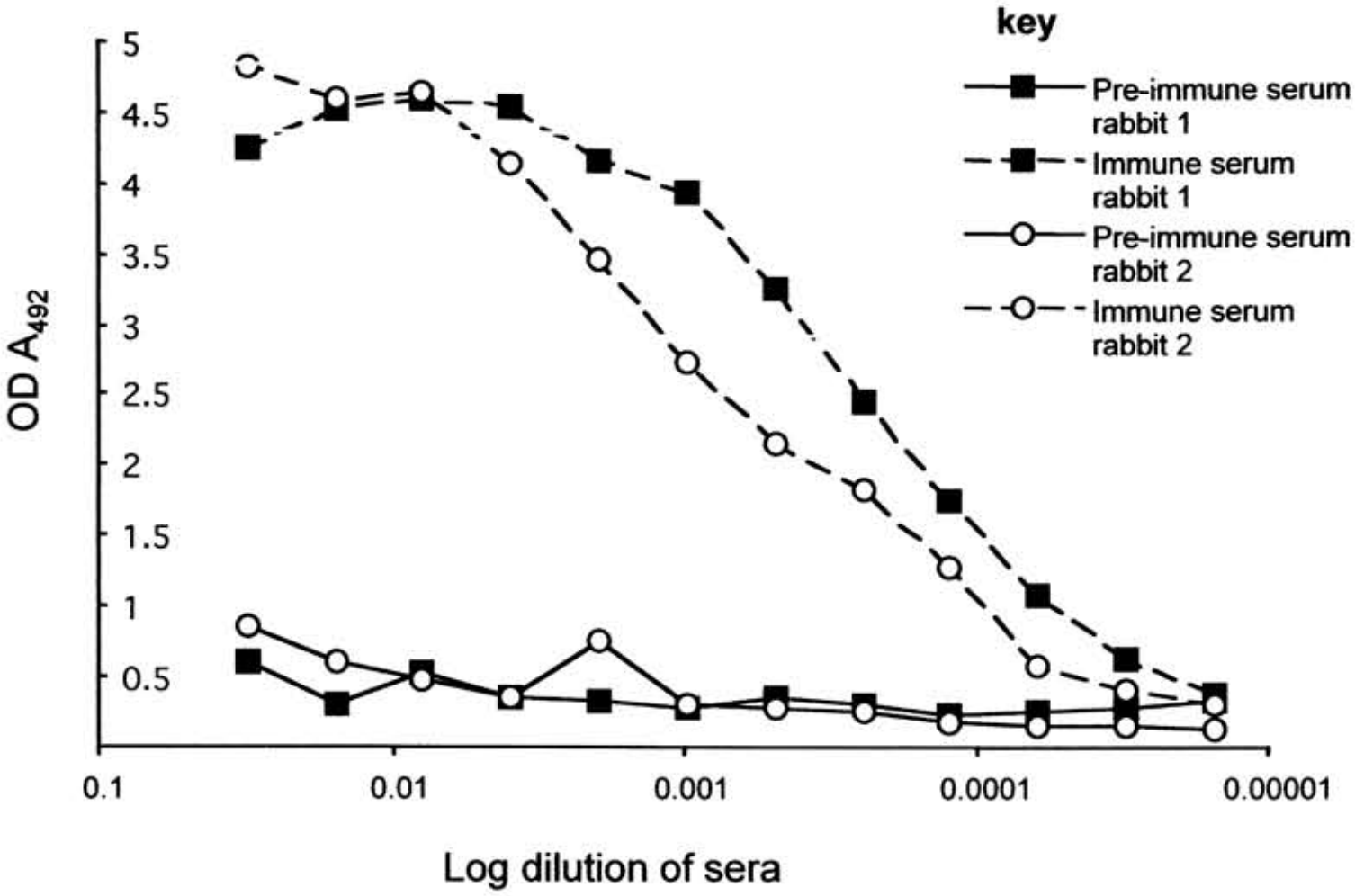
The translated amino acid sequence of NMB0345 from published MC58 genome sequence was analyzed using the PSORT and LipoP 1.0 servers. Predicted signal peptide sequence is underlined. The most likely signal peptidase I (SpI) cleavage site is indicated by an arrow between positions 20 and 21. The cysteine residue is shown in bold. The putative lipobox is shown in red and putative lipid attachment site is shown in blue. The predicted signal peptidase II signal peptide sequence is shown in brackets.

5.6 Generation of anti-NMB0345 antibodies

Two rabbits were each immunized with cobalt-column purified recombinant NMB0345 protein consisting of both the 34 kDa and 36 kDa protein bands combined with Freunds complete adjuvant (performed by CovaLab). In order to establish the titre of antibody raised against NMB0345 in serum collected from the rabbits at day 89 (following four injections of 50 μg of purified protein), a whole cell ELISA using *N. meningitidis* strain MC58 was performed (Fig. 5.12). The results indicate that following immunization of the rabbits with recombinant NMB0345 protein, high titres of anti-NMB0345 antibody were generated.

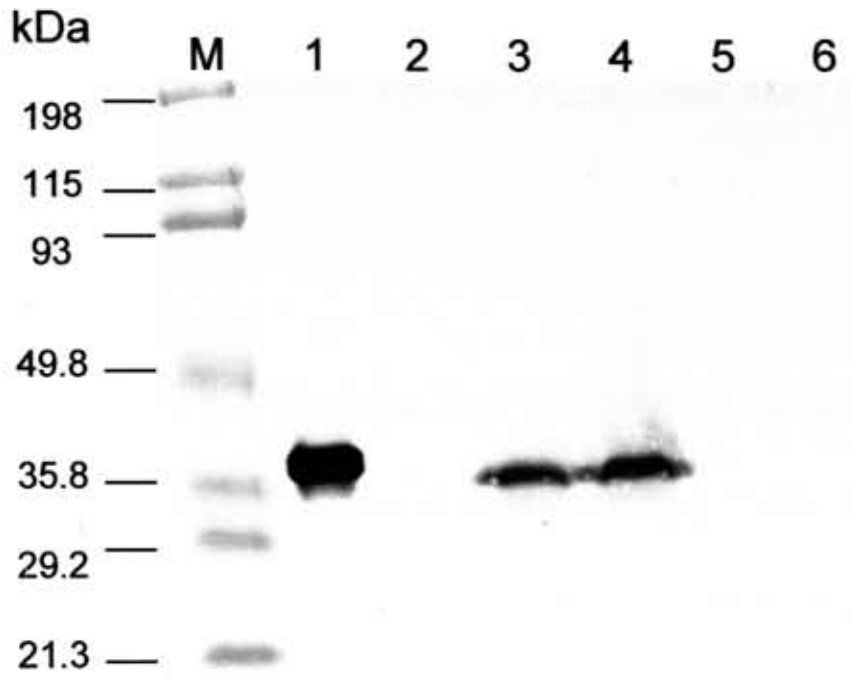
Wild-type *N. meningitidis* strains C311^{+} and MC58, NMB0345 mutants, and complemented NMB0345 mutants were analyzed by Western using polyclonal murine antibody raised against the recombinant NMB0345 protein for detection. A sample of the purified recombinant protein was also included on the blot. Two bands of approximately 36 kDa and 37 kDa were detected in the lane containing the protein purified from *E. coli* pSB10 (pLysE). A protein band of approximately 36 kDa was detected in both wild-type strains. This band may represent the processed form of the protein. No protein bands were detected in the NMB0345 mutants (Fig. 5.13).

Fig. 5.12 ELISA using sera collected from rabbits immunized with purified recombinant NMB0345 protein



Whole cell ELISA was performed to compare the binding of pre-immune rabbit sera and sera collected from rabbits immunized with recombinant NMB0345 protein to *N. meningitidis* strain MC58.

Fig. 5.13 Western blot analysis of NMB0345 mutants using polyclonal mouse serum raised against recombinant NMB0345 protein



Loading order:

M	pre-stained marker (BioRad)
1	recombinant NMB0345 (cobalt column purification)
2	-
3	MC58
4	$C311^+$
5	NMB0345.1/$C311^+$
6	NMB0345.2/$C311^+$

Purified recombinant NMB0345 protein and lysates of wild-type *N. meningitidis* strains MC58 and $C311^+$, and NMB0345 mutants were analyzed by SDS-PAGE (not shown). Western blot analysis of the same samples using serum collected from mice immunized with recombinant NMB0345 detected two bands of approximately 36 kDa and 37 kDa in the purified protein sample (lane 1) and a band of approximately 36 kDa in wild-type *N. meningitidis* strains MC58 and $C311^+$. No bands were detected in either of the NMB0345 mutants (lanes 5 and 6).

5.7 Serum bactericidal activity of anti-NMB0345 sera

Serum collected from rabbits immunized with purified recombinant NMB0345 was used to perform serum bactericidal assays (Tables 5.1a and 5.1b). No significant killing of strain MC58 was observed with these sera and no change was seen when compared to killing with pre-immune sera.

Six week-old female BALB/c mice were also immunized with recombinant NMB0345 protein according to the immunization protocol shown in Fig. 5.14. On day 35, serum was collected. Antibody titres of the murine sera were similar to those of rabbit sera. The murine sera were used to perform a serum bactericidal assay in order to determine whether antibodies raised against recombinant NMB0345 can mediate killing of the wild-type serogroup B isolate, MC58. No significant killing of MC58 was observed with this serum compared to killing with pre-immune serum collected from non-immunized BALB/c mice at any of the dilutions tested (1:2 dilution of serum 1:2-1:1024) (data not shown).

Table 5.1a **Serum bactericidal activity of anti-NMB0345 murine sera against *N. meningitidis* strain MC58**

Rabbit 1

Dilution of Serum	Pre-immune serum mean CFU	Immune serum (Day 89) mean CFU
1:2	51	36
1:4	75	6
1:8	144	9
1:16	95	33
1:32	82	29
1:64	77	47
1:128	58	34
1:256	>200	69
1:512	85	85
1:1024	95	111
No serum control	>200	>200
No complement control	>200	>200

Table 5.1b **Serum bactericidal activity of anti-NMB0345 murine sera against *N. meningitidis* strain MC58**

Rabbit 2

Dilution of Serum	Pre-immune serum Mean CFU	Immune serum (Day 89) mean CFU
1:2	52	30
1:4	19	29
1:8	56	12
1:16	99	33
1:32	59	22
1:64	92	43
1:128	84	50
1:256	119	35
1:512	35	28
1:1024	64	53
No serum control	>200	>200
No complement control	>200	>200

5.8 Assessment of NMB0345 as a vaccine candidate

To determine whether immunization of mice with recombinant NMB0345 protein confers protection against challenge with wild-type *N. meningitidis* strain MC58, groups of ten six week-old female BALB/c mice were immunized by subcutaneous injection with either 25 μg purified recombinant NMB0345 protein combined 1:1 with Freunds incomplete adjuvant, Freunds incomplete adjuvant combined with 1 x elution buffer only, or by intraperitoneal inoculation with 2 x 10^6 CFU of the attenuated strain YH102 (strain C311$^+$ with Tn*10* insertion in *rfaF* (ADP heptosyltransferase)) (Li *et al.*, 2004) on days 0 and 21 (Fig. 5.14). All groups were challenged with 1 x 10^6 CFU serogroup B strain MC58 by intraperitoneal injection on day 35. Survival was monitored for up to 60 hours following challenge (Fig. 5.15, Panels A & B). All animals inoculated with the attenuated strain survived challenge. In the first experiment (Fig. 5.15, Panel A), six out of ten animals immunized with recombinant NMB0345 protein survived challenge while only two out of ten animals immunized with Freunds incomplete combined with 1 x elution buffer survived challenge. In a second experiment (Fig. 5.15, Panel B), seven out of ten animals immunized with recombinant NMB0345 protein survived challenge, while only five out of fifteen animals Freunds incomplete survived challenge. These results suggest that immunization of BALB/c mice with the recombinant NMB0345 protein provides some protection against challenge with wild-type serogroup B *N. meningitidis* strain, MC58. Using the Chi-squared test, the P values obtained for the first and second experiments are $P = 0.15$ and $P = 0.07$ respectively. However, if the results from the two experiments are combined, a P value of 0.025 is obtained. Group sizes were selected to allow a 20% difference in survival to be demonstrated. Further experiments are required

to confirm this result. This work was carried out by S. Bakshi, Yanwen Li, and Christoph Tang.

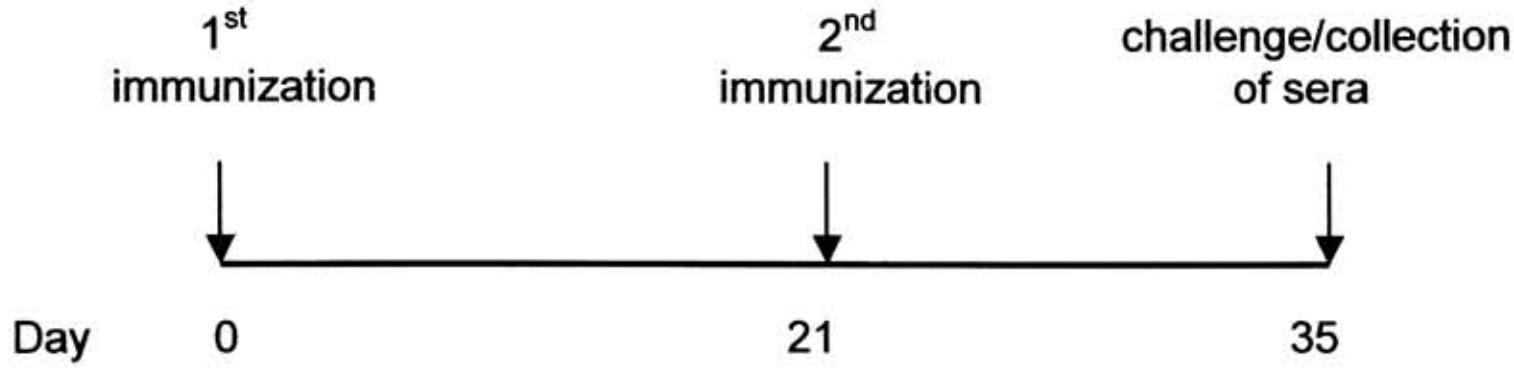
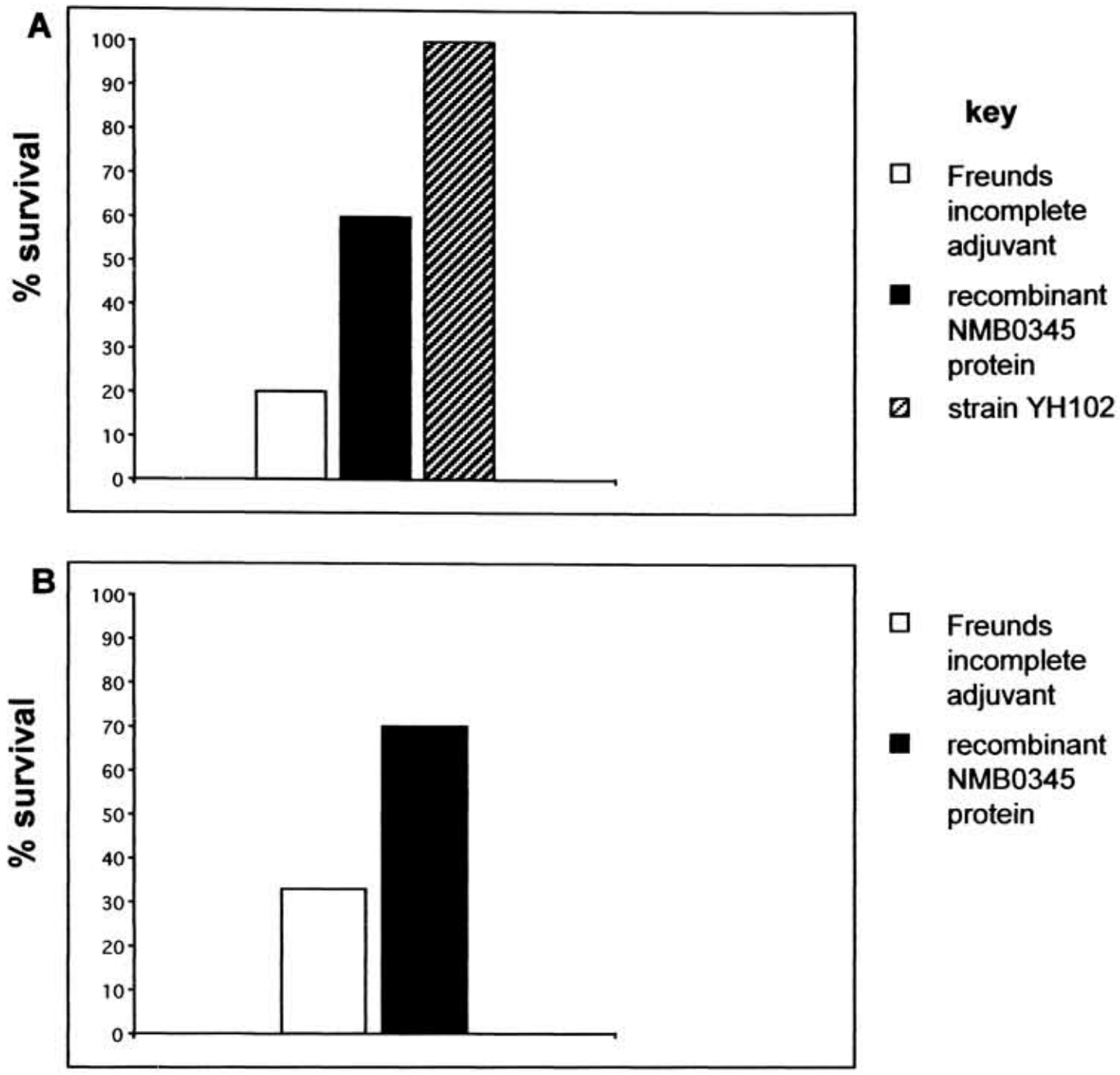


Fig. 5.14 Protocol for immunization of mice with recombinant NMB0345 protein

Fig. 5.15 Survival of mice following challenge with $1x10^6$ CFU of strain MC58

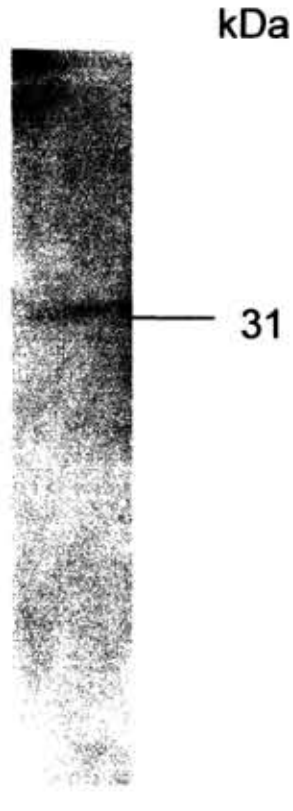


In two separate experiments (Panels A and B), mice immunized according to the protocol described in Fig. 5.14 were challenged with 1 x 10^6 CFU of strain MC58. Survival was monitored for 60 hours following challenge. In the first experiment (Panel A), all animals inoculated with attenuated strain 23A10 survived challenge. Six out of ten animals immunized with recombinant NMB0345 combined with Freunds incomplete adjuvant survived challenge. Two out of ten animals immunized with Freunds incomplete adjuvant combined with elution buffer survived challenge. In the second experiment (Panel B), seven out of ten animals immunized with recombinant NMB0345 combined with Freunds incomplete adjuvant survived challenge. Four out of fifteen animals immunized with Freunds incomplete adjuvant combined with elution buffer survived challenge.

5.9 Detection of recombinant NMB0345 protein using convalescent sera

In order to determine whether antibodies against the NMB0345 protein are generated during natural infection, Western analysis was performed using individual sera from six patients recovering from *N. meningitidis* infection. A faint band of the expected size of the NMB0345 protein (~31 kDa) was detected by sera obtained from a patient with meningitis caused by a serogroup C *N. meningitidis* isolate (Fig. 5.16).

Fig. 5.16 Detection of recombinant NMB0345 protein using convalescent serum



Western blot analysis of purified NMB0345 protein was performed using convalescent sera collected from patients recovering from infections caused by *N. meningitidis* as the primary antibody at a dilution of 1:1000. A mixture of anti-human IgM (1:10,000) and anti-human IgG antibody (1:5000) was used as the secondary antibody. A faint band of approximately 31 kDa (the expected size of the NMB0345 protein) was detected using serum from a patient recovering from meningitis caused by a serogroup B isolate of *N. meningitidis* as the primary antibody.

Discussion

The mutation in pSB5 was a frameshift in the sequence at the 3′ end of the NMB0345 gene. This problem was circumvented by designing a primer (NG375) that anneals to the sequence upstream of the poly A tract present at the 3′ end of NMB0345 (Fig. 5.3). Alternatively, a different enzyme with improved proofreading activity could have been used for PCR amplification. Although an enzyme with proofreading activity (BioXact ™ polymerase) was used in this study, different Taq polymerase enzymes have differing levels of fidelity and proofreading activity. As the requirement for the last seven amino acids for the function or immunogenicity of the NMB0345 protein is not known, it may be preferable to express the full length protein in future studies.

In order to identify the optimal conditions for the expression of recombinant NMB0345 protein, multiple conditions for expression were tested including comparison of host strains used for expression (*E. coli* BL21 pLysS and *E. coli* BL21 pLysE), temperature, and the OD at which expression of the NMB0345 protein was induced. In addition, the use of a cobalt column for purification was found to result in higher yields of protein than obtained using a nickel column. Furthermore, significantly less *E. coli* proteins were eluted using a cobalt column than when using a nickel column for purification of recombinant NMB0345 protein. Conditions for the purification of recombinant NMB0345 protein could be further optimized by titrating the concentration of imidazole used for elution of the protein from the cobalt purification column in order to reduce the level of background *E. coli* proteins that are eluted.

It is possible that the two bands may represent the processed and un-processed forms of the protein. The addition of globomycin, an inhibitor of signal peptidase II demonstrated significant inhibition of processing of recombinant *Chlamydia trachomatis* Mip-like lipoprotein resulting in the presence of two bands representing the processed and un-processed forms of the protein (Lundemose *et al.*, 1993).

The first 20 amino acids of the NMB0345 protein are predicted to comprise the signal peptide sequence. This corresponds to approximately 2.2 kDa and may account for the difference in size between the two bands observed on expression and purification of recombinant NMB0345 protein. Although both bands were detected by Western analysis, N-terminal sequencing of the lower molecular weight band may have failed due to N-terminal blocking caused by the presence of a lipid or other post-translational modification that is only present in the processed form of the NMB0345 protein. Many proteins (mainly eukaryotic) undergo post-translational modifications that render the N-terminus unavailable for Edman degradation and are effectively 'blocked'. These modifications are normally acetyl, formyl or pyroglutamyl groups (Wellner *et al.*, 1990).

The NMB0345 amino acid sequence contains a putative lipobox and is predicted to be a lipoprotein. The antibiotic globomycin, a specific inhibitor of lipoprotein signal peptidase II (Dev *et al.*, 1985), could be used to determine whether the two bands observed by SDS-PAGE analysis are due to lipidated and un-lipidated forms of the protein. Globomycin has previously been used to analyze the *Chlamydia trachomatis* Mip-like lipoprotein (Lundemose *et al.*, 1993). Alternatively, the addition of a lipid to

the protein could be monitored by the incorporation of [^{3}H] palmitate. This method has been used to demonstrate the lipidation of the recombinant *C. trachomatis* Mip-like protein and recombinant GNA33 (an *N. meningitidis* vaccine candidate) in *E. coli* (Jennings *et al.*, 2002). The immunogenicity of the recombinant NMB0345 protein could be further tested by immunizing mice with lipidated or unlipidated forms of the protein in order to determine whether the lipidated form is more immunogenic and/or protective against *N. meningitidis* challenge in mice.

Analysis by mass spectrometry has confirmed that both the 34 kDa and 36 kDa bands isolated from expression and of *E. coli* pSB10 (pLysE) are the NMB0345 protein (Fig. 5.10). Detailed analysis by mass spectrometry could be used to identify post-translational modifications of the protein that might lead to the presence of two forms on purification.

An ELISA performed to measure the binding of sera collected from rabbits immunized with recombinant NMB0345 protein to whole cells demonstrated that the sera contained a high titre of antibody (Fig 5.12). However, no serum bactericidal activity against MC58 was detected with either of these sera compared to the activity of pre-immune sera (Tables 5.1a and 5.1b). This is not unusual as high ELISA titres do not always correlate with SBA and different bactericidal activities can be observed depending on the IgG subclass of the antibodies induced. Studies have shown that while IgG1 and IgG3 are the main IgG subclasses generated following immunization with OMV vaccines (de Kleijn *et al.*, 2001; Guttormsen *et al.*, 1994; Naess *et al.*, 1996; Sjursen *et al.*, 1990; Vermont *et*

al., 2002) only IgG1 was found to show a correlation with SBA (de Kleijn *et al.*, 2001; Naess *et al.*, 1999). A further reason for weak correlation between ELISA titres and serum bactericidal activity is that antibodies may be of low affinity. Furthermore, the results of the whole cell ELISA may be misleading as epitopes that are not surface expressed in viable bacteria may become accessible during the processing of samples for ELISA. A comparison of whole cell ELISAs of $C311^+$ and $C311^+\Delta$NMB0345 (NMB0345 mutant) using sera collected from mice immunized with recombinant NMB0345 protein would allow an assessment of the specificity of the antibody raised.

Despite a lack of serum bactericidal activity, animal challenge studies suggest that immunization with the NMB0345 protein may confer protection against challenge with wild-type *N. meningitidis* (Fig. 5.15). Immunization of mice with another *N. meningitidis* antigen, GNA2132, has been shown to induce antibodies that passively protect infant rats but lack any detectable serum bactericidal activity (Welsch *et al.*, 2003). A possible reason for this lack of activity is that the *N. meningitidis* strain is grown in synthetic medium and incubated with immune serum and complement. As these conditions do not reflect the environment encountered *in vivo* during an infection, antigens expressed during infection may not be expressed by the bacterium under the conditions of the assay. Following the work of Goldschneider and colleagues, the SBA has been considered to be the 'gold standard' for the measurement of immunity against serogroups A and C (Goldschneider *et al.*, 1969a, b). Studies suggest that the SBA also correlates with protection for serogroup B (Boslego *et al.*, 1995) (Milagres *et al.*, 1994). However, a large vaccine efficacy trial indicated that the use of SBA against serogroup B as a correlate of protection may lead to an underestimation of clinical efficacy (Perkins *et*

al., 1998). This suggests that that although antibody-mediated immunity is essential for protection against serogroup B infection, other types of immunity such as cell-mediated immunity should also be considered and emphasizes the need for the use of more than one type of assay to establish the efficacy of an antigen.

Protection against serogroup B infection may be due to opsonic antibodies and innate immune responses that are not demonstrated by the SBA (Lehmann *et al.*, 1999) (Ison *et al.*, 1999). Therefore, the use of further assays such as the opsonophagocytosis assay may be informative for determining the efficacy of specific antigens. Studies have shown that the opsonic activity of serum correlates with the level of IgG antibody raised against OMVs, PorA, and PorB determined by ELISA and SBA (Aase *et al.*, 1995; Guttormsen *et al.*, 1994; Lehmann *et al.*, 1999). The whole blood assay can be used to measure the bactericidal capacity of blood collected following immunization. Although the correlation between the bactericidal activity of whole blood and protection against meningococcal disease is not known, a comparison of the bactericidal activity of convalescent sera measured by SBA and WBA indicated that the WBA is more sensitive than the SBA (Ison *et al.*, 1999). An advantage of the WBA is that in addition to complement-dependent antibody-mediated killing, it measures the opsonophagocytic activity of blood.

T-cells are required for the establishment of immunological memory and enhance the activation of phagocytic cells. Therefore, the measurement of T-cell response can be used as a further correlate of protection. T-cell responses induced by immunization can

be measured using a T-cell proliferation assay (van der Voort *et al.*, 1997). The release of cytokines by T cells in response to stimulation by Neisserial antigens can also be measured (Pollard *et al.*, 1999).

The measurement of bacteraemia following immunization of mice with recombinant NMB0345 protein is a further parameter that could be included in future experiments. This data may be particularly informative in the absence of bactericidal activity of sera collected from mice immunized with recombinant NMB0345 protein.

6 Discussion

Bacteria have evolved numerous virulence factors and mechanisms to enhance their growth and survival in the host. These include factors that are involved in the avoidance of killing, tropism of the bacterium towards specific host cell types and tissues, acquisition of key nutrients, and the production of host damage (Finlay and Falkow, 1997). The identification of virulence factors through the use of a mutagenesis strategy such as STM will identify only certain classes of factors. While toxins and other factors that may be cross-complemented during mixed infections are unlikely to be selected in such a screen, genes encoding virulence factors such as LPS and capsule that contribute to the avoidance of killing and factors that play a role in adhesion have been identified using the STM method in a range of pathogenic microorganisms including *S. typhimurium*, *M. tuberculosis*, *E. coli* K1, and *Vibrio cholerae*, and *Cryptococcus neoformans* (Hensel *et al.*, 1995) (Cox *et al.*, 1999) (Martindale *et al.*, 2000) (Merrell *et al.*, 2002) (Nelson *et al.*, 2001). STM of *N. meningitidis* identified 65 genes including NMB0342 and NMB0345 that are required during systemic infection. In addition, eight genes encoding known virulence determinants were also identified and include *siaD*, a gene encoding polysialyl transferase that is involved in capsule biosynthesis and numerous genes required for LPS biosynthesis (Sun *et al.*, 2000). The identification of these genes provides a validation of the STM method.

The NMB0342 and NMB0345 genes are arranged within a potential operon of seven genes. The close proximity of the NMB0342 and NMB0345 genes and the presence of homologues of these genes in other pathogenic bacteria suggested the possibility that other genes of the locus may also play a role in meningococcal virulence. In order to investigate whether these genes may have related functions in the virulence of *N. meningitidis*, mutations were made in each of the intervening genes of the locus and the resulting mutants were analyzed in the infant rat model of meningococcal septicaemia, the model in which the NMB0342 and NMB0345 mutants were originally identified. A number of *in vitro* models have been used to study the virulence of *N. meningitidis*. These include the infection of cultured epithelial or endothelial cells and the infection of nasopharyngeal explants (Virji *et al.*, 1995; Virji, 1996) (Townsend *et al.*, 2002) (Read *et al.*, 1995). However, as the establishment of bacteraemia is a prerequisite for all of the pathological sequelae of meningococcal infection including meningitis and septicaemia (Herrick *et al.*, 1918), this study has focused specifically on the systemic (bloodstream) stage of infection. C.I. values determined for multiple independent colonies of the NMB0342 and NMB0345 mutants confirmed their attenuation in the infant rat model of infection. Complementation of the NMB0342 mutation restored the virulence of the bacterium. In general, genes of the first part of the locus (NMB0342-NMB0345) appeared to play a greater role in virulence than those present in the latter part of locus (NMB0346-NMB0348). As only NMB0342 and NMB0345 have a major contribution to bloodstream infection, these genes were selected for further analysis.

The infant rat model of infection has been used extensively in studies of experimental *N. meningitidis* infection to demonstrate the role of cytokines (Nassif *et al.*, 1992), LPS, and capsule (Vogel *et al.*, 1996; Vogel *et al.*, 1997) in *N. meningitidis* pathogenesis, as well as the protective efficacy of monoclonal antibodies (Saukkonen *et al.*, 1989). This model has several advantages over other *in vivo* models. Firstly, there is no requirement for supplementation of the inoculum with exogenous iron as is required in mouse models (Yi *et al.*, 2003) (Holbein *et al.*, 1979). Secondly, in this model, the course of infection can be followed for at least 24 hours. This is a similar length of time to that between the asymptomatic and septicaemic stages of infection in individuals with meningococcal disease. Furthermore, infant rats infected with *N. meningitidis* can develop meningitis following septicaemia reflecting the progression of the disease in humans (Toropainen *et al.*, 1999). Initially, attempts were made to obtain CSF from infected animals in this study, but were unsuccessful. Therefore, the animal model used in this study focuses specifically on the septicaemic stage of *N. meningitidis* infection.

A disadvantage of the infant rat model is that it is not known how closely it reflects human disease. The route of inoculation used in this model bypasses the normal route of infection in the clinical situation. Studies have shown that within the nasopharynx, a number of human cellular receptors such as CD66 are specifically recognized and bound by *N. meningitidis* (Dehio *et al.*, 2000). Furthermore, the complement-regulatory protein, membrane cofactor protein or CD46, a receptor for pathogenic *Neisseria*, (Kallstrom *et al.*, 1998) is thought to determine the host specificity of *N.*

meningitidis and a recent study has shown that transgenic C57BL/6 mice expressing human CD46 are more susceptible to meningococcal disease than wild-type mice (Johansson *et al.*, 2003). Therefore, the infant rat model cannot be used for the investigation of the early stages of infection such as colonization and initial invasion, and can only be used to study later stages of pathogenesis.

As database searches revealed the presence of homologues of the genes of the NMB0342-NMB0348 locus in a range of pathogenic bacteria, further sequence analysis was carried out to examine whether the NMB0342-NMB0348 locus might constitute or be part of a pathogenicity island. PCR and Southern analyses demonstrate that while homologues of the NMB0342, NMB0345, and NMB0348 genes are present in all of the pathogenic *Neisseria* spp. tested, they are only found in a subset of the commensal *Neisseria* spp. tested. Several regions of DNA likely to have been acquired by horizontal transfer have been identified in the *N. meningitidis* genome, and include IHTs (islands of horizontal transfer), IHT-A, IHT-B, and IHT-C, described by Tettelin *et al.* and a further three regions in the MC58 genome, X1, X2, and X3, described by Vanucci *et al.*, identified using high resolution G+C plots (Tettelin *et al.*, 2000) (Lio and Vannucci, 2000). However, the NMB0342-NMB0348 locus is not located within these regions and does not have the features of a pathogenicity island (Julian Parkhill, personal communication).

The results of database searches demonstrate that homologues of NMB0345 are less common in other bacterial pathogens than homologues of NMB0342 and do not

suggest related functions for these genes. However, the close proximity of homologues of the NMB0342 and NMB0343 genes in a number of pathogenic bacteria including *H. influenzae*, *P. aeruginosa*, *V. cholerae*, and *Y. pestis*, and the involvement of the *S. flexneri* homologue of NMB0342, *ispA*, and *E. coli* homologue of NMB0343, *bolA*, in cell-division suggests that these genes may have related functions.

A potential explanation for the attenuation of the NMB0342 and NMB0345 mutants in the infant rat model of infection is that the mutants have a general growth defect. However, no difference was observed between the *in vitro* growth of the wild-type strain C311^{+} and either mutant. This does not exclude the possibility that the growth rate of the mutants may differ from that of the wild-type strain *in vivo*. Within the host, the bacterium must acquire nutrients such as iron that are essential for growth and survival. The *tonB* gene of *N. meningitidis*, a gene that plays a role in iron acquisition, has been shown to be required for replication within epithelial cells (Larson *et al.*, 2002). The quantification of bacterial replication *in vivo* is complicated by the ongoing clearance of bacteria by the defense mechanisms of the host. However, a method in which the host is inoculated with a combination of a temperature sensitive mutant that is unable to replicate in the host and the wild-type strain has been used successfully to measure the *in vivo* replication of *E. coli* and *P. aeruginosa*. This method allows the changes in the ratio of mutant and wild-type to be measured over time thereby allowing the number of wild-type generations to be calculated (Sordelli *et al.*, 1985). A similar method could potentially be used to

quantify the *in vivo* replication of *N. meningitidis* strains. However, a difficulty associated with this method is that mutations giving rise to temperature-sensitive mutants are generally spontaneous, unmarked mutations that may potentially affect the pathogenicity of the organism.

As *N. meningitidis* is exclusively a pathogen of humans, the survival of the NMB0342 and NMB0345 mutants in human whole blood and serum was also examined. The NMB0345 gene contributes to resistance against complement-mediated killing. However, the role of the NMB0342 gene is less clear and the attenuation of the NMB0342 mutant may result from multiple defects. Neither of these genes has previously been implicated in resistance to serum killing. The NMB0342 and NMB0345 mutants were not detected in a large-scale STM analysis of an *N. meningitidis* mutants of serogroup C strain in normal human serum (Geoffroy *et al.*, 2003). This result is not surprising as the STM method identifies mutants that are entirely absent at the end of the assay and not mutants with more subtle defects such as the NMB0342 and NMB0345 mutants.

Analysis of the survival of the NMB0342 and NMB0345 mutants in wild-type and C3-deficient mice confirmed the relevance of the serum-sensitivity of the NMB0345 mutant to its attenuation *in vivo*. Therefore, the use of an animal model of infection has served to further our understanding of the nature of the defect of the NMB0345 mutant *in vivo*. Results from the analysis of the NMB0342 mutant in C3-deficient and wild-type mice suggest that the mutation in the NMB0342 mutant may have pleiotropic effects; ie multiple effects resulting from the disruption of a single gene.

Based on the homology of NMB0342 to the *ispA* gene of *S. flexneri*, and the identification of homologues in only Gram-negative bacteria, a possible function of the NMB0342 gene is that it may play a role in cell division. The *S. flexneri ispA* mutant has been shown to be defective in intracellular septation within cultured epithelial cells.

Capsule and LPS are the main mediators of meningococcal serum resistance (Mackinnon *et al.*, 1993) (Kahler *et al.*, 1998; Vogel *et al.*, 1996; Vogel *et al.*, 1997). The importance of the capsule is highlighted by the observation that while invasive isolates of *N. meningitidis* are always encapsulated, isolates recovered from the nasopharynx are often unencapsulated (Nassif, 1999). A *siaD* mutant (a polysialyltransferase mutant deficient in capsule expression) and a *galE* mutant (deficient in sialylation of LPS) have previously been shown to be highly sensitive to complement-mediated lysis in normal human serum compared to wild-type *N. meningitidis* (Vogel *et al.*, 1997). The requirement for these virulence factors for serum-resistance has been further demonstrated by a large-scale analysis of the survival of *N. meningitidis* serogroup C mutants in normal human serum in which all mutants with increased susceptibility to complement-mediated lysis had mutations in either capsule or LPS biosynthesis genes (Geoffroy *et al.*, 2003). Therefore, to check for the presence of a defect in the LPS and/or capsule of the NMB0342 and NMB0345 mutants, the LPS profile and capsulation of the mutants was compared to that of the wild-type strain, $C311^{+}$. Analyses of LPS profiles detected no major differences between the LPS profiles of the wild-type strain $C311^{+}$ and either mutant by SDS-

PAGE. However, it is possible that subtle changes in LPS may not be detected by electrophoresis. Examination of the contribution (if any) of defects in LPS to the attenuation of the NMB0342 and NMB0345 mutants may be facilitated by the construction of NMB0342 and NMB0345 mutants with mutations in genes specifically required for the sialylation of LPS and comparison of the serum-sensitivity of these strains with the wild-type strain. As the reduction in survival of the NMB0345 mutant compared to the wild-type strain $C311^+$ in normal human serum was found to be similar for capsulated and un-encapsulated strains, the attenuation of the NMB0345 mutant does not appear to result from alterations in capsule expression.

It is possible that the NMB0342 and NMB0345 gene products may have an effect on serum-resistance either through direct interaction with components of the complement system or through other as yet unidentified bacterial factors. A further possibility is that mutation of the NMB0345 PPIase may lead to alterations in the conformation of target membrane proteins thereby affecting the integrity of the cell membrane. This would result in increased susceptibility of the membrane to insertion of the membrane attack complex (MAC).

The human complement system plays a crucial role in resistance to microbial infections (Brown *et al.*, 1983) (Fearon, 1998). The importance of complement-mediated killing in innate immunity is emphasized by the increased susceptibility to recurrent meningococcal disease of individuals with deficiencies in factors of the complement pathway (Biesma *et al.*, 2001) (Fijen *et al.*, 1998). Bacteria have evolved

numerous mechanisms to avoid complement-mediated killing (Rautemaa and Meri, 1999). An example of such a mechanism is the specific binding of sialic acid present in the LPS of *N. gonorrhoeae* to factor H, a key regulator of the alternative complement pathway. This results in the blocking of complement activation at the level of factor C3, thereby enhancing the serum-resistance of the bacterium (Ram *et al.*, 1998a; Ram *et al.*, 1998b).

Mice with deficiencies in other factors of the complement pathway are available and have been used for the study of *Streptococcus pneumoniae*, an organism which like *N. meningitidis* can cause both meningitis and septicaemia (Brown *et al.*, 2002) (Xu *et al.*, 2001). The use of mice with deficiencies in factors of the complement pathway would allow further characterization of the basis for the attenuation of the NMB0345 mutant.

Based on the homology of NMB0345 to previously characterized PPIases such as SurA of *S. typhimurium*, the biochemical role of the NMB0345 gene product was investigated. Expression of the NMB0345 protein was initially problematic. Expression of the protein at 37°C was found to be toxic to *E. coli* and this led to low levels of expression in initial attempts at purification of the protein. However, expression of recombinant NMB0345 protein was significantly enhanced by growing the bacteria at 30°C. This allowed purification of recombinant NMB0345 protein at a sufficient concentration for analysis of the protein's biochemical function and use in animal protection experiments.

The results of analysis of the NMB0345 mutant in wild-type and C3-deficient mice indicate that the NMB0345 PPIase may be involved in the modification of factors of the complement pathway. This possibility could be tested by performing complement-binding assays. A number of bacterial proteins including the YadA protein of *Y. enterocolitica* and the Por1A protein of *N. gonorrhoeae* have been shown to inhibit complement activation by directly binding to factors of the complement pathway. YadA binds to factor H, while Por1A binds to C4bp, a key regulator of the classical pathway (China *et al.*, 1993) (Ram *et al.*, 1998a; Ram *et al.*, 1998b).

The NMB0345 mutant is reduced in adherence to Chang epithelial cells compared to the wild–type strain. A disadvantage of using cultured epithelial cells such as Chang cells is that they are non-differentiated and are not polarized. The organ culture model, in which nasopharyngeal explants are infected with *N. meningitidis*, may be useful for the study of the ability of NMB0342 and NMB0345 mutants to colonize respiratory tissues.

Several approaches have been used for the identification and assessment of *N. meningitidis* vaccine candidates. A 'reverse vaccinology' approach was adopted by Pizza and colleagues as the basis for the selection of vaccine candidates (Pizza *et al.*, 2000). This approach relies on the availability of a genome sequence to allow the rapid identification of genes encoding proteins that are potentially surface located. Selection is not based on any evidence for the requirement of these genes during

infection. The STM method has an important advantage as genes are selected for investigation as virulence determinants based on knowledge of both their expression and requirement during infection.

The NMB0342 gene product was not selected for expression and purification as the NMB0342 protein is predicted to have numerous transmembrane regions and is therefore likely to have been difficult to purify. A study in which 570 potential vaccine candidates were cloned for expression found that expression failure was highest for proteins with more than one predicted transmembrane domain (Pizza *et al.*, 2000). However, like NMB0345, NMB0342 homologues were detected in all pathogenic *Neisseria* spp. tested and were present in only a subset of commensal species. Furthermore, the NMB0342 mutant is attenuated in the infant rat model of infection and complementation of the mutation has confirmed that this attenuation is due to the transposon insertion in NMB0342. Therefore, the NMB0342 gene product is expressed and required during infection and may have potential as a vaccine candidate.

An advantage of the NMB0345 protein in terms of its suitability as a vaccine candidate is that homologues of NMB0345 have been detected in all the pathogenic *Neisseria* spp. tested suggesting that this gene may be expressed in other strains of *N. meningitidis* and *N. gonorrhoeae*. Furthermore, the gene is required and therefore expressed both during interactions with epithelial cells and bloodstream infection.

Therefore, the NMB0345 gene product may be expressed and required during multiple stages of pathogenesis, providing an important advantage as a vaccine candidate.

The results of challenge experiments indicate that immunization with recombinant NMB0345 protein may confer protection against *N. meningitidis* serogroup B infection in mice. The Mip-related protein of *N. meningitidis*, NMB0995, has also been selected as a vaccine candidate. NMB0995 was found to be up-regulated in a microarray analysis of *N. meningitidis* serogroup B gene expression in response to contact with 16HBE14 epithelial cells (Grifantini *et al.*, 2002). The results of whole cell ELISA suggest that the NMB0345 protein is an accessible target. However, FACS analysis and/or fluorescence microscopy of the NMB0345 mutant using anti-NMB0345 mouse sera would allow a more accurate assessment of whether this protein may be surface-located as it would allow differentiation between lysed and non-lysed bacteria. The level of bactericidal activity of murine sera raised against NMB0345 protein against strain MC58 was found to be low. A number of other *N. meningitidis* vaccine candidates including *N. lactamica* OMVs and *N. meningitidis* antigens NspA and GNA2132 have been shown to protect against meningococcal challenge despite a lack of serum bactericidal activity (Oliver *et al.*, 2002) (Moe *et al.*, 2001) (Welsch *et al.*, 2003). Protection against meningococcal challenge has also been observed in mice immunized with TbpA in the absence of serum bactericidal activity (West *et al.*, 2001).

Recombinant NMB0345 protein was assayed for PPIase activity (*cis-trans*

isomerization of proline peptide bonds) *in vitro* using a method which relies on the ability of α–chymotrypsin to release p-nitroaniline from synthetic oligopeptide substrates only when the bond present on the N-terminal side of the proline residue is in the *trans* conformation (Fischer *et al.*, 1989). The results of these assays demonstrated that recombinant NMB0345 protein is an active PPIase. A difficulty associated with the PPIase assay is that a high level of background activity can be observed in reactions performed in the absence of PPIase as a significant proportion of the starting oligopeptide substrate is already in the *trans* conformation. However, use of the inhibitor FK506 results in a reduction in PPIase activity to levels similar to those obtained for reactions performed in the absence of NMB0345 protein and a clear increase in PPIase activity was observed in reactions performed in the presence of NMB0345 protein. Given that this is the second protein of *N. meningitidis* demonstrated to have PPIase activity, the first being NMB0027, (Sampson *et al.*, 1992), NMB0345 is here designated as *ppiB*. Apart from NMB0345, NMB0027, and NMB0995 (Mip-related protein), seven other genes in the genome sequence of serogroup B strain MC58 are predicted to encode PPIases based on homology to other known PPIases.

PPIase activity has been demonstrated for enzymes important for virulence that are present in a number of pathogens including *Legionella pneumophila*, *Chlamydia trachomatis*, *Salmonella typhimurium*, and *Trypanosoma cruzi*. The requirement of these enzymes for the virulence of such diverse microorganisms suggests that these enzymes may act by a mechanism that is common to both prokaryotic and eukaryotic

pathogens. The PPIases of the intracellular pathogens *L. pneumophila* (Mip), *C. trachomatis* (Mip-like protein), and *T. cruzi* (*Tc*Mip) have all been shown to enhance intracellular survival (Cianciotto and Fields, 1992) (Lundemose *et al.*, 1991) (Moro *et al.*, 1995). Although *N. meningitidis* is not considered to be an intracellular pathogen, the bacterium is capable of adhering to and invading both epithelial and endothelial cells (Virji *et al.*, 1993,Virji *et al.*, 1995). In the presence of the inhibitor FK506, FKBPs (a family of PPIases that bind to FK506) have been shown to inhibit calcineurin, a calcium and calmodulin-dependent phosphatase that is involved in signal transduction (Liu *et al.*, 1991). The ability of PPIases to modify the activity of a host cell regulatory protein such as calcineurin, represents a possible means by which these enzymes may enhance the intracellular survival of microbial pathogens.

Trigger factor of *Streptococcus pyogenes* is the only bacterial PPIase for which a target has been identified so far. The *S. pyogenes* cysteine protease (Streptococcal exotoxin B or SpeB) is folded and processed by trigger factor (Lyon *et al.*, 1998). SpeB is a multifunctional virulence factor with the ability to degrade both streptococcal surface proteins and host proteins. The action of the secreted cysteine protease on the surface of the bacterium releases an active fragment of streptococcal C5a proteinase, an enzyme that inactivates complement factor C5a (Berge and Bjorck, 1995). In addition, SpeB has the ability to cleave IgG (Eriksson and Norgren, 2003). Genetic inactivation of SpeB results in the attenuation of *S. pyogenes* in mice (Lukomski *et al.*, 1997; Lukomski *et al.*, 1998) and immunization of mice with SpeB protein protects against *S. pyogenes* infection (Kapur *et al.*, 1994). Inactivation of

SpeB also results in decreased survival of the bacterium in human whole blood (Eriksson and Norgren, 2003). In addition to cysteine protease activity, SpeB has also been shown to bind to laminin and other glycoproteins (Hytonen *et al.*, 2001). Furthermore, SpeB has been shown to bind to both fibronectin and vitronectin, extracellular matrix proteins which are known to mediate the adherence of *N. meningitidis* to endothelial cells (Unkmeir *et al.*, 2002; Virji *et al.*, 1994).

A number of similarities can be seen between the effect of inactivation of NMB0345 and inactivation of SpeB, such as reduced adhesion to epithelial cells by the NMB0345 mutant, reduced survival in whole blood, and impaired resistance to complement-mediated killing. Therefore, SpeB, the target of the *S. pyogenes* PPIase, trigger factor, may provide clues towards potential bacterial and host cell targets of NMB0345. Database searches using the BLASTP algorithm have revealed that NMB1975 has homology to the SpeB protein of *S. pyogenes*. Further studies are required to elucidate the mechanism by which the NMB0345 gene product may interact either directly or indirectly with host cells and potentially with factors of the complement pathway. Knowledge of the bacterial and/or eukaryotic target(s) of the NMB0345 PPIase would provide further insights into the specific factors required for systemic disease and may also provide new targets for intervention or potential vaccine candidates.

The role of PPIases in microbial pathogenicity is emerging as an important area of research warranting further investigation. The results of this study demonstrate that

the NMB0345 protein is a PPIase and indicate that NMB0345 may play a role at multiple stages of *N. meningitidis* pathogenesis. Therefore, PPIases may represent a previously unrecognized class of *N. meningitidis* virulence determinants. Although PPIase activity has previously been demonstrated for a meningococcal protein (NMB0027) (Sampson *et al.*, 1992), no role in pathogenesis has been shown for this enzyme. Therefore, NMB0345 is the first PPIase to be implicated in the virulence of *N. meningitidis*. It is possible that other *N. meningitidis* genes predicted to encode PPIases also play an important role in virulence. Therefore, these genes could also be analyzed for PPIase activity. Furthermore, as the results of preliminary challenge experiments indicate that immunization of mice with purified recombinant NMB0345 protein confers protection against infection with wild-type *N. meningitidis*, further PPIases of both *N. meningitidis* and other microbial pathogens could also be assessed for their potential as vaccine candidates.

Further Studies

Is the NMB0342-NMB0348 locus a polycistronic operon?

Analysis of the nucleotide sequence of the NMB0342-NMB0348 locus suggests that it may constitute a polycistronic operon. In order to establish whether these seven ORFs are transcribed as a single unit, reverse transcriptase (RT) PCR could be performed to generate a single mRNA transcript or overlapping mRNA transcripts. This information may be valuable, as genes that are part of a single operon often have related functions. RT-PCR has previously been used to analyze the *fbpABC* gene cluster of *N. meningitidis*, which is thought to mediate the transport of iron across the periplasmic space into the cytoplasm (Khun *et al.*, 2000).

What is the target of the NMB0345 PPIase?

Potential targets of the PPIase activity of the NMB0345 gene products include proteins that may be directly involved in the interaction of *N. meningitidis* with epithelial cells and/or in conferring serum resistance to the bacterium. Candidate proteins could be identified from whole cell or membrane fractions of *N. meningitidis* by resolving those proteins that are able to complex with purified NMB0345 protein by performing a 'pull down assay' (Kaelin *et al.*, 1991) (Soutoglou *et al.*, 2000). This would be achieved by binding purified recombinant NMB0345 protein to a metal chelate support and subsequently introducing a protein pool derived from a lysate of the wild-type *N. meningitidis* strain, MC58. Following washing steps, potential interacting proteins would be eluted and analyzed by SDS-PAGE or mass spectrometry.

In vitro assays of PPIase activity have previously been used to demonstrate the folding of the outer membrane proteins OmpA, OmpF, and LamB by the SurA PPIase of *E. coli* (Lazar and Kolter, 1996). The SurA protein of *E. coli* exhibits binding specificity for a peptide motif common to many integral outer membrane proteins (Bitto and McKay, 2003). As NMB0345 is predicted to encode an enzyme that assists in the folding of secreted proteins, in order to identify proteins that are either absent or migrate differently from wild-type proteins as a result of changes in conformation due to the loss of NMB0345 function, proteomic analysis could be performed to compare the 2D gel profile of the NMB0345 mutant with that of the wild-type bacterium. However, the detection of proteins by 2D gel electrophoresis is dependent on the yield of protein and can be complicated by the low expression of certain gene-products.

Is the PPIase activity of the NMB0345 protein required for virulence?

Analyses of site-specifically altered and truncated forms of the *Lp*Mip protein of *L. pneumophila* have helped to define both the requirement of *Lp*Mip PPIase activity in infection, and identify specific residues essential for enzymatic activity (Kohler *et al.*, 2003) (Wintermeyer *et al.*, 1995). The construction of *N. meningitidis* strains with defined point mutations in the NMB0345 gene would allow analysis of the contribution of NMB0345 PPIase activity to the virulence of the bacterium.

The crystal structures of bacterial PPIases required for virulence, *Lp*Mip in *L. pneumophila*, *Tc*Mip in *T. cruzi*, and SurA in *S. typhimurium*, have been solved and the active site residues determined (Riboldi-Tunnicliffe *et al.*, 2001) (Pereira *et al.*, 2002)

(Bitto and McKay, 2002). X-ray crystallography of purified recombinant NMB0345 protein would allow comparison of NMB0345 with the crystal structures of these proteins and may allow the identification of active site residues of the NMB0345 protein.

Does the NMB0345 protein confer protection against infection by heterologous strains?

The ability of recombinant NMB0345 to confer protection against challenge with other strains of serogroup B and isolates of other serogroups of *N. meningitidis* could be studied. In addition, blood collected from mice immunized with recombinant NMB0345 could be used to perform whole blood assays to measure killing of strains from a range of pathogenic serogroups of *N. meningitidis*.

Is the NMB0345 protein surface located or secreted?

Analysis of the published MC58 genome sequence of NMB0345 using the PSORT server (http://psort.ims.u-tokyo.ac.jp/form.html) suggests that the NMB0345 protein is a periplasmic protein (Nakai and Kanehisa, 1991). Anti-NMB0345 mouse sera could be used to perform fluorescence activated cell sorter (FACS) analysis to determine whether the NMB0345 protein is surface-located. Alternatively, immunofluorescence or electron microscopy could be used for immunolocalization of the NMB0345 protein. Immunogold analysis of the *Lp*Mip PPIase of *L. pneumophila* has allowed localization of the protein to both the cell surface of extracellularly grown bacteria and to host cell membranes of *A. castellanii* infected with *L. pneumophila*. The accumulation of the *Lp*Mip in host membranes is thought to increase the infection potential of the organism (Helbig *et al.*, 2001).

Anti-NMB0345 mouse sera could also be used to establish whether the NMB0345 is secreted by performing Western analysis of proteins present in the supernatant of $C311^+$ and comparison with supernatant from cultures of the NMB0345 mutant.

Does lipidation enhance the immunogenicity of the NMB0345 protein?

The NMB0345 protein is predicted to be a lipoprotein. The lipidation of GNA33 and GNA1870, *N. meningitidis* antigens being investigated as vaccine candidates, has been analyzed by monitoring the incorporation of ^3H palmitic acid (Jennings *et al.*, 2002) (Masignani *et al.*, 2003). Similar analyses could be performed to determine whether the NMB0345 protein is lipidated. Studies suggest that the attachment of a lipid may improve the immunogenicity of a protective antigen (Baier *et al.*, 2000). Lipidated and non-lipidated forms of the NMB0345 protein could be used to immunize mice. Immunization of mice with lipidated and non-lipidated forms of the *Borrelia burgdorferi* protein OspA, indicate that the lipid attachment significantly enhances the immunogenicity of this protein (Erdile *et al.*, 1993).

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Appendix A

Bacterial strains used for PCR and Southern analysis

N. meningitidis isolates:

Serogroup	Strain
A	Z2491
A	F6124
A	2059001
C	90/18311
W	A22
X	E26
Y	860800
Z	E32
B	2996
B	528
B	BZ169
B	BZ83
B	H4476
B	MC58[+]
B	C311[+]
B	B16/B6
B	1000
B	2970
B	BZ133
B	BZ147
B	BZ198
B	BZ232
B	NG3/88
B	NG6/88
B	NGE28
B	NGE31
B	NGF26
B	NGH15
B	NGH36
B	NGH38
B	NGP165
B	SWZ107

N. gonorrhoeae isolates:

Serogroup	Strain
N. gonorrhoeae	FA1090
N. gonorrhoeae	MS11

Non-pathogenic isolates:

Serogroup	Strain
N. lactamica	L13
N. lactamica	L18
N. polysaccharea	P1
N. polysaccharea	P4
N. mucosa	M7
N. cinerea	F1
N. cinerea	F3
N. elongata	I1
N. elongata	I3
N. sicca	Q29
N. perflava	U15
N. subflava	U37
N. animalis	
E. coli	DH5α

All *Neisseria* strains were provided by Martin Maiden, University of Oxford.

Appendix B

Primers used in this Study

Primers	Anneals	Direction	Sequence
NG 197	5' end of NMB0342	Forward	5'TGAAATTCGTCAGCGACCTT3'
NG 198	3' end of NMB0342	Reverse	5'TCCTCCTTTTTCAGACAGGT3'
NG 216	5' end of NMB0345	Forward	5'AAAATCCTGACTTCCGTTGC3'
NG 217	3' end of NMB0345	Reverse	5'TTGCAGGTTTGATGTTTGCC3'
NG240	3' end of NMB0341	Forward	5'TACGGTAATGCTGCCGGAAAA3'
NG241	5' end of NMB0346	Reverse	5'CCTTAATTGCCTGATAAAGCG3'
NG 290	5' end of NMB0342 starts with *Mlu*I site for ligation into complementation vector pEVori	Forward	5'ACGCGTAAATTCGTCAGCGACCTTTT3'
NG 291	3' end of NMB0342 containing *Bam*H*I* site for ligation into complementation vector pEVori	Reverse	5'GGATCCATCCTCCTTTTTCAGACAGG3'
NG 292	5' end of NMB0345 with *Mlu*I site for ligation into complementation	Forward	5'ACGCGTAAAGCAAAAATCCTGACTTC3'

	vector pEVori		
NG 327	0.5 kb downstream of 3' end of NMB0347	Reverse	5'AATCGGCAACGGTTTGGTAT3'
NG 328	3' end of NMB0345 with *Bam*HI site for ligation into complementation vector pEVori	Reverse	5'GGATCCTTTTGCAGGTTTGATGTTTG3'
NG 330	0.5kb downstream of 3' end of NMB0348	Reverse	5'TTACGCCCAAAACCTCGC3'
NG 337	5' end of NMB0345	Forward	5'ATGAAAGCAAAAATCCTGAC3'
NG 338	3' exact end of NMB0345	Reverse	5'TTTTGCAGGTTTGATGTTTG3'
NG 339 (Kan-2-FWD)	kan^R cassette of Tn*5*	Forward	5'ACCTACAACAAAGCTCTCATCAACC3'
NG 340 (Kan-2-REV)	kan^R cassette of Tn*5*	Reverse	5'GCAATGTAACATCAGAGATTTTGAG3'
NG 374	3' end of NMB0345	Forward	5'GTCAACGACAGCCGCGAGG3'
NG 375	3' end of NMB0345	Reverse	5'CTTGCCCAACAGTGCACCGAC3'
NG 376	pCRT7/CT vector to sequence from 5' end of cloned NMB0345	Reverse	5'CGTTAATCCGGATATAGTTCC3'
NG 377	5' end of NMB0348	Forward	5'TGAACGACAATACACACACCC3'
NG 378	3' end of NMB0348	Reverse	5'ATATTTGCCCGTTCGACCTCT3'
NG 379	142 bp upstream of 5' end of NMB0346	Forward	5'CTTCAGGCGGAACGGATT3'
NG 380	186 bp downstream of 3' end of NMB0346	Reverse	5'TACGCCGAGTAACACGACAAA3'

Appendix C

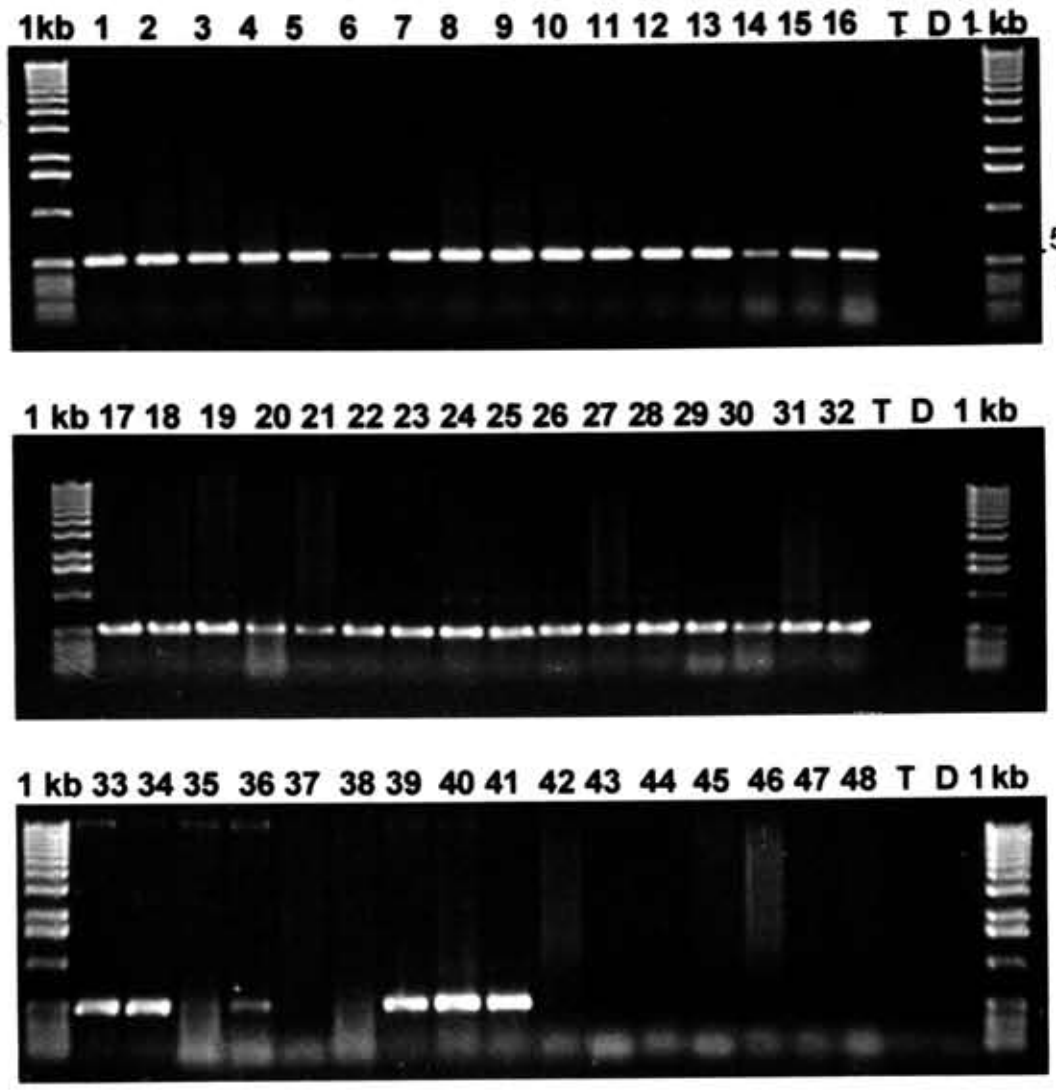


Fig. A1.1 **PCR amplification of NMB0342**

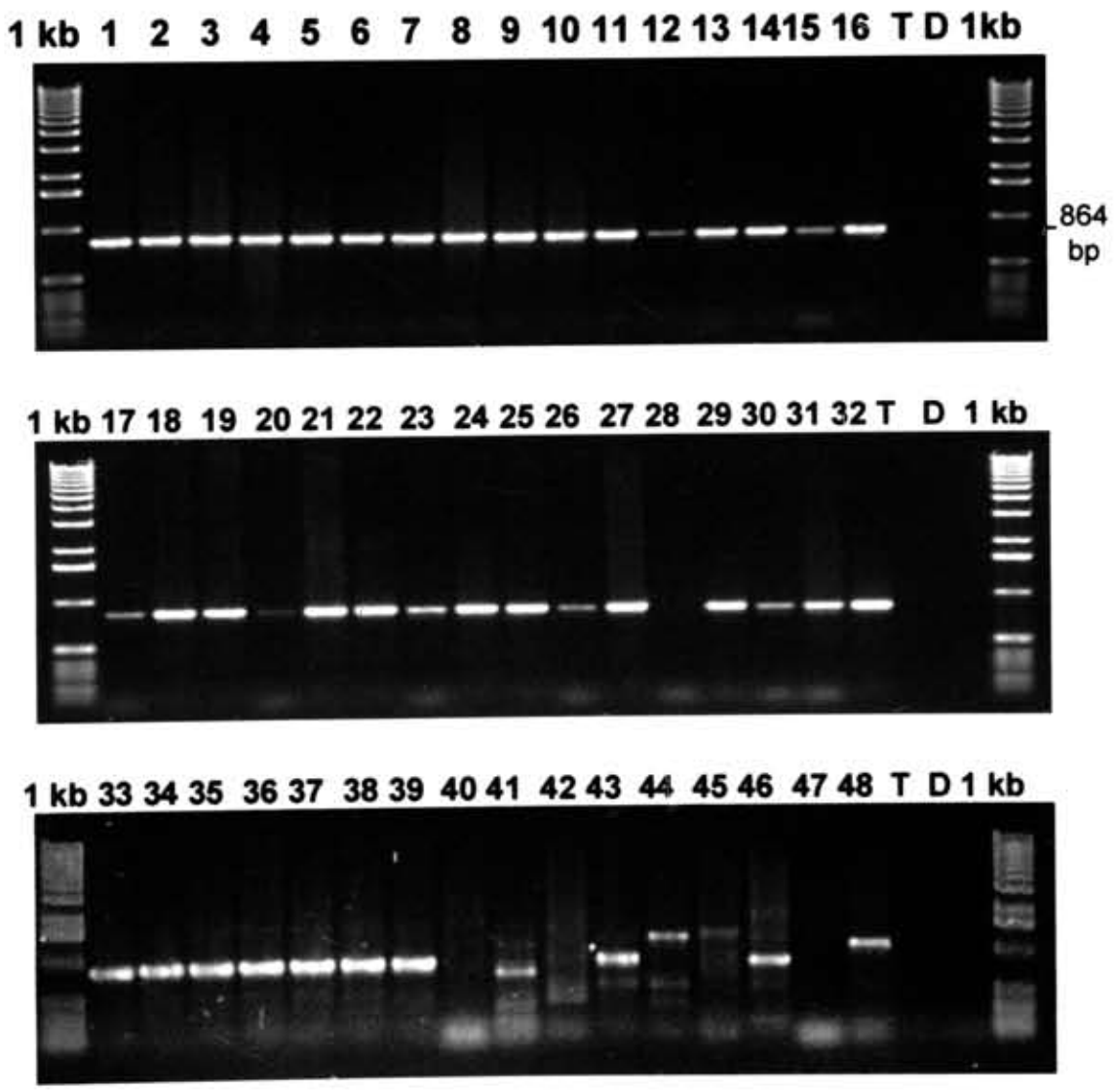
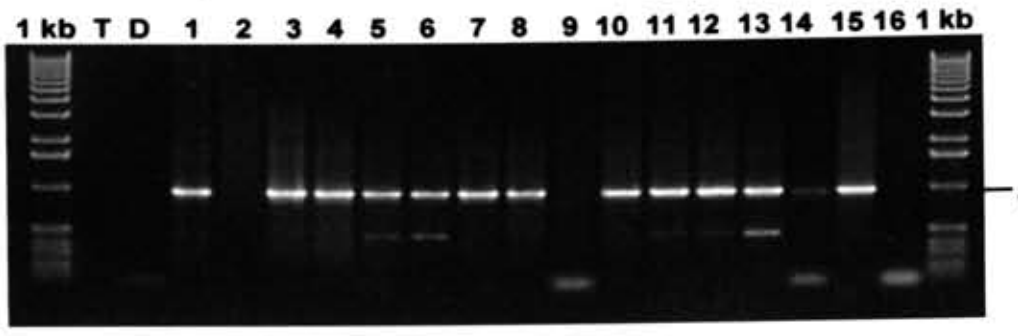


Fig. A1.2 **PCR amplification of NMB0345**

Key No.	Serogroup/ Species	Strain
1	A	Z2491
2	A	F6124
3	A	20059001
4	C	90/18311
5	W	A22
6	X	E26
7	Y	860800
8	Z	E32
9	B	2996
10	B	528
11	B	BZ169
12	B	BZ83
13	B	H4476
14	B	MC58
15	B	C311+
16	B	B16/B6
17	B	1000
18	B	2970
19	B	BZ133
20	B	BZ147
21	B	BZ198
22	B	BZ232
23	B	NG3/88
24	B	NG6/28
25	B	NGE28
26	B	NGE31
27	B	NGF26
27	B	NGH15
29	B	NGH36
30	B	NGH38
31	B	NGP165
32	B	SWZ107
33	*N. gonorrhoeae*	FA1090
34	*N. gonorrhoeae*	MS11
35	*N. lactamica*	L13
36	*N. lactamica*	L18
37	*N. polysaccharea*	P1
38	*N. polysaccharea*	P4
39	*N. mucosa*	M7
40	*N. cinerea*	F1
41	*N. cinerea*	F3
42	*N. elongata*	I1
43	*N. elongata*	I3
44	*N. sicca*	Q29
45	*N. perflava*	U15
46	*N. subflava*	U37
47	*N. animalis*	
48	*E. coli* DH5α	
1 kb	1 kb DNA ladder	
T	no Taq	
D	no DNA	

(please see figure legends on following page)

Appendix C



Key No.	Serogroup	Strain
1	A	Z2491
2	A	F6124
3	A	20059001
4	C	90/18311
5	W	A22
6	X	E26
7	Y	860800
8	Z	E32
9	B	2996
10	B	528
11	B	BZ169
12	B	BZ83
13	B	H4476
14	B	MC58
15	B	C311+
16	B	B16/B6

1 kb	1 Kb DNA ladder
T	no Taq
D	no DNA

Fig. A1.3 **PCR amplification of NMB0348**

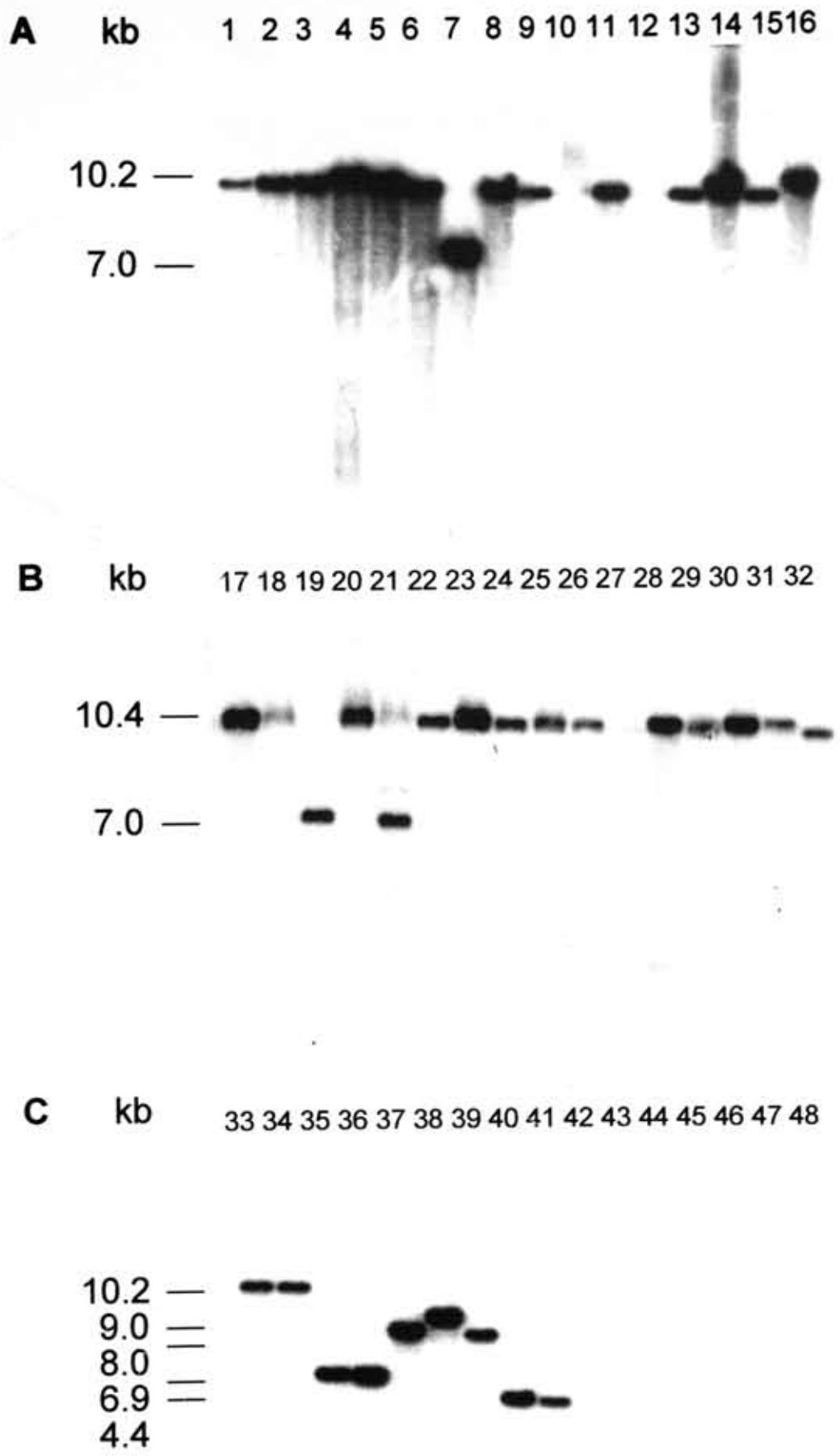
PCR analysis of pathogenic *Neisseria* spp. to check for the presence of the NMB0348 sequence.

Fig. A1.1 PCR analysis of a range of pathogenic and commensal *Neisseria* spp. to check for the presence of the NMB0342 sequence.

Fig. A1.2 PCR analysis of a range of pathogenic and commensal *Neisseria* spp. to check for the presence of the NMB0345 sequence.

(Please refer to Chapter 3, section 3.2.1 for details of PCR analysis of *Neisseria* strains).

Appendix D



Key No.	Serogroup/ Species	Strain
1	A	Z2491
2	A	F6124
3	A	20059001
4	C	90/18311
5	W	A22
6	X	E26
7	Y	860800
8	Z	E32
9	B	2996
10	B	528
11	B	BZ169
12	B	BZ83
13	B	H4476
14	B	MC58
15	B	C311+
16	B	B16/B6
17	B	1000
18	B	2970
19	B	BZ133
20	B	BZ147
21	B	BZ198
22	B	BZ232
23	B	NG3/88
24	B	NG6/28
25	B	NGE28
26	B	NGE31
27	B	NGF26
29	B	NGH15
30	B	NGH36
31	B	NGH38
32	B	NGP165
33	*N. gonorrhoeae*	SWZ107
34	*N. gonorrhoeae*	FA1090
35	*N. lactamica*	MS11
36	*N. lactamica*	L13
37	*N. polysaccharea*	L18
38	*N. polysaccharea*	P1
39	*N. mucosa*	P4
40	*N. cinerea*	M7
41	*N. cinerea*	F1
42	*N. elongata*	F3
43	*N. elongata*	I1
44	*N. sicca*	I3
45	*N. perflava*	Q29
46	*N. subflava*	U15
47	*N. animalis*	U37
48	*E. coli* DH5α	
1 kb	1 kb DNA ladder	
T	no Taq	
D	no DNA	

Fig. A2.1 **Southern analysis of pathogenic and commensal *Neisseria* spp. for the presence of the NMB0342 sequence**

Appendix D

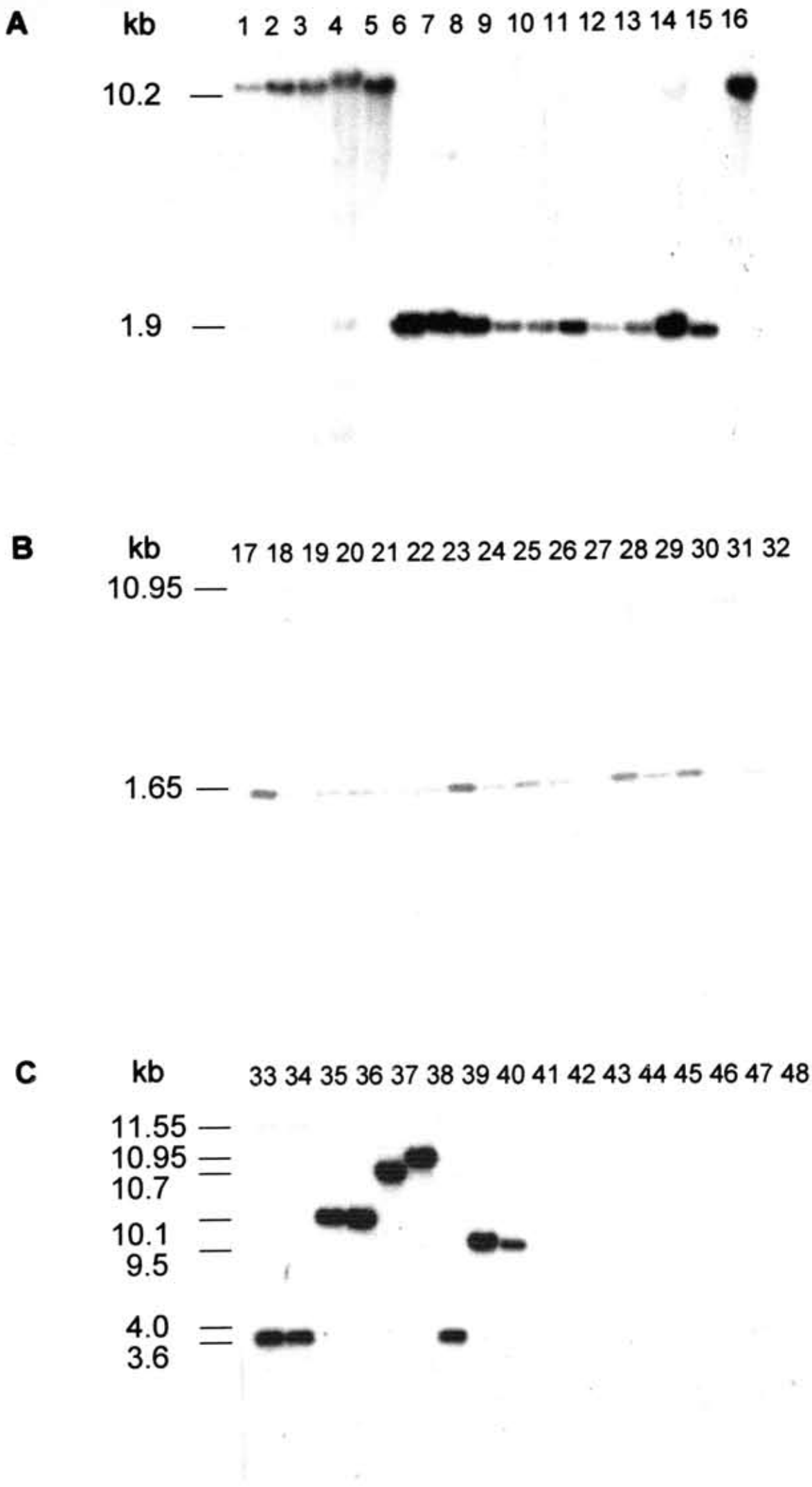


Fig. A2.2 **Southern analysis of pathogenic and commensal *Neisseria* spp. for the presence of the NMB0345 sequence**

Appendix D

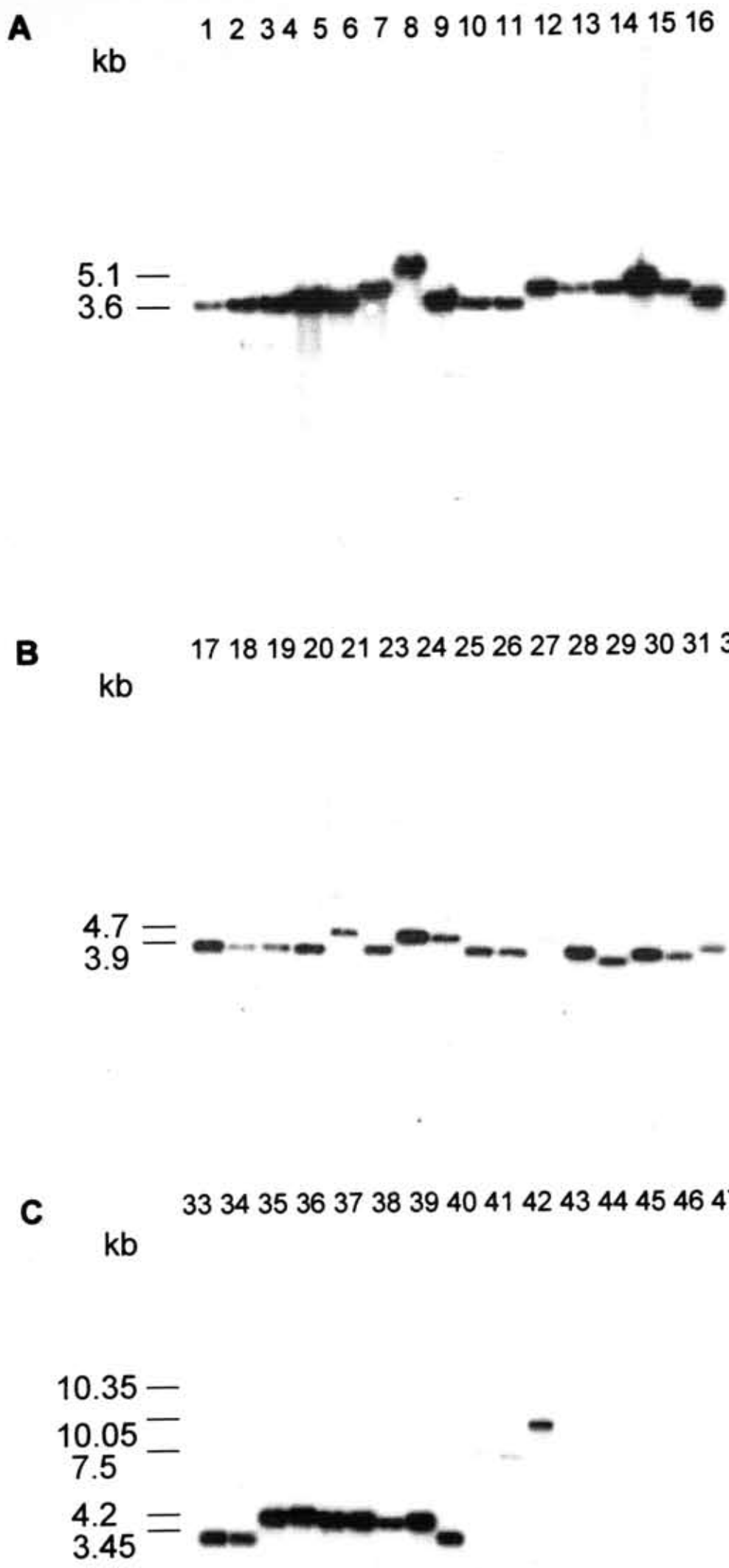


Fig. A2.3 **Southern analysis of pathogenic and commensal *Neisseria* spp. for the presence of the NMB0348 sequence**

Fig. A2.1-A2.3 Southern analysis of a range of pathogenic and commensal *Neisseria* spp. to check for the presence of the NMB0342 (Fig. A2.1), NMB0345 (Fig. A2.2), and NMB0348 (Fig. A2.3) sequences. Please refer to Chapter 3, section 3.2.1 for details of Southern analysis of *Neisseria* strains.

Appendix E

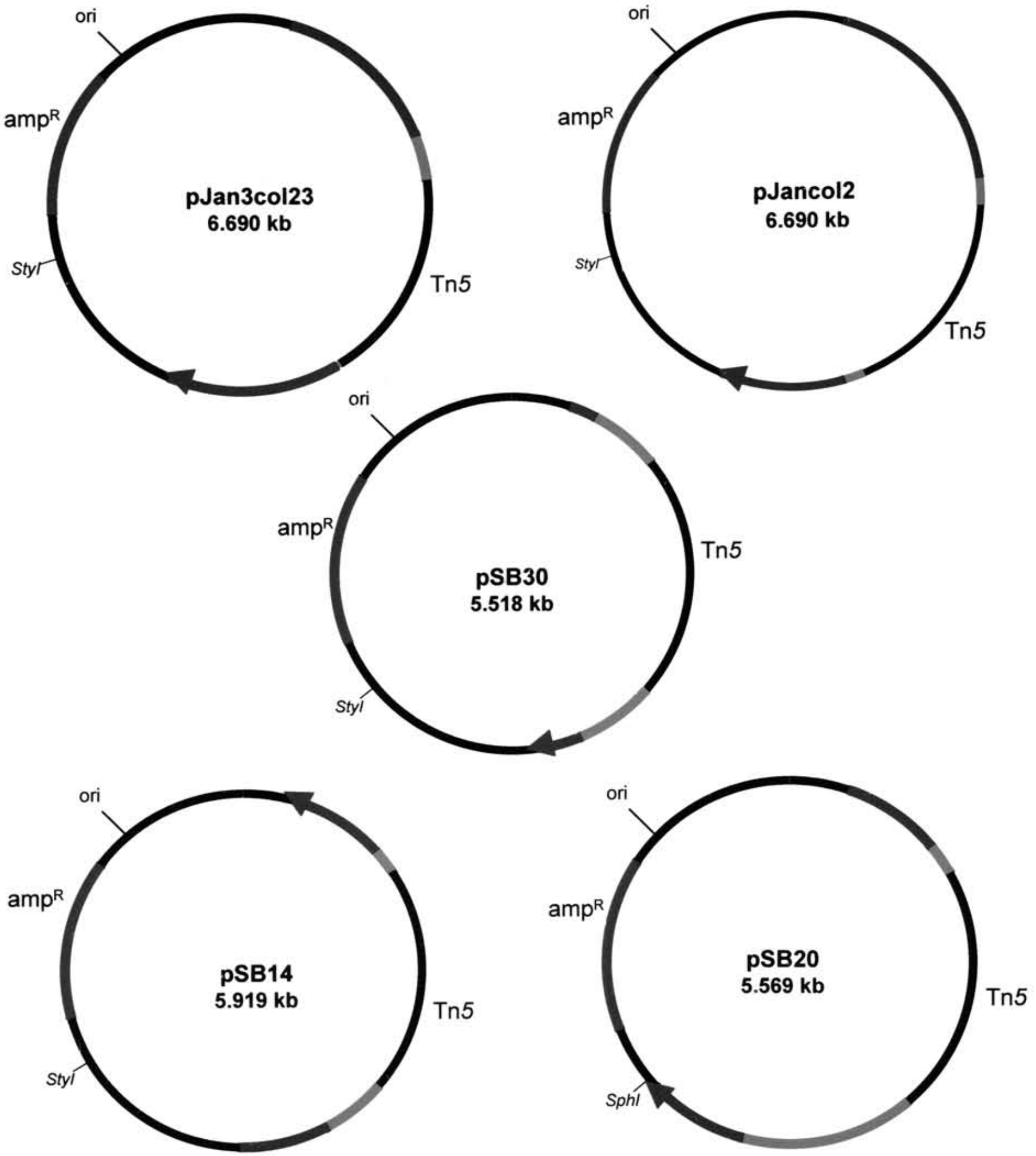


Fig. A3.1 Plasmids generated for the construction of *N. meningitidis* mutants

Fig. A3.1 **Plasmids generated for the construction of *N. meningitidis* mutants**

Plasmids generated for the construction of *N. meningitidis* mutants; pJanCol23 (Tn*5* insertion in NMB0343), pJancol2 (Tn5 insertion in NMB0344), pSB30 (Tn*5* insertion in NMB0346), pSB14 (Tn*5* insertion in NMB0347), and pSB20 (Tn*5* insertion in NMB0348). The ORF into which the transposon has inserted is represented in red. Flanking sequences are shown by a solid blue line. The transposon is represented in black. The amp^R gene is represented in green. Enzymes used for the deletion of the kan^R cassette (*Sty*I or *Sph*I) are also shown. The origin of replication is indicated as 'ori'.

Appendix F

Fig. A4.1 Sensitivity of the NMB0342 and NMB0345 mutants to saponin

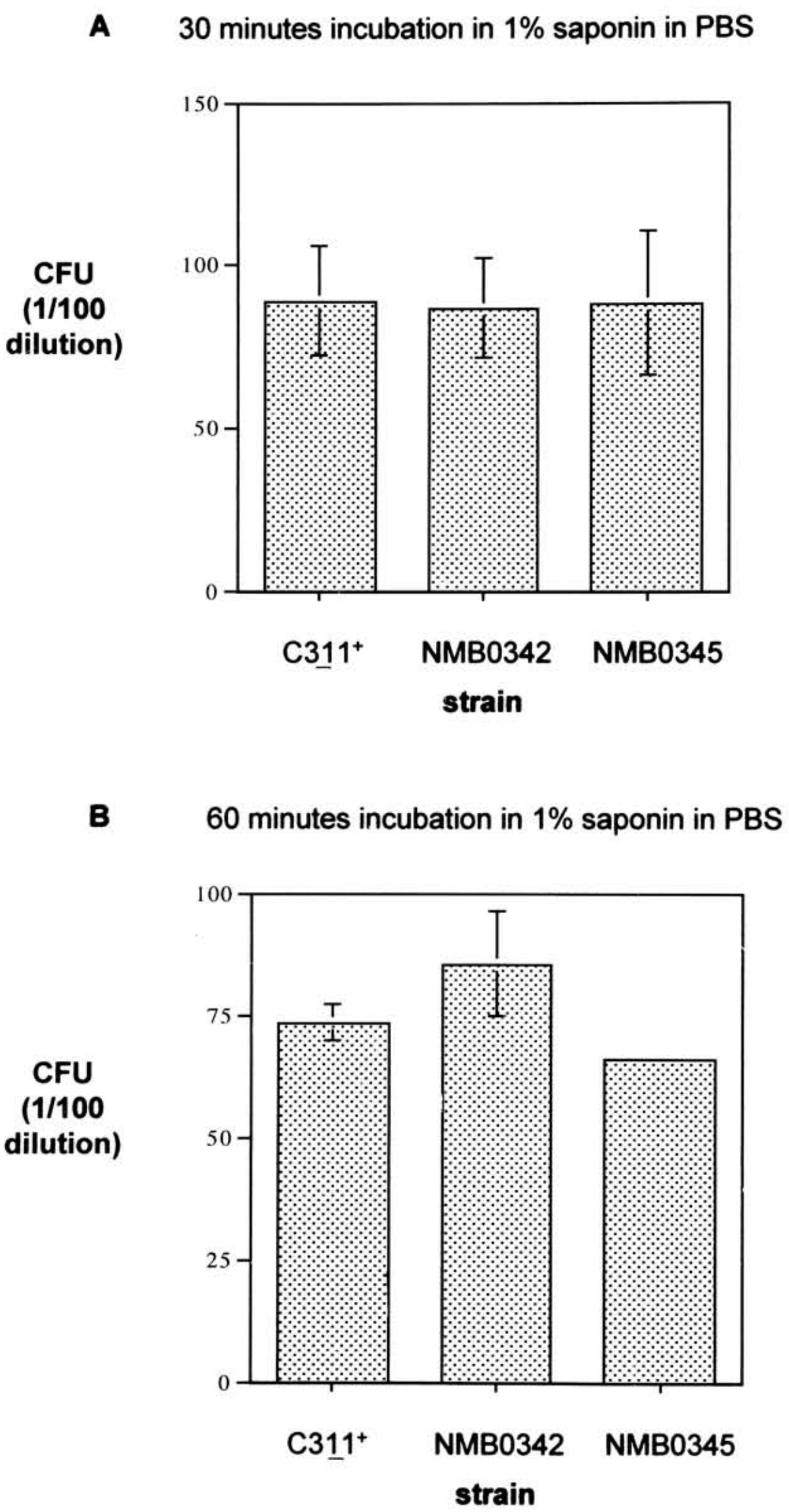


Fig. A4.1 Sensitivity of the NMB0342 and NMB0345 mutants to saponin

In order to test the sensitivity of the wild-type strain C311^{+}, NMB0342 and NMB0345 mutants to saponin, 1 x 10^{5} CFU of each strain was inoculated into 1% saponin in PBS in a 96-well microtitre plate in triplicate. Following 30 minutes and 60 minutes incubation dilutions were plated to BHI agar for enumeration. No difference in survival was seen for either mutant compared to the wild-type.

Appendix G

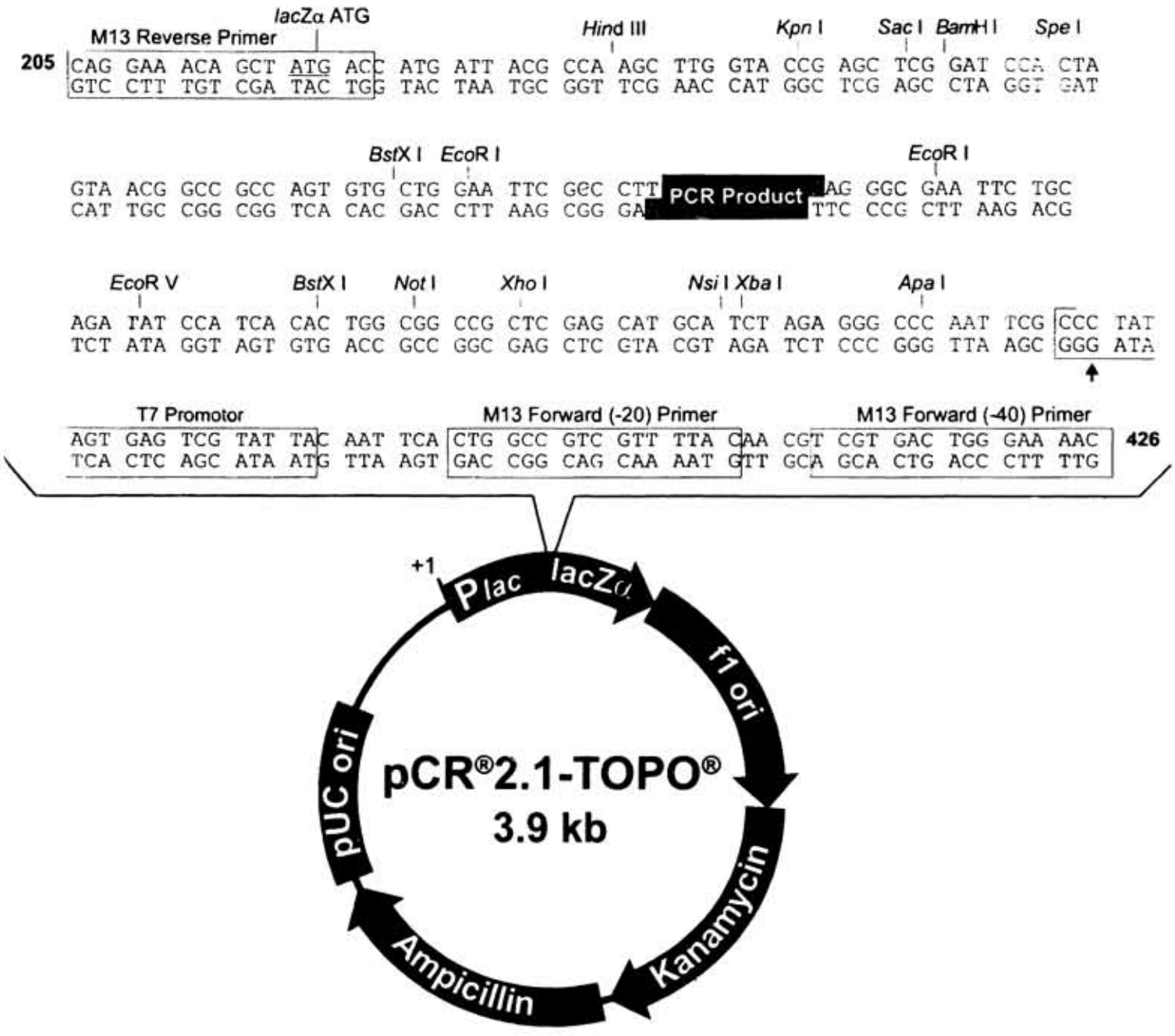
Plasmids used in this study

pCR® 2.1-TOPO®	TA cloning vector (Invitrogen)
pJancol23	pCR® 2.1-TOPO® with kan^R deleted containing NMB0343 insert (Tn*5* insertion in NMB0343).
pJancol2	pCR® 2.1-TOPO® with kan^R deleted containing NMB0344 insert (Tn*5* insertion in NMB0344).
pSB30	pCR® 2.1-TOPO® with kan^R deleted containing NMB0346 insert (Tn*5* insertion in NMB0346).
pSB14	pCR® 2.1-TOPO® with kan^R deleted containing NMB0347 insert (Tn*5* insertion in NMB0347).
pSB20	pCR® 2.1-TOPO® containing NMB0348 insert (Tn*5* insertion in NMB0348).
pEVori	Plasmid constructed by Y. Sun for complementation of *N. meningitidis* mutants.
pSB27	pEVori containing NMB0342 plasmid used for complementation of the NMB0342 mutant.
pCR®T7/CT-TOPO®	Protein expression vector (Invitrogen).
pCR®T7/CT-LacZ	Protein expression vector encoding β-galactosidase (Invitrogen).
pSB5	Plasmid used for expression of NMB0345 protein (pCR®T7/CT-TOPO® containing NMB0345 insert).
pSB10	Plasmid used for expression of NMB0345 protein (pCR®T7/CT-TOPO® containing NMB0345 insert excluding 21 nt at the 3' end of NMB0345).

Appendix G

Map and features of pCR®2.1-TOPO®

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. For the full sequence of the vector, you may download it from our web site or call Technical Service (page 19).



Comments for pCR®2.1-TOPO®
3908 nucleotides

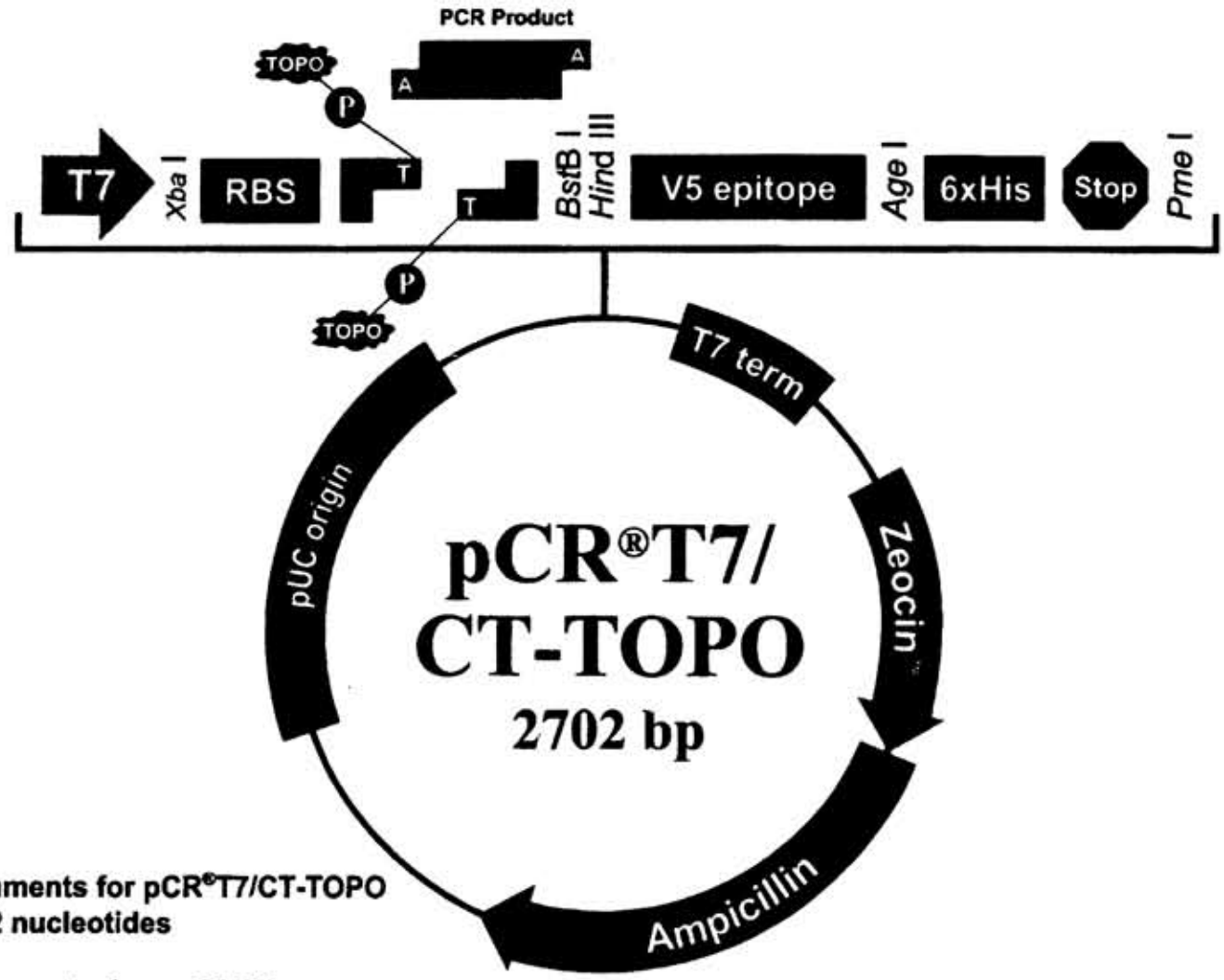
LacZα fragment: bases 1-571
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
M13 Forward (-40) priming site: bases 411-426
f1 origin: bases 548-962
Kanamycin resistance ORF: bases 1296-2090
Ampicillin resistance ORF: bases 2108-2968
pUC origin: bases 3113-3786

Appendix G

Map and Features of pCR®T7/CT-TOPO®

pCR®T7/CT-TOPO® Map

The map below shows the features of pCR®T7/CT-TOPO® . **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (page 38).**



Comments for pCR®T7/CT-TOPO
2702 nucleotides

T7 promoter: bases 21-37
T7 promoter priming site: bases 21-40
Ribosome binding site: bases 85-91
TOPO™ Cloning site: bases 100-101
V5 epitope: bases 122-163
V5 (C-term) Reverse priming site: bases 131-151
Polyhistidine (6xHis) region: bases 173-190
T7 transcription terminator: bases 240-287
Zeocin resistance gene: bases 367-830
ORF: bases 456-830
Ampicillin resistance gene: bases 834-1711
ORF: bases 851-1711
pUC origin: bases 1856-2529

Appendix G

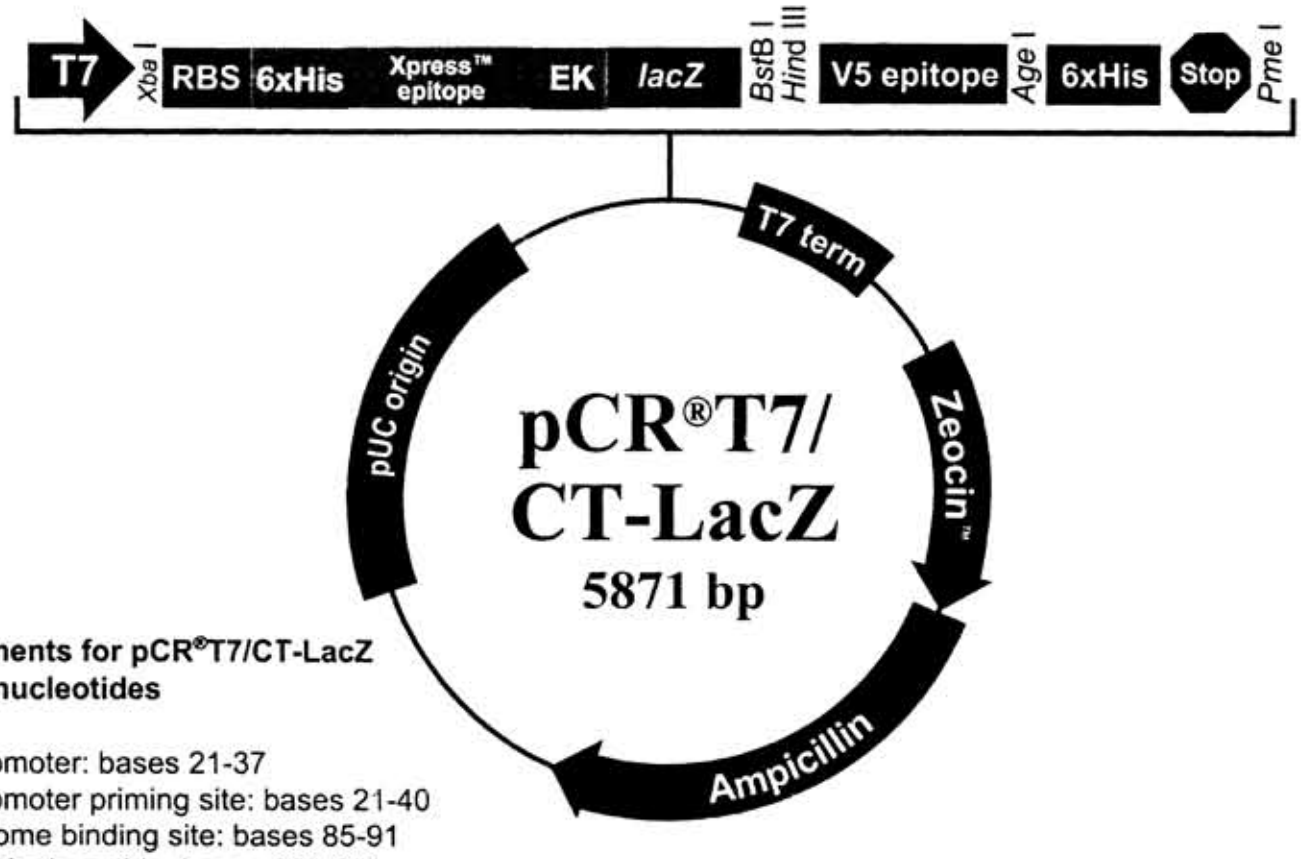
Map of pCR®T7/CT-LacZ

Description

pCR®T7/CT-LacZ is a 5871 bp control vector expressing β-galactosidase. Please note that β-galactosidase is fused to an N-terminal peptide containing the Xpress™ peptide as well as being fused to the C-terminal peptide encoding the V5 epitope and a 6xHis tag. The Xpress™ peptide contains an additional 6xHis tag, the Xpress™ epitope, and an enterokinase recognition site. The molecular weight is approximately 120 kDa.

Map of Expression Control Vector

The figure below summarizes the features of the pCR®T7/CT-LacZ vector. **The complete nucleotide sequence for pCR®T7/CT-LacZ is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 38).**

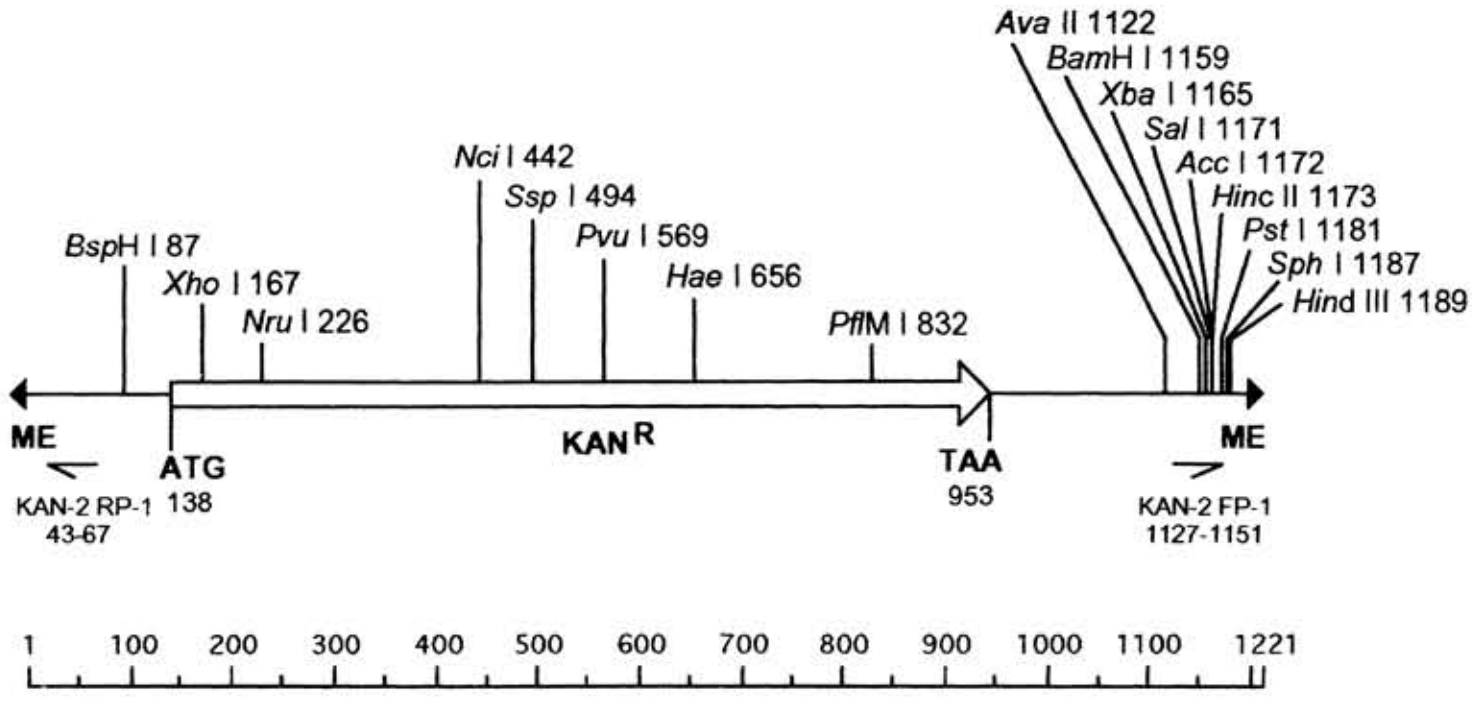


Comments for pCR®T7/CT-LacZ
5871 nucleotides

T7 promoter: bases 21-37
T7 promoter priming site: bases 21-40
Ribosome binding site: bases 85-91
N-terminal peptide: bases 100-221
    Polyhistidine (6xHis) region: bases 112-129
    Xpress™ epitope: bases 169-192
    Enterokinase recognition site: bases 178-192
LacZ coding sequence: bases 223-3268
V5 epitope: bases 3292-3333
V5 (C-term) Reverse priming site: bases 3301-3321
Polyhistidine (6xHis) region: bases 3343-3360
T7 transcription terminator: bases 3410-3457
Zeocin resistance gene: bases 3537-4000
  ORF: bases 3626-4000
Ampicillin resistance gene: bases 4004-4881
  ORF: bases 4021-4881
pUC origin: bases 5055-5635

Appendix H

EZ::TN™ <KAN-2> Transposon
(1221 bp.)



Note: Not all restriction enzymes that cut only once are indicated above.
See the following pages for further information.
Primers are not drawn to scale.

KAN-2 FP-1 Forward Primer	5' ACCTACAACAAAGCTCTCATCAACC 3'
KAN-2 RP-1 Reverse Primer	5' GCAATGTAACATCAGAGATTTTGAG 3'
ME = Mosaic End	5' AGATGTGTATAAGAGACAG 3'

Appendix H

9-d. EZ::TN <KAN-2> Transposon Sequence

EZ::TN™ <KAN-2> Transposon 1221 bp.

```
   1 CTGTCTCTTA TACACATCTC AACCATCATC GATGAATTGT GTCTCAAAAT
  51 CTCTGATGTT ACATTGCACA AGATAAAAAT ATATCATCAT GAACAATAAA
 101 ACTGTCTGCT TACATAAACA GTAATACAAG GGGTGTTATG AGCCATATTC
 151 AACGGGAAAC GTCTTGCTCG AGGCCGCGAT TAAATTCCAA CATGGATGCT
 201 GATTTATATG GGTATAAATG GGCTCGCGAT AATGTCGGGC AATCAGGTGC
 251 GACAATCTAT CGATTGTATG GGAAGCCCGA TGCGCCAGAG TTGTTTCTGA
 301 AACATGGCAA AGGTAGCGTT GCCAATGATG TTACAGATGA GATGGTCAGA
 351 CTAAACTGGC TGACGGAATT TATGCCTCTT CCGACCATCA AGCATTTTAT
 401 CCGTACTCCT GATGATGCAT GGTTACTCAC CACTGCGATC CCCGGAAAAA
 451 CAGCATTCCA GGTATTAGAA GAATATCCTG ATTCAGGTGA AAATATTGTT
 501 GATGCGCTGG CAGTGTTCCT GCGCCGGTTG CATTCGATTC CTGTTTGTAA
 551 TTGTCCTTTT AACAGCGATC GCGTATTTCG TCTCGCTCAG GCGCAATCAC
 601 GAATGAATAA CGGTTTGGTT GATGCGAGTG ATTTTGATGA CGAGCGTAAT
 651 GGCTGGCCTG TTGAACAAGT CTGGAAACAA ATGCATAAAC TTTTGCCATT
 701 CTCACCGGAT TCAGTCGTCA CTCATGGTGA TTTCTCACTT GATAACCTTA
 751 TTTTTGACGA GGGGAAATTA ATAGGTTGTA TTGATGTTGG ACGAGTCGGA
 801 ATCGCAGACC GATACCAGGA TCTTGCCATC CTATGGAACT GCCTCGGTGA
 851 GTTTTCTCCT TCATTACAGA AACGGCTTTT TCAAAAATAT GGTATTGATA
 901 ATCCTGATAT GAATAAATTG CAGTTTCATT TGATGCTCGA TGAGTTTTTC
 951 TAATCAGAAT TGGTTAATTG GTTGTAACAC TGGCAGAGCA TTACGCTGAC
1001 TTGACGGGAC GGCGGCTTTG TTGAATAAAT CGAACTTTTG CTGAGTTGAA
1051 GGATCAGATC ACGCATCTTC CCGACAACGC AGACCGTTCC GTGGCAAAGC
1101 AAAAGTTCAA AATCACCAAC TGGTCCACCT ACAACAAAGC TCTCATCAAC
1151 CGTGGCGGGG ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTCAGGGT
1201 TGAGATGTGT ATAAGAGACA G
```

Appendix H

9-c. EZ::TN <KAN-2> Transposon Restriction Data

Restriction Enzymes that cut the EZ::TN <KAN-2> Transposon 1 to 3 times:

Enzyme	Sites	Location	Enzyme	Sites	Location	Enzyme	Sites	Location
Acc I	1	1172	BstDS I	2	1093, 1154	Nci I	1	442
Aci I	1	174	BstF5 I	2	200, 826	Nla IV	1	1161
Alu I	2	1139, 1191	BstN I	2	459, 816	Nru I	1	226
Apo I	2	182, 366	BstU I	3	176, 226, 571	Nsi I	2	419, 685
AsiS I	1	569	BstY I	2	818, 1159	Nsp I	1	1187
Ase I	1	768	Btg I	2	1089, 1150	PaeR7 I	1	167
Ava I	1	167	Bts I	2	430, 517	PflM I	1	832
Ava II	1	1122	Cla I	2	29, 260	Ple I	2	801, 1177
BamH I	1	1159	Dde I	2	586, 1041	PspG I	2	457, 814
Ban II	1	224	Dsa I	2	1089, 1150	Pst I	1	1181
Bfa I	1	1166	Ear I	1	382	Pvu I	1	569
BfrB I	2	417, 683	EcoN I	1	481	Rsa I	1	404
BfuA I	1	1184	Fau I	1	1148	Sal I	1	1171
Bpu10 I	1	586	Fnu4H I	2	174, 1013	Sau96 I	1	1122
BsaW I	1	704	Hae I	1	656	Sbf I	1	1181
BsiE I	1	569	Hae III	2	173, 656	ScrF I	3	442, 459, 816
Bsm I	2	453, 530	Hinc II	1	1173	Sfc I	1	1177
BsmB I	1	585	Hind III	1	1189	Sml I	1	167
Bsp1286 I	1	224	Hpa II	3	442, 524, 705	Sph I	1	1187
BspD I	2	29, 260	HpyCH4 IV	1	159	Ssp I	1	494
BspH I	1	87	Mae II	1	159	Tli I	1	167
BspM I	1	1184	Mbo II	3	369, 480, 1058	Tsp45 I	1	716
Bsr I	3	360, 984, 1124	Mly I	2	802, 1178	TspR I	3	442, 517, 989
BsrD I	1	61	Msp I	3	443, 525, 706	Xba I	1	1165
BsrF I	1	523	Mwo I	3	281, 313, 527	Xho I	1	167
BssK I	3	440, 457, 814						

Restriction Enzymes that cut the EZ::TN <KAN-2> Transposon 4 or more times:

Alw I	BsmA I	Dpn I	HinP I	HpyCH4 III	Mbo I	Nla III	Taq I	Tsp509 I
BsaJ I	Cac8 I	Hha I	Hph I	HpyCH4 V	Mnl I	Sau3A I	Tfi I	
Bsl I	CviJ I	Hinf I	Hpy188 I	Mae III	Mse I	SfaN I	Tsp4C I	

Restriction Enzymes that do not cut the EZ::TN <KAN-2> Transposon:

Aat II	Asc I	Bsa I	BstAP I	EcoR I	MspA1 I	PpuM I	Sfo I	Tth111 I
Acc65 I	Avr II	BsaA I	BstB I	EcoR V	Nae I	PshA I	SgrA I	Xcm I
Acl I	Ban I	BsaB I	BstE II	Fse I	Nar I	Psi I	Sim I	Xma I
Afe I	Bbs I	BsaH I	BstX I	Fsp I	Nco I	PspOM I	Sma I	Xmn I
Afl II	BbvC I	BseY I	BstZ17 I	Gdi II	Nde I	Pvu II	SnaB I	
Afl III	BciV I	BsiHKA I	Bsu36 I	Hae II	NgoM IV	Rsr II	Spe I	
Age I	Bcl I	BsiW I	Dra I	Hpa I	Nhe I	Sac I	Srf I	
Ahd I	Bgl I	BspE I	Dra III	Hpy99 I	Not I	Sac II	Sse8647 I	
Ale I	Bgl II	BspLU11 I	Drd I	Kpn I	Pac I	SanD I	Stu I	
AlwN I	Blp I	BsrB I	Eae I	Mfe I	Pci I	Sap I	Sty I	
Apa I	Bme1580 I	BsrG I	Eag I	Mlu I	PflF I	Sca I	Swa I	
ApaB I	BmgB I	BssH II	Eco47 III	Msc I	Pme I	SexA I	Tat I	
ApaL I	Bmr I	BssS I	EcoO109 I	Msl I	Pml I	Sfi I	Tse I	

Appendix I

Antibodies used in this study

Antibody	Type	Experiment	Source	Working dilution
PorA	mouse monoclonal	IF	NIBSC	1:100
Texas Red	donkey anti-mouse monoclonal	IF	Calbiochem	1:200
SM1	mouse monoclonal	Western	Mumtaz Virji, University of Bristol	1:4000
Opa	mouse monoclonal	Western	Mumtaz Virji University of Bristol	1:3000
PilC	mouse monoclonal	Western	Thomas Meyer, Max Planck Institute	1:2000
Anti-capsular serogroup B	mouse monoclonal	ELISA	Murex ZM51	
Anti-Poly His Peroxidase conjugated	mouse monoclonal	Western	Sigma, U.K.	1:2000
Anti-Poly His Peroxidase conjugated	rabbit monoclonal	Western	Sigma, U.K.	1:2000
Anti-NMB0345	mouse polyclonal	Western		1:500 1:1000
Anti-NMB0345	rabbit polyclonal	Western	generated by CovaLab	