Characterisation of the interaction between *Neisseria meningitidis* and human polymorphonuclear leukocytes

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Abstract

During infection with *Neisseria meningitidis*, an important causative agent of bacterial meningitis and septicaemia, the host innate immune system clears bacteria by complement-mediated lysis and phagocytosis. In order to evade phagocytosis, the bacterium expresses a number of surface components, including a polysaccharide capsule and sialylated lipopolysaccharide (LPS). The aim of this project was to investigate the influence of bacterial metabolism and DNA repair on the interaction with polymorphonuclear leukocytes (PMNs). Results demonstrated reduced expression of the polysaccharide capsule and lowered LPS sialylation in a strain (ΔlctP) unable to acquire exogenous lactate, but no effect in a strain unable to utilise glutamate (ΔperM). These changes were associated with increased phagocytosis of fixed bacteria by the human PMN cell line, HL60. Further investigations showed that capsule expression in the ΔlctP strain was restored to wild-type levels following exposure of live bacteria to PMNs. Next, assays were established using primary human PMNs to investigate post-phagocytic events. The wild-type meningococcus survives within PMNs and is able to delay host cell apoptosis. Furthermore, the ΔlctP and ΔperM strains were significantly killed by PMNs, indicating a role for acquisition of both lactate and glutamate for survival in PMNs. Killing was dependent on actin polymerisation and the PMN oxidative burst. The role of the meningococcal Base Excision Repair (BER) DNA repair pathway was also investigated through characterisation of two Apurinic / Apyrimidinic (AP) endonuclease paralogues, NExo and NApe. Both exhibit distinct biochemical functions and are required for resistance against oxidative stress. A strain lacking both these enzymes, which is attenuated *in vivo*, was significantly killed by human PMNs as a result of the oxidative burst.
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I declare that the work in this thesis was carried out in accordance with the Regulations of Imperial College London. The work is original except where indicated by specific references in the text, and no part of the thesis has been submitted for any other degree.

Any views expressed in the thesis are those of the author and in no way represent those the Imperial College London

This thesis has not been presented to another University for examination either in the United Kingdom or overseas.
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<td>6-4PP</td>
<td>6-4 photoproduct</td>
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<td>8-oxoG</td>
<td>7,8-dihydro-8-oxo-2'-deoxyguanosine</td>
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<td>AP</td>
<td>Apurinic / Apyrimidinic</td>
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<td>APC</td>
<td>Antigen-Presenting Cell</td>
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<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
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<td>B-CSFB</td>
<td>Blood-Cerebro-spinal fluid Barrier</td>
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<tr>
<td>BER</td>
<td>Base Excision Repair</td>
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<td>BHI</td>
<td>Brain Heart Infusion</td>
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<td>bp</td>
<td>Base Pair</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
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<tr>
<td>CAMPs</td>
<td>Cationic Antimicrobial Peptide</td>
</tr>
<tr>
<td>CEACAM</td>
<td>Carcinoembryonic Antigen-related Cell Adhesion Molecule</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) Ligand</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CI</td>
<td>Competitive Index</td>
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<tr>
<td>CMP-NANA</td>
<td>Cytidine monophosphate N-acetyl neuraminic acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CP</td>
<td>Classical Pathway</td>
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<tr>
<td>CR3</td>
<td>Complement Receptor 3</td>
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<td>CREN</td>
<td>Contact Regulatory Element of <em>Neisseria</em></td>
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<td>CSF</td>
<td>Cerebro-spinal fluid</td>
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<td>CXCL</td>
<td>Chemokine (C-X-C motif) Ligand</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide Triphosphate</td>
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<td>DSB</td>
<td>Double-stranded break</td>
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<td><strong>DUS</strong></td>
<td>DNA uptake sequence</td>
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<td><strong>ECL</strong></td>
<td>Enhanced Chemi-luminescence</td>
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<td>2,6-diamino -4-hydroxy-5-formamidopyrimidine</td>
</tr>
<tr>
<td><strong>FITC</strong></td>
<td>Fluorescein 5(6)-isothiocyanate</td>
</tr>
<tr>
<td><strong>Gal</strong></td>
<td>Galactose</td>
</tr>
<tr>
<td><strong>GDH</strong></td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td><strong>GFP</strong></td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td><strong>Glc</strong></td>
<td>Glucose</td>
</tr>
<tr>
<td><strong>GlcNAc</strong></td>
<td>alpha-1,2-N-acetylglucosamine</td>
</tr>
<tr>
<td><strong>GOGAT</strong></td>
<td>Glutamate synthase</td>
</tr>
<tr>
<td><strong>GS</strong></td>
<td>Glutamine synthase</td>
</tr>
<tr>
<td><strong>GTP</strong></td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td><strong>HBMEC</strong></td>
<td>Human Brain Microvascular Endothelial Cell</td>
</tr>
<tr>
<td><strong>HBSS</strong></td>
<td>Hanks’ Balanced Saline Solution</td>
</tr>
<tr>
<td><strong>HCl</strong></td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td><strong>Hep</strong></td>
<td>Heptose</td>
</tr>
<tr>
<td><strong>HOCl</strong></td>
<td>Hypochlorous Acid</td>
</tr>
<tr>
<td><strong>HSPG</strong></td>
<td>Heparin Sulphate Proteoglycan</td>
</tr>
<tr>
<td><strong>ICAM</strong></td>
<td>Intracellular Cell-adhesion Molecule</td>
</tr>
<tr>
<td><strong>IFNγ</strong></td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td><strong>Ig</strong></td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td><strong>IL</strong></td>
<td>Interleukin</td>
</tr>
<tr>
<td><strong>Ile</strong></td>
<td>Isoleucine</td>
</tr>
<tr>
<td><strong>kb</strong></td>
<td>Kilobase</td>
</tr>
<tr>
<td><strong>KCl</strong></td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-D-manno-octulosonic acid</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LNnt</td>
<td>Lacto-N-neotetraose</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LP</td>
<td>Lectin Pathway</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane Attack Complex</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Index</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Multi-histocompatibility Complex</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MLEE</td>
<td>Multilocus Enzyme Electrophoresis</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus Sequence Typing</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NCV</td>
<td>Neisseria–containing vacuole</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NeuNAc / NANA</td>
<td>Neuraminic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer membrane vesicle</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEA</td>
<td>Phosphoethanolamine</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence Type</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Tfp</td>
<td>Type IV Pilus</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor-alpha</td>
</tr>
<tr>
<td>TSDS-PAGE</td>
<td>Tricine Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UDG</td>
<td>Uracil DNA Glycosylase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>wt/vol</td>
<td>Weight to Volume ratio</td>
</tr>
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</table>
Chapter 1

Introduction
1.1  *N. meningitidis* and meningitis

1.1.1  History and background

Bacterial meningitis was first described in 1805 following an epidemic that resulted in 33 deaths in the vicinity of Geneva (Vieusseux, 1805). In 1884, Italian pathologists Marchiafava and Celli observed intracellular micrococi in samples of cerebrospinal fluid (CSF), but it was Weischelbaum in 1887 who first confirmed that the bacterium was responsible for meningitis (Weichselbaum, 1887; Marchiafava, 1884). Originally named *Diplococcus intracellularis meningitidis*, the microorganism was renamed *Neisseria meningitidis* by Albert Neisser following the characterisation of the related bacterium *Neisseria gonorrhoeae* in 1879 (Kampmeier, 1978).

*N. meningitidis* is an aerobic Gram-negative diplococcal β-proteobacterium, and a member of the family Neisseriaceae. It is an obligate human bacterial pathogen, and an important agent of bacterial meningitis and septicaemia. The bacterium is also a commensal of the human nasopharynx. Between 10-40% of the population is colonised by the meningococcus, which is transmitted between hosts by aerosol spread (Taha and Alonso, 2008). The incidence rate of meningococcal disease is 0.39 – 7.41 per 100,000 per annum in Europe, and as high as 25 per 100,000 in developing countries (Pace and Pollard, 2007). Over 2,000 people are affected annually in the UK, where the mortality rate is approximately 10% (Health Protection Agency, 2007). Patients who recover may suffer permanent damage from meningococcal infection, including partial paralysis or deafness. The disease manifests primarily as a bloodstream and/or meningeal infection although it can also cause metastatic infections such as arthritis, pericarditis or cellulitis, endophthalmitis, conjunctivitis, pneumonia, sialadenitis (inflammation of the salivary glands), adnexitis (inflammation of ovaries and oviducts) and pelvic inflammatory disease (Wells *et al.*, 2001; Blaser *et al.*, 1984; Odegaard, 1983).
1.1.2 Symptoms and treatment of meningococcal disease

The onset of meningococcal disease can be extremely rapid. In the absence of appropriate medical care, patients can quickly lapse into septic shock. Coma, organ failure and death can follow within less than 24 hours of the onset of disease (Branco, 2007). Initial symptoms are largely non-specific, making the diagnosis problematic. Patients experience flu-like symptoms such as headache, fever, nausea and vomiting, and may display early signs of sepsis such as abnormal skin colouring and/or coldness of the extremities (Branco, 2007). These early symptoms are frequently misdiagnosed and often it is not until the patient begins to show more specific symptoms that meningococcal disease is suspected. These advanced symptoms include neck stiffness, aversion to bright light and the purpuric skin rash that is a classical manifestation of septicaemia. Meningitis refers to inflammation of the meninges of the surrounding central nervous system. If untreated, this condition can lead to coma and eventually death, usually as a result of multiple organ failure.

The diagnosis of meningitis and septicaemia relies on testing blood or cerebrospinal fluid (CSF), although the collection of CSF may not be possible if the patient is in a critical condition. Detection of bacteraemia may be by Gram stain, antigen detection or culture, and the use of polymerase chain reaction (PCR) is increasingly common. Rapid diagnosis and early treatment are key for the successful management of meningococcal disease. Prior to the 1920’s, bacterial meningitis was fatal in up to 70% of cases. Early treatments included the administration of serum from immunised horses, which reduced death rates to 30% (Flexner, 1913). The discovery of antibiotics further diminished mortality, and case fatality rates are now at 10%. The recommended antimicrobial treatment for meningococcal disease is with cephalosporin antibiotic such as ceftriaxone. Administration of antibiotics may be influenced by local antibiotic resistance patterns, although there is little clinically significant resistance to β-lactams. Patients often require from fluid replacement and resuscitation early on in disease (Branco, 2007).
1.1.3 Classification

The classification of the meningococcus was originally based on the antigens expressed on the surface of the bacterium. The polysaccharide capsule determines serogroup, the outer membrane-bound porin proteins, PorA and PorB, define sero-subtype and sero-subtype respectively, and the lipopolysaccharide (LPS) relates to the immunotype (Feavers and Maiden, 1998; Frasch et al., 1985). More recently additional classifications have been identified according to genetic differences.

There are thirteen known serogroups of *N. meningitidis* (A, B, C, E-29, H, I, K, L, M, W-135, X, Y and Z), of which six represent the majority of disease-causing strains (A, B, C, W-135, X, Y) (Stephens et al., 2007; Jodar et al., 2002; Pollard and Levin, 2000a). Serogroups B, C, W and Y all express polysialic acid-containing capsules. The sialic acid in the capsules of serogroups X and Y, which is O-acetylated, are α1-4 linked. The capsule of serogroup C strains is α2-9 linked, while serogroup B capsule is α2-8 linked, mimicking a modification of the human neural cell adhesion molecule (N-CAM) (Liu et al., 1971b; Liu et al., 1971a; Slaterus, 1961). The serogroup A capsule is composed of α1-6-linked N-acetyl-D-mannosamine-1-phosphate polymers (Swartley et al., 1998).

There are five classes of outer membrane proteins (OMPs). Class 1, designated P1, are represented by PorB, which determines sero-subtype, whilst Class 2/3, or PorA, establish the serotype. To date, over 20 serotypes and 10 sero-subtypes have been identified, although many isolates cannot be classified with this system (van Deuren et al., 2000). Immunotype according to LPS structure is determined by the groups on the α and β chains added to the inner core region (Mandrell and Zollinger, 1977; Zollinger and Mandrell, 1977). When combined, these independent methods of classification provide reference numbers describing the characteristics of a strain. For example, a serogroup B strain, of serotype 3 and subtype P1.2, with an immunotype of L3 would be designated B:3:P1.2:L3 (van Deuren et
Further classification according to the immunoglobulin A1 (IgA1) protease and pilin is also possible (Stephens et al., 1985).

Classification of the meningococcus according to the surface structures can be problematic due to antigenic variation. The capsule, porins and LPS antigens are subject to selective pressure, and thus the genes encoding them are highly variable. In addition, many carriage isolates are unencapsulated and thus cannot be serogrouped (Vogel et al., 2004). Therefore, following the advent of molecular biology techniques, strains are now classified according to variations in enzymes with metabolic functions, and by characteristics of their genomes. This is performed by Multilocus Enzyme Electrophoresis (MLEE) and Multilocus Sequence Typing (MLST). MLEE relies on separation of bacterial intracellular enzymes according to their electrophoretic mobility. The position of each enzyme following electrophoresis is compiled to give the electrophoretic type (ET) of a strain (Caugant et al., 1986). Meanwhile, MLST involves the determination of the sequence of 400-500 bp of seven housekeeping genes. Differences in the nucleotide sequence between strains allow their classification into different sequence types (ST) (Maiden et al., 1998). Both MLEE and MLST are powerful tools since they allow the typing of ambiguous and previously unclassifiable strains. MLST is generally regarded as the more useful of the two since data generated by independent laboratories can be directly compared. Disease-causing isolates from different serogroups can be grouped according to their ET or ST into clonal complexes. Data has shown that a small number of clonal complexes account for the majority of meningococcal disease (Brehony et al., 2007). For example, strains classified in clonal complex ET-37/ST-11 have been responsible for outbreaks in Canada, Europe, Australia and Israel (Jelfs et al., 2000).

1.1.4 Epidemiology

Meningococcal disease occurs worldwide as endemic disease, with the incidence varying between 1-1,000 per 100,000 people depending on location (Rosenstein et al., 2001; Tzeng and Stephens, 2000). It most frequently affects infants, due to their waning maternal passive
immunity, and young adults, although all age groups are susceptible, particularly during an epidemic (Cartwright et al., 2001; Pollard and Levin, 2000a; Goldschneider et al., 1969). Immunocompromised individuals are at a heightened risk, especially those individuals with deficiencies in the complement system (Rosa et al., 2004; Cunliffe et al., 1995; Mitchell et al., 1990; Ross and Densen, 1984). Due to the mode of transmission of the bacterium, outbreaks are more common when people live in overcrowded conditions, including military barracks and student accommodation (Yazdankhah and Caugant, 2004; Cartwright et al., 1987). In these situations, carriage of the bacterium by asymptomatic individuals is frequently as high as 40% of the population, and carriage frequencies have reached 70% in military recruits (Yazdankhah and Caugant, 2004). Bacteria are present in the nasopharynx of carriers, often residing deep within the tissue layer where detection by swabbing can be problematic (Sim et al., 2000). Carriage is affected by age and risk is associated with recent respiratory infections such as influenza, but the highest correlation is linked with social behaviour such as smoking and levels of intimate contact (MacLennan et al., 2006; Moore et al., 1990).

Of the serogroups that cause the majority of meningococcal disease, serogroups A and C are most common in Africa and Asia, whilst serogroups B and C are responsible for most cases in Europe and the US (Rosenstein et al., 1999; Caugant, 1998; Schwartz et al., 1989). The sub-Saharan Meningitis Belt in Africa, which spans ten countries from Ethiopia in the east to Senegal in the west, represents the region with the highest incidence of meningococcal disease in the world. Outbreaks of serogroup A disease are frequent, with the most severe epidemic to date occurring in 1996 when the total number of reported cases reached 152,000 with almost 16,000 deaths, although many more cases went unreported or were misdiagnosed (Mohammed et al., 2000; Ahmed et al., 1996). A number of outbreaks have also arisen in Asia, the most severe of which were caused by two serogroup A clones that originated in northern China, and eventually spread around the world (Wang et al., 1992; Cochi et al., 1987). Serogroup W-135 strains were responsible for causing outbreaks amongst pilgrims
during the Hajj Mecca, including a large-scale epidemic in Burkina Faso (Raghunathan et al., 2006; Aguilera et al., 2002).

Meningococcal disease in the UK is now predominantly the result of infection by serogroup B strains following the introduction of routine vaccination against serogroup C (Zimmer and Stephens, 2006; Ramsay et al., 2003; Maiden and Stuart, 2002). The disease affects over 2000 people in the UK each year, with a marked increase in cases during winter months (Health Protection Agency 2007). Despite available medical treatment, meningococcal disease still causes 150 deaths in the UK each year (Health Protection Agency, 2007).

1.1.5 Vaccines

The first vaccines against serogroup A and C meningococcal disease were introduced in the 1960’s (Pollard and Levin, 2000a). Raised purely against the polysaccharide capsular antigen, the vaccines were designed to elicit B-cell responses resulting in the production of antibodies against the polysaccharide (Pace and Pollard, 2007). These polysaccharide vaccines induced high levels of protection in clinical trials and were effective in controlling outbreaks within communities (van Duren et al., 2000). However, the resulting antibody production is short-lived and varies greatly with age and serogroup (De Wals et al., 2001; Gold et al., 1978). The protective effect of the serogroup C vaccine does not extend to infants and young children, the most high-risk group (Lepow et al., 1977). This is because an effective immune response is dependent on a mature T-cell response, which is lacking in young children (Morley et al., 2001). Since the vaccines do not elicit immunologic memory, repeated immunisation is required to maintain protection. This is true of the monovalent, bivalent (A/C) and tetravalent (A/C/W/Y) polysaccharide vaccines (Pollard and Levin, 2000b). Both the serogroup A and C vaccines appear to influence mucosal carriage of these serogroups, indicating a possible effect on herd immunity and transmission of the meningococcus (Zhang et al., 2001; Zhang et al., 2000). Monovalent, bivalent and tetravalent conjugate meningococcal vaccines have now been developed using the same technology employed for the Haemophilus influenzae type B.
glycoconjugate vaccine (Rennels et al., 2002; Booy et al., 1994; Costantino et al., 1992; Beuvery et al., 1983a; Beuvery et al., 1983b). Combining the polysaccharide capsular antigen by conjugation with a protein enhances T-cell responses against the polysaccharide, producing high-affinity antibodies and B-cell-dependent immunologic memory, and thus long-lasting protective immunity (Lesinski and Westerink, 2001). These vaccines also provide effective protection of young children. The conjugate vaccine against serogroup C *N. meningitidis* was introduced to the UK in 1999 in response to an epidemic caused by an ST-11 clone (Lakshman and Finn, 2002). Subsequent surveillance showed an 81% decrease in cases of meningococcal disease caused by serogroup C strains in the period between 1998-1999 and 2000-2001 (Miller et al., 2001). The protective effect of the serogroup C conjugate vaccine appears to wane after a period of four years but herd immunity also prevents disease in unvaccinated individuals and may reduce transmission rates (Trotter et al., 2004; Ramsay et al., 2003). Furthermore, despite potentially eliciting a selective pressure for capsule switching from serogroup C to B, this phenomenon does not appear to have been an issue since the incidence of serogroup B meningococcal disease has not risen following the introduction of the vaccine (Balmer et al., 2002) (Health Protection Agency 2004).

A bivalent conjugate vaccine against serogroups A and C is also available and has been widely administered in sub-Saharan Africa following successful trials in Niger, the Gambia and the UK (Chippaux et al., 2004; Fairley et al., 1996; Twumasi et al., 1995). A tetravalent A/C/W/Y conjugate vaccine that employs diphtheria toxin as the carrier protein has been successfully introduced into the United States and Canada (Bilukha and Rosenstein, 2005). The challenge for manufacturers of these vaccines is to ensure protection is higher than offered by the vaccines raised against individual serogroups. Recently, encouraging data has been released on trials performed with a *H. influenzae* type B–*N. meningitidis* serogroup C–tetanus toxoid glycoconjugate vaccine (Tejedor et al., 2008).
As a result of the mimicry by the serogroup B polysaccharide capsule of a modification of a human N-CAM, the design of vaccines against this serogroup is problematic since immunisation with the polysaccharide antigen does not elicit an IgG response and may lead to auto-immunity (Stephens et al., 2007). Vaccine development is focussed on alternative bacterial antigens. These include outer membrane proteins (OMPs), outer-membrane vesicles (OMVs) and LPS (Stephens et al., 2007). The challenge is to find a conserved antigen present in all serogroup B strains. Many meningococcal antigens are highly variable and thus are unsuitable as vaccine targets. Recent trials have shown success with a vaccine raised against a combination of OMV targets, including the Factor H binding protein expressed by the meningococcus, which is known to help subvert the host immune response (Beernink and Granoff, 2008; Beernink et al., 2006; Schneider et al., 2006). Further work is also investigating the possibility of using antigens expressed by the commensal Neisseria lactamica to produce a vaccine against the serogroup B meningococcus (Finney et al., 2008; Liu et al., 2008).
1.2 Virulence determinants

1.2.1 Capsule

An important feature that distinguishes the meningococcus from the gonococcus is expression of a polysaccharide capsule (Nassif and So, 1995). There are six distinct meningococcal capsule serogroups with differing chemical compositions. The serogroup B capsule is similar in structure to the *Escherichia coli* K1 capsule (Echarti *et al*., 1983; Hirschel *et al*., 1983). It is composed of chains of N-acetyl neuraminic acid (NeuNAc or NANA) of varying length. These monomers are linked at carbons two and eight, to form polysialic acid. At the reducing terminus of the sialic acid polymers is a diacylglycerophosphate group (Frosch and Muller, 1993).

Capsule expression is controlled by the cps locus, a 24 kb region with homology to the *E. coli* kps locus. The cps locus comprises three distinct regions that control synthesis (*siaABCD*) and transport (*ctrABCD* and *lipAB*) (Tzeng *et al*., 2001; Swartley *et al*., 1996; Frosch *et al*., 1989). The molecular basis of capsule expression was first characterised by Frosch *et al*. Expression of the cps locus in *E. coli*, followed by deletion of individual regions, enabled characterisation of the separate regions responsible for transport of capsule polysaccharide from the cytoplasm to periplasm, and thence to the cell surface (Frosch *et al*., 1989). The first stage of capsule biosynthesis involves sialic acid synthesis in the bacterial cytoplasm. D-glucosamine is modified to produce N-acetyl mannosamine, the open chain form of which combines with phosphoenolpyruvate (PEP) via the action of NANA synthetase (SiaC) and cytidine monophosphate N-acetyl neuraminic acid (CMP-NANA) synthetase (SiaB) to produce sialic acid, or NeuNAC (Gunawan *et al*., 2005; Edwards *et al*., 1994).

Polymerisation of sialic acid also occurs in the cytoplasm. The biochemistry of the initiation of this process is not yet fully understood as the initial acceptor molecule has not been identified. Following initiation, polymerisation is mediated by the glycosyltransferase
encoded by *siaD*, which transfers a Neu5Ac group from the donor CMP-Neu5Ac to the reducing terminus of the sialic acid glycan, thus elongating the oligosialyl polymer (Edwards *et al.*, 1994). The mechanism of termination of this process is also not known. However, *E. coli* K1 chain termination occurs between 160-230 units, indicating that there is active determination of chain length (Pelkonen *et al.*, 1988). It has been suggested that this may be caused by a loss of affinity once the chain reaches this length, or that abortive translocation within the catalytic site prevents further elongation. Termination may also be caused by an unknown allosteric effect.

Capsule expression is modified in response to the immediate environment within the host. Expression of capsule is phase-variable as a result of on-off switching and slipped-strand mispairing or reversible frameshift mutation in the *siaD* gene (de Vries *et al.*, 1996; Hammerschmidt *et al.*, 1996). Capsule expression appears to be up-regulated during bloodstream infection, where the capsule is known to protect the bacterium from complement-mediated killing and phagocytosis (Vogel *et al.*, 1997; Read *et al.*, 1996; Hammerschmidt *et al.*, 1994). Down-regulation occurs during colonisation to allow intimate binding of the bacterium to the epithelial monolayer (Stephens *et al.*, 1993; Virji *et al.*, 1993). Down-regulation of capsule, and also pili, was detected by Western blotting. Reverse transcriptase PCR (RT-PCR), DNA binding and footprinting analysis confirmed the regulatory role of CrgA, a transcriptional regulator belonging to the LysR family (Deghmane *et al.*, 2002). However, more recently, work using transfected cell lines expressing the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) has demonstrated invasion of cells by capsulate meningococci (Bradley *et al.*, 2005). CEACAM expression is upregulated on host epithelial cells by chemokine signalling orchestrated by cytokines Interferon-gamma (IFNγ), Tumour necrosis factor alpha (TNFα) and Interleukin-6 (IL-6). It has been suggested that the increase in receptor density allows for adhesion and invasion of capsulate bacteria, mediated by the surface-expressed Opacity proteins and, to a lesser extent, by pili (Rowe *et al.*, 2007).
1.2.2 LPS

Meningococcal LPS, also called lipooligosaccharide (LOS) is an important endotoxin and a major factor contributing to the pro-inflammatory response during disease. LPS is an amphipathic glycolipid of approximately 4.8 kDa (Jones et al., 2003), which comprises oligosaccharide chains added to the inner core, anchored to the lipid A moiety embedded in the bacterial outer membrane. Immunotypes are distinguished by differences in the modification of the oligosaccharide chains such as by acetylation and addition of phosphoethanolamine groups (Tsai et al., 1987). There are twelve distinct Neisserial immunotypes. Serogoup B strains tend to express L3,7,9 immunotype LPS (Figure 1.1) (Tsai et al., 1987). These strains are also able to add sialic acid onto the lacto-N-neotetraose (LNnt) moiety. Sialylation is achieved either from endogenously synthesised sources of sialic acid, or from exogenous CMP-NANA in the surrounding environment (Exley et al., 2005b; Smith et al., 1992).

Unlike E. coli, where the LPS biosynthesis genes are organised as large operons, the meningococcus possesses a number of small operons that are distributed throughout the genome (van der Ley et al., 2001; van der Ley et al., 1997). Localised chromosomal rearrangements at these individual loci are responsible for some of the variation seen in LPS structure, an important feature in meningococcal pathogenesis (Kahler et al., 1998; Kahler and Stephens, 1998). The first step of LPS biosynthesis is governed by the \( lpx \) locus, which synthesises the lipid A portion of the molecule (van der Ley et al., 2001). The UDP-GlcNAc acetyltransferases encoded by \( lpxA \) and \( lpxD \) acetylate UDP-GlcNAc, which is then dimerised by LpxB (Steeghs et al., 1997). Following addition of a 3-deoxy-D-manno-octulosonic acid (Kdo) group to the lipid A molecule, a secondary acetylation step is achieved by \( lpx1 \) and \( lpx2 \) (van der Ley et al., 2001).
Figure 1.1 Structure of Neisserial immunotype L3,7,9 LPS

Schematic representation of Neisserial LPS immunotype L3,7,9 comprising the inner Kdo and Lipid A core (black), heptose moieties (Hep; red), phosphoethanolamine (PEA, yellow on black), Glucose (Glc; green), Galactose (Gal, dark blue), N-acetylglucosamine (GlcNAc, light blue) and sialic acid (NeuAc, magenta). Linkages are shown for each bond (Plested et al., 1999).

Glycosylation of the moiety is performed by the Kdo transferase encoded by kdtA (Tzeng et al., 2002). Extension of the LPS molecule from lipid A is achieved by enzymes encoded by two main loci, rfa and lgt. The α-1,5 heptosyltransferase encoded by rfaC adds the first heptose group (Hepl), followed by the action of rfaF, which attaches the second heptose (HeplI) and is required for elongation of the LPS inner core (Stojiljkovic et al., 1997; Jennings et al., 1995). rfaK encodes the α-1,2-N-acetylglucosamine (GlcNAc) transferase which adds the GlcNAc group to HeplI (Kahler et al., 1996). Glycosyltransferases encoded by the lgt locus catalyse the elongation reactions of the different LPS chains (Wakarchuk et al., 1996). The products of lgtF, lgtE, lgtA and lgtB result in the addition of galactose, glucose, GlcNAc and galactose respectively to form the terminal LNnt. The UDP-glucose-4-epimerase encoded by galE is
important for the addition of the first glucose molecule in the oligosaccharide (Jennings et al., 1993). Sialylation of the LNnt terminus is achieved by the alpha-2,3-sialyltransferase encoded by \textit{lst} (Vogel et al., 1997).

Meningococcal LPS is heterogeneous and highly variable, with individual strains able to display more than one LPS immunotype (Tsai et al., 1983). Variation is achieved by sequence changes within encoding regions and alterations in transcriptional regulation of the enzymes involved. In particular, the terminal structures are subject to high-frequency switching of expression, leading to variation in immunotype. High mutation rates in the \textit{lgtA} gene of immunotype L3,7,9 strains as a result of slipped-strand mispairing in a homopolymeric tract of guanine residues, leads to variation in the terminal lacto-N-neotetraose (Jennings et al., 1995). Such modulation of immunotype may impact further upon the LPS structure. For example, serogroup B strain MC58 switches between immunotypes L3 and L8. Of these two immunotypes, only L3 can be sialylated, an effect that is important for adhesion and invasion of host cells, and avoidance of host killing mechanisms (Jennings et al., 1999).

1.2.3 Variable antigens

1.2.3.1 Type IV pilus

Type IV pili (Tfp) are dynamic, hair-like protein filaments that extend from the bacterial surface. In addition to mediating bacterial attachment to various host cell types, Tfp are involved in the generation of twitching motility that allows bacterial aggregation, and are also required for DNA transformation (Koomey, 1998; Goodman and Scocca, 1991). The pilus fibre is composed mainly of pilin, an 18-22 kDa polypeptide that is synthesised as precursor subunits with signal sequences. Processing of pilin by the PilD pre pilin peptidase / transmethylase in the periplasm produces the mature pilin (Freitag et al., 1995; Strom and Lory, 1993), which undergoes further post-translational modification by O-glycosylation and phosphorylation at external sites on the fibre (Forest et al., 1999; Jennings et al., 1998). Further
modification of the Tfp may include addition of phosphocholine or phosphoethanolamine, which has been characterised in the gonococcus (Hegge et al., 2004). Recent work has identified an alternative method of pilin glycosylation via the addition of a glyceramido-acetamidotrideoxyhexose residue (Chamot-Rooke et al., 2007). The structure of the neisserial pilus has been elucidated via a range of techniques including cryoelectron microscopy, fibre diffraction and antigenic mapping, revealing a helical cylindrical structure (Parge et al., 1995). The hydrophobic tail units form the cylinder core, providing tensile strength, while the globular head units form the helix surface (Merz and So, 2000). Within the globular domain is a surface-exposed loop structure that represents the hypervariable region of the pilus (Seifert, 1996).

Pilin biosynthesis occurs in the periplasm, and is controlled by proteins encoded by pil genes. The pilin polypeptide is composed of subunits of PilE, and assembled into the mature pilin in the periplasm by three further pilus proteins, PilD, PilF and PilG (Tonjum and Koomey, 1997). The full-length fibre is thought to emerge through pores in the cell membrane composed of twelve units of PilQ, which form rings in the outer membrane that are proposed to allow passage of the pilus (Collins et al., 2005; Collins et al., 2001). The filament is then stabilised by the membrane protein PilW, which is also involved in maintaining pilus functionality, and by PilC, which works antagonistically with PilT, which acts to retract the pilus from the bacterial surface (Yasukawa et al., 2006; Carbonnelle et al., 2005; Rahman et al., 1997).

Variation in Tfp occurs primarily within the regions of the PilE subunit that are exposed on the surface and occur at a rate of approximately $10^{-4}$ per cell division, effecting changes in post-transcriptional modification, immunoreactivity and adhesive capacity (Nassif et al., 1993; Virji et al., 1993). Recombination events occur within multiple small sequence regions within conserved elements in the pilE / pilS locus. The locus consists of one expressed gene (pilE) and eight homologous silent regions, including pilS. These non-expressed alleles recombine with the active pilE gene through gene conversion, leading to mutation within pilE (Haas et al., 1992; Aho and Cannon, 1988; Haas and Meyer, 1986). Positive selection acting on these regions
results in the emergence of novel antigenic variants (Andrews and Gojobori, 2004). In addition, pilC2 is subject to phase variation as a result of frameshift mutations in a poly-G tract within the region encoding the signal peptide (Rytkonen et al., 2004; Jonsson et al., 1991).

### 2.2.2 Opacity proteins

The opacity proteins comprise two families of neisserial outer membrane proteins, Opa and Opc. Opa proteins are more abundant than Opc, which were so named due to their homology to Opa (Merz and So, 2000). The Opa proteins were originally identified due to the effect of their expression on colony opacity and colour, which is attributed to bacterial aggregation (Swanson, 1982; Swanson, 1978). Opas are important neisserial adhesins, mediating intimate adhesion to epithelial cells via heparin sulphate proteoglycan (HSPG) receptors, and interact with the CEACAM, or CD66, receptors expressed by phagocytic host cells (Virji et al., 1996a; Lambden et al., 1979; King and Swanson, 1978).

Meningococcal strains possess up to five opa loci, with differences in sequence that occur within one semi-variable and two hyper-variable regions. Variation arises as a result of translocations, deletions and point mutations within these regions (Hobbs et al., 1998; Hobbs et al., 1994). Opas possess eight trans-membrane strands and four surface-exposed loops, three of which correspond to the variable regions in the corresponding gene (Malorny et al., 1998). The variable nature of opa gene expression may result in expression of more than one Opa per strain, allowing for a spectrum of proteins expressed at the cell surface.

### 2.2.3 Porins

Neisserial porins are the most abundant outer membrane proteins displayed on the bacterial cell surface. They fall into two discrete groups, PorA (45 kDa) and PorB (33 kDa) (Frasch et al., 1985), and contribute greatly to the antigenic variability of the meningococcus. Porins possess a β-pleated barrel structure which is embedded in the lipid membrane (Derrick et al., 1999), and mediate interactions both within the bacterial membrane and with host cells (Weel
et al., 1991). Porins act as ion channels across the plasma membrane and are modulated by adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Rudel et al., 1996).

Work with gonococcal porins has revealed a role for PorB1A in invasion of host epithelial cells (Bauer et al., 1999). As yet the role of porins in entry of the meningococcus into epithelial cells has not been elucidated. However, meningococcal porins are known to induce actin reorganisation in host cells, indicating a role in invasion (Merz and So, 2000). Gonococcal porins also play a role in interactions with phagocytic cells. PorB modulates phagosome maturation and the oxidative burst of neutrophils (Lorenzen et al., 2000; Mosleh et al., 1998), and is responsible for redistribution of late-endosomal marker LAMP1 to the plasma membrane (Ayala et al., 2002).
1.3 Pathogenesis

1.3.1 Colonisation of the nasopharynx

Colonisation of the human nasopharyngeal epithelium by *N. meningitidis* is dependent on adhesins expressed on the surface of the bacterium. Adhesion, which is limited to non-ciliated secretory epithelial cells, follows two distinct steps (Stephens and McGee, 1981). The first, initial attachment, is characterised by dispersed microcolonies of 10 – 100 meningococci on the surface of epithelial cells. This is followed by extension of microvilli, or filopodia, from the surface of the epithelial cells (Stephens *et al.*, 1983). The second stage, intimate attachment, develops eight to sixteen hours after infection. The bacteria disperse from the microcolonies and individual diplococci become intimately attached to the cell surface. This process has been investigated using an organ culture model to mimic conditions in the human host and enable dissection of the colonisation process (Stephens *et al.*, 1983). Adhesion to epithelial cells has also been investigated in the closely related pathogen, *N. gonorrhoeae*. However, extrapolation from work with the gonococcus may be unreliable due to significant differences in the species’ gene content. Crucially, the gonococcus does not express a polysaccharide capsule, which is likely to be of particular importance when considering adhesion to host cells since it might affect accessibility of bacterial adhesins to host receptors (Fox *et al.*, 1991).

Initial attachment is mediated primarily by the Tfp, and is proposed to occur through interaction with the CD46 receptor, a complement regulatory protein expressed on nearly all human cells. This interaction has been demonstrated by Western blotting with purified pili, and can also be blocked with an anti-CD46 antibody (Kallstrom *et al.*, 1997). Doubt has been cast on CD46 as the cognate receptor for Tfp since meningococcal adhesion to CD46 expressed in cell lines is very weak, and further work with *N. gonorrhoeae* indicates that a CD46-independent occurs (Kirchner *et al.*, 2005; Tobiason and Seifert, 2001). However, pilus binding mediates the transient release of calcium within the host cell that occurs immediately after
initial contact. This response is dependent on CD46 expression and the pilus protein PilC1, and is thought to aid bacterial adhesion by triggering lysozyme exocytosis (Ayala et al., 2001). Furthermore, initial attachment of gonococci to host epithelial cells results in phosphorylation of tyrosine 354 on the intracellular domain of CD46 by Src-family kinases. Inhibition of these tyrosine kinases results in a significant reduction in adhesion of the gonococcus to the epithelium (Lee et al., 2002). Despite the lack of evidence for a direct interaction between Tfp and CD46, these data support the hypothesis that the CD46 receptor is involved in adhesion to host epithelial cells.

Four hours post-infection, the bacteria proliferate and begin to form microcolonies on the epithelial surface. This is accompanied by the formation of actin-, ezrin- and moesin-rich microvilli-like extrusions from the host cell, which eventually engulf and internalise the bacteria (Edwards et al., 2000). These membrane rearrangements rely on PilT, which mediates pilus retraction (Forest et al., 2004). The mechanical force exerted upon the cell membrane by pilus retraction is equivalent to that required to induce microvilli elongation (Merz and So, 2000). Cortical plaques, rich in the integral membrane proteins CD44 and intracellular cell-adhesion molecule 1 (ICAM-1; CD54), also form beneath the adherent bacteria (Merz and So, 1997). Actin polymerisation and rearrangement is mediated by activation of GTPases Rho and Cdc42 (Eugene et al., 2002). Recruitment of ezrin, moesin and transmembrane proteins, however, is independent of GTPase activation. There is also evidence for a role for transmembrane receptor tyrosine kinase ErbB2 in internalisation of meningococci (Hoffmann et al., 2001). Infection induces clustering and phosphorylation of this receptor, which in turn activates the tyrosine kinase Src, which then phosphorylates cortactin, an actin-binding protein that is present in cortical plaques, and regulates actin assembly (Weed and Parsons, 2001). Little else is known about this process but it is clear that the resulting cytoskeletal reorganisation is essential for internalisation of meningococci into epithelial cells.
Eight to sixteen hours post-infection, host cell membrane protrusions retract, and bacteria disperse across the epithelial surface. Dispersal is mediated by PilT and down-regulation of pilus expression (Yasukawa et al., 2006; Pujol et al., 1999). A simultaneous up-regulation in the pilus component PilC and reduction in capsule expression results in intimate attachment of bacteria to the epithelial layer (Deghmane et al., 2002). Capsule down-regulation is thought to aid this process by permitting Opacity proteins to interact with CD66 receptors and HSPGs, binding bacteria to the apical epithelial surface (de Vries et al., 1998). This process is under genetic control within the meningococcus, coordinated by the negative transcriptional regulator CrgA, named for its binding affinity for the CREN (Contact Regulatory Element of Neisseria) region identified in the pilC, pilE and capsule biosynthetic sia loci, and twelve other genes of unknown function (Deghmane et al., 2004; Morelle et al., 2003). crgA itself possesses a CREN region and so is up-regulated on contact with host cells to suppress capsule expression, but is transcriptionally controlled by a negative feedback loop (Deghmane et al., 2002).

1.3.2 Invasion and traversal of the nasopharyngeal epithelium

The nasopharyngeal epithelium comprises 60–90% squamous epithelium, interspersed by 120 µm thick ciliated pseudostratified columnar cells with secretory simple columnar goblet cells (Ali, 1965). The distribution of these cell types varies according to the location in the nasopharynx, with a higher density of goblet cells in the lateral wall, and a higher proportion of squamous epithelium in the posterior wall (Ali, 1965). Networks of capillaries underlie patches of these epithelial layers, and a high density of mast cells and secretory cells are present in the tunica propria, a framework of collagenous fibres and bundles of blood and lymphatic vessels (Ali, 1965). The nasopharyngeal epithelium has barrier functions, mediated by tight junctions between cells (Pujol et al., 1997). Traversal of the epithelial layer by the meningococcus is not well understood. Transmission electron microscopy and gentamicin protection assays demonstrate that meningococci are internalised by and reside within vacuoles inside epithelial cells, and are thus protected from host clearance mechanisms.
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(Plant et al., 2006; Capecchi et al., 2005; Pujol et al., 1997). Successful invasion of the epithelial cell layer relies on a number of bacterial factors including Tfp, Opa, Opc and LPS (Plant et al., 2006; Muenzner et al., 2000; Pujol et al., 1997; de Vries et al., 1996). Truncated LPS mutants lacking the sialic acid decoration or one heptose group in the inner core show reduced invasion of epithelial cell lines, whilst a strain lacking both heptose moieties is unable to invade (Plant et al., 2006). Expression of the polysialic acid capsule hinders invasion and is down-regulated during this stage of infection (de Vries et al., 1996). Work with the gonococcus has implicated PorB in the invasion process (Rechner et al., 2007). More recently, additional invasins have been identified in the meningococcus, including NadA, a surface antigen that, when expressed in E. coli, is sufficient to induce adhesion and invasion of epithelial cell lines. A meningococcal mutant lacking the nadA gene also shows significantly reduced invasion in a capsule-negative background (Capecchi et al., 2005).

It is as yet unclear as to whether bacteria traverse the epithelial layer by a transcellular route. It has been hypothesised that the meningococcus migrates para-cellularly via the tight junctions but evidence to support this is conflicting. One study reported that the integrity of the epithelial barrier is lost during traversal of bacteria (Stephens and Farley, 1991). However, more recent work has also described bacterial traversal of the epithelial layer whilst electrical resistance across the monolayer remained intact, indicating that tight junction integrity was not affected by infection (Pujol et al., 1997; Merz et al., 1996).

1.3.3 Invasion of the Blood-Brain Barrier (BBB) and Blood-CSF (B-CSF) Barrier

Following dissemination of N. meningitidis in the bloodstream, the development of meningitis occurs as a result of invasion and colonisation of the CSF. The leptomeningeal layers consist of the pia mater, a delicate membrane composed of fibrous tissue that is closely associated with the surface of the brain and spinal cord, and the arachnoid mater, a looser membranous layer that surrounds the central nervous system. The outermost layer is the
dura mater, a thicker membranous layer that is attached to the skull itself. The mechanism by which \textit{N. meningitidis} invades the subarachnoid space is unknown but is thought to be facilitated by high levels of bacteraemia (Bergman \textit{et al.}, 2006). There are two distinct barriers through which bacteria reach the CSF. They can cross the endothelium of capillaries in the BBB and enter the subarachnoid space between the leptomeninges. Alternatively, the bacteria may traverse the B-CSFB via the endothelium of the choroid plexus, the capillary network within the ventricles where CSF is produced by specialised cuboidal cells (Levine, 1987). Both of these routes present a barrier of a monolayer of polarised endothelial cells which, unlike capillary endothelia throughout the rest of the body, possess tight junctions which confer high electrical resistance (Rubin \textit{et al.}, 1991). Thus the BBB is very similar in nature to the nasopharyngeal epithelium and much of the research performed with the meningococcus on polarised monolayers of epithelial cells is thought to be directly relevant to the invasion into CSF (Pujol \textit{et al.}, 1997). More recently, work has been performed using the human brain microvascular endothelial cell line (HBMEC). Invasion of these cells relies on Opc engaging fibronectin, which anchors the bacterium to integrins on the surface of the endothelial cells (Unkmeir \textit{et al.}, 2002b). In addition to mediating the internalisation of bacteria, this contact also activates MAP- and tyrosine kinase signalling cascades that result in the release of inflammatory cytokines (Sokolova \textit{et al.}, 2004). Microscopy confirmed the presence of bacteria residing within \textit{Neisseria}-containing vacuoles (NCV) inside HBMECs (Nikulin \textit{et al.}, 2006). Expression of the polysialic acid capsule is necessary for survival and replication of the meningococcus within the NCV (Nikulin \textit{et al.}, 2006).
1.4 *N. meningitidis* and the immune response

1.4.1 Humoral immunity

1.4.1.1 Antibody-mediated immunity

The humoral immune response against *N. meningitidis* relies on the production of antibodies by B cells. Opsonisation of bacteria by antigen-specific antibodies leads to phagocytosis and/or complement-mediated lysis. Antibody-mediated immunity against *N. meningitidis* develops over time and is age-dependent, detectable in approximately 20% of individuals aged 12 months, rising to 50–80% at 12 years of age, and reaching up to 85% in adulthood (Matsunami, 1918). Trans-placental transfer of immunoglobulins provides newborn infants with anti-meningococcal antibodies, and serum from 50% of newborns show bactericidal activity, which explains the low incidence of disease in this group (Goldschneider et al., 1969) (Health Protection Agency 2007). However, maternal immunity wanes rapidly, and susceptibility to infection is highest in children aged between 6 to 12 months (Goldschneider et al., 1969). The levels and specificity of bactericidal activity as a result of natural immunity vary between individuals, determining susceptibility to disease (Heist, 1922).

Immunity to meningococcal infection acquired following repeated transient colonisation with different strains confers a degree of cross-protection (Reller et al., 1973). Some protective immunity may also be afforded following infection by other bacterial species which express antigens related to the meningococcal outer surface antigens (Hoff and Hoiby, 1978). Colonisation by the commensal *N. lactamica* occurs frequently in childhood, and has been linked to production of antibodies against pathogenic meningococcal strains (Gold et al., 1978; Goldschneider et al., 1969). It is now thought that the close relatedness of *N. lactamica* could be exploited to design cross-protective vaccines. Work has shown that live bacteria or outer-
membrane preparations of the commensal species induce protective responses in mice (Li et al., 2006; Gorringe et al., 2005).

1.4.1.2 Complement-mediated killing
Humoral immunity also relies on antibacterial components of the blood, most importantly the complement system, which is the primary killing mechanism of *N. meningitidis*. Complement-mediated killing is particularly effective against Gram-negative bacteria, and is closely linked to the inflammatory response (Schneider et al., 2007). The complement system comprises a proteolytic cascade of a series of membrane-bound and fluid-phase components that culminates in the production of a C3 convertase, which cleaves complement component C3 to produce C3b (Walport, 2001b; Walport, 2001a). Binding of C3b to the bacterial surface results in phagocytosis by cells of the innate immune response, or lysis by formation of the membrane attack complex (MAC) (Walport, 2001b; Walport, 2001a). The complement cascade is initiated by the classical (CP) and lectin (LP) pathways, and amplified by the alternative pathway (AP) (Schneider et al., 2007). The CP is initiated by binding of the C1 complex to bacterial surface components such as lipid A, or to pathogen-specific IgG or IgA bound to the bacterium, whilst the LP is initiated by binding of mannose-binding lectins or ficolins to microbial carbohydrates (Fujita et al., 2004). The importance of the complement system in the prevention of meningococcal disease is highlighted by the increased susceptibility of individuals who lack complement components such as C3, C4, and most strikingly, elements of the MAC (Rosa et al., 2004; Figueroa et al., 1993; Fine et al., 1983).

1.4.2 Cell-mediated immunity
During disease *N. meningitidis* encounters a range of phagocytic cells, or leukocytes, that represent the cellular arm of the innate immune response. Granular leukocytes include neutrophils, basophils and eosinophils, which are characterised by the presence of granules in the cytoplasm, whilst agranular leukocytes include monocytes, macrophages and
lymphocytes. Opsonisation results in targeting of the bacterium for phagocytosis. Cells expressing the Fcγ receptor recognise particles opsonised by IgG, whilst the complement receptor 3 (CR3) binds bacteria opsonised by the complement component C3b (Mantovani et al., 1972; Messner and Jelinek, 1970; Lay and Nussenzweig, 1968). Non-opsonic phagocytosis of bacteria also occurs following engagement of phagocytic receptors by bacterial surface antigens. Phagocytosis of bacteria follows receptor engagement, and is initiated by conformational changes in the cytoplasmic part of the target receptor, often as a result of tyrosine phosphorylation (Greenberg et al., 1996; Greenberg et al., 1993). This process triggers the phagocytic signalling cascade which culminates in actin polymerisation beneath the site of receptor engagement, and mediates subsequent internalisation of the bacterium.

Phagocytosis triggers the release of proteolytic and additional degradative enzymes from cytoplasmic granules that facilitate the intracellular degradation of the bacterial cell. Certain phagocytic cells, including neutrophils and macrophages also employ an oxidative burst, characterised by the production and release of highly reactive oxygen species (ROS). The oxidative burst is mediated by a cellular NADPH oxidase (Babior et al., 1976; McPhail et al., 1976). The dormant oxidase comprises four components that reside in the cytosolic compartment and a membrane-bound b-type cytochrome component (Dewald et al., 1979). Cell activation, as a response to bacterial LPS for example, results in translocation of the cytosolic components to the membrane to form a functional electron-transfer system. NADPH is exploited to catalyse reduction of molecular oxygen, the products of which are then transported via the cytochrome domain of the enzyme to the phagosomal compartment (Babior, 1999). Within the phagosome these antimicrobial oxidising agents, including ROS such as hydrogen peroxide ($\text{H}_2\text{O}_2$), superoxide anion and hydroxyl radicals, and nitrogen species such as nitric oxide and peroxynitrite kill pathogens by damaging lipids, proteins and DNA (Dyet and Moir, 2006; Hampton et al., 1998).
Degradation of bacterial antigens by antigen-presenting cells (APC) such as dendritic cells (DC) and macrophages results in presentation of bacterial peptides by the multi-histocompatibility complex (MHC) on the APC cell surface. This process triggers maturation of DCs, leading to a characteristic change in receptor expression profile (Roake, 1995; Inaba, 1994; Schuler et al., 1985). Mature DCs and macrophages migrate to the lymph nodes and present the MHC bearing antigens, in combination with co-stimulatory molecules on the cell surface, to T- and B-cells. T-cells express the T-cell receptor (TCR) and are divided into subsets of helper, cytotoxic, regulatory and memory T-cells (Gatenby et al., 1984). They provide the host with an efficient repertoire of phagocytic cells specific to individual antigens, which are closely regulated by cytokines released by other phagocytic cells. B-cells are lymphocytes, which produce specific antibodies against different antigens. Immature B cells are activated by detection of the antigen that they are specific for, combined with the activity of helper T-cells, resulting in rapid production of specific antibodies (Kipps, 1997). This arm of the cell-mediated immune response represents an important link to humoral immunity.

1.4.2.1 Interaction between N. meningitidis and polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMN), or neutrophils, infiltrate nasopharyngeal tissue at the site of infection in response to chemoattractant cytokines, or chemokines, such as IL-8, released by infected epithelial and endothelial cells (Moller et al., 2005; Lee et al., 2003). PMNs are usually the first cell type to be recruited to the site of bacterial infection, and thus it is likely that N. meningitidis encounters PMNs during both colonisation and development of disease. Following opsonisation by C3b or IgG, meningococci are internalised by PMNs via CR3 and Fcγ receptors, respectively (Fijen et al., 2000). Non-opsonic phagocytosis of the meningococcus by PMNs also occurs but at a lower level, and would be a particularly important mechanism for individuals with complement defects (Estabrook et al., 1998). This non-opsonic interaction appears to be mediated primarily by direct interaction between Opa and CD66 (or CEACAM) receptors expressed by PMNs (Hauck et al., 1998; Virji et al., 1996a). Members of the CD66 receptor family are expressed by epithelial and endothelial cells as well as phagocytic cells.
such as PMNs. They function in cell-cell adhesion, cell cycle control and cellular differentiation. Their frequency of expression on cancer cells indicate a regulatory and sensory aspect to their adhesive role (Bos et al., 1999). In addition to this primary function, CD66a, CD66c, and CD66e also recognise neisserial Opa proteins (Virji et al., 1996b). A range of techniques have been employed to identify the residues involved in this interaction. Opas target a sequence within the centre of the N-terminal domain of the receptor that is likely to have functional importance to the host and thus is conserved (Chen et al., 1997; Virji et al., 1996b). The conserved amino acids Tyr\textsuperscript{34} and Ile\textsuperscript{91} are critical for Opa-binding although specificity is conferred by additional residues that are not shared by all CD66 family members (Bos et al., 1999). Since Opas are subject to phase variation there are distinct requirements for CD66 binding within the antigen which have not been characterised (Billker et al., 2000; Popp et al., 1999). Regulation of capsule expression may be imperative for the meningococcus to control the Opa-CD66 interaction (Dehio et al., 2000). Opa-CD66 binding enables adherence to the epithelium and thus the capsule is down-regulated to maximise contact between antigen and receptor. However capsule expression is thought to be up-regulated to evade phagocytosis. As postulated above, this response may occur in order to mask Opas on the bacterial surface, either sterically or by charge, thus preventing CD66-mediated phagocytosis.

Phagocytosis by PMNs initiates the oxidative burst signalling cascade within the cell. The fate of \textit{N. meningitidis} within PMNs has not been fully established. Studies have shown some killing of internalised opsonised meningococci by the endo-lysosomal degradation pathway and the oxidative burst (Peters et al., 1999; Estabrook et al., 1992). However, \textit{Neisseria gonorrhoeae} is able to survive and replicate within PMNs (Simons et al., 2005; Parsons et al., 1982) and it has been suggested that \textit{N. meningitidis} is also capable of intracellular survival (Estabrook et al., 1998; Estabrook et al., 1992). \textit{N. meningitidis} expresses a number of enzymes to combat the oxidative burst, such as catalase and superoxide dismutase, which neutralise hydrogen peroxide (Soler-Garcia and Jerse, 2004; Dunn et al., 2003). Glutathione peroxidise expression is required for resistance to killing by paraquat (Moore and Sparling, 1996). More
recently, it has been shown that the gonococcus is able to downregulate the oxidative burst elicited by PMNs. Infections of PMN with live and dead bacteria revealed that live gonococci inhibit the oxidative burst in a contact-dependent manner. Dead gonococci elicited an oxidative burst but PMNs challenged with both live and dead bacteria produced significantly less ROS, indicating that the live gonococci were actively inhibiting the oxidative burst (Criss et al., 2008). In addition, it has been shown that expression of porins reduces phagocytosis and modifies the oxidative burst of PMNs (Lorenzen et al., 2000).

1.4.2.2 Interaction between N. meningitidis and macrophages

The interaction of pathogenic Neisseria with macrophages has been studied with the gonococcus using Transmission Electron Microscopy (TEM) and quantitative measurement of association to show that the bacterium is internalised by macrophages, although expression of the Tfp reduces phagocytosis (Jones and Buchanan, 1978; Blake and Swanson, 1975; Ota et al., 1975). In the presence of opsonins, macrophages preferentially phagocytose opsonised gonococci via the Fcγ receptor (Jones et al., 1980). Following phagocytosis, gonococci are efficiently killed (Cooper and Floyd, 1982) although more recent work has shown that expression of porins by the gonococcus results in a delay in the maturation of the phagolysosome (Mosleh et al., 1998). Work with the meningococcus has also demonstrated that phagocytosis of N. meningitidis occurs, and revealed a crucial role for the polysialic acid capsule in modifying a number of steps in the interaction with macrophages. Capsulate bacteria associate significantly less than unencapsulated strains, and once internalised, expression of the capsule delays the initial recruitment of late endosomal and lysosomal markers to the phagolysosome (Read et al., 1996). Opsonisation of bacteria with mannose-binding lectin (MBL) enhances both association and killing (Jack et al., 2005). Interestingly, capsulate bacteria are killed more effectively than unencapsulated strains, indicating that a non-lysosomal killing mechanism may be responsible (Read et al., 1996). More recent work has revealed that the meningococcal superoxide dismutase reduces phagocytosis by macrophages and nitric oxide detoxification systems enhance intracellular survival (Stevanin et
Furthermore, the meningococcus has been shown to delay apoptosis of macrophages following phagocytosis. This process is dependent on a functional nitric oxide detoxification system, and on the PorB porin (Tunbridge et al., 2006).

The inflammatory response that characterises meningococcal disease is mediated largely by cytokine production by phagocytic cells. There is evidence that the bacterium manipulates this process during interaction with macrophages to effect changes in the release of pro-inflammatory mediators. LPS is a well-characterised molecule that induces pro-inflammatory cytokine secretion by macrophages. This process relies on the Kdo2 moiety linked to the lipid A core (Zughaier et al., 2004). N. meningitidis also expresses other non-LPS components that induce this response since LPS-deficient mutants are still able to induce IL-8 production (Pridmore et al., 2001). Non-LPS surface components are also responsible for the induction of TNFα, IL-1β and IFN-γ production (Sprong et al., 2001). Expression of Neisserial adhesin NadA results in increased release of TNF-α and IL-8 by macrophages, indicating a stimulatory function that would in turn augment the resulting inflammation and recruitment of further inflammatory cells to the site of infection (Franzoso et al., 2008). Opa expression also affects the outcome of the interaction, with different Opa variants eliciting varying levels of pro-inflammatory cytokine release (Makepeace et al., 2001). Furthermore, meningococcal metabolism of nitric oxide produced by macrophages during the oxidative burst modifies the release of nitric oxide-regulated cytokines and chemokines resulting in decreased production of TNF-α, IL-12 and CXCL8, and an increase in secretion of IL-10 and CCL5 (Stevanin et al., 2007).

### 1.4.2.3 Interaction between N. meningitidis and dendritic cells

In the presence of N. meningitidis, DCs undergo maturation and release cytokines including TNF-α, IL-1α, IL-6 and IL-12. This is mediated partly via engagement of encapsulated meningococci by the class A scavenger receptor, although unencapsulated bacteria also elicit cytokine release (Villwock et al., 2008). The pro-inflammatory DC response also relies
partly on non-LPS outer membrane components including PorA, although LPS is required to 
induce high-level release of cytokines, particularly IL-12 (Al-Bader et al., 2004; Uronen-Hansson et 
al., 2004; Dixon et al., 2001). Expression of the polysialic acid capsule, sialylation of the LPS and 
elongation of the LPS α-chain inhibit association and phagocytosis of the meningococcus by 
DCs (Kurzai et al., 2005; Unkmeir et al., 2002a; Kolb-Maurer et al., 2001). Unencapsulated strains are 
also significantly more susceptible to intracellular killing (Kolb-Maurer et al., 2001). However, 
neither expression of capsule nor LPS sialylation by intracellular bacteria affect DC cytokine 
release (Unkmeir et al., 2002a; Kolb-Maurer et al., 2001). Work has also compared the effect of live 
and dead meningococci on DC maturation and cytokine release. Phagocytosis of live 
meningococci elicits cytokine release but results in significantly reduced maturation of DCs. 
This manipulation of DC receptor expression profile may affect DC interaction with T-cells, 
and thus influence the resulting inflammatory response (Jones et al., 2007).
1.5 Neisserial carbon metabolism

1.5.1 Nutritional requirements

Iron metabolism and its role in pathogenesis has been extensively studied in the meningococcus. Bacteria grown in the absence of iron show reduced rates of growth and replication (Perkins-Balding et al., 2004). The role of iron in meningococcal virulence was demonstrated during in vivo experiments performed by injecting host mice with supplemental iron compounds followed by inoculation with N. meningitidis. Mice receiving supplemental iron developed a lethal infection while control animals suffered only transient bacteraemia (Holbein et al., 1979). The meningococcus acquires iron from a range of sources within the host by removing the metal ion from host carrier proteins such as haemoglobin, transferrin and lactoferrin (Price et al., 2004; Lewis and Dyer, 1995; Mickelsen et al., 1982). Acquisition of iron from these sources is mediated by a number of OMPs, the expression of which is regulated by iron availability (Pettersson et al., 1993). Despite being unable to produce siderophores, the meningococcus is, however, able to utilise siderophores produced by other bacteria (Rutz et al., 1991). One major iron transporter is FrpB, an OMP that works with energy-coupling protein TonB to import enterobactin, a siderophore produced by E. coli (Carson et al., 1999; Pettersson et al., 1995). Transferrin-binding proteins (TBPs) TbpA and TbpB are expressed at the bacterial surface and work antagonistically to bind human transferrin (Renauld-Mongenie et al., 1997; Irwin et al., 1993). The role of both proteins in virulence has been assessed in the murine model of bacteraemia. Mutant strains lacking either both TBPs or TbpA were significantly attenuated, whereas a strain deficient in TbpB was not (Renauld-Mongenie et al., 2004). The meningococcus also expresses lactoferrin binding proteins (LBPs) LbpA and LbpB and haemoglobin-specific receptors such as HmbR (Quinn et al., 1994; Lee and Hill, 1992; Schryvers and Morris, 1988).

With the exception of iron, little is known about the role of metabolism in meningococcal pathogenesis. N. meningitidis is a fastidious micro-organism that is able to utilise only a
limited range of carbon sources, including glucose, lactate and pyruvate (Cartwright et al., 1987). These compounds are available throughout the upper airway, bloodstream and CSF (Smith and Forman, 1994; Horwitt et al., 1949).

1.5.2 Lactate

Lactate is taken up by *N. meningitidis* via an inner membrane permease encoded by *lctP* (Exley et al., 2005a). When grown in liquid media, both the meningococcus and gonococcus emerge from lag phase earlier and grow faster when lactate is added to media containing glucose (Exley et al., 2005b; Smith et al., 2001). Addition of lactate to gonococcal cultures results in a two-fold increase in oxygen consumption and general stimulation of metabolism culminating in up to 20% greater LPS production and enhanced protein synthesis (10 to 20%) and pentose content (30 to 60%), indicating stimulation of ribosome production (Smith et al., 2007; Gao et al., 1998; Parsons et al., 1996; Britigan et al., 1988). The underlying mechanism of this metabolic stimulation was revealed by NMR studies that showed that in the presence of glucose, lactate was diverted away from gluconeogenesis and used purely as an energy source (Leighton et al., 2001). *In vivo*, analysis of gonococcal metabolism following subcutaneous infection of guinea-pigs showed a preferential use of the lactate generated by the leukocyte inflammatory response over glucose (Goldner et al., 1979).

Similarly, in the CSF, lactate is utilised preferentially by the meningococcus. As lactate levels in the CSF rise during bacterial meningitis whilst glucose levels drop, this preference for lactate may enable the bacteria to grow more rapidly during disease and therefore enhance its virulence. A mutant lacking the lactate permease showed a significant growth defect during growth in CSF (Exley et al., 2005a). Following import, lactate is converted to pyruvate by one of three lactate dehydrogenases (Fischer et al., 1994; Erwin and Gotschlich, 1993). The reason for this potential redundancy has not been elucidated, although work suggests that each enzyme is independently regulated (Erwin and Gotschlich, 1996). Pyruvate is converted to PEP via PEP synthase, and combined with *N*-acetyl mannosamine to produce NANA or sialic.
acid (Gunawan et al., 2005). The activated form of this compound, CMP-NANA, is used by the bacterium to produce the polysialic acid capsule via enzymes encoded by the capsule biosynthetic and assembly locus, *cps*. CMP-NANA can also be incorporated into LPS via the LPS-specific sialyltransferase encoded by *lst* (Gilbert et al., 1996) (Figure 1.1). As a result of its direct link with sialic acid production, mutants lacking *lctP* shows a marked reduction in sialylation of the LPS. Furthermore, this strain is significantly attenuated during bloodstream infection of infant rats, but not in complement-deficient animals. Thus, unlike in the CSF, lactate is required during bloodstream infection not for growth but for production of sialic acid to avoid complement-mediated lysis (Exley et al., 2005a).
Figure 1.2 Neisserial carbon metabolism and sialic acid production

(A) Schematic of central carbon metabolism in *N. meningitidis*. Sialic acid is manufactured from lactate via PEP. (B) Sialic acid polymer. The α(2,8)-linked polymer is bound to the outer membrane via a diglyceride moiety to form the α(2,8)-linked polysialic capsule.
1.5.3 Glutamate

Glutamate metabolism provides a crucial link between carbohydrate and nitrogen metabolism (Smith, 1975). NADP-specific glutamate dehydrogenases (GDHs) are primarily involved in assimilation of ammonia and production of glutamate, catalysing the reaction that converts 2-oxoglutarate and ammonia to L-glutamate. Meanwhile, NAD-specific GDHs catalyse glutamate catabolism (Merrick, 1988). Many bacteria also employ an alternative glutamine synthesis pathway that relies on glutamine synthase (GS) and glutamate synthase (GOGAT). This GS/GOGAT pathway operates during nitrogen-limiting conditions, whilst the NADP-GDH is only active in high nitrogen concentrations (Reitzer, 1996).

*N. meningitidis* possesses two NADP- and NAD-GDH but no glutamate synthase, and thus does not possess a functional GS/GOGAT pathway, indicating that the NADP-GDH plays a major role in ammonia assimilation (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000; Holten and Jyssum, 1973). Regulation of GDH expression is poorly understood, although work has shown that addition of glucose to media results in a decrease in NAD-GDH and an increase in NADP-GDH, indicating an increase in glutamate production (Holten and Jyssum, 1973). Screening of a library of signature-tagged meningococcal mutants revealed that a strain lacking a gene encoding a NADP-specific L-glutamate dehydrogenase *ghdA* was unable to cause systemic infection in the infant rat model (*Sun et al.*, 2000). Expression of GdhA varies across different meningococcal strains, and is highest in hypervirulent lineages ET-5 (serogroup B) and IV-1 (serogroup A) (Pagliarulo *et al.*, 2004). Further work identified a positive transcriptional regulator, encoded by *gdhR*, which controls expression of GdhA. GdhR activity was increased by the addition of glucose, rather than lactate, to glutamate-containing medium. Mutants lacking GdhR were unable to regulate carbon metabolism and exhibited a severe growth defect when grown in the presence of glucose (Pagliarulo *et al.*, 2004). More recently, GdhR has been found to regulate additional genes encoding enzymes involved in glucose catabolism and L-glutamate uptake. A previously unknown ABC-type transporter, GltT, was identified as an L-glutamate importer, operating in low sodium conditions such as in the intracellular
environment. Accordingly, a mutant lacking GltT was unable to survive within HeLa cells (Monaco et al., 2006). This importer has since been renamed PerM, and a second transporter, GltS, which is active in high sodium conditions has also been identified (Unpublished). The role of glutamate metabolism in bacterial pathogenesis has not been widely studied. Investigation of the importance of glutamate to pathogenic strains of *E. coli* has demonstrated that glutamate metabolism is required for acid-resistance whereby the glutamate decarboxylase GadA/GadB acts as a proton acceptor and the antiporter GadC exchanges the intracellular product of the decarboxylation to produce glutamic acid, which is secreted by the cell (Sayed et al., 2007; Grant et al., 2001). A similar system also operates in gastrointestinal bacterial pathogen, *Shigella flexneri* (Bhagwat and Bhagwat, 2004). Investigation of glutamate metabolism in *Brucella abortus* has also demonstrated that highly virulent strains metabolise glutamate at a significantly lower rate than strains with low virulence capabilities, although the reason for this finding is unknown (Disinger and Wilson, 1962).
1.6 DNA Repair

1.6.1 DNA damage

Repair of damaged DNA is an essential activity performed by all living cells. Loss or alterations in bases in both the coding and non-coding regions of the genome results in mutations. Mutation in DNA is the driving force of evolution, leading to new phenotypic variants which propagate or perish according to the selective pressures of an organism’s environment. In bacterial pathogens, mutation is a mechanism by which microbes adapt to their host, enabling the emergence of new traits such as antibiotic resistance (Woodford and Ellington, 2007; Denamur and Matic, 2006). However, mutations are more frequently detrimental to the bacterium, and if unchecked, the rapid accumulation of mutations in the genome would be lethal. Damage may be caused as a result of endogenous metabolic reactions, error-prone replication or exogenous mutagens such as ultraviolet (UV) light or oxidative stress. DNA is also subject to spontaneous degradation, causing deamination and hydrolytic loss of bases (Davidsen and Tonjum, 2006). Bacterial DNA is also susceptible to mutation during replication as a result of horizontal transfer and recombination events (Sargentini and Smith, 1985).

Oxidative damage to DNA occurs as a result of free radicals and ROS produced within bacteria during metabolism and following spontaneous degradation of biomolecules. Free radicals cause damage to DNA bases and sugars both directly and indirectly by generating reactive aldehydes via lipid peroxidation. The most active free radical species is the hydroxyl radical. Hydroxyl radicals react with purines to produce 7,8-dihydro-8-oxo-2’-deoxyguanosine (8-oxoG) (Devasagayam et al., 1991), open-ringed structures such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy), and cyclopurine deoxynucleosides (Dirksen et al., 1988). Hydroxyls also target double carbon-carbon bonds in pyrimidines to produce glycol and pyrimidine hydrates (Cadet et al., 1999). Furthermore, hydroxyl radicals can lead to double-strand DNA breaks following abstraction of hydrogen atoms from the DNA structure.
(Henner et al., 1983). Damaged bases such as 8-oxoG result in transitions in DNA such as replacement of guanine with thymine, while other mutations such as glycol and hydrates block replication and are therefore highly cytotoxic (Laval et al., 1998) (Figure 1.3).

Figure 1.3 DNA lesions resulting from oxidative damage

Chemical structures of the more common stable oxidative DNA base lesions. Hydroxyl radicals result in pyrimidine hydrates (5-hydroxy-dU, 5-hydroxy-dC) and glycols (thymine/uracil glycol). They react with purines to produce 8oxo-dG/A and open-ringed structures such as Fapy-dA/G. These bases are incorporated into the DNA strand and result in mutation (Dojindo.com).
In addition to endogenous sources of ROS, *N. meningitidis* is exposed to high concentrations of external oxygen during colonisation of the human nasopharynx, and encounters harmful oxidative agents released by the host immune response during disease (Davidsen and Tonjum, 2006). Phagocytosis of the bacterium by cells such as neutrophils and macrophages triggers the oxidative burst, which culminates in the production of ROS including \( \text{H}_2\text{O}_2 \) and hypochlorous acid (HOCl) (Thomas *et al*., 1988). \( \text{H}_2\text{O}_2 \) reacts with transition metals present in the bacterial cell to produce hydroxyl radicals (Almeida *et al*., 1999; Imlay and Linn, 1988), while HOCl readily breaks down into chloramines, which attacks amino-groups (Dukan and Touati, 1996). In order to maintain genome integrity and survive mutagenic events, the meningococcus possesses a series of DNA repair pathways.

### 1.6.2 DNA repair mechanisms

Due to the fundamental nature of DNA repair, genes encoding DNA repair enzymes are largely conserved in all living organisms. DNA repair pathways have been well characterised in *E. coli*. Damage to one strand of DNA is repaired using the intact complementary strand as the template. This process is carried out by three repair pathways that excise and replace the damaged or erroneous nucleotides.

The Base Excision Repair (BER) pathway is initiated by DNA glycosylases which have specificity for particular DNA substrates. For example, uracil DNA glycosylase (UDG) targets erroneous uracil residues that have been incorporated into DNA. One of the most common lesions occurs as a result of oxidation of guanine to 8-oxoG. In *E. coli* 8-oxoG lesions are targeted by the DNA glycosylase MutY, which acts with MutT and MutM to constitute the 8-oxoG repair system (Michaels *et al*., 1992). Glycosylase activity produces an abasic site, which can also arise spontaneously by depurination or depyrimidination. Abasic sites are acted upon by apurinic/apyrimidinic endonucleases (AP endonucleases) (Lindahl, 1993). These highly conserved enzymes bind the abasic site, cleaving the DNA 5’ to the lesion, and remain bound to the resulting 3’ hydroxyl group until being displaced by DNA polymerase,
which replaces the lesion with the correct base (Dianov et al., 2003; Demple and Harrison, 1994) (Figure 1.1). Many organisms possess two fully functional AP endonucleases. For example, *E. coli* expresses ExoIII and EndoIV, *Saccharomyces cerevisiae* expresses Apn1p and Uve1p, whilst the human forms are HAP1 and HAP2 (Boiteux and Guillet, 2004; Hadi and Wilson, 2000; Ljungquist et al., 1976). This duplication is thought to reflect the fundamental importance of BER to the survival of all organisms.

Mismatch Repair (MMR) recognises base-pair mismatches and insertion/deletion loops introduced into the genome by errors during DNA replication (Schofield and Hsieh, 2003). In *E. coli* these lesions are thought to be recognised by the MMR enzyme MutS which coordinates with MutL in an ATP-dependent manner to activate the endonuclease MutH (Junop et al., 2003). The endonuclease binds the lesion and directs the strand specificity of the MutS-MutL-MutH repairosome (Marti et al., 2002). MutL also facilitates binding of helicase II to the lesion to ensure the correct region is unwound (Mechanic et al., 2000). Since the damaged region of DNA can be as long as 1 kb and helicase II dissociates after 40–50 bases, MutL continues to recruit helicase II to prevent the strands from re-annealing (Mechanic et al., 2000). Following degradation by polarity-specific exonucleases, the resulting gap is repaired by DNA polymerase and ligated by DNA ligase (Marti et al., 2002). Other replicatory errors are repaired by translesion synthesis, which employs low fidelity translesion polymerases instead of the normal DNA polymerases. These enzymes, of which *E. coli* possesses three, pass over small lesions such as AP sites, allowing replication to continue, but at the risk of introducing frameshift mutations (Napolitano et al., 2000).
Figure 1.4 Base Excision Repair Pathway

Schematic representation of the BER pathway. A lesion (X) is acted on by a DNA glycosylase (blue) to produce the abasic site indicated in red. The site is targeted by an AP endonuclease (green), which bends the DNA and cleaves 5’ to the lesion. DNA polymerase (yellow) and DNA ligase (red) repair the DNA strand.
Chapter 1 Introduction

The Nucleotide Excision Repair (NER) pathway acts on damaged regions of two to 30 bases arising following exposure to exogenous agents such as UV light. The resulting bulky lesions interfere with normal base-pairing and impair transcription. The most common of these lesions are cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) caused by shortwave UV irradiation, as well as numerous chemical adducts. The lesions are highly diverse in structure and are thought to be recognised by the distorting effect they have on DNA structure (de Laat et al., 1999). In *E. coli*, NER is performed by the UvrABC complex (Seeberg, 1978). UvrB interacts with both UvrA and UvrC in an ATP-dependent manner in the presence of damaged DNA. The damaged site is recognised and targeted by UvrA, which then dissociates to allow UvrC binding and endonuclease activity (Malta et al., 2007). Incisions are made either side of the lesion, and polymerases and ligases are recruited to repair the defect.

Double-stranded breaks (DSBs) in DNA arise during replication when the replication fork encounters a blocking lesion, such as those induced by ROS, during genome rearrangements, physical stress exerted during cell division or as a result of endogenous agents such as UV light, ROS and X-rays (Shrivastav et al., 2008; Davidsen and Tonjum, 2006). Failure to repair DSBs results in deletion, translocation and accumulation of cytotoxic genome rearrangements that may stall replication altogether. DSBs are repaired by two recombinational repair pathways. Non-homologous recombinational repair relies on the specialised DNA ligase IV to ligate the free DNA ends. Recombinational repair is achieved by either the RecF or RecBCD pathways which remove the target lesion, leaving a gap which is repaired by strand exchange using a single-stranded template (Davidsen and Tonjum, 2006).

Genes encoding DNA repair enzymes in most bacterial species possess binding sites for components of the SOS response. This damage-inducible system is characterised by an up-regulation of expression of a range of components of DNA repair pathways (Michel, 2005). The
SOS response is regulated by two proteins, the repressor LexA, and the inducer, RecA. In the absence of stress, LexA binds specific SOS box sequences within the promoters of SOS-inducible genes, preventing their expression. RecA binds single-stranded DNA that is produced following DNA damage. This complex induces self-cleavage of LexA, allowing expression of the repressed genes (Michel, 2005). The SOS response has been well studied in a number of pathogenic and non-pathogenic bacterial species including *E. coli*, *Staphylococcus aureus*, *Vibrio cholerae* and *Bacillus subtilis* (Goerke et al., 2006; Michel, 2005; Wojciechowski et al., 1991; Yasbin, 1977). Interestingly, *N. meningitidis* does not possess an SOS response, and is thus thought to be unable to up-regulate expression of components of the repair pathway, even when under stress (Davidsen and Tonjum, 2006).

There has been little work devoted to investigating the role of BER in pathogenesis despite its potential importance in maintaining DNA integrity in the face of the innate immune response. *Salmonella enterica* Serovar Typhimurium requires its full complement of DNA glycosylases in order to survive effectively within host macrophages, and a putative AP endonuclease in *Brucella abortus* is necessary to resist oxidative stress (Hornback and Roop, 2006; Suvarnapunya and Stein, 2005). However, these studies relied upon the characterisation of strains lacking genes identified by sequence comparison alone and did not include any investigation of the biochemical activity of the gene products.

### 1.6.3 Repair pathways in *Neisseria*

Examination of the *N. meningitidis* genome reveals that it contains homologues of genes encoding characterised enzymes involved in BER, NER, MMR and recombinational repair. However, many of the enzymes and responses characterised in *E. coli* are absent in the meningococcus. The absence of the SOS response in *Neisseria* was confirmed by transcriptional analysis of the gonococcal genome following exposure to mutagenic agents including UV light and methyl methanesulphonate (Black et al., 1998). The absence of a LexA
homologue and of LexA binding sites within Neisserial DNA repair enzyme-encoding genes such as \textit{recA}, \textit{uvrA} and \textit{uvrB} supports this finding, indicating that RecA functions purely within recombinational repair and not as part of a damage-inducible response (Parkhill \textit{et al.}, 2000; Black \textit{et al.}, 1997; Black \textit{et al.}, 1995).

A number of Neisserial DNA repair pathways have been described, primarily in the gonococcus. It is expected that recombinational repair plays an important role in maintaining genome stability due to the absence of the SOS response and other regulatory systems. Accordingly, recombinational repair is the best characterised DNA repair system in \textit{Neisseria} (Kline \textit{et al.}, 2003). In addition to repair of replication errors, recombination is involved in DNA transformation and is involved in phase variation of surface structures including Tfp and Opa (Aho \textit{et al.}, 1991; Hagblom \textit{et al.}, 1985). RecA was identified in the gonococcal genome by complementation of an \textit{E. coli} mutant (Koomey and Falkow, 1987). Further work indicated that species-specific interactions are required for DNA repair, but not for homologous recombination. Introduction of the \textit{E. coli} gene into a gonococcal \textit{recA} mutant complemented the strain for pilin variation and partially for DNA transformation but not for repair of DNA damage (Stohl \textit{et al.}, 2002). Further \textit{rec} genes have been identified in the gonococcal genome, including intact, functional enzymes encoded by \textit{recB}, \textit{recC} and \textit{recD} (Mehr and Seifert, 1998). Strains of the meningococcus possess an inactive \textit{recB} allele, and thus is impaired in repair of DNA damage caused by UV light (Salvatore \textit{et al.}, 2002). RecN, which is known to play a structural role in positioning DNA during transformation, also appears to be active within DNA repair (Skaar \textit{et al.}, 2002), and homologues of other recombinational repair enzymes including RecG, RecR, RuvA, RuvB and RuvC, are also functional (Kline \textit{et al.}, 2003). All Neisserial genomes lack a RecF homologue, but possess the other components of the RecF repair pathway, indicating a RecF-like pathway in \textit{Neisseria}. This pathway is constitutively active, and thus is thought to partially compensate for the lack of an SOS response (Kline \textit{et al.}, 2003; Mehr and Seifert, 1998).
MMR, NER and BER in the meningococcus have been less well characterised. Examination of the meningococcal genome has revealed homologues of genes encoding the MMR enzymes MutS and MutL, but not MutH, which is likely replaced by an alternative unidentified component or mechanism. It is also thought that defects in MMR are likely to influence the virulence of the bacterium (Richardson and Stojiljkovic, 2001). NER has been studied in the gonococcus, in which a system that acts on pyrimidine dimer lesions has been identified (Campbell and Yasbin, 1984). The gonococcus also expresses UvrA, which complements the respective mutant strain of E. coli, and strains lacking a UvrB homologue have increased sensitivity to UV light (Black et al., 1997; Black et al., 1995). Interrogation of the meningococcal genome has identified homologues of all components of the BER pathway, although the glycosylases nei and alkA and endonuclease nfo are absent. The only component of BER studied in the meningococcus is the DNA glycosylase MutY, which has been characterised at a biochemical level, though there has been no work on its importance in virulence and disease. A strain lacking mutY showed an increased spontaneous mutation rate (Davidsen et al., 2005). In E. coli, MutY acts with Fpg and MutT to repair 8-oxoG. E. coli strains lacking these enzymes display mutator phenotypes, with the strain lacking Fpg showing the highest rate of mutation (Fowler et al., 2003). In contrast, a meningococcal strain lacking mutY displays a far greater mutation rate than a strain lacking fpg, and a double mutant shows considerably higher rates of mutation than either of the single mutants, indicating that MutY and Fpg act in synergy, and that MutY plays an important role in meningococcal DNA repair (Davidsen et al., 2005).

1.6.4 Meningococcal genome plasticity

In addition to the absence of an SOS response, the meningococcus also possesses fewer regulatory response elements such as σ factors and two-component regulatory systems compared with E. coli (Parkhill et al., 2000). It has been suggested that this less complex gene regulation is compensated for by a greater genome plasticity, and that this is an adaptation
to the more limited environments encountered by \textit{N. meningitidis} in the human host in comparison with the broad range of environments experienced by the non-host specific \textit{E. coli}. The higher mutation rates resulting from increased genome plasticity may allow for the emergence of more varied phenotypic traits allowing the meningococcus to adapt and survive within the human host (Davidsen and Tonjum, 2006).

Meningococcal genome instability is further enhanced by additional repeat and uptake sequences, antigenic and phase variation mechanisms and mutator alleles. Examination of the meningococcal genome has revealed hundreds of repeat sequences, ranging from a few bases to up to 1 kb or more in length (Tettelin \textit{et al.}, 2000). These elements allow for frequent duplication, deletion and recombination events. The most common of these is the 10 bp Neisserial DNA uptake sequence (DUS), 5'-GCCGTCTGAA-3', that is required for natural transformation of both the meningococcus and gonococcus (Smith \textit{et al.}, 1999; Goodman and Scocca, 1988). DNA fragments containing a DUS are preferentially incorporated into the gonococcal genome (Elkins \textit{et al.}, 1991). The role of DUSs is not fully established although they are postulated to act as transcriptional regulators, and are seen most frequently in genes encoding components involved in DNA repair, recombination, replication and modification (Davidsen \textit{et al.}, 2004; Goodman and Scocca, 1988). Genome plasticity also plays a crucial role in phase variation, which allows the bacterium to present a variety of antigenic phenotypes. Hypermutation in localised areas of the genome, known as contingency loci, is orchestrated by a number of sequence elements. Short repeat sequences such as homopolymeric tracts and microsatellites, or tandem repeats may affect regulation or expression of nearby promoter regions (Feil \textit{et al.}, 2001). Slipped-strand mispairing results in addition or deletion events, and Rec-A dependent recombination is known to control variation of virulence-associated surface components such as Opa, Tfp, LPS and capsule via the introduction of a stop codon (Rytkonen \textit{et al.}, 2004; Berrington \textit{et al.}, 2002; Jennings \textit{et al.}, 1999; Hammerschmidt \textit{et al.}, 1996; Rudel \textit{et al.}, 1995; Sarkari \textit{et al.}, 1994; Murphy \textit{et al.}, 1989; Kawula \textit{et al.}, 1988). More recently,
meningococcal mutator strains have also been shown to possess a defective MMR pathway (Richardson et al., 2002; Richardson and Stojiljkovic, 2001).

1.7 Project Aims

The aim of this project is to characterise the non-opsonic interaction between *N. meningitidis* and human PMNs. This aspect of meningococcal disease is not well understood despite its potential importance to the development and progression of the disease. The project will also investigate the role of bacterial metabolism of lactate and glutamate to the interaction with PMNs. Finally, the project will investigate two DNA repair enzymes that are thought to act within the BER pathway, and will characterise their role in the pathogenesis of *N. meningitidis*. 
Chapter 2

Materials and Methods
2.1 **Bacterial strains and plasmids**

2.1.1 *Bacterial strains* - *N. meningitidis* MC58 (ET-5, ST-32) is a serogroup B clinical isolate expressing immunotype L3,7,9 LPS and adhesins Opa and Opc (Exley et al. 2005). The isogenic mutants used in this study and their characteristics are shown in Table 1. For GFP expression, strains were transformed with plasmid pEGFP (kind gift from Myron Christodoulides (Christodoulides et al., 2000)). Bacteria were cultured on Brain-Heart Infusion (BHI) agar with Levanthal’s supplement at 37°C in 5% CO₂. Antibiotics were added as required at the following concentrations: tetracycline 2.5 µg/ml; kanamycin 50 µg/ml; erythromycin 2 µg/ml. The concentration of bacteria was calculated by measuring the OD₆₀₀ of a 1:50 dilution of bacterial suspension in 200 mM NaOH/1% SDS (wt/vol).

*E. coli* XL1-blue cells (Invitrogen, for recombinant work) and ER2566 (New England Biolabs, for protein expression) were propagated in Luria Bertani (LB) media. Antibiotics were used at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 75 µg/ml; tetracycline, 2 µg/ml.

2.1.2 *Bacterial growth conditions* - For growth in liquid media bacteria were grown overnight on solid media, harvested to phosphate-buffered saline (PBS) and used to inoculate liquid BHI media at an OD₆₀₀ of 0.15. For growth in defined media, bacteria were inoculated to Hanks Balanced Saline Solution (HBSS; 1 mg/ml D-glucose). Lactate (1 mM) and pyruvate (100 µM) were added as necessary.
### Table 1 Strains used in this study

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<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<td>Clinical isolate (wild-type strain, serogroup B)</td>
<td>Exley et al. (2005)</td>
</tr>
<tr>
<td>H44/76</td>
<td>Clinical isolate (wild-type strain, serogroup B)</td>
<td>Monaco et al (2006)</td>
</tr>
<tr>
<td>8013</td>
<td>Clinical isolate (wild-type strain, serogroup C); Opa negative</td>
<td>Pujol et al (1997)</td>
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<td>Exley et al. (2005)</td>
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<td>Exley et al. (2005)</td>
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<td>Exley et al. (2005)</td>
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<td>Exley et al. (2005)</td>
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</tr>
<tr>
<td>MC58ΔnexoΔnape</td>
<td>Deficient in NMB0399 and NMB2082</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.1.3 Assay for survival against oxidative stress - Strains were grown overnight, harvested into PBS and the bacterial suspensions adjusted to $1 \times 10^8$ CFU/ml and serial dilutions of 1:10 made up to 10 CFU/ml. To optimise oxidative stress conditions serial dilutions were spotted to solid media containing a range of concentrations of hydrogen peroxide (Biochemika) or paraquat (Sigma). The optimum concentration was described as the concentration at which 10% killing of the wild-type MC58 strain occurred. Each strain ($10 \mu$l, $10^3$ CFU/ml) was then plated to BHI agar containing optimised concentrations of either hydrogen peroxide (13 mM; Biochemika) or paraquat (0.075 mM; Sigma), or onto BHI alone. Killing as a result of oxidative stress in the presence of hydrogen peroxide or paraquat was calculated as a percentage of CFU recovered from media containing no oxidative agent. The assays were performed in triplicate on at least three independent occasions.

2.1.4 Fixation and labelling – Strains were grown on solid media overnight, harvested to PBS and fixed in 3% paraformaldehyde (PFA). Bacteria were then incubated with 2.5 mg/ml fluorescein 5(6)-isothiocyanate (FITC; Sigma) in PBS for 30 minutes at 37°C, then washed extensively in PBS to remove unbound label.

2.1.5 Construction of bacterial strains – Upstream (700 bp) and downstream (700 bp) fragments flanking NMB2082 were amplified using primers shown in Table 2. The PCR products were ligated into pCR2.1 TOPO. The upstream fragment was excised using restriction endonucleases HindIII and HpaI (Invitrogen) and ligated into the vector containing the downstream fragment. The gene encoding tetracycline resistance was removed from donor vector pCMT18 and inserted between the up- and down-stream fragments at the unique EcoRI site to produce pAC1. N. meningitidis MC58 was transformed to tetracycline resistance by standard methods to produce the $\Delta$nap strain. The $\Delta$nexo strain was constructed by Tn5 transposon mutagenesis by Megan
Table 2 Primers used in this study

All primer sequences are 5’ to 3’. Relevant restriction sites are

<table>
<thead>
<tr>
<th>Sequence (5’ - 3’)</th>
<th>Restriction site</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGGATCCATGAAAATCACCACCTGG</td>
<td>BamHI</td>
<td>Amplification of NMB0399</td>
</tr>
<tr>
<td>CCCAAGCTTTTACCAATCGAATTCTGC</td>
<td>HindIII</td>
<td>Amplification of NMB0399</td>
</tr>
<tr>
<td>CGGGATCCATGCTAAAATCATTTCCGCC</td>
<td>BamHI</td>
<td>Amplification of NMB2082</td>
</tr>
<tr>
<td>CCCAAGCTTTTCGCGCAGCATAGTCATAC</td>
<td>HindIII</td>
<td>Amplification of NMB2082</td>
</tr>
<tr>
<td>GAGTTAACGACGTTGGCGGAAAATGATTT</td>
<td>HpaI</td>
<td>Mutagenesis of NMB2082 for <em>N. meningitidis</em>: upstream fragment</td>
</tr>
<tr>
<td>GAAAGCTTTGGCTGCACAACGGCTTTAT</td>
<td>HindIII</td>
<td>Mutagenesis of NMB2082 for <em>N. meningitidis</em>: upstream fragment</td>
</tr>
<tr>
<td>GAGATATCATCCACATCTGATCCTCTCCG</td>
<td>EcoRI</td>
<td>Mutagenesis of NMB2082 for <em>N. meningitidis</em>: downstream fragment</td>
</tr>
<tr>
<td>GAGTTAAACAACATCGCCACACAAACAT</td>
<td>HpaI</td>
<td>Mutagenesis of NMB2082 for <em>N. meningitidis</em>: downstream fragment</td>
</tr>
</tbody>
</table>
Winterbotham. The double \( \Delta \text{nexo} \Delta \text{nape} \) mutant was constructed by transformation of the \( \Delta \text{nexo} \) strain with pAC1 by standard methods.

### 2.2 DNA manipulation

#### 2.2.1 DNA isolation

For isolation of genomic DNA, bacteria were grown overnight on solid media and a half loop of bacteria was harvested and resuspended in 560 µl TE buffer (10 mM Tris, pH 7.6, 1mM EDTA), 30 µl SDS (wt/vol) and 3 µl of 20 µg/ml proteinase K (Sigma, UK). The mixture was incubated for one hour at 37°C. 100 µl of 5 mM NaCl and 80 µl of cetyl-trimethyl-ammonium bromide/NaCl (10% CTAB, Sigma; 0.7 M NaCl, BDH) were added, and the solution incubated at 65°C for 10 minutes. 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. The solution was centrifuged at 16000 × g for 20 minutes and the aqueous layer retained. DNA was precipitated by the addition of two volumes of 100% ethanol followed by centrifugation at 16000 × g for five minutes. The pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer containing RNase (100 µg/ml, Sigma UK).

For plasmid purification from *E. coli*, bacteria was grown overnight in 5 ml LB media and pelleted by centrifugation at 16,000 × g for ten minutes. For *N. meningitidis*, bacteria were grown overnight on solid BHI media, a half loop harvested to PBS and pelleted by centrifugation at 16,000 × g for five minutes. Plasmid DNA was purified using the QIAprep® Spin Miniprep kit according to the manufacturer’s protocol (Qiagen).
2.2.2 Polymerase Chain Reaction – Reaction mixtures contained 1 pmol/µl of each primer, 1.5 mM MgCl₂, 200 µM dNTPs, 2 units of Taq DNA polymerase (Sigma) and 0.1 volume of 10 x PCR buffer (Gibco BRL) in a 25 µl final volume. Control reactions containing no Taq polymerase or no target DNA were also performed. Thermal cycling conditions were as follows: initial denaturation step at 94°C for five minutes, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for up to three minutes, depending on the length of the fragment to be amplified (1 kb/min), and a final extension step at 72°C for ten minutes.

2.2.3 Restriction endonuclease digestion of DNA – Restriction digests were performed with 0.01 volumes of BSA, restriction enzyme(s) and either plasmid or genomic DNA, and 0.1 volume of 10 x reaction buffer. All reagents were obtained from New England Biolabs. Reactions were incubated at 37°C for up to six hours.

2.2.4 Gel electrophoresis and purification of DNA from agarose gels – DNA was resuspended in loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and fractionated in Tris Acetate EDTA (TAE) buffer (Tris 0.4 M, EDTA-Na₂-salt 0.01 M, acetic acid 12.01 g/L, 0.2M) on 1% agarose (Gibco, BRL) gels containing a 1:100 dilution of the Cybersafe DNA gel stain. DNA was visualised using a UV transilluminator (UVP). For purification of DNA, gel fragments were excised using a scalpel and purified using the QIAluick® gel extraction kit according to the manufacturers protocol (Qiagen).

2.2.5 Ligation of DNA – DNA fragments generated by PCR were ligated into the pCR2.1 TOPO vector (Invitrogen) according to the manufacturers’ protocol. All other ligations were performed using a 1:5 ratio of insert to vector, 4 µl 5 x T4 DNA
ligase buffer and 1 µl T4 DNA ligase (1 U/µl; NEB), made up to a final volume of 20 µl with dH₂O (Sigma, UK). The mixture was incubated at 16°C overnight.

2.2.6 Transformation of chemically competent E. coli – A 10 µl ligation reaction was added to 50 µl E. coli TOP10 competent cells and incubated on ice for 30 minutes. The mixture was subjected to heat shock at 42°C for 30 seconds, then 250 µl SOC medium was added. 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 dH₂O, then 10 ml 250 mM KCl was added and the volume adjusted to 1 L, followed by autoclaving. A sterile solution of 2 M MgCl₂ (5 ml) was added to the final solution. Following addition of SOC medium to transformed bacteria, the solution was incubated at 37°C with continuous shaking (200 r.p.m) for one hour. The bacteria were then plated to solid media containing appropriate antibiotics.

2.2.7 Southern blot – Restriction endonuclease digested genomic DNA was separated on 1% agarose gels overnight at 30 V. Gels were incubated in depurination solution (0.25 M HCl) for 12 minutes, rinsed, and equilibrated in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 18 minutes, followed by incubation in neutralising solution (1.5 M NaCl, 1 M Tris pH 8) for 18 minutes. Gels were then transferred to nitrocellulose membranes (Hybond N+, Amersham) by capillary action in Saline-sodium citrate (SSC) buffer (150 mM NaCl, 15 mM NaH₂(C₃H₅O(COO)₃) pH 7). DNA was fixed to the membrane by exposure to UV light for 30 seconds. Membranes were washed in wash solution (0.5% SDS 10%, 0.1 x SSC) for 20 minutes at 65°C. DNA probe preparation and alkaline phosphatase labelling was performed using the AlkPhos Direct Labelling and Detection System with CDP-Star (GE healthcare) according to the manufacturer’s protocol. Membranes were placed in an enhanced chemiluminescence (ECL) cassette for detection.
2.2.8 DNA Sequencing – DNA sequencing was performed using 1 µl primer (3.2 pmol / µl) and dH₂O (Sigma, UK) and made up to a final volume of 10 µl. Automated sequencing was performed by the Department of Biochemistry, Hammersmith Campus, Imperial College London.

2.3 Protein preparation and analysis

2.3.1 Preparation of protein samples for Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (TSDS-PAGE) - Protein samples were prepared by harvesting 1 x 10⁹ CFU of bacteria from solid media to PBS. For analysis of bacteria post-incubation with cells or supernatant, assays were performed as described below and the entire contents of a microtitre dish well were transferred to a 1.5 ml tube. Treatment of bacteria with 50 U neuraminidase or proteinase K was performed for one hour at 37°C. For analysis of adherent PMNs, cells were dislodged from the wells by scraping with a pipette tip and thoroughly re-suspended, followed by transfer of total well contents to a 1.5 ml tube. Samples were then centrifuged at 16,000 xg to pellet the bacteria and/or PMNs, resuspended in 50 µl dH₂O, and an equal volume of 2 x SDS-PAGE sample buffer (100 mM Tris HCl, 4% SDS, 200 µM β-mercaptoethanol (BME)), followed by heat treatment at 90°C for ten minutes. To prepare samples for LPS analysis by TSDS-PAGE, 1 µl proteinase K (Qiagen) was added to the SDS-PAGE sample buffer, and incubated overnight at 37°C. A further 1 µl proteinase K was then added and incubated at 65°C for three hours.

2.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) – SDS-PAGE analysis of proteins was carried out as previously described (Sambrook et al, 1989). Polyacrylamide gels were run in SDS buffer (200 mM glycine, 248
mM Tris, 34 mM SDS) at 120 V for 1-2 hours. The reagents used to prepare polyacrylamide gels are shown in Table 3.

### Table 3 SDS-PAGE gel constituents

<table>
<thead>
<tr>
<th>Resolving Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris 1.5 M pH 8.8</td>
<td>1 ml</td>
</tr>
<tr>
<td>Tris 0.5 M pH 6.8</td>
<td>-</td>
</tr>
<tr>
<td>40% Acrylamide/Bis (Biorad)</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>40 µl</td>
</tr>
<tr>
<td>dH2O</td>
<td>1.71 ml</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED) (BDH)</td>
<td>1.6 µl</td>
</tr>
<tr>
<td>10% APS (Sigma)</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

### 2.3.3 Staining and de-staining of SDS-PAGE mini-gels – Polyacrylamide gels were stained by placing them into approximately 20 ml of Coomassie Blue Stain (0.2% Coomassie Blue R-250, 40% ethanol (v/v), 10% glacial acetic acid (v/v), 50% ddH2O) for 30 minutes at room temperature (RT) with shaking. To destain, the gels were placed in 20 ml Destain I solution (40% methanol (v/v), 10% glacial acetic acid (v/v), 50% ddH2O) for 30 minutes at RT with shaking, then replaced with Destain II solution (10% glacial acetic acid (v/v), 4% glycerol (v/v) 86% ddH2O) for a further 30 minutes.
2.3.4 Western blot analysis – Protein samples were separated by SDS-12% PAGE with a pre-stained marker (10–250 kDa, BioRad) and transferred to an Immubilon-P polyvinylidene fluoride (PVDF) membrane (Millipore) by wet transfer. A sponge soaked in dH₂O was placed onto the transfer apparatus, followed by a piece of Whatman® paper pre-wet with Transfer buffer (0.2M glycine, 0.02M Tris-HCl). The gel was then placed on top, and covered with the PVDF membrane, which was pre-wet with methanol. A further piece of Whatman® soaked in Transfer buffer, and another sponge soaked in dH₂O were placed on top, and the apparatus secured and placed into the wet transfer tank. Transfer was performed in Transfer buffer, with stirring, at 70 V for one hour. The PVDF membranes were then blocked in 5% milk (skimmed milk power dissolved in PBS) at 4°C overnight with gentle shaking. All further washes and antibody incubations were carried out in PBSTM (PBS, 0.05% Tween 20, 0.5% milk). Following blocking, the membranes were washed three times in PBSTM for ten minutes each, then incubated with the primary antibody (Table 4) for two hours unless otherwise stated. This was followed by three further washes, then membranes were incubated with the secondary antibody conjugated to horseradish peroxidise (HRP) for one hour. A final three washes were performed before membranes were removed from PBSTM. The was used to detect antibody binding according to the manufacturer’s protocol. The membrane was then blotted and placed in an ECL cassette for detection.
### Table 4 Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-L,3,7,9 (murine)</td>
<td><em>N. meningitidis</em> serogroup B LPS</td>
<td>1:2000</td>
<td>NIBSC (01/412)</td>
</tr>
<tr>
<td>ZM51mAb (murine)</td>
<td><em>N. meningitidis</em> polysialic acid capsule</td>
<td>1:10</td>
<td>Oxoid</td>
</tr>
<tr>
<td>B33 mAb (murine)</td>
<td>Neisserial Opacity protein (Opa)</td>
<td>1:10,000</td>
<td>Lammel <em>et al.</em> (1988)</td>
</tr>
<tr>
<td>3F11 IgM</td>
<td>Unsialylated LPS</td>
<td>1:10</td>
<td>Yamasaki <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>Anti-CD66 (rat)</td>
<td>Human CEACAM / CD66a,c,d receptor</td>
<td>1:100</td>
<td>Serotec</td>
</tr>
<tr>
<td>Anti-CD45 (murine)</td>
<td>Human leukocyte CD45 receptor</td>
<td>1:1000</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-CD18 (murine)</td>
<td>Human leukocyte CD18 receptor</td>
<td>1:1000</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-CD11b (murine)</td>
<td>Human leukocyte CD11b receptor</td>
<td>1:1000</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Anti-neutrophil elastase (murine)</td>
<td>Neutrophil elastase</td>
<td>1:500</td>
<td>Dako</td>
</tr>
<tr>
<td>8G10 (rabbit)</td>
<td>Cleaved and un-cleaved Caspase-3</td>
<td>1:1000</td>
<td>Cell Signalling technology</td>
</tr>
<tr>
<td>Anti-actin (rabbit)</td>
<td>C-terminal actin fragment</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-mouse (FITC / Rhodamine conjugate)</td>
<td>Mouse IgG</td>
<td>1:400</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>Anti-rat (FITC conjugate)</td>
<td>Rat IgG</td>
<td>1:400</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Isotype control</td>
<td>1:1000</td>
<td>Dako</td>
</tr>
</tbody>
</table>
2.3.5 Electrophoresis of LPS – TSDS-PAGE was performed to obtain high resolution separation of LPS. Two-phase gels were prepared as a 16.5% resolving gel containing 15% urea overlaid by a 4% stacking gel. Electrophoresis was carried out as described for SDS-PAGE. 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 the cross-linker, bisacrylamide. This method of separation utilises two electrophoresis buffers, the anode buffer (0.2M Tris, pH 8.9) and cathode buffer (0.1 M Tris, 0.1 M tricine, 0.1% SDS, pH 8.25). Electrophoresis was performed using the BioRad Mini-Protean II gel apparatus (BioRad). Samples were boiled for ten minutes, loaded under cathode buffer and subjected to electrophoresis at 30 V until the samples entered the resolving gel. Voltage was then increased to 105 V for approximately 1 hour.

2.3.6 Silver stain – LPS samples were prepared as described above and separated by TSDS-PAGE. The gel was then fixed and developed using the PlusOne Silver Stain kit (Amersham) according to the manufacturer’s protocol.

2.3.7 Protein expression and purification – NMB0399 and NMB2082 were amplified using primers described in Table 3. The genes were ligated into the vector pROEXHtb for protein expression and purification. \textit{E. coli} cultures of 3 to 6 litres were grown to an OD \( A_{600} \) of 0.4 to 0.6 in LB medium and protein expression was induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 1 mM, followed by continued growth for 3 hours at 37\(^\circ\)C. Cells were harvested by centrifugation, re-suspended in buffer containing 25 mM Tris pH 8.0, 25 mM imidazole, 0.5 M NaCl (buffer A) and lysed by pulse sonication. The lysate was cleared by centrifugation at 2907 \( xg \) for 15 minutes. His-tagged proteins were purified using a metal affinity HisTrap chelating HP column (GE Healthcare) pre-equilibrated with CoCl\(_2\). Proteins were eluted with an imidazole gradient at steps of 10% and dialysed.
into buffer containing 50 mM Tris pH 8, 0.5 mM EDTA, 1.0 mM dithiothreitol (DTT). The His tag was cleaved by the addition of TEV protease (Invitrogen) and the sample was then dialysed against buffer A. The cleaved proteins were applied to a cobalt loaded HisTrap column and the protein of interest was found in the flow-through. The NMB2082 protein was further purified by size exclusion chromatography using HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) pre-equilibrated in 10 mM Tris pH 7.0, 100 mM NaCl. Samples of the NMB0399 protein were crystallised without prior size exclusion chromatography.

2.3.8 Biochemical assays - Double-stranded DNA substrates 25U and 50U were made by mixing equimolar amounts of complementary strands, heating to 90°C and cooling slowly to room temperature. One strand contained a uracil residue and a 5’ hexachlorofluorescein residue (HEX) for visualisation; the complementary strand was unmodified. The sequence of the double-stranded 25U oligonucleotide was 5’-HEX GGA TCA CTA TUA TAG GTA GTT TAT–3’; the sequence of the double-stranded 50U oligonucleotide was 5’-HEX CGT GTA T GA CAT CTA ACT ATU ATA GCG CTC ATC GTC ATC GT C TC GGC ACC GT–3’. Oligonucleotides were synthesised and HPLC-purified by MWG Biotech (Ebersberg, Germany). The 25U and 50U substrates were reacted with 100 nM Herpes simplex virus Type-1 uracil DNA glycosylase (UDG) for 2 hours at 25°C in order to create the abasic 25AP and 50AP DNAs (Bellamy et al, 2001).

Nuclease assays were performed in a standard reaction buffer (20 mM Tris pH 7.5, 1 mM EDTA, 20 mM NaCl, 15 mM MgCl$_2$) supplemented with 15 mM MgCl$_2$ at 25°C. Aliquots were removed at specified time points, and quenched in formamide loading buffer (0.01% xylene cyanol, 0.01% bromophenol blue, 30 mM EDTA in formamide) prior to separation via denaturing polyacrylamide gel electrophoresis. All assays used
100 nM of the respective DNA substrate and an equivalent of 500 fmol of DNA substrate was loaded per lane onto acrylamide gels. Enzyme concentrations were as described in figure legends. Exonuclease assays were analysed using 15% PAGE whereas end-processing assays used 20% PAGE. Data were fitted using Grafit 5 (Erithacus software).

2.4 Cell culture and infection

2.4.1 HL60 culture and differentiation – HL60 cells, a promyelocytic leukaemia cell line, were cultured in RPMI-1640 medium without L-glutamine and without phenol red (Gibco Invitrogen), supplemented with 20% heat-inactivated foetal calf serum (TCS Cellworks Ltd), 10% glutamax, penicillin (250 U/ml) and streptomycin (250 µg/ml) (Gibco Invitrogen). Cell viability was determined by trypan blue dye exclusion. Dead cells are permeable and are stained blue, while live cells remain white, allowing live cells to be identified and enumerated. Cells were maintained by replacement with 15 ml fresh medium daily, and passaged weekly to a concentration of 0.5 x 10^5 cells/ml. Cells were differentiated by the addition of 4 µM retinoic acid following passage, six days prior to use (Pantelic et al. 2004).

2.4.2 Isolation of peripheral human PMNs – 20 ml of peripheral venous blood was taken from a healthy volunteer and heparinised with 0.1% heparin to prevent clotting. Blood was layered onto Histopaque 1119 (Sigma) in 15 ml tubes and then centrifuged at 800 xg for 20 minutes at RT. The buffy coat from each tube were retained, resuspended in Hanks Balanced Saline Solution (HBSS, Gibco Invitrogen) containing calcium and magnesium (CaCl₂ 140 mg/ml, MgCl₂ 100 mg/ml, MgSO₄ 100 mM; 0.2% BSA) and centrifuged at 200 xg for 10 minutes. The supernatant was discarded and the cell pellets were resuspended in >1 ml HBSS each and combined.
This suspension was then layered onto an isotonic percoll gradient (GE Healthcare) of increasing concentration from 65% to 85% in increments of 5%, made up with HBSS containing 0.2% BSA. The gradient was centrifuged at 800 xg for 20 minutes and the PMNs harvested from the interface between the 70% and 75% layers. PMNs were resuspended in HBSS containing 0.2% BSA and centrifuged for a further 10 minutes at 200 xg. The cell pellet was then resuspended in 3 ml HBSS. 10 µl cell suspension was added to 190 µl trypan blue solution (Sigma) and cell concentration quantified using a haemocytometer. The PMN concentration was adjusted to $1.11 \times 10^6$ cells / ml in HBSS and stored at RT until needed.

2.4.3 Phagocytosis assay – For phagocytosis assays, cells were washed twice with HBSS without CaCl$_2$, MgCl$_2$ or MgSO$_4$ containing 0.2% BSA and once with HBSS with CaCl$_2$ (140 mg/ml), MgCl$_2$ (100 mg/ml), MgSO$_4$ (100 mg/ml) and 0.2% BSA, and the concentration was adjusted to $2.5 \times 10^6$ cells/ml. Pre-treatment of cells with cytochalasin D (10 µg/ml; Sigma), or with antibodies for blocking experiments (Table 3), was performed at 37°C for 30 minutes. Phagocytosis assays were performed in HBSS with 2 µM lactate in 96- or 24-well microtitre plates for analysis by flow cytometry and microscopy respectively. Bacteria were added at an multiplicity of infection (MOI) of 12.5 bacteria:cells for experiments with fixed bacteria, and an MOI of 100 for experiments with live bacteria. For opsonisation, bacteria were incubated with heat-killed human serum (1:40 dilution in PBS) for 30 minutes at 37°C, followed by incubation with baby rabbit complement (Pelfreeze) for a further 15 minutes before addition to cells. Plates were incubated at 37°C for 25 minutes at 140 rpm on a shaker. The contents of wells with assays including live bacteria were fixed in 3% PFA before analysis.
2.4.4 Preparation and treatment of HL60 cell supernatants – The phagocytosis assay was performed as described in a 24-well plate. Following incubation, well contents were transferred to a 1.5 ml tube and pulse-spun to 6000 xg. The supernatant was removed and filtered using a 0.2 µm filter (VWR). For treatment with trypsin, supernatants were incubated with 0.1% trypsin (Sigma) for 20 minutes at RT, followed by addition of 0.1% trypsin-specific inhibitor (Sigma) for 15 minutes at RT. Size fractionation of supernatants was performed by centrifugation using filters that allow passage of proteins of 100 kDa, 50 kDa or 30 kDa. Centrifugation was performed at 2000 xg for 30 minutes at RT.

2.4.5 Assay for bacterial survival in PMNs – 500 µl PMNs were seeded to 48-well flat-bottomed plates at a concentration of 1.11 x 10⁶ cells/ml and centrifuged at 350 xg for five minutes, then allowed to rest for fifteen minutes prior to use. Treatment with inhibitors cytochalasin D (5 µM) and resveratrol (100 µM) was performed for twenty minutes at 37°C when needed. Bacterial suspensions were prepared at a concentration of 2.8 x 10⁷ in HBSS with CaCl₂ (140 mg/ml), MgCl₂ (100 mg/ml) and MgSO₄ (100 mg/ml). Medium was then removed from the adherent resting PMNs and replaced with 200 µl of the inoculum to give an MOI of 100. For each bacterial strain or species used, a control well was included without PMNs. Plates were sealed with parafilm, centrifuged at 800 xg for five minutes and incubated at 37°C in 5% CO₂. After one hour, saponin was added to a final concentration of 0.1% to each well and incubated for a further ten minutes to lyse PMNs. The base of each well was then scraped with a pipette tip, and thoroughly resuspended. Serial dilutions of each well were spotted in triplicate to solid media and incubated overnight. For each assay, counts were measured at time zero. Survival was quantified by counting the CFU for each condition at time zero and after the one-hour incubation. Percentage survival was
calculated by comparing each condition with the number of bacteria recovered at time zero.

### 2.4.6 Lactate dehydrogenase quantification

At zero, one, two and three hours post-inoculation, supernatants from cells were removed from the adherent PMNs, spun briefly three times at 6000 xg and passed through a low volume 0.2 µM filter. A positive control was also prepared by lysing 5.5 x 10^5 adherent PMNs with a final concentration of 0.1% saponin, then preparing the supernatant in the same way. The supernatants were then tested for lactate dehydrogenase (LDH) activity using the LDH Cytotoxicity Detection Kit (Roche) according to the manufacturers protocol. Percentage cytotoxicity was calculated using the following equation:

\[
\text{% Cytotoxicity} = \frac{[(\text{Infected PMNs} - \text{Bacteria alone})] - \text{PMNs alone}}{(\text{Lysed PMNs}) - \text{PMNs alone}}
\]

### 2.5 Flow cytometry

Analysis of the association of FITC-labelled or GFP-expressing bacteria PMNs was performed using the FACS Calibur (Becton Dickinson) gated to the PMN population. At least 10,000 events were counted. Association was quantified by multiplying the geometric mean fluorescence by the percentage total positive gated population to calculate the Mean Fluorescence Index (MFI).

Analysis of the expression of bacterial surface structures or cell receptors was performed by incubating bacteria (1 x 10^8 cells/ml) or cells (2.5 x 10^6 cells/ml) with a primary antibody (Table 3), followed by washes with PBS WITH 0.2% Tween. The cells were incubated with a secondary antibody conjugated to a fluorescent marker (Table
3). Samples were analysed using the FACS Calibur gating to the bacterial or HL60 population as appropriate, and at least 20,000 events were counted. Expression was quantified by the percentage total positive population. All assays were performed in triplicate on at least three independent occasions.

2.6 **Microscopy**

2.6.1 *Immunofluorescence* – For microscopy, phagocytosis assays were performed as described in 24-well plates with coverslips. Following incubation, plates were centrifuged at 35 $xg$ for 3 minutes, the cells fixed with 3% PFA and washed. Staining of cellular components was performed using antibodies shown in Table 2. To visualise internalised and associated bacteria coverslips were stained differentially pre- and post-permeabilisation with 0.2% saponin (Sigma). Phagocytosis was scored by counting the number of intracellular (green) and extracellular (red) bacteria per cell. The total number of intracellular bacteria is the Phagocytic Index; the total number of cell-associated bacteria is the Association Index. For this scoring system 50 cells were counted for each coverslip. For all other parameters, at least 100 cells were scored. Each assay was analysed in triplicate on three independent occasions.

2.6.2 *Light microscopy* – For analysis of PMN morphology and nuclear condensation, PMNs were seeded to coverslips in 24-well plates and/or infected and incubated as described. PMNs were then fixed with 3% PFA overnight and then washed in PBS. Prior to staining coverslips were air-dried. To stain, coverslips were held feather-side down without shaking in Giemsa-Wright stain (Sigma) for thirty seconds, followed by five minutes in $dH_2O$, air-dried, then mounted feather-side up on slides for visualisation by light microscopy.
2.7 In vivo analysis of virulence

Mixed litters of 5-day-old rats (Wistar) received 75 µl containing $1 \times 10^7$ CFU/ml *N. meningitidis* in PBS. The virulence of mutants was compared directly with MC58 in individual animals given a 1:1 ratio of wild-type to mutant bacteria. The number of mutant and wild-type bacteria recovered from the blood of animals 8 hours later was established by plating to media with or without antibiotics. The Competitive Index (C.I.) was calculated using the following equation:

$$\text{C.I.} = \frac{(\text{number of mutant / wild-type bacteria recovered from animals})}{(\text{number of mutant / wild-type bacteria in the inoculum})}$$

2.8 Statistics

Statistics were calculated using Microsoft Office Excel. Statistical significance was analysed by the Student’s *t*-test. P values of less than 0.01 were accepted to be significant. Unless otherwise stated, figures represent assays performed in triplicate.
Chapter 3

The role of bacterial metabolism in association with human PMNs
Chapter 3 The role of bacterial metabolism in association with PMNs

*N. meningitidis* preferentially utilises lactate during growth in the CSF. Previous work has shown that a mutant strain lacking the lactate permease enzyme, encoded by *lctP*, is attenuated in the infant rat model of infection (Exley *et al.*, 2005). A mutant lacking LctP had a defect in LPS sialylation (Exley *et al.*, 2005). This was thought to be due to a reduction in available PEP, generated via pyruvate, entering the sialic acid synthesis pathway. Loss of LctP may therefore also affect expression of the polysialic acid capsule on the bacterial surface.

The meningococcus is thought to utilise glutamate during bloodstream infection. Imported glutamate contributes to the TCA cycle, and thus it is possible that reduced availability of this carbon/nitrogen source will also affect sialic acid biosynthesis. Recent work has identified the genes involved in glutamate import by *N. meningitidis*. GdhR is a transcriptional regulator that regulates expression of *ghdA*, which encodes an L-glutamate dehydrogenase, and is essential for growth, in low sodium concentrations (Pagliarulo *et al.*, 2004). GdhR also regulates transcription of an operon encoding an ABC transporter involved in glutamate import. Inactivation of genes encoding the putative substrate-binding protein (NMB1963) and permease (NMB1965) within this operon resulted in reduced intracellular survival in epithelial cells (Monaco *et al.*, 2006). Further examination of the Neisserial MC58 genome has revealed two glutamate uptake enzymes encoded by *perM* and *gltS*, which are involved in glutamate import in environments presenting low and high sodium concentrations, respectively. The relevance of these genes in expression of outer membrane polysaccharides and on the survival of the meningococcus *in vivo* is unknown.

Meningococcal surface structures such as LPS and capsule are known to play a role in phagocytosis. However, the role of metabolism during interactions between *N. meningitidis* and phagocytic cells has not been previously examined.
The work described in this chapter was performed with the closely related *N. meningitidis* serogroup B, ST-32, ET-5 strains, MC58 and H44/76, and the human cell line, HL60. Previous studies have characterised this cell line according to its morphology and receptor expression (Pantelic et al., 2004). Therefore, this cell line was used in initial studies instead of primary isolated human cells to reduce host-to-host variation.
3.1 PerM is required for survival in vivo

The importance of the two previously identified glutamate transporters, PerM and GltS, for survival of the meningococcus in vivo had not yet been elucidated. A mutant strain lacking perM was constructed in the wild-type H44/76 strain. The mutant was reconstructed by transforming the wild-type strain with genomic DNA from the mutant. Loss of perM in the strains was confirmed by PCR analysis (not shown). Both strains were analysed for their ability to cause bacteraemia in the infant rat model. Twenty hours after inoculation with a 1:1 ratio of mutant and wild-type strain, blood was recovered and bacteraemia assessed by plating to solid media. The Competitive Index (C.I.) of each mutant was calculated by comparing the recovery of the mutants with the wild-type strain. The mutant lacking perM showed significant attenuation in vivo, indicating that the PerM glutamate transporter plays a role in survival of the wild-type strain (Figure 3.1). However, gltS expression was not necessary to cause bacteraemia as the mutant lacking this gene showed no significant attenuation (data not shown). This work was performed by Caterina Monaco and Christoph Tang.

![Figure 3.1 PerM is required for virulence in the infant rat model](image)

Infant rats were challenged with a 1:1 ratio of wild-type to mutant strain. Both the original mutant strain H44/76ΔperM and the back-crossed strain were examined. Bacteraemia was quantified at 24 hours post-challenge, and the Competitive Index calculated. Black circles represent data from individual animals. The horizontal lines indicate the mean Competitive Index. Both mutants are significantly attenuated in vivo.
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3.2 LPS sialylation is not affected in the absence of glutamate

The inability to utilise lactate has previously been shown to result in a reduction in sialylation of the LPS. Therefore, the effect of the loss of enzymes involved in glutamate import on sialylation was also investigated. Mutant strains lacking genes encoding the L-glutamate dehydrogenase GdhA and transcriptional regulator GdhR were constructed by Caterina Monaco (Monaco et al., 2006) (Unpublished). Bacteria were grown overnight on solid media and harvested to PBS prior to fixation in PFA. The extent of LPS sialylation was investigated in strains H44/76ΔgdhR, H44/76ΔgdhA, H44/76ΔperM and H44/76ΔgltS. Western blot analysis was performed with a mAb, 3F11, which recognises unsialylated LPS (Apicella et al., 1981). The amount of sample loaded was adjusted according to Coomassie staining and Western blotting with the anti-L,3,7,9 LPS immunotype mAb (Figure 3.2 A & B). There was no significant difference in sialylation between the wild-type H44/76 strain and the glutamate import-deficient mutants (Figure 3.2 B). The extent of sialylation was also examined by flow cytometry using the 3F11 mAb, with the unsialylated MC58Δlst mutant as a positive control (Figure 3.2 C). The data obtained from flow cytometry was consistent with the Western blot analysis. There was no significant difference in binding of the 3F11 mAb to any of the strains tested.
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Unsialylated LPS (3F11)

H44/76

\( \Delta \text{gltS} \)

\( \Delta \text{perM} \)

\( \Delta \text{gdhR} \)

Total protein

LPS (L,3,7,9)

Unsialylated LPS (3F11)

Figure 3.2 LPS sialylation is unaffected in strains lacking components of glutamate import systems

(A) Whole cell lysates of bacteria were separated by SDS-PAGE and the proteins stained with Coomassie Blue. Lysates were also transferred to a nitrocellulose membrane and incubated with mAbs against L3,7,9 LPS immunotype (L3,7,9) or unsialylated LPS (3F11) to determine the extent of LPS sialylation. (B) Fixed bacteria were incubated with a mAb against unsialylated LPS (3F11) followed by incubation with a FITC-conjugated secondary antibody. The LPS sialylation-deficient strain MC58\( \Delta \text{lst} \) was included as a positive control for 3F11 binding, which was quantified by flow cytometry. There was no difference in sialylation between strains. Error bars represent the standard error of the mean % positive population.
3.3 **Capsule expression is reduced in the MC58ΔlctP strain but is unaffected in mutants affected for glutamate uptake**

The first step in the biosynthesis of polysialic acid capsule is the addition of N-acetylmannosamine to PEP to produce sialic acid. PEP can be produced from glucose via the Entner Doudoroff pathway, or from imported lactate via pyruvate. The effect of the ΔlctP mutation on polysialic acid capsule production was therefore analysed by flow cytometry using mAb ZM51 against the serogroup B polysaccharide capsule. This assay was carried out with wild-type, MC58ΔlctP and MC58ΔsiaD strains. The MC58ΔsiaD strain lacks the capsule-specific polysialyltransferase and is therefore unencapsulated. FACS analysis confirmed the absence of the capsule in the MC58ΔsiaD strain (Figure 3.3 A). A significant reduction in capsule expression was seen in the MC58ΔlctP strain when compared to the wild-type MC58 (Figure 3.3 A).

The effect on capsule expression of loss of components of the glutamate import systems, PerM and GltS, and the transcriptional regulator GhdR was also examined. Mutants lacking the genes gdhR, gltS and perM were tested against the wild-type strain H44/76. There was no significant reduction in capsule expression in any of these strains (Figure 3.3 B). The MC58 wild-type strain and MC58ΔsiaD mutant were included as controls.
Figure 3.3 Capsule expression is reduced in the MC58ΔlctP strain but is unaffected in mutants lacking components of glutamate import systems.

Fixed bacteria were incubated with a mAb against the serogroup B capsule, followed by a FITC-conjugated secondary antibody. Binding to all strains was quantified by flow cytometry. The capsule-negative MC58ΔsiaD strain was included as a negative control. (A) Capsule expression was reduced in the MC58ΔlctP strain. (B) The ability to utilise glutamate has no effect on expression of capsule. Error bars represent the standard error of the mean % positive population.
3.4 Serogroup B N. meningitidis associates with HL60 cells with or without opsonisation

Due to their anti-phagocytic properties, the changes observed in the capsule and LPS sialylation state of the MC58ΔlctP mutant might be expected to influence its interactions with phagocytic cells. Furthermore, it would therefore be expected that the strains lacking the glutamate import system would behave as the wild-type strain during interactions with phagocytes since they had no detectable change in capsule or LPS expression. These hypotheses were tested with human neutrophils since they are one of the first forms of cellular immune defence encountered by pathogens, and it is known that the meningococcus comes into contact with these phagocytes during infection (Guarner et al., 2004).

To investigate the interaction between N. meningitidis and human neutrophils, initial assays were performed to confirm the association between the neutrophil cell line, HL60, and N. meningitidis MC58. Assays were performed in HBSS, which contains 1 mg/ml glucose, with PFA-fixed FITC-labelled bacteria. HL60 cells were differentiated with 4 µM retinoic acid for six days prior to use. Bacteria were incubated in the presence or absence of heat-inactivated human serum (10%) and rabbit complement (1:50), then added to differentiated HL60 cells. The association of labelled bacteria with HL60 cells was analysed by flow cytometry. Due to the fragile nature of the HL60 cells, care was taken to gate the intact HL60 population during data analysis. This was done by selecting cells of the appropriate size according to the forward- and side-scatter data produced during analysis. Association of labelled bacteria to cells was measured as the mean fluorescence index (MFI), which was calculated by multiplying the total percentage positive population with the geometric mean fluorescence intensity. Preliminary assays verified that fixed FITC-labelled MC58 strain associated with HL60 cells in the presence of serum and complement (Figure 3.4 A). Association was observed as a shift in the fluorescence of the gated population to the right. Association was
also observed in the absence of serum and complement, albeit at a lower level than for opsonised bacteria, but at a significantly higher level than background (Figure 3.4 A & B).

FACS analysis does not differentiate internalised from adherent bacteria. Phagocytosis relies on reorganisation and polymerisation of actin within the host cell (Stossel, 1976). This process can be inhibited by cytochalasin D, which prevents actin filament polymerisation by binding to high-affinity binding sites, disrupting the cytoskeletal network depolymerising the actin fibres. By binding to the termini of the resulting fragments, the drug prevents re-elongation of the actin fibre (Schliwa, 1982). Therefore, in order to determine whether phagocytosis was occurring, the assay was performed in the presence of cytochalasin D. Incubation with this inhibitor significantly reduced association detected by FACS (Figure 3.4 C). This trend was also confirmed by microscopy, which showed that bacteria associated to HL60 cells incubated with cytochalasin D to a lesser extent, and that treatment with the inhibitor prevented any bacteria from being internalised (data not shown).

All further assays were carried out with bacteria and cells alone to investigate the non-opsonic interaction between *N. meningitidis* and neutrophils. This process is of potential importance to the clearance of the meningococcus by the innate immune system. It is also of significance during colonisation and early stages of the disease. Activated neutrophils infiltrate infected tissues where they would encounter the meningococcus in the absence of opsonins. Since neutrophils employ cytokine release as a mechanism of activating the immune system and priming further cellular responses, this initial interaction may impact on immune response events and their outcomes later in disease development.
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Panel A: The figure shows the association of *N. meningitidis* with HL60 cells in the presence and absence of complement and serum. The association is quantified by flow cytometry, and MFI (Mean Fluorescence Intensity) is calculated by multiplying the mean fluorescence with the percentage positive cell population. Traces show cells alone (red) and cells with bacteria (green). (A) Wild-type *N. meningitidis* associates with HL60 cells in the presence and absence of complement.

Panel B: The graph shows the mean fluorescence intensity (MFI) of HL60 cells with and without complement. The MFI is plotted against the presence (+) or absence (-) of complement. Error bars represent standard error of the mean.

Panel C: The figure demonstrates the effect of cytochalasin D on the association of *N. meningitidis* with HL60 cells. The MFI is plotted against the presence (-) or absence (+) of cytochalasin D and the presence or absence of MC58. Error bars represent standard error of the mean.

**Figure 3.4 Serogroup B *N. meningitidis* associates with HL60 cells in the presence and absence of opsonisation**

Fixed FITC-labelled bacteria were incubated with HL60 cells in the presence and absence of heat-inactivated human serum or complement, and association with cells analysed by flow cytometry, and MFI was calculated by multiplying the mean fluorescence with the percentage positive cell population. Traces show cells alone (red), and cells with bacteria (green). (A) Wild-type *N. meningitidis* associates with HL60 cells in the presence and absence of opsonisation by human serum. (B) Association is independent of complement. (C) Assays were performed in the presence of cytochalasin D, demonstrating that association is dependent on actin polymerisation. Error bars represent standard error of the mean.
3.5 *The polysialic acid capsule reduces phagocytosis*

Previous studies have demonstrated that the polysialic acid capsule is an important bacterial structure for avoidance of phagocytosis (Kolb-Maurer *et al.*, 2001; Read *et al.*, 1996; McNeil *et al.*, 1994). Thus, association assays were performed with MC58ΔsiaD. This capsule-negative strain associated to a significantly higher degree than the wild-type strain, confirming the anti-phagocytic nature of the capsule, and validating the assay (Figure 3.5).

![Graph showing association of MC58 and ΔsiaD strains](image)

**Figure 3.5** *The polysialic acid capsule reduces phagocytosis of N. meningitidis*

Fixed FITC-labelled bacteria were incubated with HL60 cells, and the association of the wild-type strain was compared with the capsule-negative strain, MC58ΔsiaD. MFI was calculated by multiplying the mean fluorescence with the percentage positive cell population. The increased association of MC58ΔsiaD confirmed the anti-phagocytic nature of the capsule. Error bars represent the standard error of the mean.
3.6 Non-opsonic association of bacteria with HL60 cells is mediated in part by CD66a,c,d

It has been shown previously that non-opsonic interactions between *N. meningitidis* and PMNs are mediated by the CD66 receptor (Virji *et al.*, 1996). CD66 expression by HL60 cells was therefore examined by flow cytometry (Figure 3.6 A), and the association of fixed bacteria following incubation with an anti-CD66 mAb was analysed to determine whether this receptor mediates the interaction. Preliminary assays were performed to determine saturation of binding of the anti-CD66 mAb to the cell population (data not shown). Incubation of HL60 cells with a 1:50 dilution of the anti-CD66 mAb significantly reduced, but did not entirely abolish, the association of *N. meningitidis* with HL60 cells, indicating that this interaction is mediated in part by the CD66 receptor (Figure 3.6 B).
Figure 3.6 Association of *N. meningitidis* to HL60 cells is mediated in part by CD66

(A) Expression of CD66a,c,d receptors by HL60 cells was analysed by incubating cells with a mAb against the receptor followed by a FITC-conjugated secondary antibody. Binding was quantified by flow cytometry and the MFI was calculated by multiplying the mean fluorescence with the percentage positive cell population. Cells incubated with an isotype matched control are shown in magenta, while cells with the anti-CD66 mAb are in green. (B) Live bacteria were incubated with HL60 cells following pre-incubation with the mAb against CD66a,c,d. The decrease in association indicates that association with HL60 cells is mediated in part by CD66. Error bars represent the standard error of the mean.
3.7  **The inability to utilise lactate, but not glutamate, affects association to HL60 cells**

Next, the effect of the ΔlctP mutation on association with HL60 cells was investigated. HL60 cells were incubated with fixed, FITC-labelled bacteria, and association analysed by flow cytometry. Results indicate that the MC58ΔlctP strain associated with cells at a significantly higher level compared with the wild-type strain. This was observed on five different occasions with bacteria prepared at three separate times. Uptake of the mutant reverted to wild-type levels following complementation of the mutant with a single intact copy of lctP in trans (Exley et al., 2005), confirming that the loss of lctP was responsible for the enhanced uptake of the mutant (Figure 3.7 A). The influence of glutamate metabolism on association with HL60 cells was also investigated. As expected, there was no significant difference in association to the HL60 cells of any of mutants lacking glutamate import components, except for an apparent decrease in association of the H44/76ΔghdR mutant (Figure 3.7 B).

The association of the MC58 and MC58ΔlctP strains with HL60 was also determined by microscopy. The number of bacteria associated per 100 HL60 cells was scored. There was a significant increase in the number of MC58ΔlctP bacteria associated with HL60 cells compared with the wild-type strain, demonstrating an increased density of infection with the mutant strain (data not shown).

It has been shown previously that CD66 receptors interact with Opa expressed on the bacterial surface (Virji et al., 1996). Expression of Opa in the wild-type and MC58ΔlctP strains was examined by Western blot analysis to determine whether the ΔlctP mutation affects Opa expression. The serogroup C Opa-negative 8013 strain was used as a negative control. Binding of the anti-Opa mAb B33 was not altered in the MC58ΔlctP strain, showing that the difference in association was not due to changes in Opa expression (Figure 3.7 C).
Figure 3.7 The inability to utilise lactate, but not glutamate, results in increased association of bacteria to HL60 cells

(A;B) Fixed FITC-labelled bacteria were incubated with HL60 cells and the MFI was calculated by multiplying the mean fluorescence with the percentage positive cell population. The MC58\(\Delta\)lctP strain showed higher association to cells than the wild-type (n = 5). Wild-type levels of association were restored by complementation of the \(\Delta\)lctP mutation (A). Mutant strains lacking enzymes involved in glutamate metabolism showed no significant difference in the association when compared with the wild-type strain (B). Error bars represent the standard error of the mean. (C) Whole cell lysates of MC58, MC58\(\Delta\)lctP and 8013 (Opa-negative, serogroup C strain) were analysed for Opa expression. Samples were separated by SDS-PAGE then transferred to nylon membranes which were incubated with anti-Opa antibody B33 (1:10000). No differences in Opa expression were observed.
3.8 *Increased association of MC58ΔlctP is not only due to changes in capsule expression*

To determine whether the enhanced association of MC58ΔlctP was due to a reduction in capsule production alone, phagocytosis assays were repeated to include the MC58ΔsiaDΔlctP, MC58ΔsiaC and MC58ΔsiaCΔlctP mutants. While MC58ΔsiaD lacks the polysialic capsule, MC58ΔsiaC is unable to synthesise sialic acid and therefore lacks both capsule and LPS sialylation in the absence of exogenous CMP-NANA. Flow cytometry and microscopic analyses of association of these strains showed that the MC58ΔsiaDΔlctP strain associated more than MC58ΔsiaD, but that MC58ΔsiaC and MC58ΔsiaCΔlctP associated to the same degree (Figure 3.8). This indicates that the difference in MC58ΔlctP association with HL60 cells is due to changes downstream of SiaC, and is not due to changes in capsule production alone.

![Figure 3.8 The increased association of MC58ΔlctP is not only due to changes in capsule expression](image)

Fixed FITC-labelled bacteria were incubated with HL60 cells and association analysed by flow cytometry. The MFI was calculated by multiplying the mean fluorescence with the percent positive cell population (n = 5). Analysis of mutants lacking capsule (MC58ΔsiaD; MC58ΔsiaDΔlctP) or unable to produce sialic acid (MC58ΔsiaC; MC58ΔsiaCΔlctP) indicate that the increase in association of strains lacking LctP is due to changes downstream of SiaC and is not due to changes in capsule alone. Error bars represent standard error of MFI.
3.9 Live $MC58_{\Delta lctP}$ associates to HL60 cells to the same extent as wild-type

To determine whether the trends seen with fixed bacteria are also seen with live bacteria, phagocytosis assays were performed using live bacteria expressing GFP. The plasmid pEGFP (Christodoulides et al., 2000) was introduced into the strains by transformation and prior to use, expression of GFP was checked by flow cytometry (data not shown). Analysis of phagocytosis by flow cytometry showed that in contrast with the pattern seen for fixed bacteria, live $MC58_{\Delta lctP}$ associated with, and were internalised by, HL60 cells to the same extent as the wild-type strain (Figure 3.9 A). To confirm this result, phagocytosis assays were also performed in the presence of the actin polymerisation inhibitor cytochalasin D, which successfully blocked phagocytosis and significantly reduced association (Figure 3.9 B).
Figure 3.9 Live MC58ΔlctP associates with HL60 cells to the same extent as the wild-type strain

(A) Live GFP-expressing bacteria were incubated with HL60 cells and the association quantified by flow cytometry. MC58ΔlctP shows the same level of association as the wild-type strain. (B) Live bacteria were incubated with cells following pre-incubation with the actin inhibitor cytochalasin D. (C) To confirm this result, GFP-expressing live and fixed bacteria were incubated with HL60 cells and association analysed by flow cytometry. Error bars represent standard error of the mean.
3.10 Live N. meningitidis is internalised by HL60 cells

To verify data obtained by flow cytometry, HL60 cells were incubated with live bacteria and analysed by fluorescence microscopy. Inside:outside staining was performed by labelling bacteria with the anti-LPS mAb before and after permeabilisation with 0.2% saponin. (Figure 3.10 A). A total of 100 HL60 cells were scored by counting the total number of bacteria, both adherent and internalised, associated with each cell. This figure is the Association Index (AI). HL60 cells were also scored for the total number of internalised bacteria, to give the Phagocytic Index (PI). Microscopy confirmed that all strains were internalised by HL60 cells. The capsule-negative MC58ΔsiaD strain showed significantly higher levels of association and internalisation compared with the wild-type strain. However, MC58ΔlctP associated with HL60 cells to the same extent as the wild-type, and with the same level of phagocytosis (Figure 3.10 B), thus confirming the results observed by flow cytometry.
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(A) Extracellular *N. meningitidis*  Intracellular *N. meningitidis*  Merge

MC58

Δ*lctP*

Δ*siaD*

(B) Figure 3.10 Live *N. meningitidis* is internalised by HL60 cells

(A) Live MC58, MC58Δ*lctP* and MC58Δ*siaD* strains were incubated with HL60 cells and stained for microscopic analysis. To differentiate between extra- and intracellular bacteria, samples were stained pre- and post-permeabilisation with 0.2% saponin. Extracellular bacteria are red, while intracellular bacteria are green. (B) Association Index (AI; total associated bacteria; black bars) and Phagocytic Index (PI; total intracellular bacteria; grey bars) were calculated for 100 cells. Error bars represent standard error of the mean.
3.11 Summary

The work described in this chapter characterised non-opsonic interactions between serogroup B *N. meningitidis* and the human neutrophil cell line, HL60. It is known that the meningococcus interacts with neutrophils during disease, and that the non-opsonic interaction can occur via the CD66 receptor expressed by neutrophils (Virji *et al.*, 1996). We have confirmed this interaction, and the role of CD66, which mediates association. Complete blocking of CD66 by incubation with the anti-CD66 mAb cannot be assumed but association was not fully eliminated following incubation with this mAb, indicating that another receptor may also be involved. Analysis by microscopy also demonstrated that the bacterium is internalised, confirming that phagocytosis as well as adherence of bacteria to the outer surface of the HL60 cells occurs. The role of lactate metabolism in expression of the polysialic acid capsule, in addition to its previously described effect on sialylation of the LPS, has also been demonstrated. The reduction in both these surface components are likely to be the cause of the increased association MC58ΔlctP to HL60 cells compared to the wild-type. In addition, mutants lacking components of a glutamate import system showed no change in either capsule expression or LPS sialylation. As expected, these strains also showed no difference in association with HL60 cells when compared to the wild-type strain H44/76.

Interestingly, the difference in association with HL60 cells seen with fixed MC58ΔlctP is not observed with live bacteria. In addition, levels of association, as calculated by flow cytometry and microscopy, were significantly lower for all strains when infections were performed with live bacteria. A similar pattern was observed in previous work with dendritic cells (Jones *et al.*, 2007) and this was attributed to a bacterial activity that resulted in an alteration in DC function.
The next chapter will investigate the processes behind the disparity in results with live and killed bacteria and the possible reasons for the lower level of association of all strains during infection with live bacteria.
Chapter 4

Changes in bacterial surface components during interactions with PMNs
The previous chapter demonstrated that serogroup B *N. meningitidis* associates with differentiated HL60 cells. MC58ΔlctP associated more than the wild-type strain when phagocytosis assays were performed with fixed bacteria. However, when the assays were repeated with live bacteria, this difference in association was not detected. In addition, the degree of association of all strains with the HL60 cells was greatly reduced with live bacteria compared to fixed strains.

Previous work on the phagocytosis of *N. meningitidis* by DCs has also described enhanced phagocytosis of fixed meningococci compared to live bacteria (Jones *et al.*, 2007). This effect is independent of bacterial replication. It could be mediated either by the response of the bacteria to cells or by the production of a bacterial component that inhibits phagocytosis. Activated PMN release a variety of cytokines and antimicrobial compounds as a response to infection, and the meningococcus may be able to detect and respond to them. Both the polysialic acid capsule and sialylation of the LPS are anti-phagocytic (Estabrook *et al.*, 1998; Read *et al.*, 1996). Levels of capsule expression are regulated during interactions with host cells, displaying a largely unencapsulated phenotype during colonisation, but switching to a capsulated state during bloodstream infection (Vogel *et al.*, 1997; Read *et al.*, 1996; Hammerschmidt *et al.*, 1994; Stephens *et al.*, 1993; Virji *et al.*, 1993).

Work in this chapter was aimed at determining the cause for the reduced level of association of live meningococci to HL60 cells through analysis of both the polysaccharide capsule and the LPS following exposure of bacteria to HL60 cells.
4.1 The presence of live bacteria does not affect association of fixed bacteria to HL60 cells

To determine whether the reduced association of live meningococci to HL60 cells was due to a cellular response to the live bacteria, mixed infections were performed. HL60 cells were infected with a 1:1 mixture of live and FITC-labelled fixed MC58, and incubated for thirty minutes. The contents of the wells were then fixed in PFA and association was analysed by flow cytometry. No significant reduction in association of fixed bacteria was seen in the presence of live meningococci (Figure 4.1). This indicates that the reduced association of live meningococci with HL60 cells is the result of a bacterial response to infection rather than an effect on the HL60 cells elicited by the presence of live bacteria.

Figure 4.1 The presence of live bacteria does not affect association of fixed bacteria with HL60 cells

HL60 cells were inoculated with a 1:1 mixture of live and FITC-labelled dead MC58 at an MOI of 100 and incubated for thirty minutes. The contents of the wells were then fixed in PFA and association analysed by flow cytometry. The MFI was calculated as the % positive HL60 population multiplied by the geometric mean fluorescence. The presence of live bacteria did not affect the association of fixed MC58 with HL60 cells. Error bars represent standard error of the mean.
4.2 *Capsule expression increases in the wild-type and MC58ΔlctP strains in the presence of HL60 and epithelial cells and cell supernatants*

The meningococcal polysialic acid capsule is anti-phagocytic and its expression is known to be regulated according to the host environment encountered by the bacterium (Deghmane et al., 2002; Read et al., 1996). Therefore, capsule expression was analysed in bacteria incubated in the presence of HL60 cells. Infection of HL60 cells with live MC58 and Δ*lctP* strains was carried out at an MOI of 100 followed by a thirty minute incubation. MC58Δ*siaD* was included as a control. Binding of a mAb against the serogroup B capsule to bacteria was analysed by flow cytometry. Both the MC58 and MC58Δ*lctP* strains showed significant increase in capsule expression following incubation with HL60 cells when compared to bacteria incubated in media alone (Figure 4.2 A). In bacteria recovered from media alone, (which does not contain lactate), capsule expression in MC58Δ*lctP* was the same as the wild-type.

To determine whether the increase in capsule in the presence of HL60 cells was contact dependent, bacteria were incubated with supernatants from infected HL60 cells in the presence and absence of cells. HL60 cells were challenged with MC58 at an MOI of 100, and incubated as previously described. Following incubation, the contents of wells were spun to 6000 xg to pellet the cells, and then filtered with 2 µm filters to remove bacteria. Absence of bacteria in the filtrate was confirmed by plating to solid media (data not shown). Live MC58, MC58Δ*lctP* and MC58Δ*siaD* strains were then incubated in the recovered supernatant and incubated for thirty minutes at 37°C. Capsule expression was then analysed by flow cytometry. Both MC58 and MC58Δ*lctP* strains both showed a significant increase in capsule expression following incubation with HL60 cell supernatants when compared to bacteria incubated in naive media (Figure 4.2 B). This increase in capsule production was also observed when bacteria were incubated with supernatants prepared from uninfected HL60 cells (data not shown). These results indicate that *N. meningitidis*
responds to a compound(s) secreted by the HL60 cells, regardless of whether they have been infected.

Since an increase in capsule expression was observed in both the wild-type and mutant strains, subsequent work to investigate this change in capsule production was performed with the wild-type strain.

To investigate the effect of epithelial cells and their supernatants on capsule expression, Calu-3 human airway epithelial cells were grown to confluency in 24-well plates (provided by Dr Rachel Exley) and infected with MC58 at an MOI of 100. Infected Calu-3 cell supernatants were also prepared as described and these were examined for their effect on capsule expression by MC58. Binding of a mAb against the serogroup B capsule was analysed by flow cytometry. Capsule expression was significantly increased in bacteria incubated in the presence of Calu-3 cells and in cell supernatants compared with bacteria incubated in media alone (Figure 4.2 C). This result indicates that the increase in capsule expression in the presence of HL60 cells is not exclusive to this cell line.
Chapter 4 Changes in bacterial surface components during interactions with PMNs

Figure 4.2 Capsule expression increases following exposure to HL60 and Calu-3 cells and supernatants

(A) Bacteria were fixed following incubation with HL60 cells and analysed for capsule expression by FACS analysis with a mAb against serogroup B capsule. The mean fluorescence index (MFI) was calculated by multiplying the total positive population with the geometric mean fluorescence. Both wild-type and MC58ΔlctP showed a significant increase in capsule expression following exposure to HL60 cells (P < 0.05). (B) HL60 cells were inoculated with wild-type MC58. Following incubation, cells were removed by centrifugation and the supernatant filtered to remove remaining bacteria. Strains were then incubated with the filtered supernatant, and fixed, then analysed for capsule expression by FACS. Both MC58 and the MC58ΔlctP strain show an increase in capsule expression following incubation with cell supernatants (P < 0.015; P < 0.001). (C) Calu-3 cells and cell supernatants were infected with MC58 followed by fixation of bacteria and analysis of capsule expression by FACS using a mAb against serogroup B capsule. Capsule expression increases in the presence of both Calu-3 cells and their supernatant (P < 0.01; P < 0.001). Error bars represent standard error of the mean fluorescence index.
4.3 Capsule expression is not influenced by carbon source alone

The meningococcus is able to acquire a limited repertoire of carbon energy sources, predominantly glucose, lactate and pyruvate, from the immediate environment (Exley et al., 2005b; Smith et al., 2001). Work in the previous chapter demonstrated that lactate availability affects capsule expression as well as LPS sialylation (Exley et al., 2005a). The medium used to carry out infection of HL60 cells contained 1 mg/ml glucose but no lactate or pyruvate. Acquisition of these two compounds is closely linked to sialic acid biosynthesis and central intermediary metabolism. Therefore, to determine the carbon sources available to the meningococcus during incubation with HL60 cells, cell supernatants were analysed by NMR. HL60 cells were infected with MC58 and the supernatants harvested after five, ten, 30, 60 and 120 minutes. The supernatants were analysed by NMR by Dr Jake Bundy at the Department of Biomolecular Medicine at Imperial College London. The NMR spectrum shows that glucose is the most abundant molecule present in the supernatant. Glucose is present in the HBSS medium and therefore was used as the standard. The spectrum also indicates the presence of lactate and pyruvate, in addition to acetate and a number of amino acids, including alanine (Figure 4.3 A & B). Concentrations of both lactate and pyruvate increased over time in media containing HL60 cells, indicating production of these metabolites by HL60 cells (Figure 4.3 C).

To investigate whether lactate and pyruvate alone could elicit an increase in capsule expression, MC58 was incubated in media containing glucose with or without lactate (1 mM) and pyruvate (100 µM), reflecting the concentrations of these compounds in blood. Analysis of capsule expression was then performed by flow cytometry using a mAb against the serogroup B capsule. No change in capsule was seen in any of the conditions, indicating that the carbon source cannot be the sole reason for the increase in capsule expression.
following incubation with cell supernatants (Figure 4.3 D). Capsule expression was also tested in media containing a range of lactate and pyruvate concentrations, and no effect was seen (data not shown). It is interesting that no effect on capsule was observed in the presence of lactate since the MC58ΔlctP strain shows reduced capsule expression. It is likely that this is due to harvesting bacteria directly from rich solid media prior to the assay so capsule expression would be constitutively high. This assay was designed to detect any change in capsule beyond the level of expression expected after growth on rich media.
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Figure 4.3 Capsule expression is not influenced by carbon source

Bacteria were incubated with HL60 cells, and the supernatants filter-sterilised then analysed by NMR. (A) NMR spectrum of supernatant. (B) Spectral region outlined in red in (A) is magnified and known molecules of significant concentration are labelled. (C) NMR spectra of lactate and pyruvate in infected HL60 supernatants over time. Both compounds increase in concentration over time. (D) Bacteria were incubated in HBSS with additional pyruvate (200 µM) and/or lactate (20 mM). Fixed bacteria were then analysed for capsule expression by FACS with a mAb against serogroup B capsule. Mean fluorescence index (MFI) was calculated by multiplying the total positive population with the geometric mean fluorescence. No significant change in capsule was seen in the presence of any combination of carbon source. Error bars represent standard error of the mean fluorescence index.
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4.4 A proteinaceous compound in HL60 supernatants elicits an increase in capsule expression

To determine whether a proteinaceous component of the supernatant was responsible for the observed increase in bacterial capsule expression, supernatants were treated with the serine protease trypsin (0.1%) for 30 minutes followed by a specific trypsin inhibitor (From bovine pancreas; Sigma; 0.1%) for 20 minutes. Untreated supernatants and inhibitor-only controls were also included. Bacterial capsule expression was analysed by flow cytometry using a mAb against the serogroup B capsule. Supernatants treated with trypsin elicited a greatly reduced effect on capsule expression compared to untreated supernatants or those with only the added protease inhibitor, indicating that the increase in capsule observed in the presence of cellular supernatant is dependent on a proteinaceous component (Figure 4.4).

![Figure 4.4 Protein analysis of assay supernatant](image)

Infected, filtered HL60 cell supernatant was treated with trypsin, which was then inactivated with a specific inhibitor. Treated and untreated supernatants were then infected with MC58 strain. Capsule expression was analysed by flow cytometry using a mAb against the serogroup B capsule. Mean fluorescence index (MFI) was calculated by multiplying the total positive population with the geometric mean fluorescence. Trypsin treatment of the supernatants reduced their ability to cause an increase in capsule expression (P < 0.01). Error bars represent standard error of the mean fluorescence index.
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4.5 CAMPs LL-37 and HNP-1 elicit an increase in capsule expression in the meningococcus

During infection CAMPs are released into the surrounding medium by PMNs. Both LL-37 and Human neutrophil peptide-1 (HNP-1) are common PMN CAMPs, which are susceptible to degradation by serine proteases and therefore could be potential candidates for the proteinaceous effector of increased capsule expression present in the HL60 supernatant. Recent work revealed that unencapsulated meningococci are more susceptible to killing by CAMPs compared to capsulated bacteria, and that genes involved in the capsule biosynthesis pathway are upregulated in intracellular meningococci, and in bacteria exposed to CAMPs in growth media (Spinosa et al., 2007). Therefore, MC58 was incubated in medium containing LL-37 (10 µg/ml; 100 µg/ml) or HNP-1 (1 µg/ml; 5 µg/ml) for 30 minutes then fixed in PFA. Capsule expression was assessed by flow cytometry using a mAb against the serogroup B capsule. Bacterial survival was also analysed by plating aliquots to solid media. Incubation with both LL-37 and HNP-1 resulted in an increase in capsule expression in a concentration-dependent manner (Figure 4.5 A & C). Killing of bacteria also occurred in the presence of both CAMPs, and was dependent on concentration. Both concentrations of LL-37 were cytotoxic, while HNP-1 was only lethal at a concentration of 5 µg/ml (Figure 4.5 B & D). To confirm that the increase in capsule was the result of the CAMPs and not due to changes in antibody binding as the result of bacterial death, capsule expression was analysed in bacteria killed by heat-treatment prior to fixation. Capsule expression following heat-killing was not affected in comparison bacteria fixed in PFA alone (data not shown).
Figure 4.5 CAMPs LL-37 and HNP-1 induce an increase in capsule expression

(A; B) MC58 was incubated in the presence and absence of CAMPs LL-37 (10 µg/ml and 100 µg/ml) or HNP-1 (1 µg/ml and 5 µg/ml) for thirty minutes, and capsule expression analysed by flow cytometry using a mAb against the serogroup B capsule. Mean fluorescence index (MFI) was calculated by multiplying the total positive population with the geometric mean fluorescence. Bacterial survival was also analysed by plating to BHI agar. Incubation with both LL-37 and HNP-1 result in an increase in capsule expression and in bacterial killing, both in a dose-dependent manner. Error bars represent standard error of the mean survival or fluorescence index.
4.6 **The protein effector present in the HL60 supernatants has a molecular mass of 50-100 kDa**

Since addition of both LL-37 and HNP-1 effected an increase in capsule expression in the meningococcus, the HL60 supernatants were fractionated to determine the molecular mass of the active fraction. Supernatants were prepared as previously, and subjected to fractionation by centrifugation with filters that allow passage of proteins with the molecular mass of 100 kDa, 50 kDa and 10 kDa. Samples were examined by SDS-PAGE and Coomassie blue staining to confirm fractionation (data not shown). Live MC58 was added to each filtrate for thirty minutes and then examined for capsule expression. Analysis of capsule expression by flow cytometry showed that only the 100 kDa filter fraction retained activity, indicating a protein smaller than 100 kDa and larger than 50 kDa was responsible for the increase in capsule expression (Figure 4.6 A). Since CAMPs are less than 10 kDa in size, this data excludes them from being an effector candidate unless they were present in the form of a complex with another, larger protein.

The protein constituents of HL60 cellular supernatants were analysed. TCA-precipitated proteins from HL60 and Calu-3 cell supernatants, whole-cell lysates of MC58 incubated in HL60 supernatant, and naive medium were separated by SDS-PAGE and stained with Coomassie blue (Figure 4.6 B). Protein bands of a range of sizes are present in all lanes. An abundant 70 kDa protein seen in the whole cell lysate of bacteria incubated in supernatant and in the precipitated proteins of supernatant. This was identified as BSA carried over from the cell culture medium by mass spectrometry. The was performed by Professor Jun Wheeler at the National Institute of Biological Standards and Control (NIBSC). To confirm that the increase in capsule expression was not caused by BSA, MC58 was incubated in media containing 0.1% BSA for thirty minutes, followed by fixation. Analysis of capsule expression by flow cytometry showed that bacteria incubated with BSA do not show any change in capsule expression compared with bacteria in media alone (data not shown).
Figure 4.6 Fractionation and protein content of HL60 cell supernatant

(A) Infected HL60 cell supernatants were filter-sterilised and then subjected to size fractionation by centrifugation with filters allowing passage of molecules of less than 100, 50 and 30 kDa. MC58 was then incubated with the fractions for thirty minutes and analysed for capsule expression by flow cytometry using a mAb against the serogroup B capsule. Mean fluorescence index (MFI) was calculated by multiplying the total positive population with the geometric mean fluorescence. Only the 100kDa fraction retained activity, indicating that a protein of between 100 and 50 kDa elicits the increase in capsule seen with the whole supernatant. Error bars represent the standard error of the mean fluorescence index. (B) SDS-PAGE analysis of proteins present in TCA-precipitated supernatants of infected HL60 and Calu-3 cells (pS), MC58 incubated in the presence of HL60 supernatants (MC58 – S) or in media alone (MC58 – H).
4.7 Incubation with HL60 cells results in a change in meningococcal LPS

Apart from the polysialic acid capsule, sialylated LPS is also an anti-phagocytic surface component of *N. meningitidis* (Estabrook *et al.*, 1998). Thus, changes in LPS sialylation could contribute to the reduced association of live MC58 to HL60 cells. LPS sialylation was therefore analysed in bacteria in the presence and absence of cells. HL60 cells were infected with MC58 and MC58Δ*lctP* strains at an MOI of 100, and incubated for thirty minutes. The unsialylated MC58Δ*lst* mutant was included as a control. LPS sialylation was analysed by flow cytometry using a mAb (3F11) which recognises unsialylated LPS. The MC58Δ*lctP* strain showed a decrease in 3F11 binding following incubation with HL60 cells, indicating an apparent increase in sialylation (Figure 4.7 A). However, the MC58Δ*lst* strain also showed reduced binding. Since this strain is unable to sialylate LPS, this suggests that a mechanism other than sialylation may be affecting the binding of the 3F11 mAb.

To determine whether there were any other changes in the LPS, Western blot analysis was performed on bacterial strains MC58, MC58Δ*lctP* and MC58Δ*lst* incubated in the presence and absence of HL60 cells. Binding of a mAb against immunotype L3,7,9 LPS showed a change in the binding pattern, with detection of a higher molecular weight LPS in the presence of cells (Figure 4.7 B).

LPS was also analysed by silver staining of Proteinase K-treated lysates of bacteria separated by SDS-PAGE on a 15% urea gel, followed by staining. A subtle shift to a higher molecular weight LPS was consistently visible for each strain after incubation with cells (Figure 4.7 B).
Figure 4.7 Incubation with HL60 cells results in a change in bacterial LPS

(A) Bacterial LPS was analysed in bacteria following incubation in the presence and absence of HL60 cells. Reduced binding of mAb 3F11 (which recognises unsialylated LPS) was analysed by flow cytometry, indicates an increase in sialylation in MC58ΔlctP and the unsialylated strain Δlst. Extent of sialylation was quantified according to the percentage of the bacterial population that was positive for antibody binding. Error bars represent the standard error of the mean % positive population. (B) LPS was examined by Western blot analysis with an immunotype specific mAb against neisserial LPS. Incubation with cells resulted in an alteration in the pattern of LPS in all strains. Sialylation was also analysed by silver staining. The shift towards a higher molecular weight LPS was seen in all strains.
4.8 The change in meningococcal LPS on incubation with HL60 cells is caused by interaction with a protein

To determine whether the change in LPS was the result of alterations in LPS sialylation, the binding of the anti-L3,7,9 mAb was analysed by Western blotting following incubation of bacteria with neuraminidase. Neuraminidase is a glycoside hydrolase which specifically cleaves sialic acid residues from substrates such as glycolipids and glycoproteins. MC58, MC58ΔlctP and MC58Δlst strains were incubated in the presence or absence of 50 U neuraminidase at 37°C for one hour. Initially the binding of mAb 3F11 was analysed to verify the activity of the neuraminidase. mAb 3F11 binding was entirely absent in samples of MC58 and MC58ΔlctP not treated with neuraminidase. However, following treatment with this enzyme, mAb 3F11 bound at equal levels with all strains including MC58Δlst, thus confirming that incubation with neuraminidase removes all sialic acid from the LPS (Figure 4.8 A). Furthermore, there was no change in binding of the mAb against L3,7,9 LPS in the presence or absence of neuraminidase, indicating that the shift in binding seen in the presence of HL60 cells is not due to LPS sialylation (Figure 4.8 A). Since the shift in LPS was observed in both the wild-type and mutant strains, all further work to investigate the nature of this change was performed with the wild-type strain.

To investigate whether the shift in LPS was mediated by a proteinaceous compound, whole-cell lysates of MC58 strain incubated in the presence and absence of HL60 cells were treated with the serine protease proteinase K. Binding of the mAb against L3,7,9 LPS was analysed by Western blot. The shift in binding that occurs in the presence of HL60 cells was lost in bacterial lysates treated with proteinase K, indicating that the shift was caused by interaction with a protein (Figure 4.8 B). Control bacterial lysates prepared in the absence of HL60 cells showed no change in binding following incubation with proteinase K.
Figure 4.8 The change in LPS is due to a protein interaction

(A) Bacteria were incubated with neuraminidase to remove sialic acid from the LPS. Bacterial lysates were then analysed by Western analysis incubating with mAbs against against unsialylated LPS (3F11) and against L3,7,9 immunotype LPS. Following treatment of MC58 and MC58ΔlctP with neuraminidase, binding of mAb 3F11 to samples of LPS was indistinguishable from MC58Δlst, confirming the activity of neuraminidase. There was no change in binding of the anti-LPS mAb, indicating that the change in LPS on incubation with HL60 cells is not due to sialylation. (B) Bacterial lysates were prepared from bacteria incubated in the presence and absence of HL60 cells. The lysates were then incubated in the presence or absence of proteinase K and the LPS analysed by Western blot with an antibody against the Neisserial LPS. The shift in binding seen in the presence of HL60 cells is partially removed by incubation with proteinase K, indicating an interaction with a protein.
4.9 **Incubation with CAMP LL-37 also causes a change in meningococcal LPS**

CAMPs are released by PMN during infection, and are also known to bind bacterial LPS (Golec, 2007; Rosenfeld *et al.*, 2006). Therefore, the effect of two CAMPs, and lysozyme, a common PMN secretin, on meningococcal LPS was analysed. MC58 was incubated with LL-37 (100 µg/ml), HNP-1 (5 µg/ml) and lysozyme (100 µg/ml) and bacteria lysates analysed by Western blot. Binding of the mAb against L3,7,9 immunotype LPS was unaffected in the presence of HNP-1 and lysozyme, but showed a significant shift in binding following incubation with LL-37 (Figure 4.9 A).

The effect of LL-37 on meningococcal LPS was analysed further. To determine whether the effect was the result of an active bacterial function, both live and fixed bacteria were incubated with LL-37 (100 µg/ml). Analysis by Western blot showed that incubation with LL-37 results in a shift in binding, regardless of whether the meningococcus is live or dead, indicating that this shift cannot be responsible for the difference in association of live and dead bacteria to HL60 cells (Figure 4.8 B). To confirm that the CAMP LL-37 was binding the LPS, bacteria lysates were treated with proteinase-K before analysis by western blot. The shift in binding of the mAb against L3,7,9 LPS was partially reduced following proteinase-K treatment of bacteria incubated with LL-37, indicating that the LL-37 binds to LPS directly (Figure 4.9 C).
Figure 4.9 CAMP LL-37 causes a change in LPS

LPS in bacterial lysates was analysed by Western blotting with the L-3,7,9 mAb. (A) Bacteria were incubated with CAMPs LL-37 (100 µg/ml) and HNP-1 (5 mg/ml) and lysozyme (100 µg/ml). Incubation with LL-37, but not HNP-1 or lysozyme, elicits a change in binding of the α-LPS antibody. (B) Live or PFA-fixed bacteria were incubated in the presence and absence of LL-37. (C) Bacteria were incubated in the presence and absence of LL-37. Lysates were then incubated in the presence or absence of proteinase K. The change in LPS occurs when the incubation is performed with both live and dead bacteria. The change is partially removed following proteinase K treatment, indicating an interaction with a protein.
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4.10 Summary

The work presented in this chapter demonstrates that changes occur in capsule expression and LPS structure following exposure of the meningococcus to HL60 cells. Incubation with cells and cellular supernatants leads to an increase in capsule expression. It is possible that this trend is the mechanism behind the reduced association of live bacteria with HL60 cells compared with dead bacteria. It might also be the reason for the loss of the difference in association between the wild-type and MC58ΔlctP strains when performed with live bacteria since the strain lacking LctP also showed a significant increase in capsule expression to wild-type levels. However, there is no direct evidence for this. Interestingly, the change in capsule expression was also observed in bacteria incubated with epithelial cell line, Calu-3, and with supernatants prepared with these cells. Previous studies have shown a down-regulation of capsule expression on contact with epithelial cells (Deghmane et al., 2002), and this also suggests that the increase in capsule is due to a component in the supernatant rather than the result of direct contact with the cells themselves. Analysis of the supernatant indicated that the increase in capsule expression was due to a protein component of between 50–100 kDa. This excludes all known CAMPs released by PMNs, including LL-37, which also elicits an increase in capsule, because they are significantly smaller molecules. However, it is possible that the supernatant protein represents a complex that includes a CAMP such as LL-37. Alternative candidate proteins may include the LPS binding proteins LBP and BPI (Tobias et al., 1997).

Analysis of LPS following incubation with HL60 cells shows a change in LPS structure resulting in binding of an anti-L3,7,9 immunotype antibody to a higher molecular weight LPS. This does not occur in the presence of cell supernatants alone, indicating either a contact-dependent mechanism, or that the effect relies on the microenvironment that occurs in close
proximity to the HL60 cells. Proteinase treatment indicated that the change in LPS is the result of binding with a protein, and that the CAMP LL-37 also elicits the same change in LPS structure. However, no direct link between the two could be established as attempts to detect LL-37 by Western blot analysis were unsuccessful (data not shown). The nature of the change in LPS structure has not yet been elucidated and would require detailed analysis by mass spectrometry. Attempts to prepare samples for this analysis were also unsuccessful due to technical difficulties and were abandoned due to time constraints.
Chapter 5

Identification of the Neisserial AP endonuclease and its role in pathogenesis
The Base Excision Repair (BER) pathway is responsible for the repair of DNA lesions induced by oxidative damage. Bacterial pathogens are likely to encounter damage of this nature during disease development, particularly during interactions with phagocytic cells which generate an oxidative burst in response to phagocytosis of microbes (Babior et al., 1976). Despite this, the importance of BER in bacterial virulence is not well understood. Previous studies have identified homologues of known BER enzymes in bacterial species such as *Brucella abortus* and *Salmonella typhimurium* (Hornback and Roop, 2006; Suvarnapunya and Stein, 2005). A *B. abortus* mutant strain lacking an Exonuclease III homologue is sensitive to oxidative stress, whilst *S. typhimurium* strains lacking a range of BER enzyme homologues, including a putative AP endonuclease, are significantly attenuated for intracellular survival in macrophages and the murine model of typhoid (Hornback and Roop, 2006; Suvarnapunya and Stein, 2005). The crystal structure of some BER components has been determined, including the human and *E. coli* AP endonucleases, HAP1 and ExoIII respectively (Mol et al., 2000; Gorman, 1997; Mol et al., 1995). Work has also characterised the biochemical activity of some bacterial BER enzymes, predominantly from *E. coli*, such as ExoIII, MutY and Fpg (Francis and David, 2003; Mol et al., 1995). However, there have been no studies on the impact of biochemically characterised BER enzymes on virulence.

The trEMBL database was examined for homologues of the *E. coli* AP endonuclease, ExoIII, in the genome of *N. meningitidis* gene strain MC58. This revealed NMB0399, whose predicted product shared 37% amino acid identity with ExoIII (Carpenter et al., 2007). Recombinant NMB0399 has similar activities to other known AP endonucleases, exhibiting 3′-5′ exonuclease and 3′phosphodiesterase (3dRp) activity, the process of removing phosphoglycolates from the 3′ terminus of the DNA strand (Carpenter et al., 2007). However, the protein did not exhibit AP endonuclease activity and was therefore named NExo. Resolution of the crystal structure of NExo showed that a histidine at position 167 within the DNA binding site could prevent binding of the abasic site, and thus inhibit AP endonuclease
activity (Figure 5.1). Substitution of His167 with serine or glycine conferred AP endonuclease activity on NExo. This work was performed by Julien Bergeron and Elisabeth Carpenter at the Centre of Structural Biology at Imperial College London. Biochemical analysis was carried out by Hellen Thompson in the Biochemistry Department of the Faculty of Life Sciences.

Figure 5.1 His$^{167}$ is a structural determinant for the lack of AP endonuclease activity in NExo

The abasic ribose binding site of HAP1 with a 15mer DNA complex is shown with the electrostatic potential surface of the HAP1 protein (blue and red representing positive and negative charge respectively) and the superimposed NExo protein (yellow), showing the presence of His$^{167}$ of NExo in the proposed abasic ribose binding site. Atoms in the DNA structure are coloured cyan for carbons, red for oxygen, blue for nitrogen and orange for phosphate. Residues Arg$^{177}$ and Met$^{270}$ were removed from the HAP1 structure to reveal the binding site beneath. This work was performed by Elisabeth Carpenter, and this image is reproduced with her kind permission.

This chapter describes the subsequent search for an active Neisserial AP endonuclease, and the investigation into its role in the pathogenesis of meningococcal infections.
5.1 Identification and purification of NMB2082, a Neisserial AP endonuclease paralogue

The *N. meningitidis* serogroup B MC58 genome was interrogated on the trEMBL database (Boeckmann *et al.*, 2003) using the program BLAST (Altschul *et al.*, 1997), at the European Bioinformatics Institute (www.ebi.ac.uk), for further AP endonuclease paralogues using the sequence of the human AP endonuclease HAP1. This search identified NMB2082, which shares 34% amino acid identity with HAP1, 27% identity with ExoIII and 25% identity with NExo (Figure 5.2 A & B). NMB2082 was amplified and ligated into the expression vector pROEXHtb. The recombinant protein was expressed in *E. coli* strain ER2566, followed by lysis of the cells by pulse sonication. The His-tagged proteins were then separated using a metal affinity column, followed by elutions with imidazole. The His tag was cleaved from the recombinant protein which was further purified by dialysis and size exclusion chromatography. The resulting fractions were separated by SDS-PAGE to verify purity (Figure 5.2 C).
Chapter 5 Identification of the Neisserial AP endonuclease

(A) Orientation of NMB2082, a putative AP endonuclease in the MC58 genome. Size bar indicates 1 kb.

(B) Structure-based multiple sequence alignment of the complete sequences of NExo (NMB0399), ExoIII (E. coli endonuclease), HAP1 (human endonuclease) and NApe (NMB2082). Positions in the alignment are colour coded in darker blue according to the number of identical residues at that position. Residues involved in metal binding, catalysis and abasic ribose binding are coloured pink, green and yellow respectively. The amino acid sequence numbers of NExo are indicated under the sequence. The predicted secondary structure is indicated in red for α-helices and green for β-strands.

(C) Protein fractions from the final purification step of NMB2082 were analysed by SDS-PAGE to verify protein size and purity. The predicted size of NMB2082 is 30 kDa.

Figure 5.2 Identification and purification of NMB2082, a Neisserial AP endonuclease homologue

(A) Orientation of NMB2082, a putative AP endonuclease in the MC58 genome. Size bar indicates 1 kb. (B) Structure-based multiple sequence alignment of the complete sequences of NExo (NMB0399), ExoIII (E. coli endonuclease), HAP1 (human endonuclease) and NApe (NMB2082). Positions in the alignment are colour coded in darker blue according to the number of identical residues at that position. Residues involved in metal binding, catalysis and abasic ribose binding are coloured pink, green and yellow respectively. The amino acid sequence numbers of NExo are indicated under the sequence. The predicted secondary structure is indicated in red for α-helices and green for β-strands. (C) Protein fractions from the final purification step of NMB2082 were analysed by SDS-PAGE to verify protein size and purity. The predicted size of NMB2082 is 30 kDa.
5.2 Structural and biochemical analysis of NMB2082

Crystallisation of NMB2082 was achieved by hanging-drop experiments with 20 mg/ml protein in a well solution made up from 20% PEG 20000, 0.1M bicine pH 9.0, 2% dioxane. The crystal structure of NMB2082 was solved at a 1.5 Å resolution under the conditions outlined in Figure 5.2 A and showed a high degree of structural similarity with NExo, HAP1 and ExoIII, including the conservation of key catalytic residues (Figure 5.3 B). Notably, NMB2082 possessed glycine (Gly\textsuperscript{170}) at the equivalent position to NExo His\textsuperscript{167}, suggesting that NMB2082 could be a functional AP endonuclease (Figure 5.3 B). Determination of the crystal structure was performed by Dr Elisabeth Carpenter.

To analyse the AP endonuclease activity of NMB2082, the purified protein was incubated with an abasic DNA substrate. The substrate was produced from a double-stranded DNA template, 25U, which was made by mixing equimolar amounts of complementary 25 bp strands, one of which contained a uracil residue at position 11, and a 5’-hexachlorofluorescein label (HEX) for visualisation. The resulting double-stranded DNA substrate was incubated with \textit{Herpes simplex} virus type-1 uracil DNA glycosylase (UDG, a gift from Dr Geoff Baldwin) to excise the uracil residue, producing the abasic DNA substrate 25AP, containing an AP lesion at the 11 bp position. The NMB2082 protein was incubated with both the 25AP and 25U substrates. Concurrent control reactions were performed in the absence of the NMB2082 protein, and with the \textit{E. coli} AP endonuclease ExoIII as a positive control. Aliquots were then separated by denaturing polyacrylamide gel electrophoresis. AP endonuclease activity was detected by the appearance of a 10 bp product resulting from cleavage of the AP site at position 11 on the DNA strand (data not shown). Like other active AP endonucleases, NMB2082 displayed 3’-5’ exonuclease activity when incubated with the 25U substrate (Figure 5.3 C). NMB2082 also showed 3’dRp diesterase activity (data not shown; work performed by Hellen Thompson). The NMB2082 protein also displayed significant specific AP endonuclease activity (Figure 5.3 C). Kinetic constants were
generated by fitting the data from the gel-based assay to the Michaelis-Menten steady-state
equation to give a turnover rate, $k_{AP}$ of $0.651 \pm 0.085 \text{ s}^{-1}$ and $K_M$ of $15.71 \pm 17.17 \text{ nM}$ (Figure 5.2D). The rate of AP endonuclease activity exhibited by ExoIII did not vary and thus the
data from assays with this enzyme could not be fitted to the same equation for comparison.
However, the ExoIII $k_{AP}$ was approximated as $0.083 \pm 0.013 \text{ s}^{-1}$ from the average rate,
indicating that NApe has a faster turnover of AP sites than *E. coli* equivalent ExoIII. *N.
meningitidis* NMB2082 was therefore designated NApe (*Neisseria* Apurinic / apyrimidinic
endonuclease).
Figure 5.3 NMB2082 is the Neisserial AP endonuclease (NApe)

(A) Crystallographic statistics for NMB2082. (B) The crystal structure of NMB2082 (green) was resolved and compared with HAP1 (red), ExoIII (magenta) and NExo (yellow). (C) Substrates 25AP (100 nM) and 25U (100 nM) were incubated with NApe (NMB2082; concentrations indicated) for the time periods shown and separated by denaturing PAGE. Control lanes show the unreacted 25AP and 25U substrates. NApe shows both 3′-5′ exonuclease (with the 25U substrate) and AP endonuclease activity (with the 25AP substrate). AP endonuclease activity was confirmed by the appearance of the 10 bp product following cleavage at the AP site at position 11 in the 25AP DNA substrate. (D) The rate of AP endonuclease activity for NApe was measured by the appearance of the 10 bp product band over time. Data are shown with the best fit to the Michaelis Menten equation with a $k_{AP}$ of $0.651 \pm 0.085$ s$^{-1}$ and $K_M$ of $15.71 \pm 17.17$ nM.
5.3 Construction and characterisation of MC58Δnexo, MC58Δnape and MC58ΔnexoΔnape in N. meningitidis

To insertionally inactivate NMB2082, a tetracycline resistance cassette was inserted into the target gene to produce MC58Δnape (Figure 5.4 A). 700 bp fragments up- and downstream of the NMB2082 gene were amplified and a gene encoding tetracycline resistance from plasmid pCMT18 was inserted between them. The resulting plasmid was used to transform wild-type N. meningitidis strain MC58 to antibiotic resistance. An insertional inactivation of the gene encoding NExo was also performed by Tn5 mutagenesis to produce the MC58Δnexo strain. This strain was constructed by Megan Winterbotham (Figure 5.4 A). A double mutant strain, MC58ΔnexoΔnape was also constructed. The identity of strains was confirmed by Southern analysis (data not shown). To determine whether NExo and Nape are expressed by wild-type strain MC58, and to verify the loss of expression in the mutant strains, whole-cell lysates of the wild-type and mutant strains grown overnight on solid medium were separated by SDS-PAGE and analysed by Western blot using antibodies raised in mice immunised with recombinant NExo and NApe (Figure 5.4 B). Immunisation of mice was performed by Yanwen Li. To confirm the absence of any growth defect as a result of the mutations liquid BHI medium was inoculated with each strain taken from solid medium, to give an initial O.D. $A_{600}$ of 0.15. The O.D. $A_{600}$ was measured every hour over a period of 8 hours to produce a growth curve. None of the mutant strains exhibited a significant growth defect (Figure 5.4 C).
Figure 5.4 Construction of mutants MC58Δnexo, MC58Δnape and MC58ΔnexoΔnape in *N. meningitidis*

(A) Construction of mutant strains. 700 bp regions up- and down-stream of NMB2082 were amplified, ligated into a plasmid vector and a tetracycline resistance cassette inserted between them to produce plasmid pAC2. NMB0399 was amplified, inserted into a vector and subjected to mutagenesis with Tn5 to produce plasmid pMW1. These constructs were then used to transform MC58. (B) Whole cell lysates of MC58, MC58Δnexo, MC58Δnape and MC58ΔnexoΔnape were analysed for expression of NExo and NApε. Samples were separated by SDS-PAGE following transfer to nylon membranes and incubated with sera against NExo or NApε (at a dilution of 1:1000). Expression of both proteins is seen in the wild-type strain but not in the corresponding mutants. (C) Liquid BHI media was inoculated with MC58, MC58Δnexo, MC58Δnape and MC58ΔnexoΔnape, and OD₆₀₀ measured hourly. None of the mutant strains display a growth defect in liquid media.
5.4 Both NExo and Nape are required for resistance against oxidative stress

Oxidative stress is an important aspect of innate immunity against bacterial pathogens, and AP endonucleases are critical for repairing DNA lesions induced by oxidative stress. Therefore the role of NApe and NExo in bacterial survival under conditions of oxidative stress was examined.

To analyse the importance of NExo and NApe in resistance to oxidative stress, mutants lacking these enzymes were compared with the wild-type strain for survival in media containing oxidising agents hydrogen peroxide (H$_2$O$_2$) and paraquat, which generate free radicals in the periplasm and cytoplasm, respectively (Pacello et al., 2008; Turner et al., 2003). H$_2$O$_2$ is produced naturally as a by-product of oxygen metabolism and is catabolised by peroxidases to produce water and oxygen. However, at higher concentrations it is readily converted into highly reactive hydroxyl radicals which lead to extensive DNA damage (Balasubramanian et al., 1998). Paraquat reacts with cellular NADPH to produce superoxide radicals, disrupting cellular biochemistry, particularly pathways that rely on NADPH. Superoxide radicals promote hydroxyl radical production, and thus contribute to damage to cellular DNA (Keyer and Imlay, 1996). Known numbers of bacteria were plated onto media with or without these oxidising agents. The percentage survival was calculated by comparison with the number of CFU after overnight growth. The mutants lacking NExo or NApe were significantly more sensitive to oxidative stress compared with the wild-type strain. Both MC58$\Delta$nexo and MC58$\Delta$nape were recovered at 70% of the wild-type in the presence of paraquat, and at 60% and 80% of the wild-type respectively when incubated with H$_2$O$_2$ (Figure 5.5). Furthermore, the survival of the double mutant under conditions of oxidative stress was significantly impaired (40% of wild-type in paraquat and 30% in H$_2$O$_2$) compared with both the single mutants (Figure 5.5)
Figure 5.5 Both NExo and NApe are necessary for resistance to oxidative stress

(A) Bacterial strains were grown on solid media in the presence and absence of the oxidising agents $H_2O_2$ (13 mM) or Paraquat (0.075 mM). The image shown is representative of strains grown on media containing $H_2O_2$. (B) Killing was calculated as a percentage of CFU recovered of strain on media without these agents. Strains lacking NExo and NApe were more sensitive to $H_2O_2$ and Paraquat; the strain lacking both enzymes is significantly more sensitive than the single mutants alone. Error bars represent Standard error of the Mean.
5.5 *NExo* and *NApe* are necessary for virulence

The generation of oxidative stress is an important aspect of defence against microbial infection *in vivo*. Therefore the physiological relevance of the sensitivity to oxidative stress of the *NExo* and *NApe* mutant strains was evaluated by assessing their ability to cause bacteraemia. Five-day old infant rats were challenged with a 1:1 ratio of mutant and wild-type strain, and 24 hours later the relative survival of the strains was determined by plating venous blood to media with and without antibiotics. The Competitive Index was calculated by comparing the recovery of the mutants with the wild-type strain. Both MC58Δnexo and MC58Δnape were recovered from the bloodstream of infected infant rats at significantly lower levels than the wild-type strain (Figure 5.6). The double mutant displayed significantly higher attenuation than either of the single mutant strains, indicating an additive effect of the loss of both *NExo* and *NApe*.

**Figure 5.6 NExo and NApe are required for virulence in the infant rat model**

Infant rats were challenged with a 1:1 ratio of wild-type to mutant bacteria. Following bleeding 24 hours later, serial dilutions were plated to solid media and incubated overnight. Competitive Index (C.I) was calculated following quantification of CFU recovered. Attenuation is shown as a negative log.CI value. The single mutants were significantly attenuated, while the double mutant was more attenuated than either of the single mutants.
5.6 Summary

The data presented in this chapter constitute the first study to combine structural and biochemical analysis of predicted DNA repair enzymes with analysis of their role in survival and pathogenesis (Carpenter et al., 2007). NExo is the meningococcal protein that is most closely related to the *E. coli* AP endonuclease and has exonuclease activity. However, this enzyme is not an AP endonuclease due to the His\textsuperscript{167} blocking the DNA binding site. The His residue is present in the genome so did not arise due to a point mutation during cloning or expression of the NMB0399 gene. This work highlights the importance of characterising the gene product rather than relying on the known activity of homologues in other genomes. Instead, NApe, which is more closely related to a human than the *E. coli* AP endonuclease, was demonstrated to be a functional AP endonuclease. Assays to determine the role of these two enzymes demonstrated that NExo and NApe are expressed by *N. meningitidis* and play independent roles in the survival and pathogenesis of the bacterium.

Both NExo and NApe are required for resistance against oxidative stress, and for survival *in vivo*. The additive effect seen in assays with the double mutant strain indicates that each enzyme acts independently and in separate pathways. The meningococcus is known to interact with phagocytic cells during development of disease, and thus is exposed to the oxidative burst. It is likely that NExo and NApe play a role in ameliorating the effects of the oxidative burst by repairing the resulting lesions in the bacterial DNA. Their differing range of substrate specificities means that both enzymes would be necessary for efficient DNA repair function.
The next chapter examines the interaction of the meningococcus with human neutrophils. The double mutant MC58\textit{ΔnexoΔnape} is included in this work to further analyse the role of these two DNA repair enzymes in meningococcal disease.
Chapter 6

Characterisation of the non-opsonic interaction between *N. meningitidis* and primary human PMNs
Chapter 3 described work in which non-opsonic phagocytosis of *N. meningitidis* by the human neutrophil cell line HL60 was investigated. Live meningococci associated with and were internalised by HL60 cells in a manner that is independent of lactate acquisition by the bacterium. Previous work has also described phagocytosis of *N. meningitidis* by PMNs and macrophages, identifying the anti-phagocytic role of the polysialic acid capsule (Read *et al.*, 1996) and some host cell receptors involved in internalisation (Virji *et al.*, 1996). However, nothing is known about the fate of the meningococcus following uptake by PMNs. It has been shown that the gonococcus survives and replicates within PMNs, and that it utilises lactate generated by the cells. It has been suggested that lactate utilisation is a mechanism by which *N. gonorrhoeae* reduces the efficacy of the oxidative burst. Recent work has confirmed that the gonococcus does inhibit the oxidative burst elicited by PMNs (Criss *et al.*, 2008). Other mechanisms of reducing the effect of the oxidative burst have also been identified. The meningococcus expresses functional catalase, peroxidase and cytoplasmic superoxide dismutases (Seib *et al.*, 2005; Wilks *et al.*, 1998; Archibald and Duong, 1986). Genes encoding a manganese import transporter MntABC, which is involved in protection of the gonococcus against ROS, and SodB are also present in the meningococcal genome (Tettelin *et al.*, 2000). Expression of the gonococcal porin PorB has also been shown to reduce the efficacy of oxidative burst (Lorenzen *et al.*, 2000).

The gonococcus also delays apoptosis of neutrophils following phagocytosis (Simons *et al.*, 2006). It has been suggested that this process enables the bacterium to replicate intracellularly and thus aid in the propagation of disease. This process has not yet been investigated in the meningococcus. Neutrophils containing multiple intracellular meningococci have been recovered from the bloodstream of patients (Guarner *et al.*, 2004). It is possible that the bacterium may survive within neutrophils to aid dissemination within the host, but as yet there is no direct evidence for this. To survive within a phagocytic cell, the
meningococcus would require the means to both withstand or manipulate PMN killing mechanisms, and/or to repair the resulting damage.

Work in this chapter investigates the fate of *N. meningitidis* and human PMNs following phagocytosis. Furthermore the role of the bacterial lactate permease LctP, glutamate uptake enzyme PerM, and BER enzymes NExo and NApe is examined. Since work with the gonococcus suggests that survival of the wild-type strain in PMNs relies on its ability to scavenge intracellular lactate from the host cell (Simons *et al.*, 2006; Britigan *et al.*, 1988), the survival of the MC58ΔlctP strain was analysed. PerM is a component of an ABC-type glutamate transporter that operates at low sodium conditions such as those that may be experienced by the meningococcus within PMNs. Bacterial glutamate is predicted to be converted to glutathione, a low molecular weight, water-soluble antioxidant. In its reduced state, glutathione acts as a scavenger of free radicals and as an electron donor to restore oxidised macromolecules (Carmel-Harel and Storz, 2000). High concentrations of glutathione have been identified in the gonococcus and it is thought to be an important first-line defence against oxidative stress (Archibald and Duong, 1986). For this reason, the H44/76ΔperM strain was also analysed in PMNs. BER is the primary pathway by which organisms repair DNA damage caused by oxidative stress (Wilson *et al.*, 2003). NExo and NApe are required for resistance to oxidation by H₂O₂ and paraquat, and for survival *in vivo* (Carpenter *et al.*, 2007) & (Chapter 5). Thus, the influence of these enzymes in survival in PMNs was also examined.
Chapter 6 The non-opsonic interaction with primary human PMNs

6.1 Characterisation of isolated primary PMNs

Venous blood was obtained from healthy volunteers, and PMNs were purified by histopaque separation. PMNs were separated on a dextran gradient, and the concentration of cells was adjusted to $1 \times 10^6$ cells/ml in HBSS. The viability of PMNs was assessed using trypan blue exclusion, and was calculated to be >99% (not shown). The homogeneity of PMNs was confirmed by microscopy and flow cytometry. For microscopy, $5.5 \times 10^5$ PMNs were stained on coverslips with Giemsa-Wright stain and scored for the presence of a multi-lobed nucleus; purity was calculated as >95% by this method (Figure 6.1 A). PMN receptor expression was also examined by flow cytometry. Following activation with 100 ng/ml PMA, $5.5 \times 10^5$ PMNs were incubated with antibodies against CD45, CD18 or CD11b. CD45 is also termed leukocyte common antigen (Thomas, 1989). The CD18 integrin forms a heterodimer with CD11b, generating CR3, which is expressed by activated PMNs (Mazzone and Ricevuti, 1995). Binding of antibodies against CD45, CD18 and CD11b was analysed by flow cytometry. All activated PMNs were recognised by antibodies against these cell markers but not by an isotype control (Figure 6.1 B).

Expression of CEACAM-1 (CD66) was analysed by both fluorescence microscopy and flow cytometry using an antibody against the a,c,d isoforms of CD66. This receptor mediates non-opsonic interactions with *N. meningitidis* via the meningococcal Opa proteins (Virji et al., 1996) (Figure 6.1 C). Expression of the granular protein, neutrophil elastase, was also analysed by fluorescence microscopy. Cells were scored (n = 100), of which >95% were positive for neutrophil elastase (Figure 6.1 D & data not shown). Collectively, these data demonstrate that the procedure for isolating PMNs from peripheral blood was successful.
PMNs were isolated from the venous blood of healthy volunteers and prepared for analysis by microscopy and flow cytometry. (A) PMNs were stained with Giemsa-Wright stain and scored for the presence of multi-lobed nuclei. Purity was confirmed as >95% Magnification is shown above each image. (B) PMN receptor expression was analysed by flow cytometry using mAbs against CD45, CD18 and CD11b (as indicated). Antibody binding (green trace) was compared with cells incubated with an isotype control (purple trace) and confirmed expression of each receptor. (C) Expression of the CD66 receptor was analysed by microscopy and flow cytometry using a mAb against the CD66a,c,d isoforms. (D) Neutrophil elastase expression was analysed by microscopy using a mAb against this granule protein. Scale bars represent 20 µm.
6.2 Primary PMNs associate with N. meningitidis strains in the absence of opsonins

Prior to investigating the survival of bacteria during and after phagocytosis, it was necessary to determine whether phagocytosis of N. meningitidis by primary PMNs occurred in a similar manner as observed with HL60 cells (Chapter 3). PMNs were challenged with live GFP-expressing MC58, MC58ΔlctP and MC58ΔsiaD at an MOI of 100 in the absence of opsonins, and incubated for thirty minutes. Following fixation, the association of bacteria with PMN was analysed by flow cytometry. All strains associated to similar levels to those seen with HL60 cells. MC58 and MC58ΔlctP strains associated with primary PMNs to the same extent, whilst the unencapsulated MC58ΔsiaD strain showed significantly increased association (Figure 6.2). Microscopic analysis was also performed to verify internalisation of bacteria (data not shown). These assays were performed on at least three independent occasions with PMNs isolated from different donors.
Figure 6.2 Primary PMNs phagocytose *N. meningitidis* in the absence of opsonins

(A) Primary PMNs were incubated with live GFP-expressing MC58, MC58ΔlctP and MC58ΔsiaD strains and association analysed by flow cytometry. MFI was calculated by multiplying the mean fluorescence with the percentage positive cell population. All strains associated with PMNs. MC58ΔsiaD associated to a significantly higher degree than either wild-type or MC58ΔlctP strains. The MC58ΔlctP strain associated to PMNs to the same level as the wild-type. Error bars represent standard error of the mean fluorescence index.

(B) Primary PMNs were incubated with live GFP-expressing MC58. FACS trace shows the uninfected PMN population (purple trace) and confirms association of the wild-type strain with PMNs (green trace).
6.3 Wild-type N. meningitidis MC58 survives in the presence of human PMNs

To determine the fate of bacteria following interaction with PMNs, assays were established to examine bacterial survival using adherent PMNs. Optimisation of this assay included verifying that a confluent layer of PMNs was produced by centrifugation of \(5.5 \times 10^5\) PMN onto flat-bottomed 48-well plates, where they adhered. This was necessary to ensure maximum contact between PMNs and bacteria. Further optimisation was performed to analyse the influence of altering the MOI (data not shown). Assays were performed by inoculating the adherent PMNs with bacteria at an MOI of 100 in the absence of opsonins, and incubating for one hour. Bacterial survival in the presence and absence of PMNs was analysed by plating serial dilutions of the contents of the wells to solid media following lysis of PMNs with 0.1% saponin which does not kill \(N. meningitidis\). Survival was calculated as the percentage of bacteria recovered after incubation with PMNs compared with the number of bacteria in the input. \(E. coli\) DH5α was used as a control. If DH5α was not killed in the presence of PMNs, results were not examined. Wild-type MC58 strain was not killed in the presence of PMN (Figure 6.3). This was confirmed with PMNs from at least three different donors, on at least three independent occasions with each donor.
Figure 6.3 Wild-type *N. meningitidis* MC58 strain survives within PMNs

Adherent primary human PMNs were challenged with bacteria at an MOI of 100. Survival of *E. coli* DH5α was compared with MC58 by recovery of bacteria from PMNs following lysis with saponin. The percentage survival was calculated after a one-hour incubation by comparing the number of bacteria recovered with the number in the input. *E. coli* DH5α was killed by PMNs (P < 0.005), while MC58 was not. Error bars represent the standard error of the mean. This figure is a representative dataset from an assay performed on PMNs isolated from one donor on three independent occasions.
6.4 *Infection with N. meningitidis delays PMN apoptosis*

To investigate the effect of *N. meningitidis* on PMN viability, markers of cell death and apoptosis were analysed in PMNs in the presence and absence of bacteria. Lactate dehydrogenase (LDH) is a membrane-bound enzyme located on the cytoplasmic face of the plasma membrane that converts lactate to pyruvate (Holten and Jyssum, 1974). Due to its location on the plasma membrane of eukaryotic cells, LDH is found in the surrounding medium following loss of membrane integrity during early stages of apoptosis and cell death. LDH release into the medium is therefore a commonly used marker of apoptosis. Adherent PMNs were infected with live or dead MC58 at an MOI of 100, and incubated for up to three hours. Supernatants of both infected and non-infected cells were harvested and analysed for LDH activity. Percentage cytotoxicity was calculated by comparing results with wells in which all cells had been lysed by the addition of saponin as described in Material and Methods (Chapter 2). Uninfected cells showed a baseline level of between ten to 14% cytotoxicity over three hours (Figure 6.4 A). This indicates that in all assays involving infection of PMNs, >85% are viable on infection with *N. meningitidis* as described in this chapter. PMNs infected with both live and dead MC58 strain show reduced cell death compared to uninfected cells. This trend was most marked in PMNs infected with the live meningococcus (Figure 6.4 B).

Apoptosis was also analysed by examining the activation of caspase-3. Caspases are a family of cysteine proteases that include both initiators and effectors that coordinate the cellular destruction and disassembly that occurs during apoptosis (Slee et al., 1999). Caspase-3, also known as apopain or CPP32, is a key effector of apoptosis in mammalian cells (Fernandes-Alnemri et al., 1994). It is present in an inactive form as a 37 kDa pro-caspase that is cleaved to produce the active 15 kDa and 17 kDa fragments. Adherent PMNs were inoculated with non-opsonised MC58, or with the *Saccharomyces cerevisiae* particle
zymosan, with or without 10% human serum. The total contents of the wells were harvested following a 4-hour incubation, and the proteins separated by SDS-PAGE. Equal protein loading was verified by Western blot analysis with a mAb against actin. Caspase-3 activation was then analysed by Western blot analysis using mAb 8G10 that recognises both the cleaved and uncleaved forms of caspase-3. Caspase-3 was detected in both its active and inactive forms under all conditions. PMNs inoculated with opsonised and non-opsonised zymosan showed significantly higher activation of caspase-3 compared with uninfected PMNs, indicating that phagocytosis of these particles initiated apoptosis. In contrast, PMNs infected with MC58 showed less caspase-3 activation than those infected with zymosan, or than uninfected cells, indicating that live *N. meningitidis* appears to down-regulate apoptosis of PMNs (Figure 6.4 C).
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Figure 6.4 Infection with *N. meningitidis* leads to a delay in PMN apoptosis

(A; B) Supernatants from non-infected (A) and infected (B) PMNs were harvested at one, two and three hours post-challenge and analysed for lactate dehydrogenase (LDH) activity. The percentage cytotoxicity was calculated by comparison with uninfected cells and results obtained after all cells had been lysed with saponin. Cytotoxicity is reduced in cells infected with both live (white bars) or dead (black bars) bacteria, although this trend is more marked in cells infected with live MC58. Error bars represent the standard error of the mean. (C) Adherent PMNs were inoculated with MC58, and with opsonised and non-opsonised zymosan. The contents of the wells were harvested four hours post-challenge. Proteins were separated by SDS-PAGE and PMN caspase activation analysed by Western blot with a mAb that recognises both cleaved (active) and uncleaved (inactive) Caspase-3. A mAb that recognises actin was used as a loading control. Cells inoculated with MC58 show lower caspase activation than those with zymosan, indicating an inhibition of this apoptotic pathway.
6.5  Infection with N. meningitidis leads to a delay in nuclear condensation

A feature of apoptosis in PMNs is a change in morphology of nuclei from the characteristic multi-lobed form to a more condensed, rounded shape. To investigate whether infection with *N. meningitidis* also affects this marker of apoptosis, adherent PMN were infected with MC58 or non-opsonised zymosan on coverslips and incubated for three hours. Cells were fixed with PFA, and the coverslips were stained with the Giemsa-Wright stain. Qualitative assessment of the appearance of PMN nuclei indicate that PMNs infected with MC58 show less condensation of the nucleus when compared with cells infected with zymosan (Figure 6.5). This result provides further independent evidence indicating that wild-type *N. meningitidis* delays the apoptosis of PMNs.

![Image of PMN nuclei](image)

**Figure 6.5 Infection with N. meningitidis leads to a delay in PMN nuclear condensation**

Adherent PMNs were challenged with non-opsonised zymosan or *N. meningitidis* MC58 and incubated for three hours. Coverslips were prepared for analysis of nuclear condensation by microscopy. PMNs were stained with Giemsa-Wright stain and nuclear morphology compared with uninfected PMNs. Cells stained at time zero show normal multi-lobed nuclear morphology. Three hours post-challenge, uninfected and zymosan-infected cells show clear nuclear condensation. Cells infected with MC58 show a lesser degree of condensation. Scale bars represent 30 µM.
6.6 *N. meningitidis* requires the lactate permease LctP, glutamate transporter PerM and BER enzymes, NExo and NApe, for survival in PMNs

Due to the potential biological roles of LctP, PerM and NExo and NApe in resistance against the oxidative burst, mutant strains MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM were assessed for survival in the presence of PMNs. Adherent PMNs were inoculated with bacteria at an MOI of 100 and incubated for one hour. Wild-type strains MC58 and H44/76 were included, along with *E. coli* DH5α. Following lysis of PMNs with 0.1% saponin, the survival of each strain was assessed by plating to solid media. Percentage survival was calculated as the ratio of bacteria recovered compared with the input at time zero. This assay was performed with at least three different PMN donors, on at least three independent occasions for each donor. *E. coli* DH5α was significantly killed by PMNs in every assay. Both wild-type MC58 and H44/76 strains survived in the presence of PMNs. However, MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM strains were all significantly killed by PMNs even in the absence of opsonins (Figure 6.6). This indicates that LctP, PerM and a combination of NExo and NApe are required for survival in the presence of PMNs.
Figure 6.6 *N. meningitidis* requires the lactate permease LctP, glutamate transporter PerM, and BER enzymes NExo and NApe for survival in PMNs

Adherent primary human PMNs were challenged with bacteria at an MOI of 100. *E. coli* DH5α was included as a control. The survival of strains was analysed by plating serial dilutions of the total contents of wells after lysing cells with saponin. The percentage survival was calculated after a one-hour incubation by comparing the number of recovered bacteria with the input. Neither wild-type strain was killed by PMNs. All three mutant strains were sensitive to PMNs. The figure shows representative results from assays performed with PMNs from one donor on three independent occasions. Error bars represent the standard error of the mean.
6.7 MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM are killed by PMN in an actin- and ROS-dependent manner

MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM were attenuated in the presence of PMNs unlike the corresponding wild-type parental strains. To elucidate the mechanism of sensitivity of each strain, PMNs were treated with cytochalasin D or resveratrol. Cytochalasin D inhibits actin polymerisation, and thus prevents phagocytosis of bacteria (Schliwa, 1982). Resveratrol is a phytoalexin produced by plants in response to infection with bacteria and fungi. It has antioxidant, anti-inflammatory and immunomodulatory activities, and has been shown to reduce the oxidative burst of PMNs, monocytes and T-cells by inhibiting myeloperoxidase activity and interfering with phosphatidylinositol 3-kinase (PI3-K) signalling to NADPH oxidase (Castro et al., 2008; Deby-Dupont et al., 2005; Poolman et al., 2005). Prior to use of these inhibitors with PMNs, their potential antibacterial activity was determined. MC58 strain was incubated with cytochalasin D (5 µM) and resveratrol (100 µM) for one hour, and survival was calculated as the number of bacteria recovered as a percentage of the input. Neither cytochalasin nor resveratrol inhibited bacterial growth at these concentrations (data not shown). This assay was also attempted with the standard ROS inhibitor, Diphenyleneiodonium chloride (DPI), however this agent was bactericidal and therefore could not be used (data not shown). Therefore, assays were performed with adherent PMNs in the presence of cytochalasin (5 µM) or resveratrol (100 µM). PMNs were pre-treated with each inhibitor for 20 minutes prior to challenge with E. coli DH5α, MC58, MC58ΔlctP, MC58ΔnexoΔnape, H44/76 and H44/76ΔperM. Inhibitor- and PMN-free controls were included for each strain. Following a one-hour incubation, PMNs were lysed with 0.1% saponin, and surviving bacteria recovered by plating. This assay was performed with at least three different PMN donors, on at least three independent occasions for each donor. In the absence of cytochalasin D or resveratrol, neither MC58 or H44/76 were killed, whilst E. coli, MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM had reproducible significant defects for survival in PMNs. However, in the
presence of both cytochalasin D and resveratrol, survival of MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM was restored to wild-type levels (Figure 6.7 A & B). *E. coli* DH5α also survived in the presence of the actin and ROS inhibitors. The effect of cytochalasin D on survival indicates that the attenuation of strains relies on actin polymerisation. The effect of resveratrol in restoring wild-type levels of survival indicate that killing of MC58ΔlctP, MC58ΔnexoΔnape and H44/76ΔperM was the result of ROS-dependent killing mechanisms. Therefore the wild-type MC58 and H44/76 strains rely on the activity of LctP, PerM and BER enzymes NExo and NApe to survive ROS-mediated killing by PMNs.
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Figure 6.7 *N. meningitidis* mutants are killed in an actin- and ROS-dependent manner

Adherent primary human PMNs were pre-treated with either 5 µM cytochalasin (A) or 100 µM resveratrol (B) for twenty minutes prior to challenge with bacteria at an MOI of 100. Bacteria were recovered after a one-hour incubation by plating to solid media after lysing PMNs. The percentage survival was calculated after a one-hour incubation by comparing the number of recovered bacteria with the input. Both cytochalasin D and resveratrol restored the survival of MC58ΔictP, MC58ΔnexoΔnape and H44/76ΔperM to wild-type levels, indicating that sensitivity of these mutant to PMNs is dependent on actin polymerisation and ROS production. The figure shows a representative dataset from assays performed with PMNs from one donor on three independent occasions. Error bars represent the standard error of the mean.
6.8 Summary

The work described in this chapter investigated the fate of both the bacterium and host cells when primary human PMNs were infected with *N. meningitidis*. Following validation of the procedure to isolate primary PMNs, assays demonstrated that wild-type *N. meningitidis* MC58 is able to survive in the presence of human PMNs, and to delay apoptosis of the host cells. The same observation has been made previously in work with the gonococcus (Simons et al., 2006). The ability to survive intracellularly in PMNs is likely to be important for development of meningococcal disease since intracellular bacteria are not exposed to complement-mediated killing in the bloodstream. It is possible that delayed PMN apoptosis may influence cytokine signalling by PMNs, thus affecting the innate immune response to the infection, and prolonging the protection afforded to the bacteria by the live PMN. It is also possible that meningococci residing within circulating PMNs may contribute directly to development of meningitis since PMNs are able to cross the BBB via cell adhesion molecules (CAMs) expressed by activated endothelial cells as a result of the inflammatory response to infection (Wong et al., 2007). Manipulation of phagocyte function is well described in bacterial pathogenesis. For example, work with dendritic cells (DC) has shown that infection with live meningococci results in a different outcome than infection with dead bacteria. Live meningococci were phagocytosed to a lesser extent and despite eliciting cytokine release, they impeded DC maturation more than fixed bacteria (Jones et al., 2007). It is suggested that the resulting lower density of maturation marker receptors expressed by DCs may influence the subsequent T-cell response, and thus progression of the inflammatory response (Jones et al., 2007).

The role of LctP, PerM and NExo and NApe in survival within PMNs was also investigated in this chapter. Mutant strains lacking the genes encoding these enzymes were attenuated for
survival in the presence of PMNs. Treatment with cytochalasin D restored survival of all three strains to wild-type levels, indicating that the attenuation of these mutants relied on actin polymerisation. It may therefore be extrapolated that killing therefore relies upon phagocytosis, but the effects of cytochalasin D on NADPH oxidase signalling pathways and extracellular release of anti-microbial peptides is not known and therefore care should be taken when making this assumption. Treatment with resveratrol to inhibit ROS production also restored survival of mutant strains to wild-type levels, indicating that the attenuation seen with each mutant relied on the oxidative burst. Work with the gonococcus has demonstrated that the bacterium utilises lactate produced by PMNs (Goldner et al., 1979), and it has been suggested that this process depletes the intracellular lactate reserves, thus potentially reducing the extent of the oxidative burst. The gonococcus is known to inhibit the oxidative burst of PMNs and it is possible that the same activity is performed by the meningococcus. Attempts were made to quantify the production of ROS by infected PMNs. However, the detection methods used did not allow the use of resveratrol as a negative control due to cross-reaction between resveratrol and the detection agent, and therefore this analysis was not possible. The mechanism of inhibition of ROS production by the gonococcus has not yet been elucidated but it may be that the ability to utilise lactate is necessary for this activity. Previous work has also shown that lactate is required for sialylation of the LPS and production of the polysialic acid capsule (Exley et al., 2005) & (Chapter 4). The role of these surface components for survival within phagocytes is not well understood, although work has demonstrated a role for the capsule in survival within both phagocytic and non-phagocytic cells (Spinosa et al., 2007). It is possible that the inability to acquire lactate results in a reduction of the defence of this mutant strain to the oxygen-dependent killing mechanisms employed by the PMNs. Glutamate uptake is mediated by PerM in low sodium conditions such as in the intracellular environment. Glutamate is required for the production of the antioxidant molecule glutathione (Unpublished). Thus it is
likely that the H44/76ΔperM mutant lacks intracellular glutathione and is less efficient at ameliorating the effects of free radicals, making it more susceptible to the oxidative burst (Unpublished).

Attenuation of the mutant lacking BER enzymes NExo and NApe highlights the importance of the BER pathway in repairing damage inflicted on the bacterial genome by ROS. Accumulation of mutations will occur rapidly during exposure to high concentrations of ROS and in the absence of a functional repair pathway, represented by these two enzymes, the mutations are cytotoxic.
Chapter 7

Discussion
7.1 Overview

PMNs are voracious killers of bacterial pathogens and a critical part of the innate immune system. They are activated by cytokines in their environment, or by certain pathogen-associated molecule patterns (PAMPs), most notably LPS (Saluk-Juszcak and Wachowicz, 2005; Harada et al., 1994). *N. meningitidis* encounters PMNs in the host, and has been recovered from within the phagocytic cells of patients with meningococcal disease (Guamer et al., 2004; Peters et al., 2003). PMNs are recruited to sites of infection and are able to migrate through cellular layers to gain access to invading pathogens (Spanaus et al., 1997). It is therefore likely that the first point of contact of *N. meningitidis* with these cells occurs during colonisation of the nasopharyngeal epithelium. PMNs also circulate in the bloodstream, the route by which the meningococcus disseminates within the host (Exley et al., 2005a). PMNs express a wide range of receptors, some of which (including CR3 and Fcγ receptors) recognise opsonised particles (Fijen et al., 2000). Other receptors exhibit specificity for structures expressed on the surface of the meningococcus, such as CD66 and CD14, which recognise Opa and LPS respectively (Ahren et al., 2001; Virji et al., 1996). Following engagement by these receptors, the pathogen can be internalised regardless of opsonisation. Expression of surface antigens such as the polysialic acid capsule and sialylation of LPS play an important role in mediating interactions with phagocytic cells (Estabrook et al., 1998; McNeil and Virji, 1997; Read et al., 1996). Production of these structures is closely linked to bacterial carbon metabolism and it is known that lactate is required for wild-type levels of LPS sialylation (Exley et al., 2005b; Smith et al., 2001). The role of the metabolism of the carbon sources lactate and glutamate was therefore investigated during interactions with PMNs.

In meningitis and meningococcal septicaemia, killing of the bacterium primarily occurs via complement-mediated lysis. Initiation of the classical (CP), alternative (AP) or lectin pathways of the complement cascade leads to deposition of C3b on the bacterium, and
subsequent insertion of the membrane attack complex (MAC) in the bacterial outer membrane, and bacterial lysis (Schneider et al., 2007). The complement component C3bi also acts as an opsonin through recognition by CR3 on phagocytic cells (Messner and Jelinek, 1970). For these reasons, individuals who possess defects in the complement system are particularly susceptible to meningococcal disease (Rosa et al., 2004; Figueroa et al., 1993; Fine et al., 1983). In the absence of this important killing mechanism, hosts must rely on other defensive immune mechanisms. One such mechanism is non-opsonic uptake and killing by PMNs. It has been shown that serogroup C N. meningitidis is taken up by PMNs in a non-opsonic manner (Estabrook et al., 1998). Work with N. gonorrhoeae has shown that the gonococcus survives and replicates within PMNs, and results in a delay in PMN apoptosis (Simons et al., 2005; Parsons et al., 1982) but this interaction has not previously been studied with the meningococcus.

PMNs also play an important role in the inflammatory response, releasing pro-inflammatory cytokines and anti-microbial compounds, and producing ROS in response to engagement and phagocytosis of bacterial pathogens. ROS cause oxidative damage to the bacterial cell, including the formation of lesions in DNA. The BER pathway is dedicated to ameliorating the effects of oxidative damage (Wilson et al., 2003). A robust repair system would be required for the bacterial genome to withstand attack by the ROS produced by PMNs. Therefore, two putative meningococcal AP endonuclease homologues were identified and their role in pathogenesis and survival in the presence of PMNs was investigated.

The aim of this thesis was to characterise the non-opsonic interaction of N. meningitidis with human PMNs. This included investigation of the role of lactate and glutamate metabolism, and the importance of the BER DNA repair pathway in the meningococcus.
7.2 Outcome of interaction between wild-type *N. meningitidis* and PMNs

The interaction between serogroup C *N. meningitidis* and PMNs has been studied previously. Both opsonised and non-opsonised meningococci associate with, and are internalised by PMNs (Fijen *et al.*, 2000; Estabrook *et al.*, 1998). In this study, the association of fixed serogroup B MC58 strain to HL60 cells was shown by flow cytometry, and phagocytosis confirmed by microscopy. It is known that the neisserial Opa proteins interact with the CD66 receptor (de Jonge *et al.*, 2003; Popp *et al.*, 1999; Chen *et al.*, 1997). Analysis of differentiated HL60 cells confirmed expression of this receptor and pre-incubation of the cells with an anti-CD66 antibody significantly reduced the association with bacteria. This indicates that the CD66 receptor mediates non-opsonic interaction between Opa-expressing meningococci, although additional receptors may also be involved. No alternative receptors have yet been identified although candidates include sialic acid-specific receptors that could mediate lectinophagocytosis, such as sialic acid-binding Ig-like lectins (siglec), which are expressed by all leukocytes (Monteiro *et al.*, 2004; Zhang *et al.*, 2000). Phagocytosis of meningococci is also affected by anti-phagocytic components expressed on the surface of the bacterium. Expression of the polysialic acid capsule and sialylation of LPS are known to reduce association and internalisation by phagocytes (Read *et al.*, 1996; Estabrook *et al.*, 1992). An unencapsulated mutant lacking the polysialyl-transferase SiaD associated with HL60 cells significantly more than the wild-type strain, confirming the previously described anti-phagocytic role of the capsule, and validating this flow cytometric method for analysis of the interaction between HL60 cells and *N. meningitidis*.

When phagocytosis assays with HL60 cells were repeated with live bacteria, association and phagocytosis were greatly reduced in comparison with fixed bacteria. This effect has been observed previously in work with DCs where it was attributed to manipulation of the cell by the bacteria, thus inhibiting uptake (Jones *et al.*, 2007). Here, a mixed infection of HL60 cells
with live and FITC-labelled fixed bacteria was performed to investigate whether the reduced uptake was due to a cellular response to the live bacteria. However, the presence of live bacteria did not significantly affect the uptake of fixed bacteria by HL60 cells. This data indicates that the reduced association of live bacteria is due to a bacterial response to host cells. Examination of bacteria following exposure to HL60 cells showed that capsule expression in live, but not fixed, *N. meningitidis* increased significantly. This pattern was also observed in bacteria incubated in supernatants taken from HL60 cells infected with *N. meningitidis*, indicating that the increase in capsule expression occurs as a result of a contact-independent mechanism. The same response was seen in bacteria exposed to the epithelial cell line Calu-3 and to supernatants from infected Calu-3 cells.

Previous work has demonstrated a down-regulation in capsule expression on contact with epithelial cells (Deghmane *et al.*, 2002). This is in keeping with the increase in capsule in the presence of epithelial cells or neutrophils being dependent on a mechanism that does not involve contact with the cells themselves. Supernatants from infected HL60 cells were analysed by NMR to determine potential candidates that may induce this increase in capsule expression. The NMR spectra indicated the presence of both lactate and pyruvate, in addition to alanine, acetate and some unidentified organic acids. Sialic acid production by *N. meningitidis* is closely linked to the metabolism of lactate and pyruvate and further investigation of the availability of these carbon sources in cell supernatants showed an increase in concentration of both over time, indicating that they are secreted by HL60 cells. However, there was no change in capsule expression in bacteria incubated with lactate and/or pyruvate in addition to glucose. This does not exclude their potential importance in the bacterial response to HL60 cells since it remains possible that the source of PEP for the production of sialic acid originates from the lactate and/or pyruvate present in the media. However, this result indicates that the increase in capsule expression is not due to carbon
source availability alone. Furthermore, NMR may be insufficiently sensitive to detect significant changes in alternative candidate compounds in the cellular supernatants.

Supernatants were analysed further to determine whether a protein was responsible. Treatment with a protease resulted in a significant reduction in the response of bacteria to HL60 supernatants, indicating a role for a proteinaceous component. PMNs release CAMPs such as LL-37 and HNP-1 in response to bacterial pathogens (Borregaard, 1984). Epithelial cells also secrete a range of CAMPs including LL-37 (Weinberg et al., 1998). Previous work has demonstrated that capsule expression increases resistance to killing by CAMPs and that expression is increased in intracellular bacteria within epithelial cells (Spinosa et al., 2007). Incubation of MC58 with LL-37 or HNP-1 elicited an increase in capsule expression in a dose-dependent manner. However, the potential role of CAMPs in the response to HL60 and Calu-3 cells is dubious since size fractionation of supernatants showed that the fraction containing proteins of 50-100 kDa was active rather than lower molecular weight fractions. LL-37 and HNP-1 are both <10 kDa in size, and therefore could not be the effector of the observed capsule expression response unless they were present in complex with a larger protein. LL-37 is known to bind LPS, which in turn forms complexes with LPS-binding protein (LBP), a 55 kDa protein secreted by PMNs. However, there is no evidence that this potential complex might be present in the supernatants, nor that it would induce the effects seen in capsule expression. Further investigation into the active component of HL60 cell supernatants might include analysis of the effect of LBP on capsule expression in the presence and absence of LL-37, and work to detect this protein in cell supernatants. Also, detailed dissection of the protein components of cell supernatants could be performed by HPLC to identify candidates that could then be tested for their effect on capsule expression. 

*N. meningitidis* expresses a number of two-component regulatory systems such as PhoPQ, which is known to be linked to alterations in genes encoding components of the bacterial cell surface in response to detection of endogenous proteins, such as CAMPs (Newcombe et al., 171).
It is possible that the protein effector responsible for the change in capsule expression is detected via one such regulatory system, leading to transcriptional or structural alterations in capsule biosynthesis or in utilisation of available carbon sources. Therefore, further work might involve investigation of the importance of known two-component regulatory systems in the response to cells and cell supernatants (Tzeng et al., 2008; Newcombe et al., 2005). Identification of candidate genes involved in the response might also be achieved using microarray analysis of bacteria exposed to cells (Claus et al., 2007). In addition, mass spectrometric analysis of the capsule following exposure to cells and supernatants may provide an indication of the nature of the change in capsule. Since the increase in expression was measured by antibody binding it is not clear whether the response observed is due to an increase in the amount of capsule produced or as a result of a change in capsule structure that would affect binding of the mAb against the serogroup B capsule.

LPS sialylation is known to influence the outcome of interactions with phagocytic cells and was also analysed in bacteria following incubation with cells. Both antibody binding assays and silver staining indicated an increase in sialylation following exposure to cells, but not to cellular supernatants. However, the mutant lacking the LPS sialyltransferase Lst also showed this trend. Since this strain is unable to sialylate its LPS, this result was unexpected. Interestingly, analysis of the LPS of strains exposed to HL60 cells indicated a change in the LPS itself following exposure to cells. The nature of the change has not been elucidated and requires detailed analysis of the LPS by mass spectrometry. Preparation of LPS samples for mass spectrometric analysis was attempted but was unsuccessful. Sialic acid is bound to the terminal Gal of the lacto-\(N\)-neotetraose (\(\text{Gal} \beta 1-4\text{GlcNAc} \beta 1-3\text{Gal} \beta 1-4\text{Glc}\)) on the LPS \(\alpha\) chain. It is possible that alteration of the LPS on incubation with cells could influence the level of this sialylatable LPS substrate. However, my results indicate that there may be a fundamental modification in the structure of the LPS that may modify the interaction with the HL60 cells. It is unlikely, however, that this change in LPS is responsible for the difference in
association of live and dead bacteria with HL60 cells since the change was observed in LPS analysed from both live and dead bacteria. Alternatively, the change in LPS may be due to protein binding. Treatment of LPS samples taken from bacteria exposed to HL60 cells with a protease resulted in a loss of the shift in binding of the anti-L3,7,9 LPS mAb, indicating that a protein interaction plays a role. Incubation of bacteria with CAMPs was also performed and a clear shift in LPS was seen following incubation with LL-37. This shift was reduced following incubation with a protease. LL-37 has previously been shown to bind bacterial LPS (Golec, 2007; Rosenfeld et al., 2006), and it is possible that this interaction is responsible for the shift in LPS observed in the presence of HL60 cells. It may not be significant that the shift is not seen with cellular supernatants since it is likely that such an interaction could be dependent on higher concentrations of CAMPs that might only arise in the micro-environment in close proximity to PMNs. However, there is no direct evidence for this. Attempts were made to detect LL-37 in HL60 cells and cell supernatants. However, the sensitivity of a commercially available mAb against LL-37 was too low to enable this analysis to be performed successfully. Western blot analysis was performed with a positive control lane containing 10 µg LL-37, a far higher concentration than could be expected in a normal preparation of 2.5 x 10^5 PMNs. The mAb used for this analysis was sensitive enough to detect this control, but only to a low degree. Thus when lysates of PMNs were tested using the same method, no detection of LL-37 was seen. It is unknown whether this was due to the absence of the CAMP or the low sensitivity of the mAb. Identification of additional candidates responsible for the change in LPS might also be achieved by microarray analysis and investigation of the potential role of known meningococcal two-component systems.

The events following phagocytosis of \textit{N. meningitidis} by PMNs were studied using primary human PMNs from a number of donors instead of HL60 cells to ensure the data were as physiologically relevant as possible. The intracellular fate of the meningococcus following non-opsonic uptake by PMNs has not previously been studied, although work with the
gonococcus has shown that the bacterium survives and replicates within PMNs, and result in a delay in PMN apoptosis. Association of meningococci with primary PMNs was confirmed prior to any further work with these cells to ensure that phagocytosis of bacteria occurred in the absence of opsonins as described with HL60 cells (Chapter 3). Bacterial survival assays were then performed and showed that wild-type MC58 and H44/76 strains were able to survive in the presence of adherent PMNs whilst *E. coli* DH5α did not.

A number of meningococcal enzymes have been previously characterised that are likely to contribute to survival in PMNs. These are predominantly enzymes that act to ameliorate the effects of oxidative stress on the bacterium, and include superoxide dismutase, glutathione peroxidise and catalase (Soler-Garcia and Jerse, 2004; Dunn *et al.*, 2003; Moore and Sparling, 1996). Intracellular survival of the meningococcus in PMNs has potential importance in the progression of disease. The host utilises PMNs to eradicate bacterial infection and to prevent dissemination and systemic disease. The ability to survive PMN killing may be one explanation for the rapid progression of meningococcal disease and septicaemia. Also, since PMNs circulate within the bloodstream and are known to cross damaged regions of the BBB (Wong *et al.*, 2007), it is possible that bacteria could hijack PMNs and use them as a route into the CSF, and a mechanism to avoid bactericidal components within the bloodstream, most crucially the complement pathway. However, as yet there is no direct evidence for this.

The fate of PMNs infected with *N. meningitidis* was also investigated. Analysis of apoptosis by LDH release, caspase-3 activation and cell morphology demonstrated that PMNs infected with wild-type meningococcus showed delayed apoptosis. This trend has also been observed in PMNs infected with the gonococcus (Simons *et al.*, 2005). Inhibition of apoptosis in PMNs may have a dual effect on infection. Firstly, increased survival of the host cell may provide the bacteria with more protection from the hostile environment of the plasma, thus
improving bacterial survival and aiding in progression of the disease. Second, manipulation of the natural apoptotic pathways of PMNs, which are usually very short-lived, is likely to affect the profile of cytokine and chemokines, with a larger number of viable PMNs available to respond to infection. This could lead to a more rapid and intense inflammatory response.

Previous studies have also shown that nitric oxide-regulated expression of cytokines by macrophages is affected by metabolism of nitric oxide by the meningococcus, thus influencing the cytokine release profile and potentially modulating the immune response as a result (Stevanin et al., 2005; Dunn et al., 2003). It is possible that a similar effect occurs in PMNs, and this may also play a role in the uncontrolled inflammatory response that occurs on infection with this bacterium. Further investigation into this effect on PMN viability would include analysis of the cytokine release profiles of PMNs in the presence and absence of the meningococcus and the potential effects on the subsequent inflammatory response. This could include work with additional phagocytic cells such as DCs and lymphocytes such as T- and B-cells. A comparison of the effect of live and dead bacteria in these systems would also be an interesting addition to the trend of reduced uptake of live bacteria by phagocytes described here (Chapter 3) and previously (Jones et al., 2007) and might help explain the difference in response of phagocytes to live and dead bacteria. Further work with mutant strains deficient in genes known to encode key virulence factors and surface components such as capsule, different LPS structures and OMPs would also aim to identify which bacterial components are required to prolong PMN viability. Finally, it would be interesting to analyse bacterial survival and PMN activity in the context of the upper airway organ culture model (Exley et al., 2005a) or in combination with a polarised layer of epithelial cells to mimic the conditions present in the nasopharynx. This work would allow a greater understanding of the role of PMNs and their interactions with host cells during colonisation and the early stages of meningococcal disease.
7.3 Role of lactate

The role of metabolism in interactions of *N. meningitidis* with phagocytic cells has not previously been examined. It has been shown that the ability to utilise lactate via the lactate permease is necessary for a wild-type level of LPS sialylation (Exley et al., 2005a). Analysis of the lactate permease mutant showed that it produces less polysialic acid capsule, indicating that the importance of lactate to the meningococcus is also extended to this critical surface structure. Due to its anti-phagocytic properties, the reduction in capsule may have resulted in the increased association of the MC58ΔlctP strain to HL60 cells compared with the wild-type strain. To verify that the increased association of this mutant was not the result of changes in Opa expression that might affect the interaction with the CD66 receptor, Opa expression was analysed in MC58ΔlctP. However, no effect was seen. It can therefore be postulated that the increased association of the MC58ΔlctP mutant occurs in part as a result of the reduced capsule expression in this strain. As has been previously hypothesised, it is possible that the capsule reduces recognition of the Opa proteins by the phagocytic receptors. The reduced capsule expression in MC58ΔlctP would result in increased exposure of the antigens and thus increased association and internalisation of the bacteria. This hypothesis could be tested by performing phagocytosis assays with strains lacking LctP in an Opa negative background. The phagocytosis assays were performed with fixed strains containing mutations in a range of genes within the sialic acid synthesis pathway. Results indicated that although capsule is involved in preventing phagocytosis by neutrophils, that mutations affecting biosynthesis of sialic acid had a more profound effect than those leading to loss of capsule alone. Additionally, the effect of the lactate permease mutation was not entirely masked by the loss of polysialyl-transferase SiaD. Mutants lacking the sialic acid synthase SiaC associated with HL60 cells to a greater extent than those lacking capsule alone. SiaC is necessary for sialic acid synthesis which is then incorporated either in the capsule or in LPS.
The differential uptake between live and dead bacteria, and the increase in capsule expression as described above for the wild-type strain was also observed with MC58ΔlctP. This suggests that the meningococcus is capable of producing capsular components in response to cells in both the presence and absence of lactate. Interrogation of carbon metabolism of the meningococcus presents two possible carbon sources that may be recruited as alternatives to lactate. Glucose is readily utilised by the bacterium during aerobic respiration whilst pyruvate is also available in the blood and CSF (Siesjo et al., 1968), albeit in far lower concentrations. It is possible that either of these carbon sources may be re-directed in the wild-type and MC58ΔlctP strains, via PEP, to produce sialic acid in the absence of lactate. It is also of note that the reduction in capsule expression in MC58ΔlctP is only seen in bacteria grown on nutrient-rich medium. Analysis of capsule expression following incubation in minimal media such as HBSS showed that there was no difference in expression between the wild-type strain and MC58ΔlctP. It is likely that this is due to reduced capsule expression by the wild-type strain since the only carbon source provided by minimal media is glucose so the inability to utilise lactate in the mutant strain has no effect. This might also explain the loss of the difference in association of the MC58ΔlctP strain with HL60 cells when analysed with live bacteria.

Despite associating with PMNs to the same degree as the wild-type, the MC58ΔlctP strain was significantly attenuated for intracellular survival in primary PMNs. Inhibition of actin polymerisation and ROS production restored survival of the mutant strain to wild-type levels, indicating that the killing mechanism is likely to rely predominantly on the oxidative burst following phagocytosis of the bacteria. There are a number of potential explanations for the attenuation of this strain. It may be the result of a growth defect within PMNs caused by the inability to utilise lactate. *N. gonorrhoeae* is known to utilise cellular lactate for growth so the attenuation may partly be the result of reduced growth rate (Goldner et al., 1979). Also, the
scavenging of lactate from PMNs might be the mechanism by which the gonococcus might reduce the oxidative burst since PMNs require lactate to generate ROS (Topley et al., 1996; Britigan et al., 1988). The same may be true of the meningococcus and this is a possible explanation for the attenuation of this mutant since it would be unable to reduce the oxidative burst by depriving the PMNs of their lactate supply. Unfortunately this could not be demonstrated here as assays to quantify ROS production were unsuccessful due to technical difficulties. Both available ROS production inhibitors, DPI and resveratrol, cross-reacted with the detection agent used to quantify ROS. A variable decrease in ROS detection was observed in the presence of MC58ΔlctP compared to the wild-type, but this could not be validated in the absence of control samples. Finally, the enhanced killing of the MC58ΔlctP strain by PMNs may be due to its reduced ability to express capsule and sialylate LPS. Available intracellular lactate would provide wild-type strains with a substrate for sialic acid production in a similar way to growth in rich medium. Capsule expression and LPS sialylation are thought to be important for intracellular survival and capsule also promotes resistance to CAMPs (Spinosa et al., 2007; Nikulin et al., 2006). However, it is unlikely that this is the reason for attenuation since MC58ΔlctP bacteria recovered from HL60 cells show wild-type levels of capsule expression.

The importance of lactate acquisition to the meningococcus is clearly multi-faceted. Previous studies have shown its importance in resistance against complement-mediated killing and virulence (Exley et al., 2005a) and the work described here has indicated the role of lactate in survival within PMNs. However, it appears that the contribution of lactate in these distinct locations is likely to be different. Since lactate is an important carbon source for the meningococcus, and is present in blood, CSF and intracellularly, metabolism of this carbon source is clearly crucial to survival of the meningococcus and plays a role in more than one aspect of virulence. The work described here may also be relevant to the interaction of the
meningococcus with other phagocytic cells such as macrophages and DCs, demonstrating the wide-reaching effects of metabolism to this bacterial pathogen.

### 7.4 Role of Glutamate

The importance of glutamate metabolism in interactions of *N. meningitidis* with phagocytic cells has not been previously studied. Production of sialic acid for capsule expression and LPS sialylation, which are key components in the interaction between the meningococcus and host cells, relies on carbon metabolism. A clear link between lactate metabolism and sialic acid production has been demonstrated and this was also investigated in strains deficient in four components of glutamate metabolism. The mutant strains were lacking either one of the two glutamate import enzymes, PerM or GltS (which operate in low sodium and high sodium concentrations respectively) GdhA, the glutamate dehydrogenase, and GhdR, the transcriptional regulator of GhdA. Analysis of capsule expression and LPS sialylation in each of these mutants demonstrated no change in either of these surface components. When analysed for association with the HL60 cell line, there was also no difference in association of any of the mutants compared with the wild-type H44/76 strain, with the possible exception of the H44/76ΔgdhR strain, although the variation observed with this strain means it is difficult to draw conclusions about this result.

The absence of an effect of glutamate metabolism on capsule expression and LPS sialylation, and on association with HL60 cells is not surprising. During metabolism, some glutamate is redirected to produce 2-oxoglutarate, a component of the citrate cycle, which indirectly provides oxaloacetate for the production of PEP (www.genome.ad.jp/kegg/pathway). However, metabolism of glutamate is not closely linked to sialic acid production in the same way as lactate, which does play a role in capsule biosynthesis and LPS sialylation. It is therefore likely that even if glutamate does contribute to the PEP required for normal sialic acid production, that in the absence of glutamate import or production, this loss could be
compensated for by a more immediate carbon sources, such as lactate, pyruvate or glucose. Since there was no effect on capsule or LPS, it would therefore be expected that association of bacteria with HL60 cells would not be affected in the absence of an intact glutamate metabolism pathway.

The ability of the H44/76ΔperM strain to cause bacteraemia in the infant rat model was analysed. Despite the absence of an effect on capsule and LPS sialylation in this strain, it was significantly attenuated *in vivo* compared to the wild-type strain, indicating an effect of the mutation somewhere other than in the bacterial surface components. Interrogation of the metabolic pathway associated with glutamate metabolism revealed a potential link between glutamate and glutathione production (Archibald and Duong, 1986). In its reduced state, glutathione is known to act as a scavenger of free radicals and as an electron donor to restore oxidised macromolecules (Carmel-Harel and Storz, 2000), and in the gonococcus glutathione is thought to play an important role in defence against oxidative stress. Thus it was suggested that the attenuation of H44/76ΔperM may be due to an inability to resist oxidative damage due to low cellular content of glutathione resulting from the mutation in the glutamate transporter PerM. Subsequent analysis of resistance of the H44/76ΔperM strain to oxidative agents H$_2$O$_2$ and paraquat performed by Caterina Monaco at The University of Lecce revealed that this strain is significantly more sensitive to oxidative stress than the wild-type strain. The oxidative burst elicited by PMNs relies on production of ROS that exert oxidative stress on bacterial pathogens. Therefore, the strain deficient in PerM was analysed for intracellular survival in PMNs, where it showed a significant defect in survival compared to the wild-type strain. Inhibition of actin polymerisation and ROS production in PMNs restored survival of the mutant to wild-type levels, indicating that the susceptibility of the mutant relied upon internalisation and subsequent ROS production by PMNs. This result therefore indicates that the attenuation of H44/76ΔperM *in vivo* is likely to be due to its inability to withstand the oxidative burst of phagocytic cells such as PMNs and
macrophages. This represents an interesting link between carbon/nitrogen metabolism and oxidation resistance mechanisms. Analysis of glutathione production in the H44/76ΔperM strain has been performed at The University of Lecce. This work has shown that the strain lacking PerM has significantly reduced levels of intracellular glutathione compared to the wild-type strain, indicating that this is a likely cause of the attenuation of this mutant in the face of oxidative stress (Unpublished). Glutathione was also analysed in mutants lacking the glutathione synthetase Gsh. In this background, a deficiency in PerM had no additional effect on glutathione concentrations, indicating that these enzymes act in the same pathway (Unpublished). Additional work has also been completed by Christoph Tang, with the assistance of the Max Plank Institute in Berlin, to analyse the survival of the H44/76ΔperM strain in mice lacking the gp91 phox subunit of the NADPH oxidase. The PMNs of these animals are unable to elicit an oxidative burst. This work has shown that survival of the mutant strain is restored to wild-type levels during infection of these knockout animals, confirming the role of the oxidative burst in the attenuation of this mutant (Unpublished). Further investigation into the importance of glutamate metabolism would require complementation of the mutants described here, and quantification of ROS production during assays with PMNs and in vivo.

7.5 Role of DNA repair

Investigation into the interaction between N. meningitidis and PMNs indicates that the bacterium is capable of responding to the presence of PMNs by altering certain surface structure components in order to evade phagocytosis. However, analysis by microscopy shows that a significant number of bacteria are still internalised. Initiation of phagocytosis triggers the signalling pathways involved in the PMN oxidative burst that culminates in the release of potent bactericidal compounds into the phagosome (Babior et al., 1976). Even if the bacterium was to survive this assault, the resulting damage to the bacterial DNA would likely
be cytotoxic. AP endonucleases act within the BER pathway to ameliorate the effects of oxidative damage to cellular DNA (Wilson et al., 2003). Therefore the neisserial AP endonuclease would be predicted to play a role in protecting the bacterial genome from this attack. Searches of the serogroup B MC58 genome revealed two putative AP endonucleases, NExo (NMB0399) and NApe (NMB2082). Despite possessing the closest identity with the E. coli enzyme ExoIII, NExo does not display AP endonuclease activity. Instead NExo appears to be specialised for exonuclease and efficient 3′dRp activity (Carpenter et al., 2007). Surprisingly, the human HAP1 paralogue, NApe, is actually the functional AP endonuclease. Interrogation of the structures of NExo and NApe revealed that their differing activities can be attributed to an amino acid change in the DNA binding site. Substitution of His₁⁶⁷ in NExo with glycine or serine, the residues found in ExoIII and HAP1 respectively, conferred AP endonuclease activity (Carpenter et al., 2007). This work highlights the importance of combining biochemical and structural characterisation of hypothetical proteins with investigation of their function. Most previous studies have relied on identification of proteins purely by sequence homology, followed by characterisation of mutants lacking the genes encoding those proteins. The work described here has demonstrated that although NExo is the closest bacterial AP endonuclease homologue, it is NApe that fulfils this role and therefore care should be taken in assuming that the closest homologues of known proteins will perform an identical function in vivo.

NApe is an AP endonuclease and it is therefore thought to act within the BER pathway. However, since NExo acts independently, despite its homology to ExoIII, its role in DNA repair is more ambiguous. Exonuclease activity has previously been ascribed to AP endonucleases and DNA polymerases while 3′dRp activity is restricted to AP endonucleases. NExo is therefore a novel enzyme. It is possible that it may act within BER, within a pathway distinct from NApe. Alternatively, NExo may act in a different pathway altogether, or may potentially act alone to clear blocked ends and cleave terminal
mismatched bases. Both NExo and NApe are required for resistance to oxidative stress and for virulence. A mutant lacking both enzymes is significantly more attenuated under oxidative stress and \textit{in vivo}, indicating that despite NApe possessing the same activities, that NExo has a specific role independent of NApe. The double mutant was also significantly attenuated for intracellular survival in PMNs. Survival was restored to wild-type levels when ROS production and actin polymerisation was inhibited in PMNs, indicating that attenuation of the mutant lacking NExo and NApe was due to killing by the oxidative burst following phagocytosis. This supports the hypothesis that NApe plays a role in BER since BER acts on damage caused by oxidative stress, such as that elicited by the oxidative burst. This work also suggests that NExo is linked with BER since a single mutant deficient in NExo is sensitive to oxidative stress.

The roles of each enzyme cannot be extrapolated individually to their importance in survival in PMNs since survival of only the double mutant strain, MC58\textit{ΔnexoΔnape} was investigated and further work would require characterisation of the MC58\textit{Δnexo} and MC58\textit{Δnape} to identify the role each enzyme plays in survival in the presence of PMNs. Complementation of these mutations would also be required for further work, and it would be interesting to analyse the survival of these strains in mice lacking the gp91 phox subunit of the NADPH oxidase to confirm their role in ameliorating the effects of the oxidative burst. However, the data obtained from analysis of survival in the presence of oxidative agents and \textit{in vivo} would suggest that both NExo and NApe are likely to be involved in resistance to the oxidative burst. Attenuation of mutants lacking NExo and NApe in the presence of oxidative stress is likely to be the result of an inability to repair damage to DNA that would usually be repaired by BER. Accumulation of lesions such as 8-oxoG, Fapy, pyrimidine dimers and glycols, and AP sites would result in cytotoxic mutations. Bacteria deficient in NApe would lack an AP endonuclease, and thus would be unable to excise AP lesions resulting either directly from oxidation or from the activity of glycosylases. The strain lacking NExo could have severely
impaired ability to repair blocked ends and terminal mismatched bases. This process may still be carried out by NApe, although the rate of exonuclease activity of NExo is far higher. Further analysis of the role of NExo within DNA repair would involve the analysis of a range of bacterial mutants lacking different enzymes known to act within BER and other DNA repair pathways. These include MutT, MutY, Fpg, RecA, UvrA and UvrB (Davidsen et al., 2005; Black et al., 1997; Black et al., 1995). It would also be interesting to analyse the extent and nature of DNA damage occurring within the genomes of the wild-type and mutant strains following exposure to oxidative stress to determine the lesions that NExo and NApe act on.

The pairing of NExo- and NApe-like enzymes is not unique to the meningococcus. A search of the genomes of 297 bacterial species performed by Elisabeth Carpenter at the Centre for Structural Biology at Imperial College revealed the NExo-NApe pairing in widely divergent species (Figure 7.1). These include other strains of *N. meningitidis*, *N. gonorrhoeae* and other pathogenic species such as *Pseudomonas aeruginosa* and *Bordetella pertussis*. The pairing also extends into non-pathogenic species. There are three main hypotheses to explain the occurrence of this pairing. First, it is possible that one of the genes was acquired by horizontal transfer from a different species or from a eukaryotic host. However, analysis of the GC content of *nexo* and *nape* (data not shown) show that there is no significant difference between them and the remainder of the genome, providing no evidence that they originated by horizontal transfer. A second possible explanation is that one of the genes could have arisen by duplication. As shown by the NExo substitution mutants, a single change in the amino acid sequence of the DNA binding site alters the binding specificity, and therefore the activity, of the enzyme. Such a mutation may have become established in the genome if it confers an evolutionary advantage and would therefore have been selected for and established within the population by genetic drift. However, this hypothesis is also unlikely since *nexo* and *nape* are more similar to their *E. coli* and human counterparts than they are to each other. The most likely explanation is that the NExo-NApe pairing has arisen
as a result of convergent evolution. The diversity of species in which the pairing is found suggests it is likely that it arose numerous times, supporting the importance of both enzymes to survival. It has been conserved within these species as it confers an evolutionary advantage. In the case of the meningococcus this advantage is translated as the ability to resist killing by oxidising agents. This could also be the case for the other pathogenic species with the enzyme pairing, and may protect species that are not in contact with eukaryotic hosts from alternative forms of oxidative stress.

Figure 7.1 The NExo / NApe pairing is found in divergent bacterial species

Phylogenetic analysis of 297 sequenced bacterial genomes was performed to determine the frequency of the NExo / NApe pairing. The difference between NExo and ExoIII paralogues was defined by the presence of a small or large sidechain in the position equivalent to NExo His167. The number of species with both NExo and NApe paralogues is given after the phylum name, and the number of genomes searched is in brackets. Phyla with pairs of these enzymes are shown in red. The NExo/NApe pairing found in three bacterial phyla, the actinobacteria, gammaproteobacteria and betaproteobacteria. This phylogenetic analysis was performed by Elisabeth Carpenter at the Centre for Structural Biology at Imperial College London, and this image is reproduced with her kind permission.
Interestingly, a conflicting clue towards the origin of the NExo-NApe pairing in the meningococcus exists within the gene encoding NExo. Interrogation of the NExo gene sequence reveals the DNA Uptake Sequence, 5′-GGCGGCGGCG-3′ within the coding region. Despite the similarity in GC content between *nexo* and *nape*, it is possible that NExo was acquired from an external source, most likely of bacterial origin due to its homology with ExoIII. The novel activity of NExo endows the meningococcus with efficient exonuclease and 3′dRp activity in addition to that performed by NApe. The work described here has demonstrated the importance of BER and its related enzymes to survival of oxidative stress and the addition of NExo to the meningococcal enzyme profile may have aided in the ability of the bacterium to survive in the hostile intracellular environment encountered within phagocytic cells, thus contributing to its virulence. The meningococcal genome, like many other bacterial pathogens, shows a high degree of plasticity, allowing high mutation rates leading to rapid evolution and adaptation to the human host. However, in circumstances such as those encountered within the intracellular compartment, the bacterium would require strict control of DNA damage to prevent build-up of cytotoxic mutations. It is likely that this role is fulfilled by NExo and NApe, and that this was the reason for the evolution of the NExo-NApe pairing in the meningococcus.

### 7.6 Summary

The aim of this project was to characterise the non-opsonic interaction between *N. meningitidis* and PMNs. This was performed using the human PMN cell line HL60 to investigate the events involved in initial contact and phagocytosis of the meningococcus, and primary human PMNs to investigate the fate of the bacteria and PMNs during the interaction. This work has demonstrated that although the meningococcus is internalised by PMNs, and that live bacteria actively respond to the presence of the cells, resulting in increased expression of the anti-phagocytic polysialic acid capsule and a change in LPS structure. It is
possible that these responses result in reduced uptake of bacteria by PMNs. Intracellular meningococci are able to survive within PMNs. This ability relies on the ability to utilise lactate, to import glutamate in low sodium conditions, and to perform DNA repair via the newly identified NExo-NApe enzyme pairing. The presence of the meningococcus also results in a delay in apoptosis of PMNs.

The implications of this work for the human host are that since PMNs are unable to eradicate the meningococcus, their primary role in defence against disease is compromised. The additional manipulation of the life span of PMNs through delaying apoptosis suggests that this may be a mechanism behind the uncontrolled inflammatory response that is a hallmark of infection with \textit{N. meningitidis}. PMNs may therefore represent a safe haven for bacteria in the bloodstream, allowing them to avoid complement-mediated killing, and potentially providing an alternative route into the CSF. The absence of an efficient cell-mediated immune response combined with this manipulation of PMN function may partly explain the rapid progression of meningococcal disease in many cases, particularly in individuals lacking components of the complement pathway.

Carbon metabolism has been previously shown to play a crucial factor in the pathogenesis and phenotypic traits of the meningococcus, particularly in growth and expression of surface structures. The work described here has demonstrated additional roles for the metabolism of both lactate and glutamate in the progression of disease by identifying the link between carbon metabolism and resistance to killing by intracellular ROS. Clearly the role of bacterial metabolism in disease is multi-faceted and closely linked to the immediate environment within the host.
DNA repair has not been extensively studied in the meningococcus and the identification of the NExo-NApe pairing raises important questions about the validity of work where the function of enzymes is assumed from the activity of the closest genetic homologue. This work has also shown that despite the extensive plasticity of the meningococcal genome and frequency of mutator phenotypes in highly virulent strains, that DNA repair is also crucial to the survival of the bacterium. The meningococcus expresses an additional enzyme, NExo, that appears to work in parallel to BER to allow for survival in the hostile intracellular environment. The frequency of the pairing for NExo and NApe homologues in diverse bacterial strains indicates that this is an important and valuable acquisition.

It is unlikely that this work will contribute to efforts to develop or improve current vaccine design. Although enzymes involved in metabolic pathways have previously been suggested as vaccine candidates, LctP and PerM are located in the membrane and therefore are not exposed in the bacterial surface (Exley et al., 2005a; Pagliarulo et al., 2004). NExo and NApe are also unlikely candidates due to their intracellular location and close homology to the human AP endonuclease. However, this work does contribute towards a better understanding of the mechanisms behind the pathogenesis of *N. meningitidis* and the ways in which meningococcal disease develops. Such knowledge aids in the development of more effective treatment regimes and may help in more accurate planning of vaccination strategy.
Bibliography


