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**Investigating the Therapeutic Potential of Genetically Modified *Neisseria lactamica***

by

**Zoe Caroline Pounce**

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# ABSTRACT

FACULTY OF MEDICINE

Infection and Immunity

Thesis for the degree of Doctor of Philosophy

**Investigating the Therapeutic Potential of Genetically Modified *Neisseria lactamica***

Zoe Caroline Pounce

*Neisseria meningitidis* colonises the human nasopharynx and can invade through the epithelium to cause rapidly progressing, potentially fatal disease. Carriage of the commensal *Neisseria lactamica* correlates with decreased colonisation with *N. meningitidis* and reduced invasive meningococcal disease (IMD), making *N. lactamica* a candidate for an anti-meningococcal vaccine or probiotic. Intranasal inoculation of *N. lactamica* showed a good safety profile, but only 34% of those challenged with this species became colonised. Intramuscular injection of *N. lactamica*-derived outer membrane vesicles (OMVs) demonstrated their potential as a mucosal adjuvant but with limited anti-meningococcal immunogenicity. This project investigated the use of genetically modified (GM) *N. lactamica* to enhance the potential of this organism to protect against IMD.

*N. lactamica* previously proved resistant to genetic modification. Here we showed that a significant barrier to transformation is the activity of restriction enzymes, which was partially overcome by the incorporation of exclusively hypermethylated cytosine residues into donor DNA, by bespoke PCR amplification. Although it was observed that *N. lactamica* Y92-1009 is highly resistant to heterologous genetic recombination *in vivo, in vitro* and in biofilms, this technique allowed us to accurately target genetic modifications to various chromosomal loci in this strain. Multiple factors affecting the efficiency of transformation were identified, including the extent of homology between donor and chromosomal DNA and the number of DNA uptake sequences. Transformation was found to be dependent on the presence of type 4 pili, which were also required for efficient interaction of *N. lactamica* with epithelial cells.

Meningococcal immunogen PorA was selected for the proof of principle that heterologous antigens could be expressed in *N. lactamica.* With the view to progress this strain to human challenge, the protein was introduced in an antibiotic-resistance-free manner, using the deletion and re-introduction of β-galactosidase as a marker of transformation on X-Gal-containing agar. PorA P1.7,16 was expressed at modest levels but the expression of other PorA types was unsuccessful. PorA P1.7,16 was presented on the surface of bacteria and on OMVs. Intraperitoneal injection of mice with PorA-containing OMVs induced production of serum bactericidal antibodies (SBA) that were active against both *N. meningitidis* H44/76 and MC58.

A genetic enhancer sequence was identified upstream of PorA in *N. meningitidis* and was used to modulate PorA expression in *N. lactamica.* In order for *N. lactamica* to tolerate phase variable expression of PorA, the enhancer sequence was truncated and expression reduced. At this reduced level, a panel of three PorA-expressing strains of *N. lactamica* were assessed for progression to human challenge. Whilst strains were comparable, genetically stable and antibiotic susceptible, the low-level PorA expression meant that phase variation could not be detected and OMVs derived from these strains did not induce SBA activity in mice.

This thesis presents the first steps in developing GM strains of *N. lactamica* with the potential to reduce nasopharyngeal colonisation with *N. meningitidis* and to vaccinate against invasive meningococcal disease.

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# Academic Thesis: Declaration Of Authorship

I, Zoe Pounce, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Investigating the Therapeutic Potential of Genetically Modified *Neisseria lactamica*

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: .....

Date: .....

# Acknowledgements

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This thesis is a summary of my PhD project but it is a product of the hard work and support of many different people. Thank you to all of them.

# Definitions and Abbreviations

| | |
|---|---|
| AEC | 3-amino-9-ethylcarbazole |
| β-gal | β-galactosidase |
| bp | base pair |
| BSA | Bovine Serum Albumin |
| CBA | Columbia Blood Agar |
| CCUG | Culture Collection of the University of Göteborg |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CFU | Colony Forming Units |
| CREN | Cintact Regulatory Element of *Neisseria* |
| dCTP | deoxycytidine triphosphate |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| dNTP | deoxyribonucleotide triphosphate |
| dOMV | Deoxycholate-extracted OMV |
| DUS | DNA Uptake Sequence |
| FAM | 5'-fluorescein amidite |
| FCS | Foetal Calf Serum |
| fHbp | factor H binding protein |
| gDNA | genomic DNA |
| GM | Genetically Modified |
| GMP | Good Manufacturing Practice |
| hmPCR | hympermethylated Polymerase Chain Reaction |
| HRP | Horseradish Peroxidase |
| Ig | Immunoglobulin |
| IMD | Invasive Meningococcal Disease |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| LB | Luria-Bertani Broth |
| LOS | Lipooligosaccharide |
| LPS | Lipopolysaccharide |
| MALT | Mucosa Associated Lymphoid Tissue |
| MC.7 | Modified Catlin media |
| MenB | Serogroup B *Neisseria meningitidis* |
| MFI | Mean Fluorescence Intensity |

| | |
|---|---|
| MHB | Mueller-Hinton Broth |
| MIC | Minimum Inhibitory Concentration |
| MOI | Multiplicity of Infection |
| Ncin | *Neisseria cinerea* |
| NHBA | *Neisseria* Heparin Binding Antigen |
| NHCIS1 | Non-Homologous Chromosomal Insertion Site 1 |
| Nlac | *Neisseria lactamica* |
| Nmen | *Neisseria meningitidis* |
| OD | Optical Density |
| OM | Otitis Media |
| OMV | Outer Membrane Vesicle |
| ONPG | *Ortho*-Nitrophenyl-β-Galactoside |
| ORF | Open Reading Frame |
| PAMP | Pathogen-Associated Molecular Pattern |
| PBS | Phosphate-Buffered Saline |
| PBS-t | 0.05 % Tween-20 in PBS |
| PCR | Polymerase Chain Reaction |
| PHE | Public Health England |
| PV | Phase Variable |
| RMS | Restriction/Modification System |
| RNA | Ribonucleic Acid |
| RPM | Revolutions per Minute |
| SA | β-galactosidase Specific Activity |
| SBA | Serum Bactericidal Antibody |
| SDM | Site-Directed Mutagenesis |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| S-DUS | Scrambled DUS |
| SEM | Scanning Electron Microscopy |
| SNP | Single Nucleotide Polymorphism |
| ssDNA | single stranded DNA |
| SSM | Slip-Strand Mispairing |
| STI | Sexually Transmitted Infection |
| T4P | Type 4 Pili |
| TEM | Transmission Electron Microscopy |
| Tm | Melting Temperature |
| TSB | Tryptone Soy Broth |

| | |
|---|---|
| UAS | Upstream Activation Sequence |
| USS | Uptake Signal Sequence |
| WT | Wild Type |
| W/V | Weight/Volume |
| X-Gal | 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside |

# Chapter 1 Introduction

Mucosal surfaces of the human body, such as the gut, respiratory tract and genitourinary tract, are home to a wide variety of microorganisms. Although these environmental niches may act as reservoirs for potential pathogens, the milieu of commensal organisms plays an important role in regulating metabolic functions, supporting immune homeostasis and contributing to the prevention of autoimmune disease. Furthermore, by occupying otherwise vacant niches, commensal organisms can protect against potential pathogens, generating competition for nutrients, receptor binding and other resources. Studies have shown that some bacteria, either directly or indirectly, attack other species, preventing co-colonisation in a given habitat (Dawid et al., 2007; Deasy et al., 2015; Shak et al., 2013). Biodiversity at mucosal surfaces is believed to promote a 'healthy' phenotype, whereas a high density of some species has been associated with disease. For example, in the nasopharynx of non-viral pneumonia patients, a high abundance of *Haemophilus influenzae* was observed, whilst the nasopharynx of healthy subjects contained a wider variety of microorganisms (Sakwinska et al., 2014). This demonstrates the role of microbial colonisation at mucosal surfaces in maintaining health, as well as in initiating disease.

## 1.1 The Nasopharynx

### 1.1.1 Anatomy of the Nasopharynx

The nasopharynx is shown in Figure 1. It forms part of the upper respiratory tract, situated between the anterior nares (or nostrils) and oropharynx. Its mucosal surface is home to various lymphoid tissues and is made up of pseudostratified ciliated columnar epithelium containing goblet cells, which secrete mucin. Areas of stratified squamous epithelium appear in the nasopharynx with age (Ali, 1965). The Eustachian tube directly connects the nasopharynx to the middle ear.

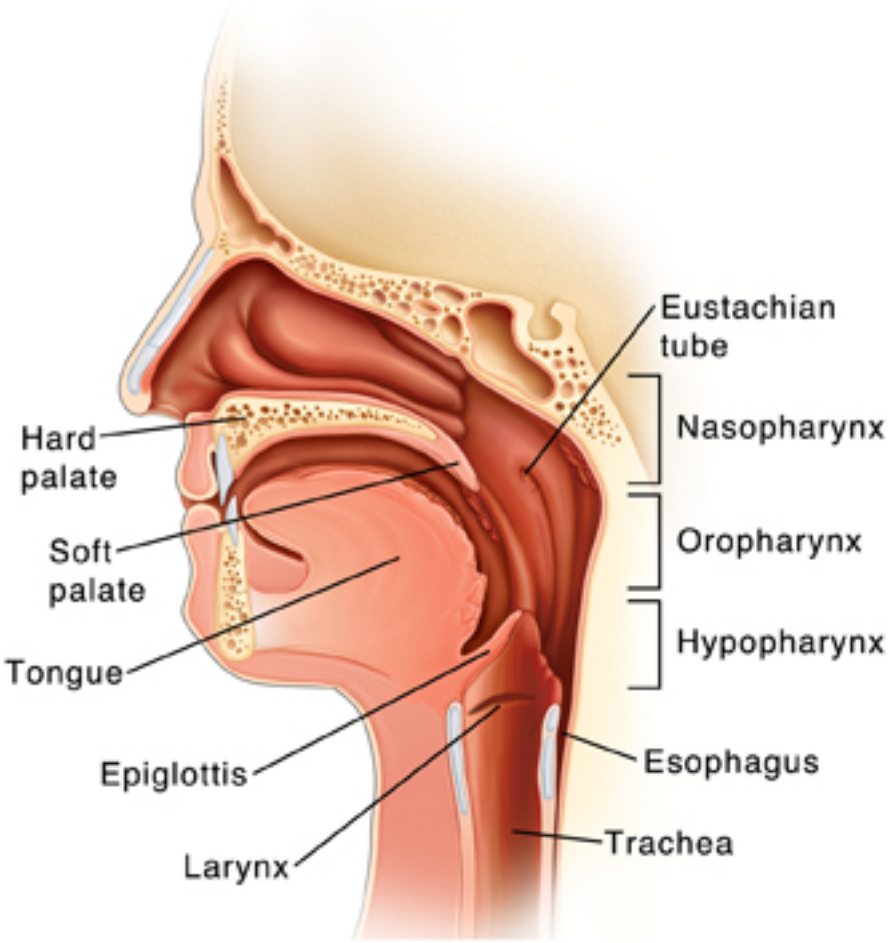



Figure 1. Components of the Human Upper Respiratory Tract

The nasopharynx is situated behind the nose and above the oropharynx. It contains the opening of the Eustachian tube, which connects the nasopharynx to the middle ear.
Image taken from https://www.fairview.org/HealthLibrary/Article/84507

### 1.1.2 The Nasopharyngeal Microbiome

Due to its close proximity to the middle ear, the nasopharynx is a frequent reservoir for causative agents of middle ear infection, such as *H. influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis* (Revai et al., 2008). Middle ear infection, or otitis media (OM), is characterised by inflammation and a build up of fluid behind the eardrum, which causes earache and fever (NHS Choices, 2016). The global incidence of acute OM is 10.85%, with over 50% of cases occurring in children under the age of 5 (Monasta et al., 2012). OM is thought to affect 25% of children by the age of 10 in the UK (NHS Choices, 2016). It is usually self-limiting, or can be treated with antibiotics, and mortality is very low in the developed world (Monasta et al., 2012).

Colonisation of the nasopharynx can also lead to the development of diseases with much higher mortality. Nasopharyngeal colonisation is necessary, but not sufficient, to initiate invasive disease by *S. pneumoniae* or *Neisseria meningitidis.* Following nasopharyngeal colonisation, these bacteria can invade through the epithelium, which may result in bacteraemia, meningitis and/or pneumonia. Whilst invasion is an infrequent consequence of colonisation by these species, there is a significant risk of mortality or permanent disability as a result (Drijkoningen and Rohde, 2014; Jafri et al., 2013; Ladhani et al., 2015). These diseases are much less prevalent than OM, however their high mortality has made them a target for vaccination (Marshall et al., 2016; Torres et al., 2015). Invasive meningococcal disease will be discussed more in Section 1.2.3.

The four major potentially pathogenic bacteria known to occupy the nasopharynx (namely *H. influenzae, S. pneumoniae, M. catarrhalis* and *N. meningitidis*) are constantly carried asymptomatically by a subgroup of the population. These bacteria generally act as commensal organisms and in all cases, disease is a relatively infrequent consequence of carriage.

In healthy children, the most prevalent colonisers of the nasopharynx are a variety of Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria (Bogaert et al., 2011; Teo et al., 2015). These include high proportions of members of the genus *Streptococcus, Rothia, Prevotella, Gemella, Veillonella, Fusobacteria, Haemophilus* and *Neisseria* (Stearns et al., 2015). The diversity of the nasopharyngeal microbiome appears to increase between childhood and maturity, with a concurrent large increase in bacterial load (Stearns et al., 2015). One analysis in 10 healthy adults (aged 18-21) revealed that *Corynebacterium* (Actinobacteria) and *Dolosigranulum*, *Staphylococcus* and *Streptococcus* (Firmicutes) were the most prevalent genera (Cremers et al., 2014). A more recent study, analysing both children and their parents identified high levels of the following in the adult population: Lachnospiraceae, *Staphylococcus and*

*Streptococcus* (Firmicutes), *Sphingobacterium* and *Prevotella* (Bacteriodetes) and *Bifidobacteria*, *Rothia* and *Propionibacterium* (Actinobacteria) (Stearns et al., 2015).

### 1.1.3 The Immune System and the Nasopharyngeal Microbiome

Despite the nasopharynx being a harbourer of potential pathogens, relatively little is known about regulation of the microbiome and the involvement of the immune system at this site.

Waldeyer's ring is comprised of multiple areas of lymphatic tissue in the nasopharynx and oropharynx, as shown in Figure 2. The adenoid, tubal tonsils, palatine tonsils and lingual tonsil function as part of Mucosa Associated Lymphoid Tissue (MALT) and are strategically located to come into contact with both airborne and alimentary pathogens.

MALT comprises secondary lymphoid tissue and effector sites. In secondary lymphoid tissues, B cells undergo clonal expansion, following antigen-specific T-cell activation. This causes Immunoglobulin A (IgA) class switching and migration of B and T cells to effector sites where antibodies bind antigen (Cesta, 2006). MALT is not entirely isolated from distinct anatomical sites. Antigen presentation and B-cell activation at one mucosal surface has been shown to induce IgA secretion in MALT elsewhere (Hiroi et al., 1998; Kiyono and Fukuyama, 2004; Rudin et al., 1998). It has been proposed that mucosal vaccination primes the most appropriate immune response against mucosal pathogens, as the greatest antibody response is observed at mucosal surfaces, where the pathogen will most likely be encountered (Rudin et al., 1998). Despite this, the majority of vaccines for mucosal pathogens continue to be designed for parenteral administration, which also presents a multitude of logistical issues for under-developed and developing countries, including the requirement for trained healthcare professionals, sterile needles and a cold chain.

As a mucosal surface, the majority of immunological protection in the nasopharynx is thought to be conferred by IgA. IgA is secreted by B cells and then transported across the epithelium into the lumen. When bound to bacteria in the nasopharynx, IgA is thought to interact with specific receptors and immune mediators either to eradicate pathogens and prevent infection, or to promote colonisation of commensals. It is not in the interest of the host to promote sterilising immunity in response to commensal colonisation, as this may result in the vacation of a niche for potential pathogenic attack. Some bacteria have developed mechanisms to overcome IgA activity, such as the IgA binding proteins produced by many strains of Group A *Streptococci* (Johnsson et al., 1994).

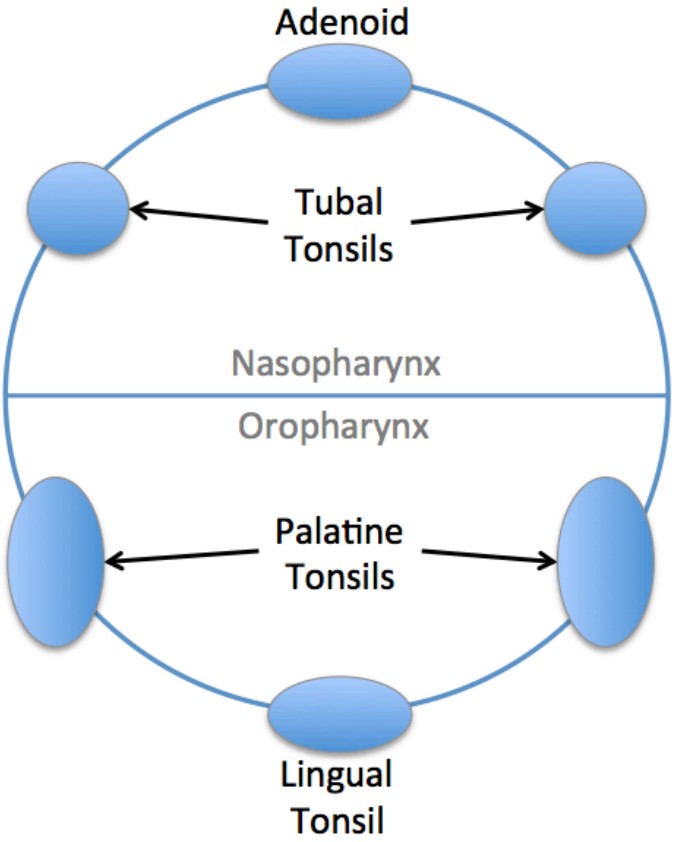



Figure 2. Waldeyer's Ring

Waldeyer's Ring comprises secondary lymphoid tissue split between the nasopharynx (adenoid and tubal tonsils) and oropharynx (palatine tonsils and lingual tonsil). This collection of mucosa-associated lymphoid tissue (MALT) comes into contact with both respiratory and alimentary pathogens.
Image re-created and adapted from http://epomedicine.com/medical-students/applied-anatomy-of-palatine-tonsils/

It is becoming increasingly understood that IgD also plays a substantial role in immunity at mucosal surfaces. IgD is often co-expressed with IgM on B cells, where it functions as an antigen receptor (Finkelman et al., 1976). However, IgD is also found in blood and mucosal secretions and on the surface of basophils (Finkelman et al., 1979; Rowe and Fahey, 1965). IgD cross-linking induces the release of pro-inflammatory and antimicrobial factors from basophils and triggers B-cell activation (Chen et al., 2009). IgM−IgD+ B cells have also been identified, and are abundant in tonsillar and nasal tissues (Chen et al., 2009). IgM−IgD+ B cells are unusual, in that the IgD is biased towards the λ light chain (Arpin et al., 1998). Those in the upper respiratory tract also express hypermutated and clonally related V(D)J genes (Liu et al., 1996) and are polyreactive (Koelsch et al., 2007). These cells are therefore highly differentiated and specialised immune components for protection in the upper respiratory tract mucosa.

IgD-binding proteins have been identified in *M. catarrhalis* and *H. influenzae*. This binding of respiratory organisms to B cells is thought to activate pro-inflammatory and B cell-activating pathways in basophils (Chen et al., 2009). A putative IgD-binding protein has recently been identified in the nasopharyngeal commensal organism, *Neisseria lactamica,* and it appears that its interaction with IgD is biased towards the λ light chain (A. Vaughan, unpublished data).

## 1.2 The Pathobiont: *Neisseria meningitidis*

### 1.2.1 Microbiology

A pathobiont is defined as an organism that ordinarily lives symbiotically with the host by has the potential to become pathogenic. The genus *Neisseria* contains two species with the potential to cause disease in humans: *N. meningitidis* and *N. gonorrhoeae,* alongside a number of purely commensal species. Whilst *N. gonorrhoeae* colonises and infects the urogenital tract and is generally considered pathogenic, *N. meningitidis* colonises the nasopharynx, where it is ordinarily symbiotic. On rare occasions, *N. meningitidis* is capable of invading through the epithelium and causing invasive meningococcal disease (IMD).

*N. meningitidis*, or the meningococci, are Gram-negative, oxidase-positive, aerobic diplococci and grow optimally at 35-37°C. They are human specific and exclusively colonise the nasopharynx, except during pathogenesis. Some strains express polysaccharides, which form a capsule around the outer membrane, as shown in Figure 3. The capsule allows the organism to resist phagocytosis or complement-mediated killing in the bloodstream (Uria et al., 2008) and is therefore required in

invasive strains (with few rare exceptions (Findlow et al., 2007; Hoang et al., 2005; Johswich et al., 2012)). The meningococci express diverse capsule polysaccharides, which are the basis for serogroup classification. The majority of meningococcal serogroups are not associated with disease and the capsule is not required for nasopharyngeal colonisation. In fact, *N. meningitidis* down-regulates capsule production upon contact with epithelial cells (Deghmane et al., 2002). IMD cases worldwide are almost exclusively caused by six serogroups of *N. meningitidis*: serogroups A, B, C, W, X and Y (Abio et al., 2013). The meningococci are further classified into subtype and serosubtype according to variations in the cell surface exposed regions of outer membrane proteins porin B (PorB) and porin A (PorA) respectively (Frasch et al., 1985).

The surface structures of meningococci are constantly changing in their expression levels and antigenic epitopes, making them particularly elusive to the immune system (Alamro et al., 2014). Temperature-dependent differential gene expression has been observed in three virulence genes in *N. meningitidis*, where it has been demonstrated that RNA secondary structures prevent translation of genes necessary for capsule biosynthesis, sialylation of lipopolysaccharide (LPS) and expression of factor H binding protein (fHbp) at 30°C, but these structures denature and permit translation at 37°C (Loh et al., 2013). The bacteria experience temperatures of around 37°C in the blood during invasive disease, but during colonisation of the nasopharynx, the temperature is lower. This suggests that the expression of these temperature-regulated genes is beneficial during invasive disease only.

Surface-expressed proteins in the meningococci are also subject to phase variation. The meningococcal genome includes multiple tracts of repeated bases, which can be either intragenic or in the promoter region of a gene (Alamro et al., 2014). For example, PorA has a tract of around 11 guanosine residues (poly-G tract) in the promoter and a 7 base pair (bp) tract of adenosine residues (poly-A tract) near the 5' end of the coding region (Tauseef et al., 2013). During DNA replication and transcription, these polymeric tracts are vulnerable to errors by polymerase enzymes, where the polymerase slips and either increases or decreases the number of residues in the tract. This process, known as slip-strand mispairing (SSM), can result in a change of gene expression levels (when the poly tract is in the promoter) or complete abrogation of gene expression (when it is in the coding region). Phase variation causes mixed populations, whereby a proportion of bacteria are expressing a gene and the remaining cells are not expressing it, or expressing it at different levels, at any given time. With 82 candidate genes for phase variation in *N. meningitidis* MC58 alone (Snyder et al., 2001), there is the potential for incredibly varied populations, expressing each of these genes at different levels. This means that should a selective pressure arise for any state, at least a subset of cells will survive, providing a valuable mechanism

of immune escape. The presence of phase variable methyltransferases further amplifies the potential for variation amongst *Neisseria* populations. Phase variation of these genes results in global changes to DNA methylation, therefore altering the expression of multiple genes (Seib et al., 2017).

Surface structures of the meningococci are also subject to antigenic variation. Some antigenic epitopes are highly variable, allowing them to avoid recognition and binding by host antibodies. This is particularly well documented in meningococcal Type 4 pili (T4P), which are shown in Figure 3. Pili are a key component of meningococcal pathogenesis due to their role in cell adhesion and are therefore considered a virulence factor (Snyder and Saunders, 2006). However, they are more likely evolved for colonisation of and persistence in the nasopharynx, as they are the major meningococcal adhesin and pilus-deficient *N. meningitidis* are severely impaired for epithelial cell binding (Nassif et al., 1994; Tang et al., 2016; Virji et al., 1995a). Like other members of the *Neisseriaceae*, *N. meningitidis* are naturally competent (Putonti et al., 2013), meaning they can assimilate DNA from their extracellular environment and incorporate it into the chromosome. T4P are instrumental in the binding and internalisation of extracellular DNA (Berry et al., 2013; Cehovin et al., 2013), which may be crucial for adaptation to environmental stresses and survival of meningococci in the nasopharynx. T4P are highly immunogenic but due to their diverse and essential roles, reduced expression of these extracellular structures may be detrimental to the fitness of the organism. Instead, pathogenic *Neisseria* are able to swap out the gene for the major protein component of pili (*pilE*), with a number of silent, unexpressed variants (*pilS*) by genetic recombination (Hagblom et al., 1985; Miller et al., 2014).

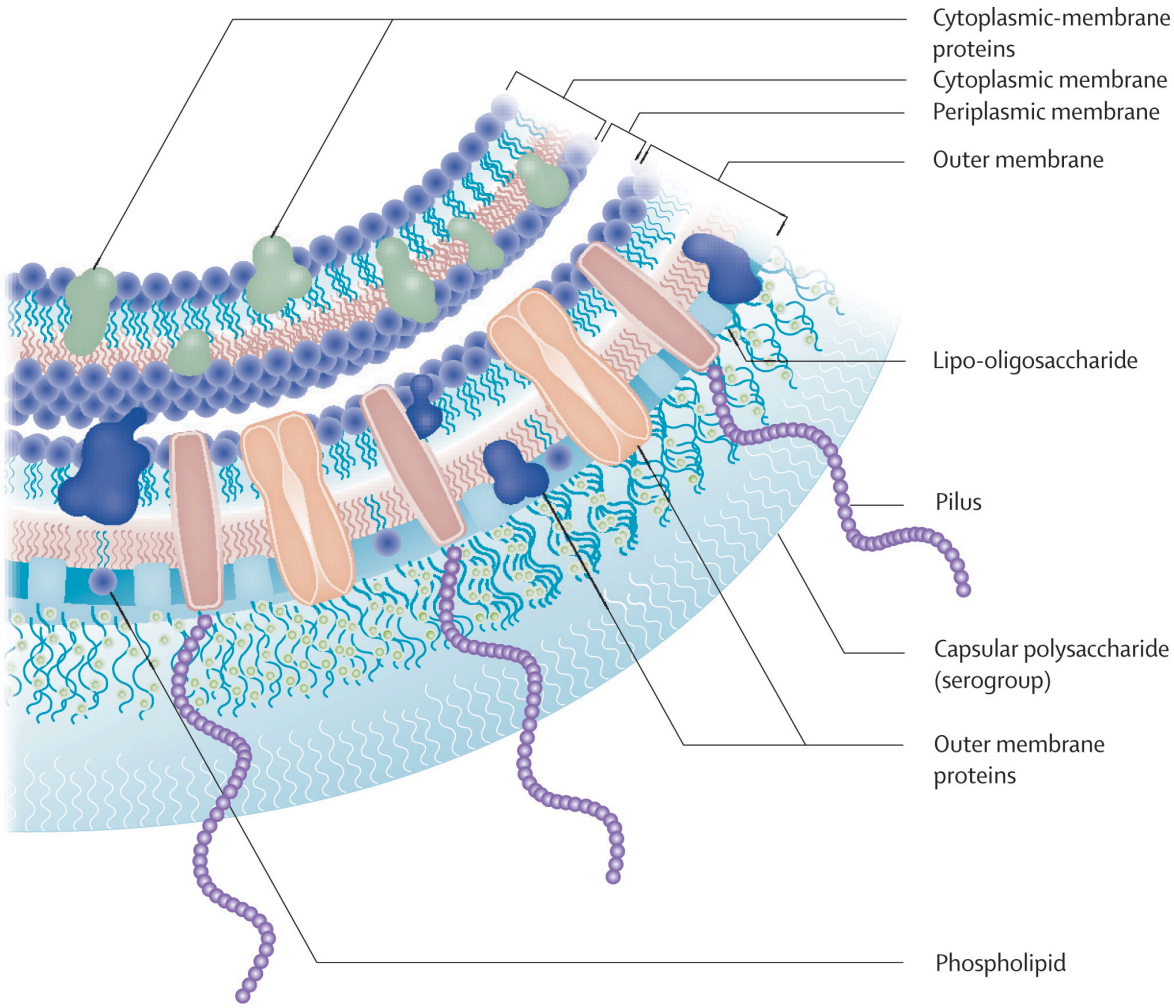



Figure 3. Cell Membrane and Surface Features of *N. meningitidis*

*N. meningitidis* express phospholipid cytoplasmic and outer membranes, both of which harbour proteins. Outer membrane proteins include porins (PorA and PorB) and adhesins, such as opacity proteins. Some strains express capsular polysaccharide, which varies greatly between strains and coats the outer membrane. Type 4 pili extend from the outer membrane, through the polysaccharide capsule.
Image taken from http://www.thelancet.com/journals/lancet/article/PIIS0140-6736(07)61016-2/fulltext?elsca1%3DSEMINAR-LP%26elsca2%3Demail%26elsca3%3D

### 1.2.2 Epidemiology

The exclusive environmental reservoir of *N. meningitidis* is the human nasopharynx. Asymptomatic carriage occurs either chronically or transiently in approximately 10% of the population at any time (Cartwright et al., 1987; Greenfield et al., 1971; Stephens, 1999) and is spread by respiratory droplets. Nasopharyngeal colonisation is necessary for *N. meningitidis* to cause disease but progression from acquisition to invasive disease is rare (Gold et al., 1978). In a small proportion of cases, colonisation by a new strain is shortly followed by invasive disease, which can present as bacteraemia and sepsis, meningitis or, less frequently, pneumonia. Invasion has been shown to reduce transmission (Taha, 2002) and therefore presents a fitness cost to the organism, suggesting that disease is a result of an accidental breakdown in host-commensal dynamics, and provides no evolutionary advantage. *N. meningitidis* is endemic in the UK, with IMD incidence at approximately one per 100,000 each year (Public Health England, 2015). It is also responsible for frequent meningitis epidemics in the 'meningitis belt' between Senegal and Ethiopia (World Health Organisation, 2015). The mortality rate of invasive disease by *N. meningitidis* in Europe is approximately 8% (Trotter et al., 2007), and debilitating sequelae, such as amputations, hearing loss and neurodevelopmental disabilities, are suffered by approximately 15% of survivors (Kirsch et al., 1996).

### 1.2.3 Clinical Presentation

Initial symptoms of IMD are generic, including upper respiratory symptoms, fever, headache, nausea and vomiting. The infection will then generally develop into either meningitis (infection in the meninges) or septicaemia (systemic bloodstream infection). Symptoms of meningitis include a distinctive rash, extreme headache, neck stiffness and photophobia, followed by seizures, delirium and unconsciousness. Drowsiness, lower limb pain and signs of poor circulation are indicative of septicaemia, which may also present with a distinctive rash. Later signs of septicaemia include hypotension and cerebral hypoperfusion (Pace and Pollard, 2012). Bacterial replication in the bloodstream results in the mass release of outer membrane vesicles (OMVs), which contain endotoxin, phospholipid and proteins, and are derived from the bacterial outer membrane in a process known as blebbing (Post et al., 2005). OMVs contain pathogen-associated molecular patterns (PAMPs) (Post et al., 2005), which, when in the bloodstream, induce a mass immune response, leading to damage to the microvasculature, disseminated intravascular coagulation and eventually multi-organ failure (Pace and Pollard, 2012). Progression of IMD is extremely rapid and can develop from initial onset of symptoms to death in a few hours.

With immediate clinical attention, IMD can often be treated with antibiotics (cefataxime and ceftriaxone are frequently used), however due to the rapid progression of disease and high risk of sequelae, medical intervention may be too late and vaccination to prevent disease is undoubtedly preferable.

### 1.2.4 Vaccination

One of the main difficulties with identifying vaccine candidates in the meningococci is that the majority of surface-exposed proteins are not universally expressed amongst invasive strains. One target that has proved successful is the capsule, as it is essential for survival of the bacteria during invasive disease. The capsule polysaccharides for 5 out of 6 invasive serogroups are immunogenic. Effective vaccines have been developed based on the capsules of serogroups A, C, W, X and Y (Morelli et al., 2014; Pace et al., 2009). Unfortunately this has not been possible with the group B capsule polysaccharide, as it displays molecular mimicry of human polysialic acid (Lo Passo et al., 2007). Vaccination against this antigen may induce an insufficient protective immune response, or may lead to the development of autoimmunity; therefore an alternative vaccination approach is required.

Strain-specific OMV vaccines have been used to combat epidemics caused by serogroup B *N. meningitidis* (MenB) (O'Hallahan et al., 2009; Tappero et al., 1999) but provide limited cross-reactive immunity against other strains due to antigenic variability in the immunodominant protein, PorA (Sacchi et al., 1992; Tappero et al., 1999), whose expression is also subject to phase variation (Tauseef et al., 2013). In an attempt to combat IMD caused by multiple MenB strains, the Netherlands Vaccine Institute generated two strains of *N. meningitidis*, each genetically modified to express three different PorA types. A vaccine combining OMVs derived from both strains showed good safety in Phase 1 clinical trials (Cartwright et al., 1999; Peeters et al., 1996; Rouppe van der Voort et al., 2000). An additional meningococcal strain, expressing a further three PorA types, was generated. OMVs derived from all three strains, containing a total of nine different PorA types were tested in preclinical trials, however immunogenicity against some PorA types was poor (van den Dobbelsteen et al., 2007; Kaaijk et al., 2013).

Pizza et al. used reverse vaccinology to identify conserved, surface-expressed, immunogenic proteins in MenB (Pizza, 2000), which led to the recent introduction of serogroup B meningococcal vaccine, Bexsero (*Novartis Vaccines, GSK*), into the UK national immunisation schedule. This vaccine was predicted to prevent disease caused by 78% of the MenB disease

isolates recovered in Europe (Vogel et al., 2013). Bexsero consists of a MenB disease strain-derived OMV containing porin A (PorA), alongside recombinantly produced *Neisseria* Heparin Binding Antigen (NHBA) fused with accessory protein 953, factor H binding protein (fHbp) variant 1.1 fused with accessory protein 936 and Neisserial adhesin A (NadA) (Su and Snape, 2011). Presence of serum bactericidal antibody (SBA) in the blood has previously been used to demonstrate protective immunity against IMD (Brookes et al., 2013; Goldschneider et al., 1969; Tappero et al., 1999; Uria et al., 2008). While Bexsero does induce SBA (Findlow et al., 2015), this is primarily directed against PorA, which, as mentioned previously, is highly variable between strains (Sacchi et al., 1992; Tappero et al., 1999). Human trials support the safety of this vaccine (Findlow et al., 2015), and initial analysis suggests that it is effective in reducing disease incidence (Findlow and Borrow, 2017; Parikh et al., 2016), however there is currently insufficient data to assess its longevity or its effect on carriage and herd immunity. Without this information, the cost of the vaccine is currently too high for many countries to justify its introduction into their immunisation programs (Andrews and Pollard, 2014; Izquierdo et al., 2015).

A number of cross-reactive antigens exist between *N. meningitidis* and *N. gonorrhoeae,* including some of those present in Bexsero. Hadad et al. (2012) found that the genome-derived *N. meningitidis* antigens in Bexsero have over 60% amino acid identity with the equivalent proteins in gonoccocal strains, when comparing to over 100 *N. gonorrhoeae* isolates. Implementation of OMV meningococcal vaccines have preceeded a reduction in gonorrhoea incidence, both with VA-MENGOC-BC implementation in Cuba (Petousis-Harris, 2017) and the NZ98/254 OMV vaccine in New Zealand (Petousis-Harris et al., 2016), with no change in other sexually transmitted infections (STIs), suggesting that meningococcal OMVs are capable of eliciting protective, cross-reactive immunity against *N. gonorrhoeae.* This is extremely beneficial considering there has been very little progress in developing an anti-gonococcal vaccine and antibiotic-resistance is rising in this species (Quillin and Seifert, 2018).

Whilst PorA expression is unique to *N. meningitidis,* a number of other surface-expressed proteins in *N. meningitidis* are also shared by non-pathogenic *Neisseria* (Muzzi et al., 2013). Some commensal Neisserial species, such as *Neisseria lactamica* and *Neisseria cinerea* also colonise the human nasopharynx. Concerns have been raised regarding the effect of meningococcal OMV-based vaccines, like Bexsero, on these species (Lucidarme et al., 2013), especially considering their apparent effect on *N. gonorrhoeae*. It is not yet known whether Bexsero induces a cross-reactive immune response against commensal *Neisseria,* as is likely with *N. gonorrhoeae,* or whether the immune response could promote clearance from the nasopharynx. Such cross-reactivity may

prevent beneficial commensals from colonising an environmental niche, where they may otherwise provide a valuable contribution to the microbiome.

A probiotic approach, whereby commensal species are used to occupy a biological niche and therefore prevent colonisation with *N. meningitidis*, may be a safer approach to preventing IMD.

## 1.3 The commensal: *Neisseria lactamica*

*N. lactamica* is a commensal organism, closely related to the pathogenic *Neisseria*: *N. gonorrhoeae and N. meningitidis*. Studies based on sequence identity of housekeeping genes and on DNA relatedness have clustered *N. lactamica* and *N. meningitidis* closely (Figure 4; Guibourdenche et al.; Smith et al., 1999), and *N. lactamica* possess 67% of 127 so-called virulence genes that were identified in *N. meningitidis* (Snyder and Saunders, 2006). *N. lactamica* and *N. meningitidis* also occupy the same anatomical site - the human nasopharynx, however, *N. lactamica* does not cause disease (with the exception of a few cases in immunocompromised patients (Denning and Gill, 1991; Hansman, 1978; Lauer and Fisher, 1976)). A notable difference between the two bacteria is that invasive strains of *N. meningitidis* express a polysaccharide capsule, which is known to provide protection against phagocytosis and complement-mediated killing in the blood (Uria et al., 2008), and the relevant gene operon is absent in *N. lactamica*. Other distinctions between the two species are the expression of two porins in *N. meningitidis* (PorA and PorB), but only PorB in *N. lactamica*; and the expression of β-galactosidase (β-gal) in *N. lactamica* only.

The relationship between *N. lactamica* and *N. meningitidis* has been well studied. Gold and Goldschneider observed an inverse correlation between the carriage of *N. lactamica* and meningococcal disease at different ages (Gold et al., 1978; Goldschneider et al., 1969). The peak in colonisation by *N. lactamica* coincides with the induction of meningococcal SBA in children, which signifies protective immunity. Carriage of *N. lactamica* increases to a peak at 2 years of age, before beginning to decline. Carriage of *N. meningitidis* does not increase until much later, peaking in late adolescence. A recent epidemiological study by Diallo et al. demonstrated an inverse correlation between carriage of *N. meningitidis* and non-pathogenic *Neisseria* by age group, gender and season in the meningitis belt, and showed that *N. lactamica* was the most prevalent non-pathogenic *Neisseria* throughout the meningitis belt (Diallo et al., 2016). Another study in the Faroe Islands demonstrated an inverse correlation between carriage of *N. lactamica* and carriage of *N. meningitidis* and also between carriage of *N. lactamica* and incidence of IMD

(Olsen et al., 1991). Together, these studies led to the hypothesis that carriage of *N. lactamica* prevents colonisation with *N. meningitidis* and may prime cross-protective immunity.

Vaughan et al. (2009) quantified the peak memory T cell proliferative response to *N. lactamica*- and *N. meningitidis*-derived OMVs, and quantified numbers of memory B cells in the tonsillar mononuclear cell population of children and adults. There was an absence of T cell and B cell memory to *N. lactamica* in children during the peak age of colonisation, calling into question its ability to prime a cross-reactive adaptive immune response to *N. meningitidis*. T cell memory responses increased to *N. lactamica* in older children, but B cell memory remained very low into adulthood, in contrast with *N. meningitidis*. Cross-reactive B cell epitopes between *N. lactamica* and *N. meningitidis* were observed in immunized mice, which were immunologically naive for both species (Troncoso, Sanchez, Moreda, Criado, & Ferreiras, 2000). However, due to the availability of only convalescent sera from humans, it cannot be concluded from this study that there are cross-reactive B cell epitopes in humans. Other studies have shown that there is indeed some cross-reactivity in B cells (Evans et al., 2011; Gorringe et al., 2009), although the quality of the antibody response with regards to inducing cross-protective immunity is questionable. There is also no direct evidence that colonisation by *N. lactamica* primes cross-protective immunity to *N. meningitidis*.

Vaccination with *N. lactamica*-derived OMVs has been shown to protect against lethal challenge with *N. meningitidis* in mice (Oliver et al., 2002). Following these findings, *N. lactamica* OMV vaccines were shown to elicit a broad but weak humoral response to *N. meningitidis* strains in a phase I clinical trial in humans (Gorringe et al., 2009). This study highlighted the potential for *N. lactamica* OMVs as a mucosal adjuvant. The adjuvant properties of *N. lactamica*-derived OMVs have also been demonstrated in mice, where strong immunity directed against co-administered Hepatitis B surface antigen was observed following intranasal vaccination (Sardiñas et al., 2006). The PorB protein alone from *N. lactamica* has also demonstrated immune adjuvant potential in mice (Liu et al., 2008).

In an experimental human challenge study by Deasy et al. (2015), acquisition of *N. lactamica* in the nose following intranasal inoculation of adult volunteers with live bacteria was shown to displace existing meningococci and inhibit acquisition of new strains. These results suggest that there may be some form of competitive inhibition between the two species. Two distinct fratricidal mechanisms have been identified in *N. meningitidis*, involving the TspA and MafB proteins (Arenas et al., 2013, 2015; Jamet et al., 2015). These contact-dependent growth inhibition systems involve genomic islands that co-express a toxin and associated immunity

protein(s). The toxin can be delivered to neighbouring cells via a type V secretion system. They facilitate competition but also cooperative behaviour between kin (Ruhe et al., 2013). A search of the *N. lactamica* genome reveals that homologous genes also exist in *N. lactamica,* suggesting that both species are capable of attacking the other to gain an advantage in their shared environmental niche.

Considering that nasopharyngeal carriage of *N. meningitidis* is necessary for the initiation of invasive disease, the protective effect of *N. lactamica* against *N. meningitidis* carriage could have significant therapeutic potential, however carriage of *N. lactamica* was only achieved in 34% of inoculated volunteers, and the same effect was not observed in those who did not become colonised (Deasy et al., 2015).

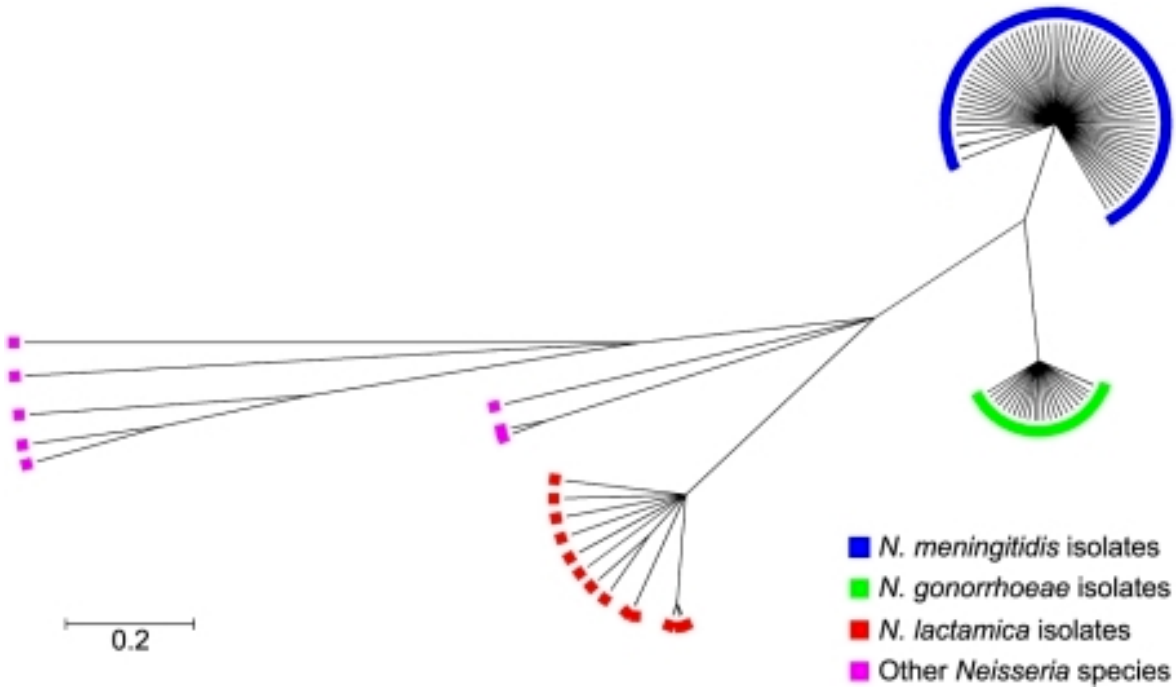



Figure 4. Phylogenetic Tree of *Neisseria*

The two pathogenic *Neisseria* species *N. meningitidis* and *N. gonorrhoeae* are genetically similar. Their closest relative is the commensal species *N. lactamica,* which shares the same environmental niche as *N. meningitidis.* Multiple other commensal *Neisseria* have been identified and are related to different extents to the pathogenic species.
Image taken from:
https://openi.nlm.nih.gov/detailedresult.php?img=PMC2924362_ppat.1001055.g002&req=4

## 1.4 Natural Competence and Genetic Transformation

Genetic competence is the ability to internalise extracellular DNA. It is widespread among bacteria but the mechanisms and regulation differ between species. The *Neisseriaceae* are naturally competent. They are capable of incorporating extracellular DNA into the chromosome, so that it is stably inherited. This property has been exploited for research purposes *in vitro* in *N. meningitidis, N. gonorrhoeae* and some commensal species (Berry et al., 2013; Higashi et al., 2011; O'Dwyer et al., 2004; Seifert et al., 1990; Tang et al., 2016).

Horizontal gene transfer benefits bacteria, as it is a rapid mechanism of evolution, promoting diversity and survival. For example, it has contributed to the spread of antibiotic resistance in *N. meningitidis* (Bowler et al., 1994; Fermer et al., 1995; Qvarnstrom and Swedberg, 2006). However, it is likely that the majority of random changes to the genome come with a fitness cost to the organism, removing or altering the function/expression of beneficial genes, or introducing an unnecessary metabolic burden. For this reason, some bacterial species have evolved to selectively uptake homotypic DNA, which is DNA from species closely related to self, therefore reducing the probability of deleterious mutations. Neisserial genomes contain multiple copies of a 10-12 bp sequence, known as the DNA Uptake Sequence (DUS) (Frye et al., 2013), which comprises approximately 1% of the genome (Cehovin et al., 2013). This sequence allows *Neisseria* to recognise and preferentially uptake homotypic DNA. A similar system, involving the Uptake Signal Sequence (USS) exists in the *Pasteurellaceae* (Redfield et al., 2006).

Our understanding of genetic competence in *Neisseria* is based on research performed predominantly using *N. meningitidis* and *N. gonorrhoeae*. The majority of the following characteristics are yet to be confirmed in *N. lactamica* or other commensals. In fact, prior to commencing this project, there had been no published examples of *in vitro* genetic manipulation of *N. lactamica,* despite attempts using a protocol that proved successful in other *Neisseria* species (O'Dwyer et al., 2004) and evidence of genetic exchange *in vivo* (van Passel et al. 2006, A. Pandey, awaiting publication). Recently, Perez-Ortega et al. (2017) demonstrated integration of green fluorescent protein alongside a gentamicin resistance cassette into *N. lactamica* strain NL-1, from the Utrecht University collection, and ATCC strain 23970, to study biofilms. In both strains, the insert was targeted to a high rate of transformation region (*hrtA),* which was initially identified in *N. meningitidis* (Claus et al., 1998).

Gene transfer appears to be enhanced within biofilms. For example, *S. pneumoniae,* a species to colonise the human nasopharynx, has been shown to upregulate competence genes within

biofilms relative to a planktonic growth state (Oggioni et al., 2006; Trappetti et al., 2011), which results in a drastically increased rate of genetic recombination (Marks et al., 2012). In clinical samples, *N. gonnorhoeae* biofilms have been identified on cervical biopsies (Steichen et al., 2008) and intrauterine devices (Pruthi et al., 2003). Genetic exchange was also shown to increase in biofilms compared to planktonic growth in this species (Kouzel et al., 2015). *N. meningitidis* microcolonies, which are considered an early stage in biofilm formation, have been observed in cerebral capillaries (Mairey et al., 2006; Pron et al., 1997) and in purpura fulminans skin lesions (Harrison et al., 2002) during disease. *N. meningitidis* microcolonies were also identified in the tonsillar tissue of healthy carriers (Sim et al., 2000) and in multi-species biofilms on 50% of examined laryngotracheal stents (Simoni and Wiatrak, 2004). A number of papers have demonstrated biofilm formation by *N. meningitidis in vitro* (van Alen et al., 2010; Apicella et al., 2012; Arenas et al., 2016; Lappann and Vogel, 2010; Lappann et al., 2006; Neil et al., 2009; Yi et al., 2004a) and one study also demonstrated biofilm formation by *N. lactamica* (Pérez-Ortega et al., 2017).

All pathogenic and human commensal *Neisseria* species described to date express T4P, which protrude from the cell surface (Aho et al., 2000). T4P contain a DNA binding protein called ComP, which preferentially binds DNA containing a DUS (Berry et al., 2013; Cehovin et al., 2013). Once DNA is bound, pili are retracted in an active process requiring the hydrolysis of ATP by PilT (Berry et al., 2013). The retractive force generated by a single T4P is incredible (50-100 picoNewtons)(Maier et al., 2002), and multiple pili are able to form bundles, which cooperate to generate forces in the nanonewton range (Biais et al., 2008). Retraction of T4P pulls the associated DNA fragment inside the cell. Based on findings in other bacteria, it is believed that one DNA strand is degraded and the other is internalised (Johnston et al., 2014; Puyet et al., 1990). Once inside the cell, single stranded DNA (ssDNA) is bound by DNA-protecting protein DprA, which recruits RecA (Mortier-Barrière et al., 2007). Identified as a DNA-repair enzyme, RecA performs a homology search throughout the genome and guides the incorporation of DNA into a site of homology. However, a novel gene or DNA fragment may be incorporated using the same system, if flanked by regions of homology to the chromosome (Johnston et al., 2014).

It has been demonstrated that regions of homology are required either side of a heterologous insert, and cross-over events between the chromosomal and transforming DNA must occur either side of the heterologous region for efficient transformation (Ambur et al., 2012). In naturally competent species, including *Streptococcus* species and *Xylella fastidiosa,* it was observed that the length of the homologous region of DNA flanking the foreign insert can affect the efficiency of transformation (Kung et al., 2013; Morrison et al., 2015; Salvadori et al., 2016). In these

organisms, transformation efficiency was shown to improve with increasing lengths of homologous DNA. In *X. fastidiosa,* a plateau in transformation efficiency was reached with 1000 bp homology either side of an approximately 1000 bp heterologous insert. Further increases in the homologous flanking length did not affect the efficiency of transformation. This is not a function of the overall size of the donor DNA because in a different study the length of exogenous DNA (between 3 and 17.5 kb) had little effect on its affinity to *N. gonorrhoeae*. It was noted, however, that there was a positive correlation between the number of DUSs in exogenous DNA and its affinity to *N. gonorrhoeae* (Goodman and Scocca, 1991).

The effect of DUSs is not limited to its affinity for *Neisseria.* Increasing the number of DUSs present within exogenous DNA has also been shown to positively influence transformation efficiency in both *N. meningitidis* (Ambur et al., 2012; Berry et al., 2013) and *N. gonorrhoeae* (Duffin and Seifert, 2010). In these organisms, sequences containing two or three DUSs had significantly greater transformation efficiencies than those containing only one, however it is not yet known if the same is true in *N. lactamica.* Also in *N. meningitidis*, Budroni et al. (2011) observed a positive correlation between the density of DUSs in any given region of the genome and the frequency of recombination at that site. They also noted more recombination in the core genome when compared to the dispensable genome (Budroni et al., 2011), which agrees with a previous observation by the same group, that the density of DUSs is higher in the core genome than the dispensable genome (Treangen et al., 2008). Furthermore, in both *N. meningitidis* and *N. gonorrhoeae,* intragenic DUSs are biased towards genome maintenance genes (Davidsen et al., 2004) and in both *Bacillus* and *Neisseria* species, transformation has been found to increase with the extent of homology between transforming and chromosomal DNA (Ambur et al., 2012; Roberts and Cohan, 1993). Taken together, these findings suggest that rather than allowing rapid evolution and adaptation to environmental stresses, DUS-directed recombination has a role in preserving genome stability.

It has been suggested that DUSs may affect the recombination phase of transformation as well as DNA recognition and uptake, and can affect different strains in different ways. Experiments were performed in two strains of *N. gonorrhoeae*: FA1090 and MS11, where DNA uptake was measured by observing the movement of labelled DNA into the cell whereas transformation was measured by acquisition of antibiotic resistance. Whilst the uptake of both DUS-containing and DUS-negative labelled DNA was comparable between the two strains, the overall rate of transformation by DUS-negative DNA was significantly higher in FA1090 compared to MS11 (Duffin and Seifert, 2010). Therefore although uptake was equivalent between the two strains, the processing of the DNA varied, resulting in different levels of recombination and

transformation. The location of DUS relative to the heterologous insert has been shown to affect transformation efficiency (Ambur et al., 2012), but it is not known whether this effect occurs at the binding, internalisation or recombination phase of transformation.

Restriction Modification Systems (RMSs) are prevalent among *Neisseria* species and are another factor to play a role in transformation. Restriction endonucleases digest DNA at defined recognition sequences, unless that sequence has already been modified by a corresponding methyltransferase – thereby inhibiting the restriction enzyme's endonuclease activity. Possessing corresponding pairs of endonuclease and methyltransferase allows an organism to distinguish between self and non-self DNA so was initially considered a primitive immune system to protect against phage. To support this hypothesis, some phage have evolved DNA modification mechanisms to evade restriction (Vasu and Nagaraja, 2013). However this doesn't appear to be the only role that RMSs play in bacteria.

Whilst methyltransferases serve to protect self-DNA, restriction enzymes present a considerable barrier to transformation, as they digest incoming DNA that does not possess the necessary methylation pattern. Naturally competent species, including *Neisseria,* have been found to express more RM genes than those that are not competent (Vasu and Nagaraja, 2013), suggesting that RMSs play an important role in genetic exchange. RMSs are associated with the development and divergence of phylogenetic clades within *Neisseria*, as bacteria are more likely to be transformed with DNA from strains possessing the same RMS (Budroni et al., 2011).

By modifying the transforming DNA in an attempt to inhibit digestion by restriction enzymes, Laver et al. have recently demonstrated successful *in vitro* genetic transformation of this important commensal organism (awaiting publication). Using PCR amplification, hypermethylated cytosine residues were inserted in place of all cytosine residues in the donor DNA. When *N. lactamica* was exposed to these 'hypermethylated' constructs under defined conditions, targeted *in vitro* genetic transformation could be achieved.

This novel technology presents a variety of opportunities to exploit *N. lactamica* in a therapeutic context. At its most basic level, the ability to insert or knockout genes is a valuable tool in molecular microbiology, allowing analysis of gene/protein function. It may also be possible to modify *N. lactamica* in a way that enhances its colonisation potential. In the human challenge study by Deasy et al. (2015), inoculation with *N. lactamica* led to carriage in 34% of volunteers. If the colonisation rate could be increased with a Genetically Modified (GM) strain, it could potentially be used as a probiotic to reduce carriage of *N. meningitidis.* This may be of particular

value in an epidemic outbreak response, to limit the spread of meningococci throughout a population. Furthermore, the introduction of heterologous antigens into this commensal organism could render it capable of eliciting an immune response, which could be used as a vaccine, either as whole cells or antigen-containing OMVs.

## 1.5 Therapeutic Use of Genetically Modified Bacteria

There is some precedence for using GM bacteria for therapeutic benefits. *Escherichia coli* has been used to produce insulin for human use since 1982 (Becker, 2016). Research into the therapeutic use of live GM bacteria has focussed mainly on lactic acid bacteria and research has predominantly been performed in mice so far, as reviewed by LeBlanc et al. (2013). Potential targets that have been investigated include weight loss, inflammatory and gastrointestinal disease, vaccination for leishmaniasis, rotavirus and HPV, a microbicide for HIV-1 and cancer. In humans, there has been a phase 1 clinical trial using attenuated *Lactococcus lactis* expressing IL-10 for the treatment of Crohn's disease (Braat et al., 2006). The attenuation meant that this strain required thymidine for growth. There has also been a lot of research into using *Salmonella* and *Clostridium* for the targeted attack of cancers (Mowday et al., 2016; Zheng and Min, 2016), which has resulted in a Phase 1 clinical trial using attenuated *Salmonella* Typhimurium for the treatment of metastatic melanoma (Toso et al., 2002).

There are currently no published examples of the use of unattenuated live GM bacteria in humans. This project will investigate the potential to introduce heterologous antigens into unattenuated *N. lactamica* for human challenge, with a view to reducing the incidence of IMD.

## 1.6 Research Aims

Previous studies demonstrate a clear link between the carriage of *N. lactamica* and reduced IMD. Therefore it is hypothesised that a *N. lactamica*-based probiotic would provide protection against disease. *N. lactamica* is a predominantly paediatric species and only colonises 34% of inoculated adult volunteers, however artificially-induced long-term carriage of *N. lactamica* by genetic modification may serve to reduce meningococcal carriage and IMD. An alternative approach to inducing long-term carriage of *N. lactamica* is to use this species as a vector for the delivery of heterologous antigen. *N. lactamica*-derived OMVs have shown strong potential as a safe mucosal adjuvant and therefore may constitute an effective vaccine if expressing a surface-exposed heterologous antigen.

The aims of this project are thus to optimise a protocol for genetically modifying *N. lactamica*; to further understand the mechanisms by which *N. lactamica* interacts with and colonises the host and to investigate the potential of expressing heterologous antigen in this species. Future goals include augmenting host colonisation to increase carriage frequency and duration, thereby optimising probiotic efficacy and using either live GM *N. lactamica* or an OMV vaccine derived from GM *N. lactamica* to alter the immune profile in humans.

These aims will require the following:

To characterise the transformation process of *N. lactamica*, specifically:

- Understanding the mechanisms underlying natural competency in this species, as a basis for *in vitro* transformation
- Comparing the efficiency of transformation under various conditions
- Determining the accuracy of targeting genetic modifications to a particular locus
- Considering the effect of transformation on the fitness of the organism
- Developing a means to genetically modify this species in an antibiotic-resistance free manner.

To better understand the role of the assumed primary adhesin, T4P, in *N. lactamica*, with a view to enhancing colonisation potential, including:

- Generating a pilin-deficient *N. lactamica* mutant
- Assessing the involvement of T4P in adhesion to epithelial cells

To examine the potential of generating GM *N. lactamica* and OMVs derived from GM strains, which will require:

- Generating a GM *N. lactamica* strain expressing a heterologous antigen. We will use meningococcal PorA as a proof of principle
- Assessing and optimising surface expression of PorA in GM *N. lactamica*
- Deriving OMVs from the GM strain
- Examining the immune response in mice following immunisation with OMVs derived from PorA-expressing *N. lactamica.*

Considering PorA is highly immunogenic and is phase variable in *N. meningitidis,* it is hypothesised that expressing PorA under the control of a phase variable promoter in *N. lactamica* may lead to increased tolerance and persistence in the nasopharynx relative to constitutive expression. To test this hypothesis, we require comparable strains of *N. lactamica* with constitutive and phase variable expression of PorA. For progression of these strains to human challenge, we must confirm the following:

- Except the PorA promoter sequence, the constitutive and PV strains must be otherwise comparable in terms of genetics, fitness, PorA expression level and interaction with epithelial cells
- The strains must be genetically stable, susceptible to relevant antibiotics and immunogenic
- When placed under selective pressure, the population of bacteria expressing phase variable PorA undergoes a reduction in PorA expression (ie. phase variation occurs).

# Chapter 2 Methods

## 2.1 Bacterial Strains and Culture Methods

All culture media was obtained from *Fisher Scientific* (Loughborough, UK). Stocks of Wild Type (WT) *N. lactamica* Y92-1009, which formed part of the GMP-manufactured cell bank developed for the original human challenge study by Evans et al. (2011), were stored at -80°C. Broth cultures of *N. lactamica* (Nlac) WT and its GM derivatives were grown in tryptone soya broth supplemented with 0.2% yeast extract (TSB). *Neisseria cinerea* (Ncin) 346T was sourced from CCUG, Goteborg and was transformed using genomic DNA from 364T Δ*pilE*1/2 (Tang et al., 2016). Both Ncin strains were also grown in TSB. *N. meningitidis* (Nmen) NZ98/254 was kindly provided by Holly Humphries and Andrew Gorringe at Public Health England, Porton Down. Nmen 8013 and Nmen 8013 Δ*pilE* were kindly provided by Rachel Exley and Chris Tang at the Sir William Dunn School of Pathology, Oxford University. All meningococcal strains were grown in Mueller-Hinton broth (MHB) or TSB. Competent *E. coli* DH5α were purchased from *New England Biolabs* (*NEB*, Hitchen, UK) and grown in Luria-Bertani broth (LB). All media was supplemented with bacteriological agar (1.5%), Ampicillin (100 μg $ml^{-1}$), Kanamycin (50 μg $ml^{-1}$) and/or 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal) (20 μg $ml^{-1}$) where relevant. Columbia blood agar (CBA) was also used for culture of all strains. A list of all *Neisseria* strains used in this project is shown in Table 1.

Bacterial stocks were produced by culturing the organisms to logarithmic growth phase at 37°C, 5% $CO_2$, 320 RPM, then combining log phase culture (50 %) with sterile glycerol (30 %) in phosphate buffered saline (PBS). After allowing the bacteria to acclimatise to the stock solutions for 10 min at room temperature, they were stored at -80°C. When required, a loop of bacteria was removed from the stock and streaked onto agar plates, without allowing the stock to thaw. For downstream applications, colonies were transferred for growth in liquid culture at 37°C, 5 % $CO_2$ unless otherwise stated. For Neisserial strains, a minimum of 10 colonies was transferred from streak plates to liquid media, to minimise the effects of phase variation.

Table 1. Neisserial Strains

| Strain | Relevant Features | Reference |
|---|---|---|
| *Neisseria lactamica* | | |
| Y92-1009 | WT | (Evans et al., 2011) |
| Y92-1009 *ΔlacZ* | *lacZ* deficient | JR. Laver, Unpublished |
| Y92-1009 *ΔlacZ* NHCIS1:*lacZ* | *lacZ* controlled by *lst* promoter in NHCIS1 locus | JR. Laver, Unpublished |
| Y92-1009 *ΔpilE* | *pilE* deficient, $Spec^R$ | This project |
| Y92-1009 2Pp7a | *porA* P1.7,16 and lacZ in NHCIS1 locus | JR. Laver, Unpublished |
| Y92-1009 4PA1 | *porA* P1.7,16 with upstream activating sequence (UAS) and *lacZ* in NHCIS1 locus | This project |
| Y92-1009 4YB2 | GM control. No additional genes | JR. Laver, Unpublished |
| Y92-1009 4PnzG4<br>Y92-1009 4PnzG5 | *porA* P1.7-2,4 and *lacZ* in NHCIS1 locus with PorA AA substitutions | This project<br>This project |
| Y92-1009 ON(100)<br>Y92-1009 PV(100)A<br>Y92-1009 PV(100)B | *porA* P1.7,16 (with 100 bp upstream activation sequence (UAS) and constitutive (ON) or phase variable (PV) promoter) and lacZ in NHCIS1 locus | This project<br>This project<br>This project |
| *Neisseria cinerea* | | |
| 364T | WT | CCUG, Goteberg |
| 364T *ΔpilE* | *pilE1* and *pilE2* deficient, $Kan^R$ | (Tang et al., 2016) and this project |
| *Neisseria meningitidis* | | |
| H44/76 | Serogroup B WT | (Piet et al., 2011) |
| H44/76 *ΔsiaD* | Capsule-free, $Kan^R$ | JR. Laver, Unpublished |
| NZ98/254 | Serogroup B WT, basis for Bexsero OMV component | Public Health England |
| 8013 | Serogroup C WT | (Tang et al., 2016) |
| 8013 *ΔpilE* | *pilE* deficient, $Kan^R$ | (Tang et al., 2016) |
| MC58 | Serogroup B WT | (Cartwright et al., 1987) |

## 2.2 Assessing Bacterial Load in *Neisseria* Cultures

### 2.2.1 Viable Counts

To assess the concentration of bacterial cultures, multiple 10-fold serial dilutions of the culture were performed in PBS. Aliquots of each dilution (10 µl) were spotted onto CBA, in triplicate. Agar plates were incubated at 37°C, 5 % $CO_2$ and colonies were counted the following day. Counts were multiplied by the dilution factor, and then by 100 to adjust for volume, to provide the number of Colony Forming Units in 1 ml of the original cell suspension (CFU/ml).

### 2.2.2 Estimations based on Culture Turbidity

The viable counts of Neisserial cultures at various time-points were correlated with the culture turbidity at that time, to generate a standard curve for estimating bacterial concentration using spectrophotometry (Figure 5). During logarithmic growth, there is a linear relationship between the Optical Density (OD) of the culture at 600 nm ($OD_{600nm}$) and the number of bacterial cells present. When $OD_{600nm}$ = 0.3, there are approximately 3.325 x$10^8$ CFU/ml. These values were used to generate the following formula for calculating culture viability, which was used to estimate bacterial concentrations in *Neisseria* cultures during logarithmic growth:

If $OD_{600nm}$ = 0.3, bacterial concentration (CFU/ml) = 3.325 x$10^8$
Therefore, when $OD_{600nm}$ = 1, bacterial concentration (CFU/ml) = 1.108 x$10^9$
Therefore **bacterial concentration (CFU/ml) = $OD_{600nm}$ x 1.108 x$10^9$**

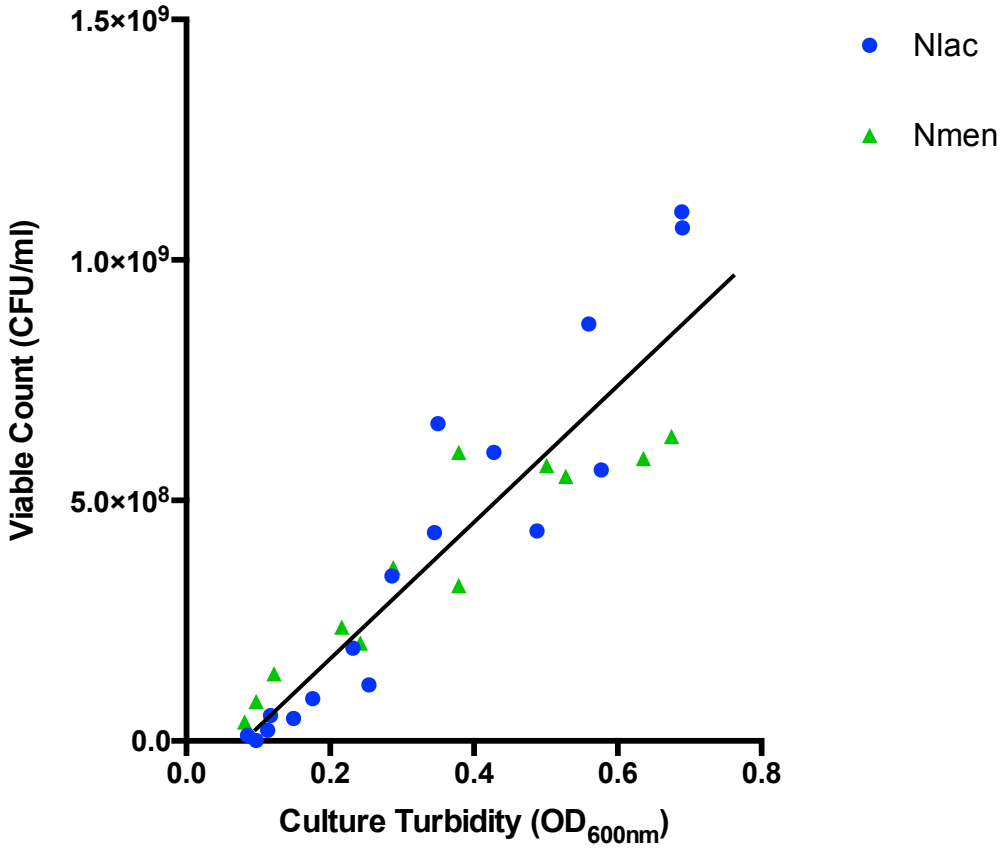



Figure 5. Standard Curve for Estimating Culture Viability by Spectrophotometry

Multiple cultures of *N. lactamica* and *N. meningitidis* were grown in TSB at 37°C, 5 % $CO_2$ with shaking. At various times during logarithmic growth, a viable count was performed on a culture and its $OD_{600nm}$ measurement was recorded. The resulting values were correlated to allow an estimation of culture viability at any given $OD_{600nm}$ during logarithmic growth.

## 2.3 Growth Curves

Growth curves were performed to assess and compare *in vitro* fitness of bacterial strains. Multiple colonies were transferred from streak plates into culture media and the turbidity of the culture was adjusted to produce a 10 ml culture with $OD_{600nm}$ of 0.1. At hourly intervals, culture turbidity was recorded and viable counts were calculated as described above. Cultures were maintained at 37°C, 5% $CO^2$, 320 RPM between time points.

## 2.4 Serial Passage

Bacterial strains were serially passaged to assess their genomic stability and observe microevolution over 28 days. Strains were streaked on CBA and incubated overnight at 37°C, 5% $CO_2$. Ten colonies were selected, resuspended in 20 µl TSB and streaked onto a new plate, which was again incubated until the following day at 37°C, 5% $CO_2$. Strains were passaged daily in this manner for 28 days, with stocks made every 7 days. Day 0 and day 28 isolates were sent to MicrobesNG for whole genome sequencing and analysed as described in Section 2.29.

## 2.5 Biofilm Growth

*N. lactamica* and *N. meningitidis* were grown to log phase in TSB at 37°C, 5% $CO_2$ with shaking before measuring the absorbance and adjusting the concentration to 1x $10^8$ CFU/ml in 50% TSB (i.e. TSB diluted 1:1 with sterile water). Bacterial cultures were then transferred as monocultures or co-cultures to a 12-well plate (2 ml per well) and incubated at 37°C, 5% $CO_2$ for 72 h. For co-cultures, 1 ml of each strain was used. Media was replaced daily with fresh 50% TSB and biofilms were observed by light microscopy. After 48 or 72 h, biofilms were disrupted by scraping and pipetting. Cells were harvested and pelleted, before being resuspended in PBS. Viable counts were performed on CBA containing X-Gal with kanamycin and/or spectinomycin to determine the species (by colony colour) and antibiotic resistance profile. After disruption and removal of biofilms, wells were confirmed as empty by light microscopy.

## 2.6 Primers

Primers (*Sigma-Aldrich,* Gillingham, UK) were designed to amplify sections of plasmid or genomic DNA and, where necessary, provide additional nucleotides. In this project, primers contained a

minimum of 20 nucleotides homologous to the target DNA with a maximum of 20 additional heterologous nucleotides. The melting temperature (Tm) difference of primer pairs did not exceed 5°C and where possible, primers contained C or G residues at the termini to optimise binding to their target sequence. Please see Appendix A for a complete list of primers used in this project.

## 2.7 Plasmids

Heterologous genes for transformation into *N. lactamica* were codon-optimised and synthesised as 'gBlock' gene fragments (*Integrated DNA Technologies (IDT)* Glasgow, UK). The heterologous gene and its promoter were flanked by sequences of DNA homologous to *N. lactamica* chromosomal loci, to direct the site of recombination. These constructs were cloned into pUC19 or pSC101 for high (500-700) or low (5) copy numbers, respectively.

Isothermal Assembly was used to assemble multiple DNA fragments into plasmids. This required double-stranded, linear DNA with a homologous overlap between adjacent fragments. Overlaps of at least 30 bp were used. The vector fragment (100 ng) was combined with 3-fold molar excess of all other fragments and diluted 1:1 with 2x Gibson Assembly master mix (*NEB*). This was incubated at 50°C for 1 h to allow the activity of exonuclease, DNA polymerase and DNA ligase to form covalent bonds between fragments.

Plasmids were transformed into chemically competent *E. coli* DH5α using the manufacturer's guidelines (*NEB)* and selected for by growing in the presence of ampicillin. Stock cultures were generated as described above.

## 2.8 DNA Extraction

Plasmid DNA was extracted from *E.coli* using the GeneJET Miniprep kit and genomic DNA was extracted from *N. lactamica* using the GeneJET genomic DNA purification kit (both from *Thermo Scientific*) according to the manufacturer's instructions. Approximate DNA concentrations were calculated by Nanodrop (*Thermo Scientific*) to inform the required volume for downstream applications. If not required immediately for downstream applications, DNA was stored at -20°C.

## 2.9 DNA Digestion

To cut linear or plasmid DNA at a specific restriction site, 1000 ng DNA was incubated with 1x Cutsmart buffer and 20 units of the relevant endonuclease (*NEB*) for a minimum of 30 min at 37°C. Endonucleases were deactivated for 20 min at 65°C.

## 2.10 DNA Amplification

Template DNA (10-1000 ng per reaction) was amplified using the Polymerase Chain Reaction (PCR). PCR mix incorporated 1x Q5 buffer, relevant primers (0.5 µM), dinucleotide triphosphates (dNTPs, 200 µM) and Q5 DNA polymerase (0.02 U/µl) in RNAse-free $H_2O$. Where appropriate, 3 % dimethyl sulfoxide (DMSO) was added to reduce non-specific DNA binding and to prevent secondary structure formation in primers.

For 'hypermethylated' PCR, deoxycytidine triphosphate (dCTP) in the dNTP mix was replaced with 5-methyl deoxycytidine triphosphate (mdCTP), Q5 DNA polymerase was replaced with Phusion DNA Polymerase, and the reaction was carried out in 1x GC buffer (all reagents from *NEB*). Table 2 indicates the thermocycling conditions used to amplify template DNA.

## 2.11 PCR Purification

PCR products were purified using the GeneJET PCR purification kit (*Fisher Scientific)* according to the manufacturers instructions. Approximate DNA concentrations were calculated by Nanodrop (*Thermo Scientific*) to inform the required volume for downstream applications. If not required immediately for downstream applications, PCR products were stored at -20°C.

## 2.12 DNA Gel Electrophoresis

DNA (8 µl) was combined with 2 µl 5x DNA binding buffer (*NEB*) and loaded into a 0.7% agarose gel (*Fisher Scientific*) made in TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA), containing 0.005% Midori green (*Nippon Genetics,* Dueren, Germany). Gels were run in TAE buffer at 90 V for approximately 40 min before imaging under UV light.

Table 2. PCR Amplification Thermocycling Conditions

| | | | |
|---|---|---|---|
| Denature | | 95°C | 10 min |
| | Repeat 18 - 25 times | 98°C | 20 s |
| Anneal | | Primer dependent* | 15 s |
| Extend | | 72°C | 30 s per kb standard/ 1 min per kb hypermethyl |
| | | 72°C | 10 min |
| | | 10°C | ∞ |

*Tm (of the primer with lower Tm) + 3°C

## 2.13 GeneScan Analysis

Single colonies were isolated after overnight growth on agar and each inoculated into 100 µl $dH_20$ before heating to 95°C for 10 min. Samples were centrifuged to remove insoluble material and the resulting supernatant was used as template for PCR amplification of the *porA* promoter using primers 39 & 37. The forward primer contains a 5'-fluorescein amidite (FAM) modification, allowing fluorescent detection of the PCR product. Product (1 µl) was added to 9.25 µl formamide and 0.25 µl DNA ladder before sending to the Protein Nucleic Acid Chemistry Lab (PNACL) at the University of Leicester for GeneScan analysis, which provides a measurement of the PCR product length. Multiple peaks for product length may be observed in one sample but results were only considered acceptable if the ratio of primary to secondary peak area was greater than 1.2. A number of isolates were sequenced to correlate the Gene-Scan-reported PCR product size with the length of the polymeric tract, which could then be extrapolated to other samples.

## 2.14 Site-Directed Mutagenesis

Site-Directed Mutagenesis (SDM) was used to incorporate small changes into pre-existing plasmids using the Q5 site-directed mutagenesis kit (*NEB*), according to the manufacturers instructions and as summarised in Figure 6. Back-to-back forward and reverse primers were designed to incorporate the desired change using the NEBaseChanger online tool (NEB, 2017). The uncut plasmid was amplified using these primers to generate linear PCR product, which was circularised by the action of kinase and ligase. The template was digested with Dpn1, and the new plasmids were transformed into *E. coli* DH5α.

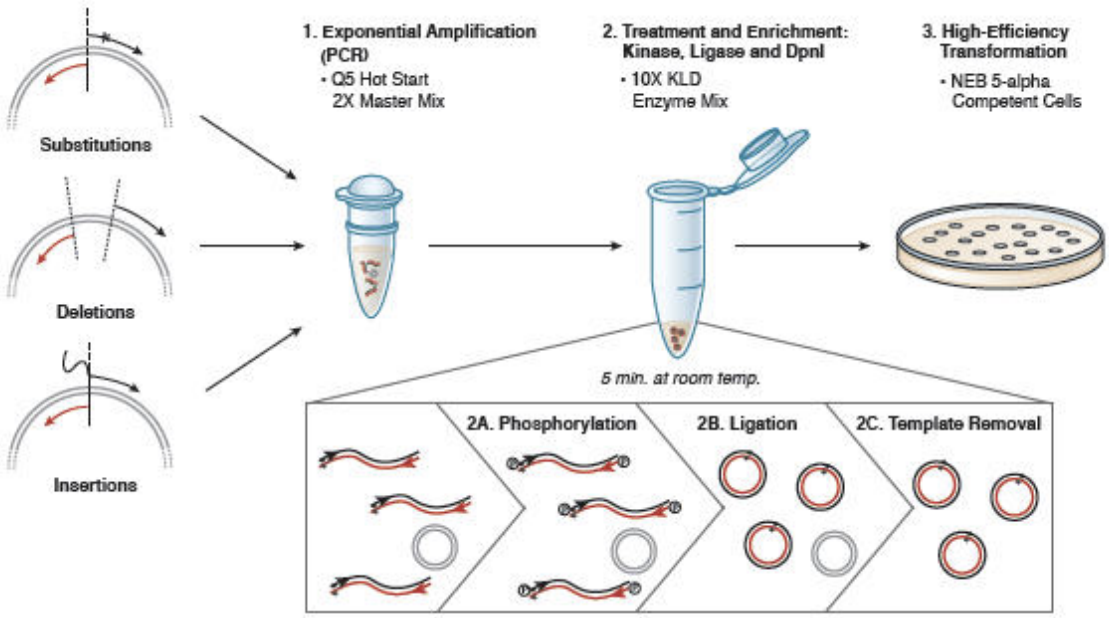



Figure 6. Site-Directed Mutagenesis Overview

Circular plasmid template is PCR amplified using SDM primers to produce a double-stranded linear product with the desired sequence. PCR products are phosphorylated and ligated to form novel plasmids, before digesting the original template with Dpn1. Finally; plasmids are transformed into competent *E. coli.*
Figure taken from (NEB, 2016)

## 2.15 Transformation of *N. lactamica*

Recipient strains of *N. lactamica* were streaked onto TSB agar and incubated at 37°C overnight. Several colonies (>10) were inoculated into 5 ml TSB and incubated at 37°C, 5 % $CO_2$ with shaking (320 RPM) to log phase ($OD_{600nm}$ = 0.3). The culture was diluted x100 in TSB and 10 µl aliquots were spotted onto TSB agar plates. Plates were then incubated upright for 6 h at 30°C, 5 % $CO_2$ before colonies were overlaid with 10 µl of DNA in elution buffer (0.46 pmol unless otherwise stated). Hypermethylated PCR product was used unless otherwise stated. Following addition of DNA, TSB plates were incubated upright at 30°C, 5 % $CO_2$ for 9 h. Bacterial patches were each resuspended into 1ml TSB. Following appropriate dilutions, 100 µl spread plates were performed to screen for successful transformants on TSB agar supplemented with either a selective antibiotic or X-Gal.

To assess transformation efficiency, the total viable count following transformation was calculated, as described in Section 2.2.1. The number of transformants was divided by the total viable count and, where relevant, this was adjusted for the amount of DNA added.

## 2.16 Transformation of *N. cinerea*

*N. cinerea* was grown on TSB overnight at 37°C, 5 % $CO_2$ prior to inoculation into 200 µl PBS. A 10 µl aliquot of the culture was spotted onto TSB and allowed to dry, then overlaid with 10 µl (500 ng) genomic DNA (gDNA) from *N. cinerea ΔpilE1/2* (Tang et al., 2016). Cells were incubated for 5 h at 37°C, 5 % $CO_2$ then resuspended in TSB. Transformants were selected on TSB agar supplemented with kanamycin.

## 2.17 Assay for β-galactosidase Activity

The β-galactosidase (β-gal) Specific Activity assay (*Invitrogen*) provides a measure of β-gal function (Miller, 1972). Bacterial cells were washed, pelleted by centrifugation at 13 000 x *g* for 3 min and resuspended in bacterial lysis buffer (0.05 M Tris HCl pH 8, 10 % glycerol, 0.1 % Triton x-100) supplemented with 100 µg/ml lysozyme and 1 % protease inhibitor cocktail. Cells were then sonicated 3 times at 5 microns amplitude, for 15 s each, and stored on ice between sonicating. Cells were re-pelleted and the supernatant retained. The supernatant of each sample was stained with protein assay dye (*BioRad*, Hemel Hempstead, UK) and protein concentrations were calculated by measuring $OD_{595nm}$, relative to known concentrations of bovine serum albumin (BSA). Supernatants were then diluted to 3 different concentrations in 30 µl RNAse-free $H_2O$.

Cleavage buffer (200 µl) and β-mercaptoethanol were added to solutions. Finally, *ortho*-Nitrophenyl-β-galactoside (ONPG -70 µl) was added immediately prior to incubation of the samples at 37°C. The time for samples to go pale yellow was recorded, at which point, stop solution (500 µl) was added to each sample. $OD_{420nm}$ measurements were made with the spectrophotometer and the following equation was applied to adjust values for the incubation time and total protein concentration, leaving final values for β-galactosidase Specific Activity (SA).

Specific Activity= nmoles of ONPG hydrolysed/t/mg protein

nmoles of ONPG hydrolysed= $\frac{(OD_{420})(8x10^5 \text{ nanolitres})}{(4500 \text{ nl/nmoles-cm})(1 \text{ cm})}$

## 2.18 Outer Membrane Vesicle Production

Deoxycholate-extracted OMVs (dOMVs) were produced using a method adapted from that described by Mukhopadhyay et al. (2005). All reagents were obtained from *Fisher Scientific* (Loughborough, UK) unless stated otherwise. See Appendix B for media and buffer recipes.

A bacterial lawn was grown on TSB plates. A streak plate was also performed to assess culture purity. Plates were incubated overnight at 37°C, 5% $CO_2$. The bacterial lawn was transferred to Modified Catlin media (MC.7) and grown in conical flasks at 37°C, 5% $CO_2$, 320 RPM until $OD_{600nm}$ ≥2. Bacteria were heat killed at 56°C for 45 min then centrifuged at 4,500 xg for 1 h. The pellet was resuspended in 5 ml $g^{-1}$ Buffer 1, homogenised, then centrifuged at 20,000 xg for 30 min to pellet whole cells and large debris. The supernatant was retained and centrifuged at 100,000 xg for 2 h to pellet OMVs. OMVs were washed in buffer 2 at 100,000 xg for 2 h then resuspended in buffer 3 and homogenised.

## 2.19 SDS-PAGE

OMV samples were stained with protein assay dye (*BioRad*, Hemel Hempstead, UK) and protein concentrations were calculated by measuring $OD_{595nm}$, relative to known concentrations of BSA.

OMVs (2.5 µg total protein) were loaded into NuPAGE 4-12% Bis-Tris gels with LDS sample buffer (25%) and reducing agent (10%) in water (final volume = 20 µl per well). Novex pre-stained protein ladder (12 µl) was added to a separate well. Gels were run at 200 V for 35 min in MES SDS running buffer (all reagents from *Thermofisher*).

For silver staining, gels were processed using SilverQuest silver staining kit (*Thermofisher*), according to manufacturer's instructions, which allows visualisation of proteins and lipooligosaccharides (LOS).

For western blotting, gels were transferred for 90 min in the XCELL blot module in NuPAGE transfer buffer (*Thermofisher*) with 20% methanol. Transfer membranes were then blocked in 5% milk PBS for 1 h with rocking before interrogation with primary antibody in 5 ml of 5 % milk PBS at 4°C, overnight, with rotation. Primary antibodies were washed from membranes for 3 x 15 min in 0.05 % Tween-20 in PBS (PBS-t), with rocking. Membranes were incubated with 1:5000 secondary antibody (goat-derived anti-mouse IgG-Horse Radish Peroxidase (HRP)) in 5 ml 5 % milk PBS for 1 h at room temperature, with rotation, before washing as previously. Chemiluminescent substrate was added to membranes. In a dark room, films were exposed to membranes prior to development and fixing using Bio-Rad ECL western blotting reagents (*Thermofisher*).

## 2.20 Colony Immunoblot

Bacteria were spread onto agar and grown overnight at 37 °C, 5 % $CO_2$. Nitrocellulose membranes were placed onto the surface of the agar to transfer colonies to the membrane and were then removed and allowed to dry. Membranes were washed in PBS-t before blocking for 1 h in 5 % milk PBS. Membranes were probed with mouse anti-PorA (P1.7) primary antibody at 1:1000 in 5% milk PBS for 2 h with rocking. After washing three times in PBS-t, membranes were probed for 1 h with 1:2000 goat anti-mouse IgG-HRP in 5% milk PBS. Membranes were washed three times as previously. Finally a solution of AEC (3-amino-9-ethylcarbazole) and $H_2O_2$ in sodium acetate was prepared according to manufacturers instructions (*Sigma-Aldrich*) and 2 ml was added per membrane. Membranes were left to develop at room temperature for 10 min before washing in $dH_2O$.

## 2.21 Flow Cytometry

To detect the presence of PorA on the surface of bacteria, 2 x $10^7$ CFU were washed in 1 ml wash buffer (5% FCS in PBS) by centrifugation at 13 000 x *g* for 3 min. Bacteria were resuspended in 100 µl wash buffer before staining with primary antibody for 30 min at 4°C. Bacteria were then washed again in wash buffer and resuspended in 100 µl. Secondary antibody (1 µl anti-mouse IgG Alexaflour-488) was added and bacteria were incubated for a further 30 min at 4°C. Another wash was performed in wash buffer before resuspending bacteria in 100 µl formalin solution (3.75%

formaldehyde + 1% methanol in PBS) and incubating in the dark at room temperature for 15 min. Once fixed, a final wash was performed in wash buffer and bacteria were resuspended in 200 µl wash buffer for analysis. Samples were kept at 4°C until acquisition on BD FACS Calibur. Flow cytometric analysis was performed using FlowJo.

## 2.22 Serum Bactericidal Activity Assay

OMVs were used to immunise mice using a protocol described previously (Brookes et al., 2013). Briefly, ten mice per group were immunised intraperitoneally with OMV containing 10 µg total protein and 0.33% Alhydrogel on days 0, 21 and 28. Sera were collected and pooled on day 35. Bacteria were grown overnight on CBA and inoculated into 10 ml Frantz medium, where they were grown at 37°C with shaking for 3 h. Bacteria were transferred to bactericidal buffer (Hanks buffered saline solution (*Invitrogen*) with 1% BSA) then diluted in bactericidal buffer to $6 \times 10^4$ CFU/ml. A series of 1:2 dilutions of heat-inactivated serum (inactivated by heating to 56°C for 30 min) were performed (20 µl) and mixed with 10 µl *N. meningitidis* and 10 µl human complement. These mixtures were incubated at 37°C, 900 RPM for 1 h before plating samples on CBA and counting colonies the following day. Titres were calculated as the reciprocal dilution which gave >50% killing compared with colonies at t=0.

## 2.23 Serum Killing Assay

Bacteria were grown to log phase in TSB before measuring the $OD_{600nm}$ and adjusting the concentration to $10^8$ CFU/ml in Frantz media. Aliquots of the $10^8$ CFU $ml^{-1}$ bacterial culture (20 µl) were transferred to a 96-well plate. Human serum (10%) and/or anti-PorA (P1.7) antibody (1%) were added to wells, which were made up to a final volume of 200 µl with Frantz media. All conditions were performed in duplicate. Viable counts were performed before and after incubating for 1 h at 37°C, 5% $CO_2$. Following the first passage, 100 µl per well were transferred to a new 96-well plate containing fresh serum (20 µl) and/or antibody (2 µl). Wells were made up to a final volume of 200 µl with Frantz media before re-incubating at 37°C. A total of four passages were performed and viable counts were measured between each passage. Where relevant, an inactivated serum control was performed using human sera heated to 56°C for 30 min.

## 2.24 Adherence Assays

HEp2 cells were grown in DMEM + 10 % FCS at 37°C, 5 % $CO_2$ and seeded in 24 well plates at 5 x$10^5$ cells per well. The following day, media was removed and wells were washed 3x with PBS prior to infection. Detroit 562 cells were grown in MEM + 10 % FCS at 37°C, 5 % $CO_2$ and seeded in 24 well plates containing sterile glass coverslips at 6 x$10^5$ cells per well. Media was changed daily until cells were confluent (2-3 days), then removed before washing wells 3x with PBS and infecting. Cell counts were performed prior to infection: media was removed, cells were washed with 1 ml PBS and 100 µl of trypsin-EDTA (0.25%, *Sigma*) was added before incubating at 37°C, 5 % $CO_2$ until all cells had detached. The contents of the well was mixed with 800 µl cell culture media and 100 µl trypan blue (0.4%, *gibco*), then counted in duplicate on a haemocytometer. An additional 'coverslip only' cell count was performed when using glass coverslips: Cells were washed with PBS and the coverslip was transferred to a new well before adding trypsin to the coverslip.

Bacteria were grown from stock cultures overnight on CBA, before resuspending multiple colonies in 3 ml TSB (*N. lactamica* and *N. cinerea*) or MHB (*N. meningitidis*). Bacteria were then grown to logarithmic phase at 37°C, 5 % $CO_2$, 320 RPM, before measuring culture turbidity ($OD_{600nm}$). Bacterial concentrations were adjusted to allow for a Multiplicity of Infection (MOI) of 30 in 0.5 ml cell culture media. Wells containing cells and cell-free controls were infected in duplicate for 0.5, 1.5 or 3 h, alongside non-infected controls.

Following infection, HEp2 cells and corresponding cell-free controls were washed 3x with PBS before adding 1 ml 1 % saponin in PBS. Detroit cells and corresponding cell-free controls were washed 3x in PBS before transferring the coverslip to a new well containing 1 ml 1 % saponin in PBS. Samples were incubated in saponin for 10 min at 37°C, 5 % $CO_2$, 320 RPM and viable counts were performed on CBA, as described in Section 2.2.1.

## 2.25 Gentamicin Protection Assay

Detroit 562 cells were seeded at 6 x$10^5$ cells per well and grown to confluence in MEM + 10 % FCS in a 24-well plate. Bacteria were grown to log phase in TSB at 37°C, 5 % $CO_2$ with shaking. Detroit cells were washed in PBS and the number of cells present in a representative well were enumerated using a haemocytometer. Bacteria at an MOI of 100 in 500 µl cell culture media were then used to infect each well. Infections were performed in duplicate for 2, 4 or 6 h. Following infection, viable counts of bacteria in the supernatant were performed. Media was then

removed and cells were washed in PBS before lysing with 1 % saponin in PBS for 10 min and performing a viable count of total cell-associated bacteria. For internalised bacteria, cells were treated with gentamicin (100 μg $ml^{-1}$) and penicillin (100 μg $ml^{-1}$) in cell culture media for the final 30 min of infection before washing cells, lysing with 1 % saponin in PBS and performing viable counts. All viable counts were performed on CBA and incubated overnight at 37°C, 5 % $CO_2$.

## 2.26 E-test

E-testing was performed for ciprofloxacin, ceftriaxone and rifampicin by a Public Health England biomedical scientist. A lawn of bacteria was spread at a standardised density on Muellar Hinton agar with lysed blood before adding the E-test strip and incubating for 24 h at 37 °C, 5 % $CO_2$. The E-test strip contains a concentration gradient of a particular antibiotic and the Minimum Inhibitory Concentration (MIC) was determined by the lowest concentration at which bacterial growth was prevented. Testing was performed in accordance with EUCAST guidelines (EUCAST, 2018).

## 2.27 Transmission Electron Microscopy

Preparation of samples for transmission electron microscopy (TEM) was adapted from methods described previously (Tang et al., 2016). Bacteria were streaked onto CBA and grown overnight at 37°C, 5 % $CO_2$. Formvar-coated grids were touched onto individual colonies, then washed 3 times for 1 min each by resting the grid on 10 μl drops of water. Finally, bacteria were stained with 5% ammonium molybdate plus 0.5 % trehelose (aqueous) for 10 s before visualising on a Hitachi H7000 TEM.

## 2.28 Statistical Analysis

All statistical analyses were performed using *GraphPad Prism 6* (San Diego, USA).

## 2.29 Whole Genome Sequence Data Analysis

Bacteria were sent to MicrobesNG for whole genome illumina sequencing using HiSeq or MiSeq. MicrobesNG used 2 x250bp paired-end reads and trimmed using Trimmomatic. The reference genome was identified using Kraken and reads were mapped to this using BWA mem for assembly. Sequences were re-annotated by Anish Pandey using Prokka V1.12 (Seemann, 2014),

with the *Neisseria* genus and a list of proteins (FASTA format) derived from two, previously annotated, complete *N. lactamica* genomes used as a basis for annotation.

To compare strains, each GM strain was compared to the *N. lactamica* Y92-1009 reference strain (Pandey et al., 2017) using snippy V2.2 (Seemann, 2018). Trimmed reads (fastq files) of GM strains were mapped against the annotated reference to identify any insertions, deletions, recombination events or Single Nucleotide Polymorphisms (SNPs). The core snippy function was then used to concatenate these comparisons into one core genome.

Following 28-day serial passage of isolates *in vitro*, snippy was also used to map the fastq files from day 28 isolates of each strain to the annotated genbank files of their respective day 0 isolates and identify any mutations. All snippy analysis was performed using the default analysis settings, where minimum mapping quality is 60, minimum coverage of variant site is 10 and the minimum proportion for variant evidence is 0.9. Mutations were screened manually and tabulated.

# *Chapter 3* Characterising Transformation of *N. lactamica*

*Note: Data in Figures 8 and 10 were partially obtained during my MRes degree in Infection and Immunity, University of Southampton (2014-2015).*

## 3.1 Introduction

*In vitro* transformation of *N. lactamica* has historically proven difficult. RMSs are a defence mechanism used by bacteria to prevent transformation with heterologous and potentially deleterious DNA, and they are abundant in *Neisseria*.

Out of 22 RMSs identified in various strains of *N. meningitidis*, NlaIV is one of the two most commonly occurring restriction enzymes, present in 19 out of 20 strains analysed (Budroni et al., 2011). It has a 4-nucleotide recognition sequence (GGNNCC), which is shorter than the majority of RMS recognition sequences, meaning it cuts at relatively high frequency. In *N. meningitidis,* the presence of NlaIV restriction sites in heterologous transforming DNA was shown to reduce transformation efficiency. Interestingly, the presence of NlaIV restriction sites had no effect on transformation when transforming DNA had greater homology to chromosomal DNA (Ambur et al., 2012). The mechanism of selectively digesting heterologous DNA is unclear, however it is possible that DNA with greater homology is more rapidly recombined into the chromosome, therefore allowing less time for restriction.

As well as being prevalent among *N. meningitidis,* NlaIV is also expressed in *N. lactamica,* along with one other restriction enzyme with a 4-nucleotide recognition sequence - NlaIII. Whilst NlaIV is expressed in multiple Neisserial species, a BLAST search of the NlaIII gene (*nlaIII*) revealed that it is not present in any other sequenced *Neisseria*, including *N. meningitidis*. However, homologues exist in *Campylobacter jejuni* and *Helicobacter spp.* All other known RMSs in *N. lactamica* have recognition sequences greater than 4 nucleotides, so are likely to cut DNA at a lower frequency, assuming equal distribution of bases. This research focuses on NlaIII and NlaIV, as they are deemed to have the most potent restriction activity of the restriction enzymes identified in *N. lactamica,* and are therefore likely to present the greatest barrier to transformation in this species.

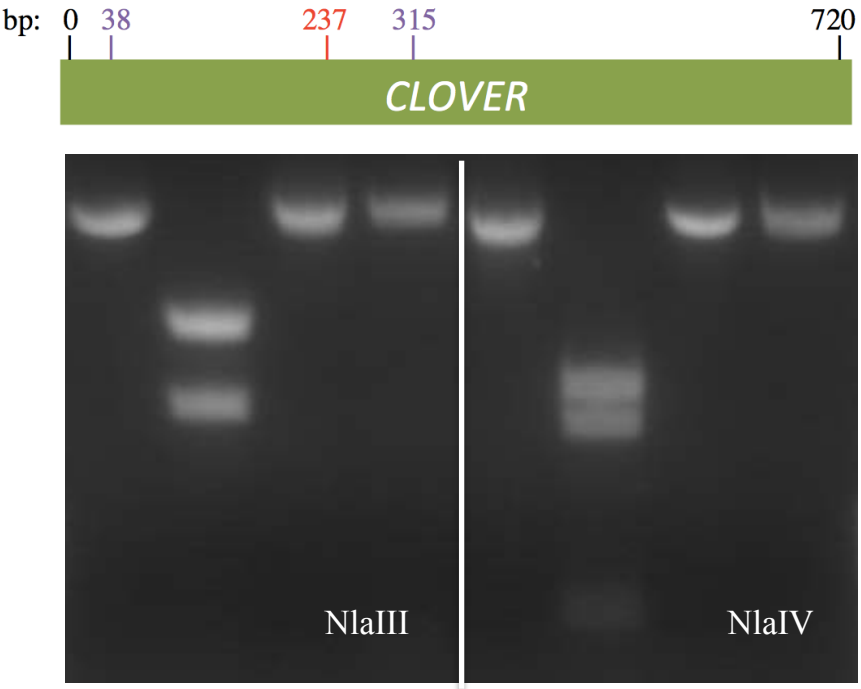
## 3.2 Restriction Activity in *N. lactamica* Transformation

In an attempt to replicate the protective effect of the methyltransferase component of a RMS in our donor DNA, we incorporated 5-methyl deoxycytosine (5m-dCTP) in place of cytosine during PCR amplification, hereby referred to as hmPCR. This process results in donor DNA containing exclusively hypermethylated cytosine residues.

The gene encoding the green fluorescent protein, CLOVER, known to contain both NlaIII and NlaIV restriction sites (CATG and GGNNCC, respectively) was amplified with primers 3 & 4 using either PCR or hmPCR. Products were then exposed to the relevant restriction enzyme, before visualising DNA by gel electrophoresis (Figure 7). The hmPCR products were resistant to digestion by both NlaIII and NlaIV. This was in contrast to DNA amplified from the same templates by standard PCR, which, under identical conditions, was cut by both enzymes to produce the expected band sizes.

These results suggest that DNA generated by hmPCR would be less susceptible to digestion by restriction enzymes in *N. lactamica.* This would increase the opportunity for DNA to be incorporated into the chromosome. It was therefore hypothesised that exposing *N. lactamica* to hmPCR products would result in an increased rate of transformation relative to normal PCR products.

To examine the effect of cytosine-hypermethylation on transformation of *N. lactamica,* a construct was developed to compare transformation with DNA produced through standard PCR or hmPCR. A cassette containing kanamycin-resistance gene *aphA3,* flanked either side by DNA homologous to the *N. lactamica nlaIII* locus was constructed within a pUC19 vector (Figure 8a). The *nlaIII* locus is naturally devoid of the NlaIII recognition sequence (CATG) and *aphA3* was modified to retain the cognate amino acid sequence, whilst removing any NlaIII recognition sites. The *ΔnlaIII:aphA3* cassette was transformed into and maintained in *E. coli*. Plasmids were extracted and then linearised by digesting with Xba1. The *ΔnlaIII:aphA3* cassette was amplified from the Xba1-cut plasmid by standard or hypermethylated PCR with primers 1 and 2, as shown in Figure 8a.




| Exposure to endonuclease | - | + | - | + | - | + | - | + |
|---|---|---|---|---|---|---|---|---|
| DNA amplification method | PCR | | hmPCR | | PCR | | hmPCR | |

Figure 7. Effect of Hypermethylation of PCR Products on NlaIII and NlaIV Restriction Endonuclease Activity

The *CLOVER* gene, containing NlaIII (shown in red) and NlaIV (shown in purple) recognition sequences (CATG and GGNNCC, respectively) was amplified by standard or hmPCR with primers 3 & 4. Products were incubated with NlaIII or NlaIV in CutSmart buffer at 37°C for 1 hr, prior to deactivating enzymes at 65°C for 20 min. DNA products were loaded into 0.7% agarose gels and run at 90V for approximately 40 min.

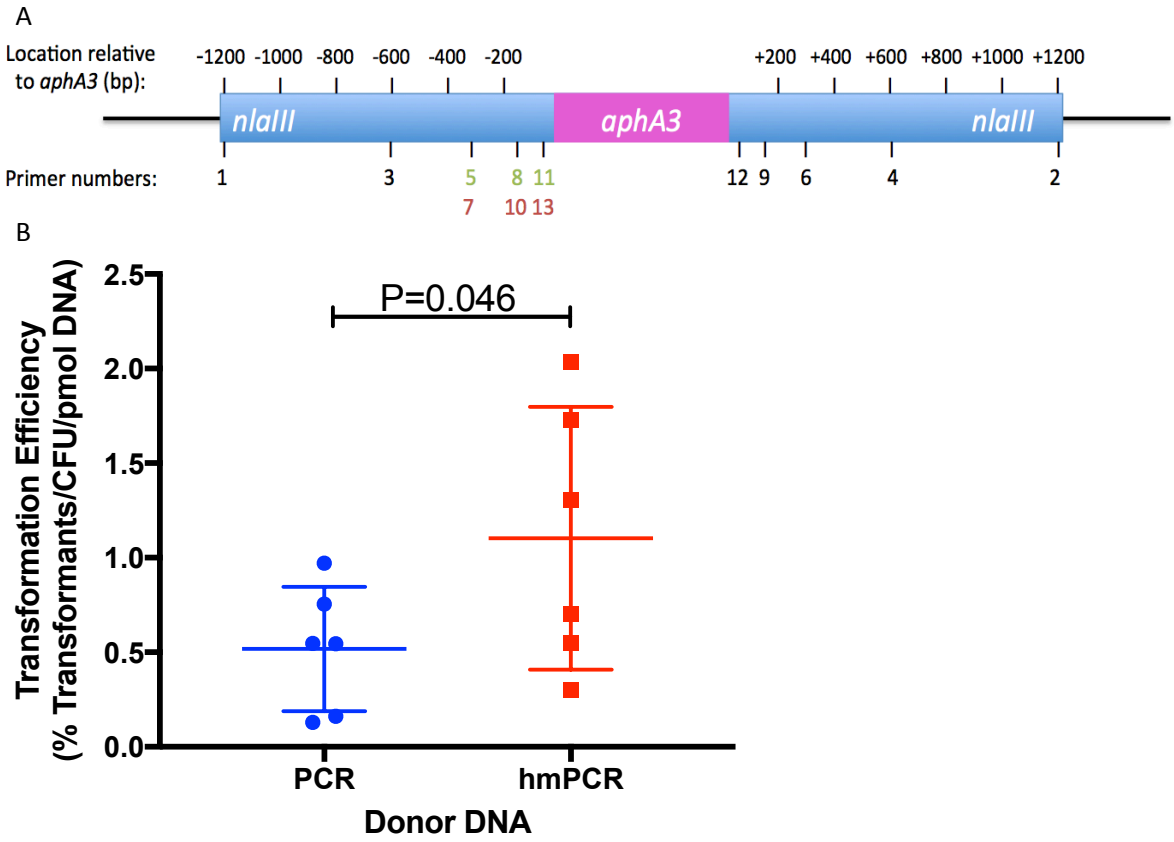



Figure 8. Testing the Effect of Cytosine Hypermethylation in Donor Material on Transformation of *N. lactamica*

A) The *ΔnlaIII:aphA3* cassette (containing kanamycin-resistance gene *aphA3,* flanked by regions of homology to the *N. lactamica nlaIII* chromosomal locus), was maintained in a pUC19 vector in *E. coli.* Binding locations of primers 1-13, relative to *aphA3* are shown. Primers to the left of *aphA3* bind to the 5'-3' strand and primers to the right bind to the reverse strand. Primers in red contain a DUS and in green contain a scrambled DUS (S-DUS). B) *ΔnlaIII:aphA3* was amplified by PCR and hypermethylated PCR (hmPCR) with primers 1 & 2, before transforming 0.46 pMol into *N. lactamica*. Transformants were selected on TSB + kanamycin. Transformation efficiencies are shown. Mean±SD. One-tailed T-test (n=6).

PCR products were used as a donor material to transform *N. lactamica.* Viable counts were performed before selecting for transformants on kanamycin-containing TSB agar. Transformation efficiencies were calculated by dividing the number of transformants by the total viable count. Transformation efficiencies were adjusted for DNA concentration (pMol) and expressed as a percentage of total bacteria (Figure 8b). A one-tailed Student's T test comparison of the transformation efficiencies shows that transformation was significantly enhanced with hmPCR product when compared to standard PCR product (P=0.046).

To further understand the interaction of RMSs and their recognition sites during transformation of *N. lactamica*, an additional gene, *CLOVER*, was added to the *ΔnlaIII:aphA3* construct for use as donor material for transformation. CLOVER is a green fluorescent protein and its gene contains two NlaIII recognition sites. The new construct, *ΔnlaIII:CLOVER-aphA3* (Figure 9b), containing *CLOVER* followed by *aphA3*, inserted into *nlaIII*, was maintained in *E. coli* within pUC19. The two NlaIII recognition sites were sequentially removed from pUC19 *ΔnlaIII:CLOVER-aphA3* by site-directed mutagenesis (SDM) with primers 33-36, whilst maintaining the cognate amino acid sequence. Resulting plasmids contained 0, 1 (in two different locations) or 2 CATG sequences in *CLOVER.*

To confirm the presence or absence of CATG sites, inserts were amplified from pUC19 *ΔnlaIII:aphA3-CLOVER* by PCR and hmPCR, using primers 3 and 4. PCR products were digested with NlaIII at 37°C before observing product sizes by DNA gel electrophoresis (Figure 9a). PCR-amplified constructs containing CATG sequences were digested and formed multiple bands on the gel when exposed to NlaIII, whereas those amplified by hmPCR remained full length. Where CATG sequences were removed from the construct, PCR-amplified constructs were no longer digested and remained the same length as those amplified by hmPCR or those not exposed to the endonuclease. Figure 9a confirms where NlaIII recognition sites are present in the *ΔnlaIII:aphA3-CLOVER* constructs and further supports that the activity of NlaIII is inhibited by cytosine-hypermethylation.

The *ΔnlaIII:CLOVER-aphA3* inserts, with varying numbers of NlaIII recognition sites, were amplified by PCR and hmPCR with primers 3 and 4, then transformed into *N. lactamica.* Transformation efficiencies were calculated as before (Figure 9b). When transforming DNA was amplified by standard PCR (black), the number of CATG sites negatively impacted the efficiency of transformation, however the location of the recognition site in the gene made no observable difference. These differences were not found to be significant by one-way ANOVA with post-hoc Tukey analysis ($p>0.05$). When transforming DNA was generated by hmPCR (red), transformation

efficiencies were significantly increased ($p<0.05$) and any effect of the presence of CATG sequences was completely abrogated.

To characterise the role of NlaIII in transformation, an NlaIII-deficient strain of *N. lactamica* was generated, without introducing any novel genes. First, a construct was generated in pUC19, containing sequences homologous to the regions immediately 5' and 3' of *lacZ*, which encodes the enzyme β-galactosidase (β-gal). The *lacZ* gene was omitted from the plasmid. The insert, containing the regions surrounding *lacZ,* was amplified by hmPCR using primers 14&15 and transformed into *N. lactamica,* with the aim of truncating *lacZ. N. lactamica* expressing β-gal form blue colonies on X-Gal-containing media, whereas *lacZ*-deficient colonies are white. White colonies were selected on TSB X-Gal and the resulting strain was termed Nlac *ΔlacZ*. A pUC19 construct was then made to encode *lacZ* flanked by regions of homology to the *nlaIII* locus, termed pUC19 *nlaIII:lacZ*. The insert was amplified by hmPCR using primers 1 & 2 and transformed into Nlac *ΔlacZ,* thereby restoring β-gal activity and knocking out NlaIII by insertional inactivation. Transformants were selected as blue colonies on TSB X-Gal. The resultant strain: Nlac *ΔnlaIII,* is NlaIII-deficient with no other genes added or removed.

Next, the *aphA3* and *CLOVER* genes from pUC19 *ΔnlaIII:aphA3-CLOVER* were transferred to a new pUC19 plasmid, where they were flanked by DNA homologous to a non-coding intergenic region of the *N. lactamica* chromosome, hereafter referred to as Non-Homologous Chromosomal Insertion Site 1 (NHCIS1). This resulted in the production of plasmid pUC19 NHCIS1:*aphA3-CLOVER* (Figure 9c). NlaIII recognition sequences were again removed from *CLOVER* in this plasmid by SDM, using the same primers as previously and the resulting plasmids formed the next templates for donor DNA.

NHCIS1*:CLOVER-aphA3* inserts containing 0 or 2 CATG sequences were amplified by PCR and hmPCR with primers 16 & 17. PCR products were transformed into Nlac WT and *ΔnlaIII,* and transformation efficiencies were calculated (Figure 9c).

Cytosine hypermethylation increased the transformation efficiency by approximately $10^3$, to a level comparable to that observed in the *nlaIII* locus. However, neither the presence of CATG sequences in donor DNA, nor NlaIII activity in the recipient strain had any effect in the NHCIS1 locus, contrary to observations in the *nlaIII* locus.

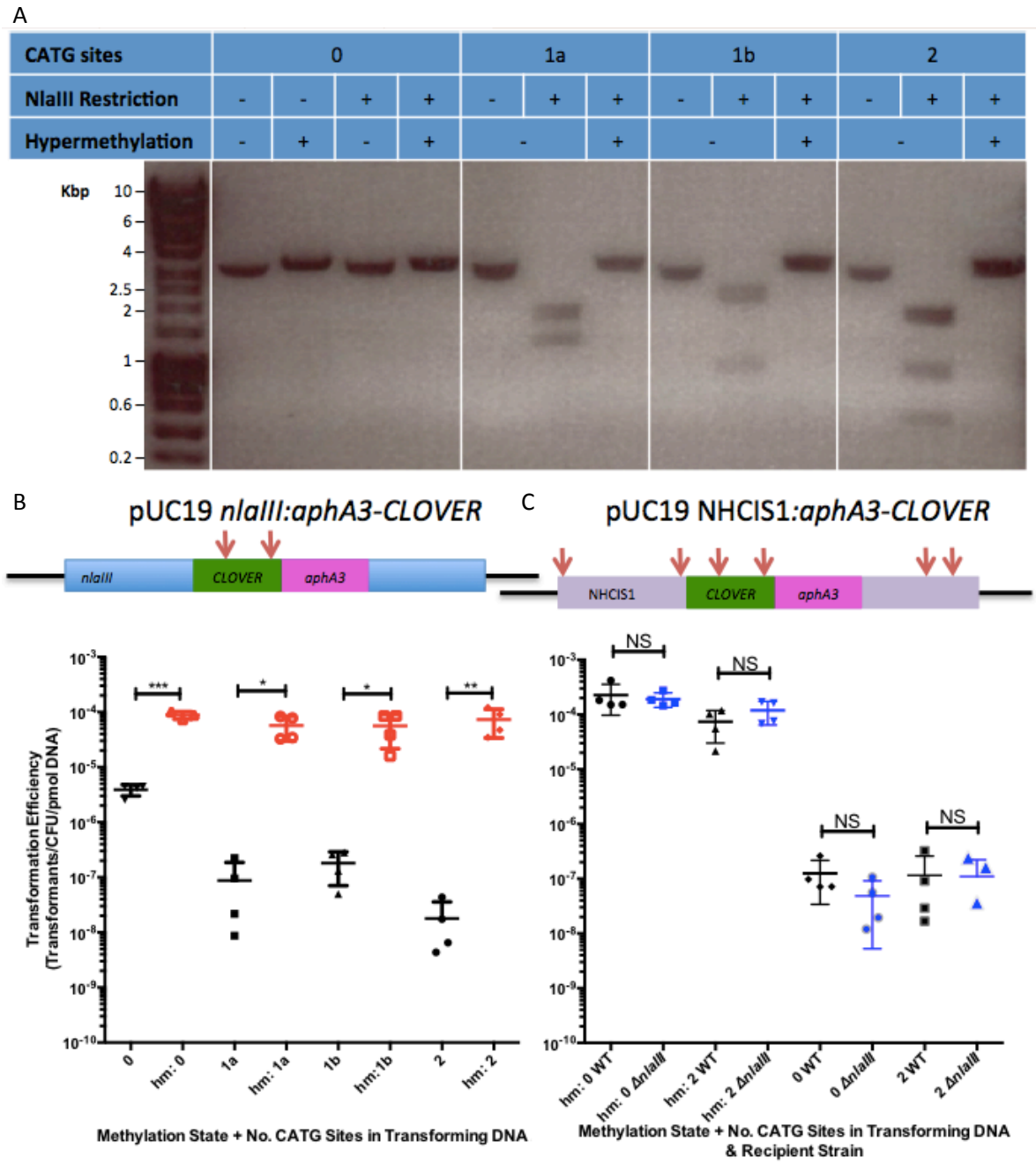



Figure 9. Effect of Hypermethylation and NlaIII Activity on Transformation of *N. lactamica*

A *CLOVER-aphA3* insert was flanked by DNA homologous to *N. lactamica nlaIII* or NHCIS1 loci and maintained in a pUC19 vector in *E. coli.* Two CATG sequences (NlaIII recognition sites) in *CLOVER* were removed by site directed mutagenesis. A) *ΔnlaIII:CLOVER-aphA3* constructs containing 0, 1 (1a and 1b) or 2 CATG sequences were amplified by PCR and hmPCR with primers 3 & 4. Products were incubated in the presence or absence of NlaIII for 1 h at 37°C before deactivating the enzyme at 65°C for 20 min. Products were run on 0.7% agarose at 90V for approximately 40 min. B) *ΔnlaIII:CLOVER-aphA3* constructs were amplified by PCR and hmPCR with primers 3 & 4, before transforming 0.46 pMol PCR product into Nlac WT. Transformations with standard PCR product are shown in black and with hmPCR product in red. C) NHCIS1:*CLOVER-aphA3* constructs were amplified by PCR and hmPCR with primers 16&17, before transforming 0.46 pMol PCR product into Nlac WT (black) or Nlac *ΔnlaIII* (blue). B & C) Transformants were selected on TSB kanamycin. Transformation efficiencies are shown. One-way ANOVA with post-hoc Tukey analysis (Not Significant (NS) >0.05, *≤0.05, **≤0.01, ***≤0.005, n=4). Diagrammatic representations of each construct include the locations of NlaIII recognition sites, shown with red arrows.

## 3.3 Other Factors Affecting *N. lactamica* Transformation

To examine the effect of various other factors on transformation efficiency in *N. lactamica,* the *ΔnlaIII:aphA3* cassette described in Section 3.3 was amplified from the Xba1-cut plasmid by PCR or hmPCR and transformed into *N. lactamica.* For these experiments, viable counts were performed before selecting for transformants on kanamycin-containing TSB agar. Transformation efficiencies were calculated by dividing the number of transformants by the total viable count. Where relevant, transformation efficiencies were adjusted for DNA concentration (pMol) and expressed as a percentage of total bacteria.

A diagrammatic representation of the *ΔnlaIII:aphA3* cassette, along with the primers used to amplify it in this section, is shown in Figure 8a.

### 3.3.1 Length of Homologous Regions Flanking the Heterologous Insert

The following primer pairs were used to amplify the *ΔnlaIII:aphA3* cassette by hmPCR before transforming *N. lactamica* with 0.46 pMol of PCR product: 1 & 2, 3 & 4, 6 & 7, 9 & 10 and 12 & 13. Primer pairs generated PCR products with the length of homology to the *N. lactamica nlaIII* locus ranging from 75 to 1200 bp either side of *aphA3*, as shown in Figure 8*.* Where PCR products contained 600 or 1200 bp homology either side of *aphA3,* there were 2 DUS contained within the construct, however for smaller constructs, only one DUS was present. To prevent the number of DUS acting as a confounding factor, an additional DUS was added to the smaller constructs on the forward primer. As a result, all PCR products contained 2 DUS.

There was a linear increase in transformation efficiency with increasing lengths of homology (Figure 10a), which was found to be significant by one-way ANOVA ($P<0.0001$).

### 3.3.2 DNA Concentration

Primers 3 and 4 were used to amplify the cassette by hmPCR. The DNA concentration of the PCR product was measured and diluted before transforming *N. lactamica* with 10 µl at various concentrations, from 0.06 to 1.06 pMol (Figure 10b). Whilst the difference between 0.26 and 0.46 pMol donor DNA was found to be significant by one-way ANOVA with post-hoc Tukey analysis ($P\leq0.05$), there was no apparent correlation between DNA concentration and transformation efficiency, and the level of transformation was comparable between all other concentrations.

### 3.3.3 Number of DNA Uptake Sequences

To determine the effect of the number of DUS in donor DNA, *N. lactamica* was transformed with DNA containing either one DUS and one scrambled DUS (S-DUS) or two DUSs. In the *ΔnlaIII:aphA3* constructs containing up to 300 bp of homologous DNA either side of the *aphA3* insert, there is one DUS within each construct, which is located immediately 3' of the *aphA3* coding sequence. During hmPCR amplification of these constructs for transformation, either an additional DUS or a S-DUS was incorporated at the 5' terminus. The S-DUS comprised of the same bases in the DUS but the order was rearranged. This experiment was performed with three different homologous flanking lengths. The *ΔnlaIII:aphA3* construct was amplified by hmPCR using primers 6 & 7, 9 & 10 and 12 & 13 to generate products that each contained 2 DUSs, with homologous flanking lengths of 300, 150 and 75 bp either side of *aphA3* respectively. The 5' terminal DUS was incorporated by the forward primers: 7, 10 and 13. Primers 5, 8 and 11 bind to the construct at the same locations as 7, 10 and 13, as shown in Figure 8a, but incorporate a S-DUS rather than a DUS. When primers 5, 8 and 11 were used in place of primers 7, 10 and 13, products contained one DUS and one S-DUS.

*N. lactamica* was transformed with 0.46 pMol hmPCR product and transformation efficiencies were calculated Figure 10c. Transformation was enhanced when DNA contained 2 DUS rather than one with all three homologous flanking lengths, however the effect was only significant with 300 bp of homology.

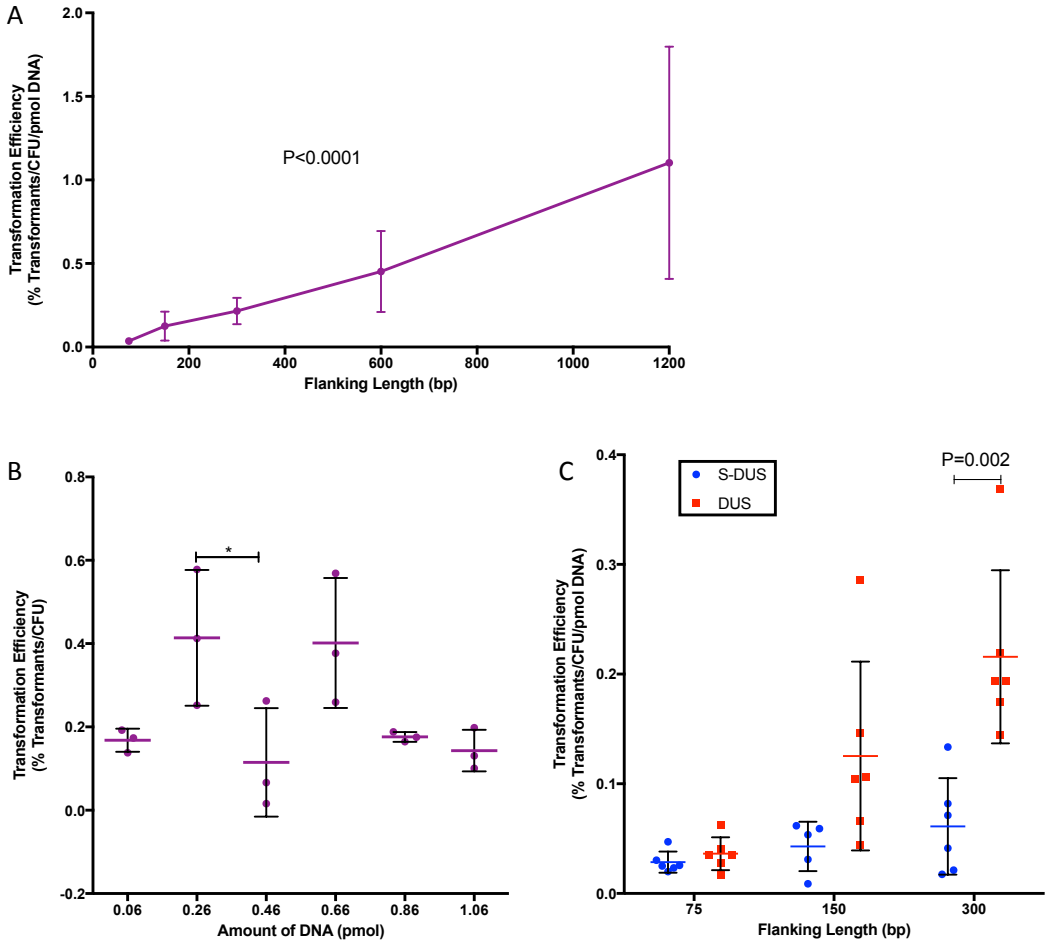



Figure 10. Factors Affecting Transformation Efficiency of *N. lactamica*

A) *ΔnlaIII:aphA3* with various lengths of homology to *nlaIII* was amplified by hmPCR, before transforming 0.46 pMol into *N. lactamica*. All inserts contained 2 DUSs. One-way ANOVA (n=6). B) *ΔnlaIII:aphA3* was amplified by hmPCR with primers 3 & 4 before transforming *N. lactamica* with 10 µl product at various concentrations. One-way ANOVA with post-hot Tukey analysis. (*P≤0.05, n=3). C) *ΔnlaIII:aphA3* with flanking lengths of 75, 150 and 300 bp was amplified by hmPCR. The insert contained 1 DUS, and forward primers incorporated an additional DUS or scrambled (S-)DUS. Products (0.46 pMol) were transformed into *N. lactamica*. Multiple T-tests (Q=1, n=6). A-C) Transformants were selected on TSB kanamycin. Transformation efficiencies are shown.

## 3.4 Transformation of *N. lactamica* is Pilus-Dependent

As transformation of *N. lactamica* is positively influenced by the number of DUSs, it was hypothesised that the competence mechanism in this species is the same as that observed in other *Neisseria*. Competence has been shown to be pilus-dependent in *N. meningitidis, N. gonorrhoeae, Neisseria elongata* and *N. cinerea* (Chen and Dubnau, 2004; Higashi et al., 2011; Seifert et al., 1990; Tang et al., 2016). To confirm whether transformation is pilus-dependent in *N. lactamica*, the *pilE* gene, which encodes the major pilin subunit, PilE, was disrupted. A gBlock was designed to encode *pilE*, with *aadA1* (a spectinomycin-resistance gene) spliced into the centre of the gene (Figure 11a). The gBlock also contained 30 bp homology to the pSC101 plasmid vector, either side of *pilE.* The pSC101 vector component was PCR amplified from a pre-existing plasmid using primers 18 & 19 and combined with the gBlock by Gibson Assembly. The insert was amplified by hmPCR with primers 20 & 21 and transformed into *N. lactamica,* generating strain Nlac *ΔpilE,* which was selected on TSB spectinomycin. This PilE-deficient strain is further characterised in Chapter 4.

The *ΔnlaIII:aphA3* cassette described previously (Figure 8a) was amplified by hmPCR with primers 3 & 4 and transformed into Nlac WT and *ΔpilE.* Viable counts were performed before selecting for transformants on kanamycin-containing TSB agar. Transformation efficiencies were calculated, adjusted for DNA concentration (pMol) and expressed as a percentage of total bacteria (Figure 11b).

Whilst transformation was consistently observed in the WT strain, no kanamycin resistant isolates were recovered when Nlac *ΔpilE* was exposed to hmPCR-amplified *ΔnlaIII:aphA3* on five separate occasions. The difference in transformation efficiency between the two strains is highly significant ($P<0.0001$). This data demonstrates that transformation in *N. lactamica* is entirely pilus-dependent.

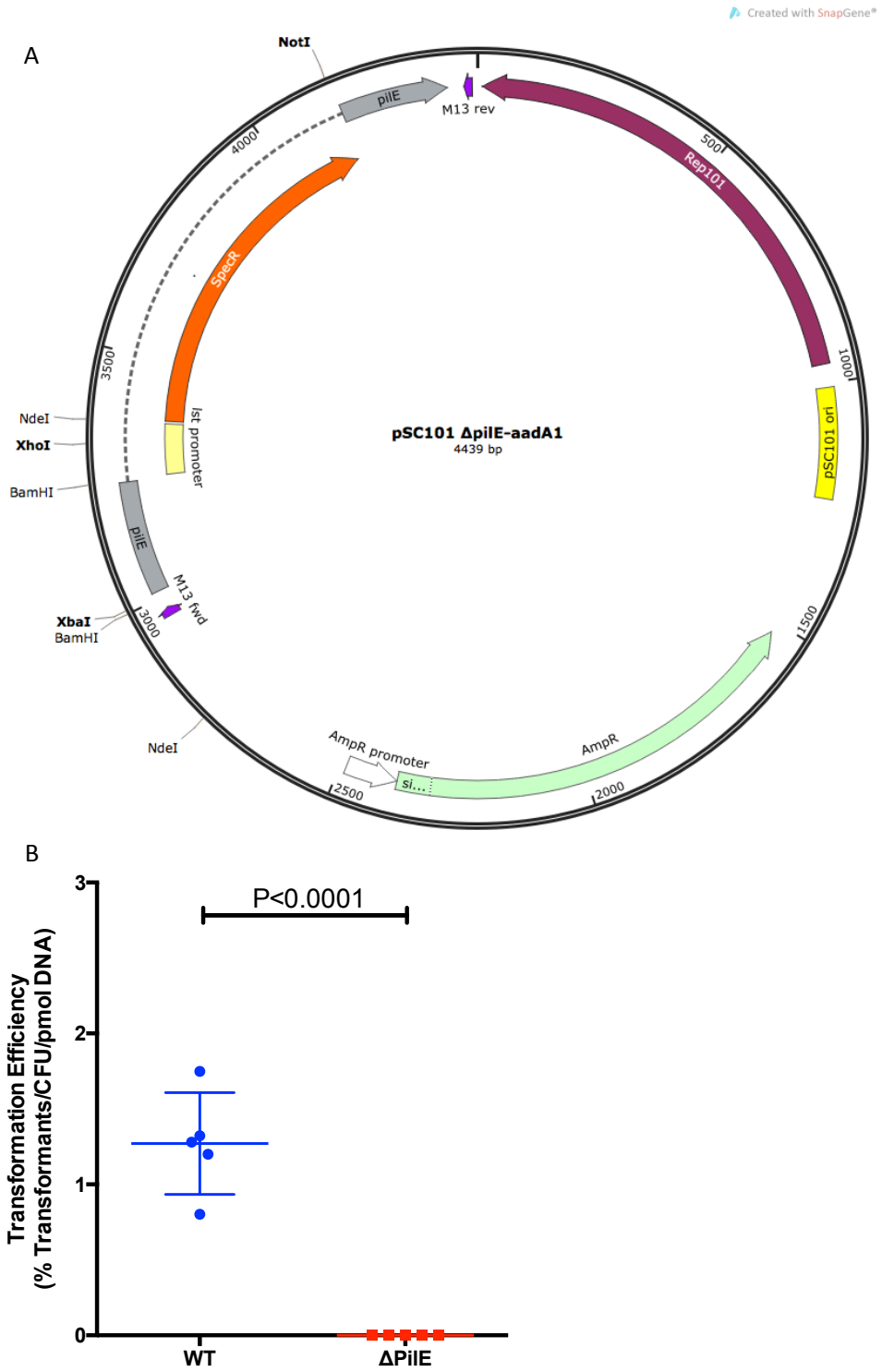



Figure 11. Transformation in PilE-deficient *N. lactamica*

A) A gBlock containing *aadA1* in the centre of *pilE* was combined with a pSC101 vector by Gibson Assembly. pSC101 *ΔpilE-aadA1* was maintained in *E. coli.* For insertional inactivation of *pilE, ΔpilE-aadA1* was amplified by hmPCR and transformed into *N. lactamica.* Transformants were selected on TSB spectinomycin, generating strain Nlac *ΔpilE*. Genes, promoters and relevant restriction sites are shown. Created using SnapGene. B) *ΔnlaIII:aphA3* was amplified by hmPCR with primers 3 & 4, before transforming 0.46 pMol into Nlac WT and Nlac *ΔpilE.* Transformants were selected on TSB kanamycin and transformation efficiencies are shown. Mean±SD. One-tailed T-test (n=5).

## 3.5 Accurate Targeting of Genetic Modifications to a Chromosomal Locus

To confirm that transformation of *N. lactamica* involved the accurate targeting of modifications to its desired chromosomal locus, analysis was performed on strains of *N. lactamica* that gained kanamycin resistance following exposure to hmPCR-amplified DNA from the *ΔnlaIII:aphA3* construct. Isolates selected for analysis had been exposed to donor DNA containing 600 bp (35 isolates) or 1200 bp (25 isolates) of chromosomal homology either side of *aphA3*. Putative transformants, selected by their ability to grow in the presence of kanamycin, were grown from stock cultures and washed in PBS before extracting genomic DNA (gDNA). The *nlaIII* chromosomal loci of Nlac WT and putative transformants were PCR amplified from gDNA using primers 11 & 12. The size of PCR products was analysed by gel electrophoresis (Figure 12).

The PCR product resulting from a Nlac WT gDNA template yielded a single band at approximately 800 bp. Using the same primers to amplify gDNA from 100% of (60) putative transformants, an insert of approximately 900 bp was detected in the *nlaIII* locus, resulting in a single band of approximately 1600 bp.

## 3.6 Genetic Exchange in Biofilms

Having determined that *N. lactamica* is naturally competent and appears to share similar characteristics with other species in its genus, it was hypothesised that the competence machinery could facilitate genetic exchange between *Neisseria* species. Considering the close genetic relationship between *N. lactamica* and *N. meningitidis,* and the fact that they both colonise the nasopharynx, these two species were deemed an appropriate pair to test this hypothesis. To best facilitate genetic exchange, these two bacterial strains were grown in biofilms. An acapsulate strain of *N. meningitidis* MC58 was chosen, as previous studies have demonstrated that capsule inhibits biofilm formation on abiotic surfaces (Lappann et al., 2006; Yi et al., 2004b). In this strain, the capsule polymerase gene *siaD* has been inactivated by the insertion of kanamycin-resistance gene *aphA3.* A GM strain of *N. lactamica* was produced to express spectinomycin-resistance gene *aadA1* in the NHCIS1 locus.

After 48 and 72 hours of growth, biofilms were disrupted and antibiotic resistance was assessed. Viable counts were performed on CBA in the presence of X-Gal with kanamycin and/or spectinomycin. At both time points, the biofilms consisting of exclusively *N. meningitidis* were larger in bacterial number than those consisting of exclusively *N. lactamica.* Colonies recovered

from Nlac biofilms appeared blue, as expected, and were resistant to spectinomycin. Colonies recovered from Nmen biofilms appeared white and were resistant to kanamycin. No isolates that were resistant to both antibiotics were recovered from monoculture biofilms. When both species were grown in co-culture, inoculated with an equal number of each strain, approximately equal numbers of colonies were recovered on kanamycin-containing agar and spectinomycin-containing agar. All colonies on kanamycin-containing agar were blue, so were deemed to be Nlac, and all colonies on spectinomycin agar were white, so were deemed to be Nmen. A small number of isolates were recovered from co-culture biofilms on agar containing both antibiotics, which is indicative of genetic exchange. In all cases, colonies resistant to both antibiotics appeared white in colour, suggesting they were *N. meningitidis* that has acquired spectinomycin-resistance from *N. lactamica.*

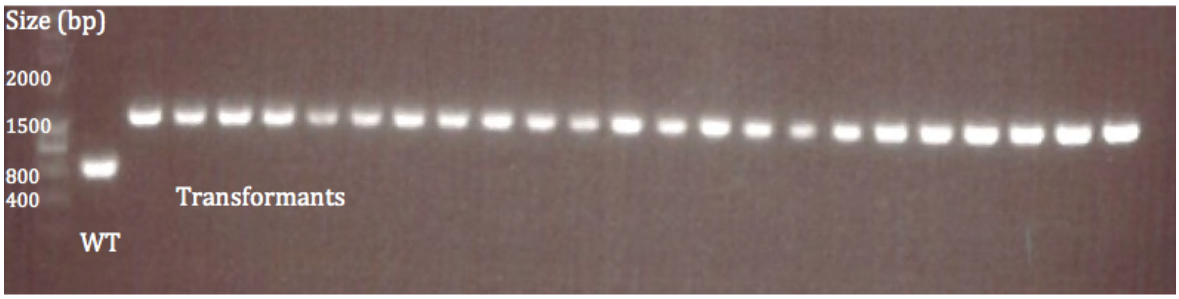



Figure 12. PCR Products Amplified from the *nlaIII* Locus of GM *N. lactamica* gDNA

The *nlaIII* loci of gDNA from Nlac WT and transformants were PCR amplified with primers 11 & 12, before running products on 0.7% agarose at 90V for 40 min. Transformants were generated using hmPCR-amplified *ΔnlaIII:aphA3* with 600 bp (35 isolates) or 1200 bp (25 isolates) flanking length. Representative samples shown.

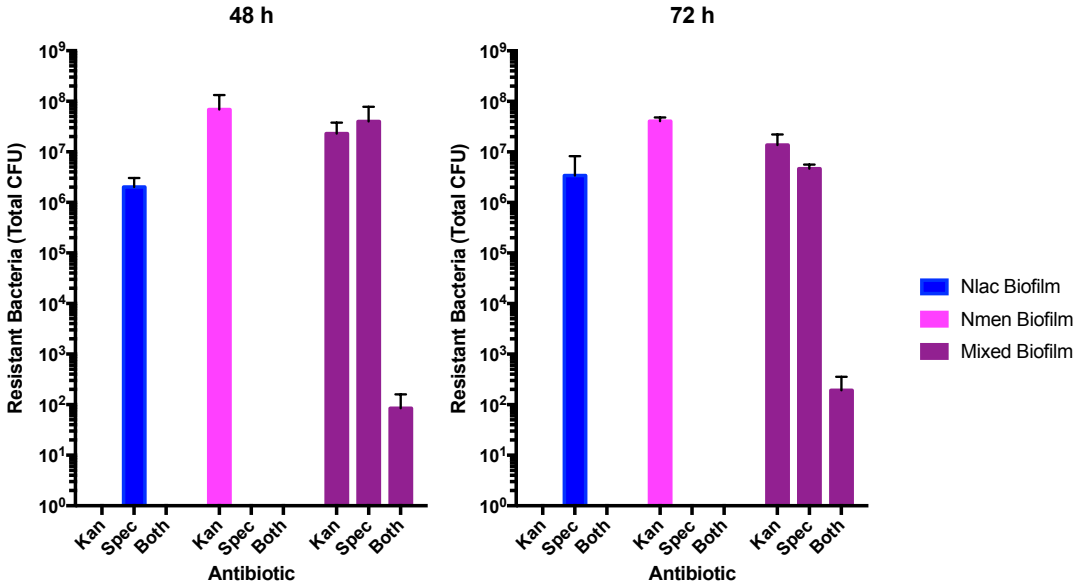



Figure 13. Genetic Exchange between *N. lactamica* and *N. meningitidis* in Biofilms

Nlac NHCIS1:*aadA1* and Nmen MC58 *ΔsiaD:aphA3* were grown to log phase in TSB, before adjusting the bacterial concentration to 1 x $10^8$ CFU/ml in 50% TSB (diluted in sterile water). Cultures were grown in 12-well plates containing 2 x $10^8$ CFU per well at 37°C, 5% $CO_2$, replacing 50% TSB daily. Biofilms were disrupted after 48 or 72 h and viable counts were performed on CBA with X-Gal, spectinomycin and/or kanamycin. Bacterial count and colony colours were recorded.

## 3.7 Antibiotic Resistance-Free Transformation

Human challenge with WT *N. lactamica* Y92-1009 has been performed previously and exhibited a good safety profile (Deasy et al., 2015; Evans et al., 2011). The data so far in this chapter demonstrate that targeted genetic modification of this strain is possible. It is therefore feasible that a GM strain of *N. lactamica* Y92-1009 could be developed for human challenge. Amongst the ethical considerations of this proposal is a requirement to curtail the release of antibiotic resistance markers into the nasopharyngeal microbiome. As evidenced in Section 3.2, the ability of *N. lactamica* to metabolise lactose can be exploited as an alternative means of screening for transformants on X-Gal-containing agar.

Another important consideration is to target modifications to a non-coding, intergenic locus, in order to prevent disruption to other genes, which might affect the fitness or behaviour of the organism. One such locus, NHCIS1, was targeted in Section 3.2. Whilst this locus does include small putative open reading frames (ORFs), which may code for non-coding RNAs, it was deemed the most appropriate locus for minimal disruption to existing genes.

To test the validity of NHCIS1 as a site for the expression of exogenous genes, using β-gal as a screening marker, a construct was designed to reintroduce *lacZ*, under the control of the *lst* promoter, into the NHCIS1 locus of Nlac *ΔlacZ.* This promoter ordinarily controls a protein involved in sialylation of the polysaccharide capsule in *N. meningitidis.* The pUC19 vector contained the *N. lactamica lacZ* gene flanked by DNA homologous to the *N. lactamica* NHCIS1 locus. The insert was amplified by hmPCR and transformed into Nlac *ΔlacZ.* Screening on TSB X-Gal revealed predominantly white colonies, with a small number of blue, putative Nlac NHCIS1:*lacZ* transformants. PCR amplification was performed on the *lacZ* (primers 14 & 15) and NHCIS1 (primers 16 & 17) loci from gDNA extracts of Nlac WT, *ΔlacZ* and putative NHCIS1:*lacZ*. PCR products were analysed by gel electrophoresis, which confirmed removal of *lacZ* from its original locus and insertion at the NHCIS1 locus (Figure 14a).

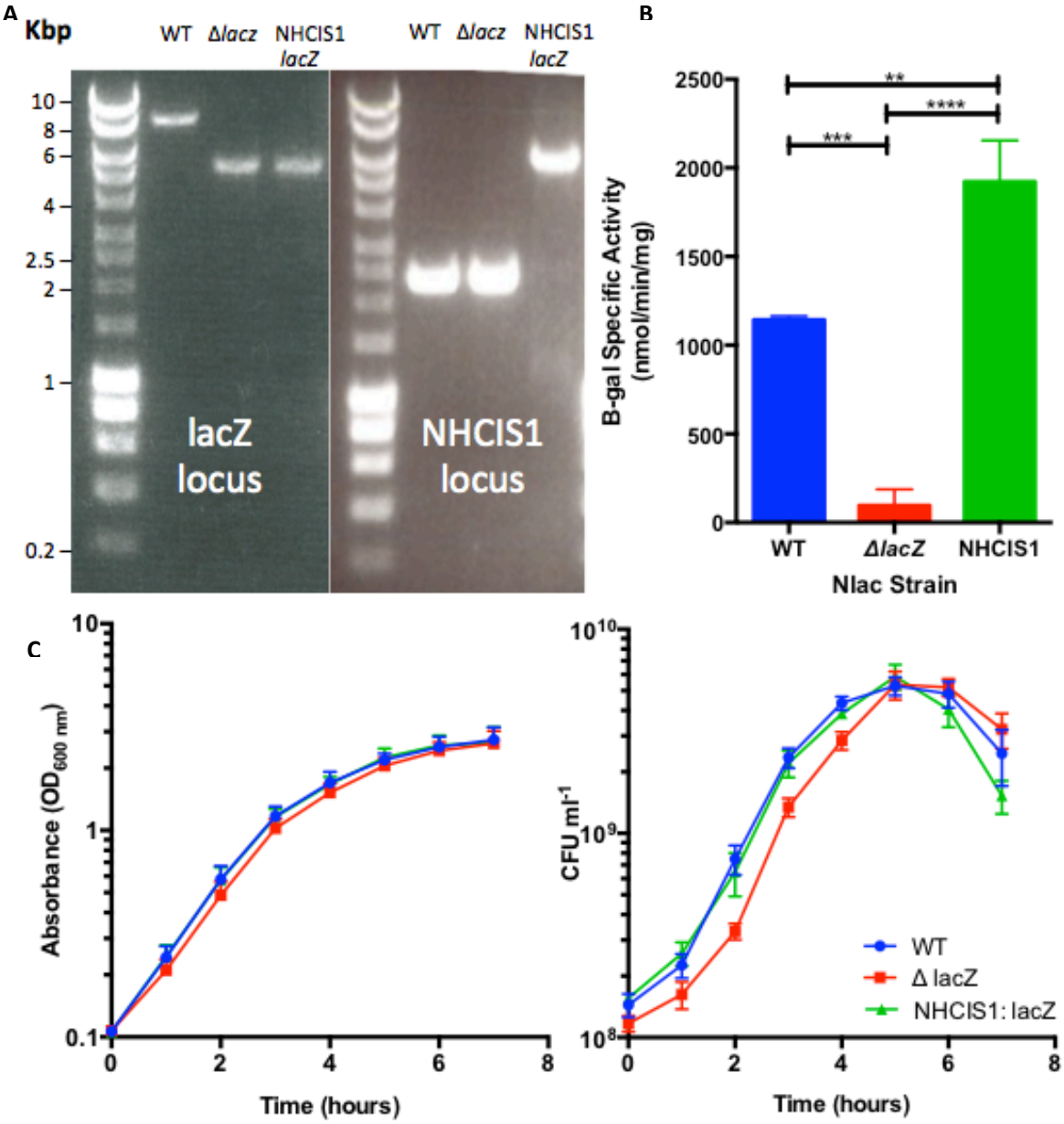



Figure 14. β-galactosidase Activity as an Alternative to Antibiotic Resistance for Marking Transformation

A) The *lacZ* locus from gDNA of Nlac WT, *ΔlacZ* and NHCIS1:*lacZ* was PCR amplified with primers 14&15 (left). The NHCIS1 locus was PCR amplified from gDNA of the same strains with primers 16&17 (right). Products were run on 0.7% agarose at 90V for approximately 40 min. B) β-gal Specific Activity was measured in Nlac WT, *ΔlacZ* and NHCIS1:*lacZ.* One-way ANOVA with post-hoc Tukey analysis (**≤0.01, ***≤0.001, ****≤0.00001, n=3). C) Colonies of Nlac WT, *ΔlacZ* and NHCIS1:*lacZ* were grown on TSB agar overnight, then resuspended in liquid TSB at a starting $OD_{600nm}$ of 0.1. The cultures were incubated at 37°C, 5% $CO_2$, 320 RPM for 7h, with hourly readings of $OD_{600nm}$ (left) and viable counts (rights).

To demonstrate that the inserted copy of *lacZ* was functional in this locus, the Specific Activity of β-gal was quantified using the Miller assay. It showed the effective loss of β-gal activity in Nlac *ΔlacZ* compared to WT, and the restored activity in Nlac NHCIS1:*lacZ* (Figure 14b).The β-gal activity in Nlac NHCIS1:*lacZ* actually exceeded that of WT, demonstrating that the change in locus of β-gal is not detrimental to its function.

No change in the fitness of the bacteria was observed, as measured by growth curves in TSB (Figure 14c). There was a slight reduction in growth rate in Nlac *ΔlacZ* compared to WT, but this was completely restored in Nlac NHCIS1:*lacZ.*

When introducing genetic modifications, selection would be preferable to screening because it allows high efficiency identification of putative transformants (those that survive application of a selective pressure); whereas screening requires multiple dilutions to observe a small proportion of individual colonies that express the desired phenotype. As such, the means to select for successfully transformed bacteria on the basis of β-gal expression would greatly simplify and streamline the transformation procedure.

Not only does β-gal metabolism of X-Gal cause a change in colony colour, β-gal is also responsible for the utilisation of lactose as a carbon source in various bacteria. The aim was to produce a formulation for selective agar to exclusively support the growth of transformed, β-gal-expressing strains on the basis of lactose metabolism. *Neisseria* are relatively fastidious and a search of the literature revealed no completely defined media that supports growth of *N. lactamica.* However, GC Agar is relatively well defined and is frequently used for the growth of *N. meningitidis.* The complete recipe for GC agar is provided in Appendix B. GC agar contains glucose, starch and proteose peptone as potential carbon sources. Proteose peptone is also likely to provide a variety of other nutrients.

Multiple variations of GC agar were made, as outlined in Table 3. Where indicated, 100 µl of 40mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), which induces β-gal expression in *E. coli,* was spread onto the surface of agar and allowed to dry prior to addition of bacteria. Both Nlac WT and *ΔlacZ* were streaked onto separate plates of each formulation and incubated overnight at 37°C, 5 % $CO_2$.

Table 3. Growth of Nlac WT and Δ*lacZ* on Modified GC Agar

| Glucose/ Lactose | Starch | IPTG | Proteose peptone (%)* | Growth | |
|---|---|---|---|---|---|
| | | | | WT | Δ *lacZ* |
| Glucose | ✓ | ✓ | 100 | ✓ | ✓ |
| Glucose | ✓ | | 100 | ✓ | ✓ |
| Lactose | | ✓ | 100 | ✓ | ✓ |
| Lactose | | | 100 | ✓ | ✓ |
| Lactose | | ✓ | 50 | ✓ | ✓ |
| Lactose | | | 50 | ✓ | ✓ |
| Lactose | | ✓ | 25 | ✓ | ✓ |
| Lactose | | | 25 | ✓ | ✓ |
| Lactose | | ✓ | 20 | ✓ | ✓ |
| Lactose | | | 20 | ✓ | ✓ |
| Lactose | | ✓ | 15 | ✓ | ✓ |
| Lactose | | | 15 | ✓ | ✓ |
| Lactose | | ✓ | 10 | ✓ | ✓ |
| Lactose | | | 10 | ✓ | ✓ |
| Lactose | | ✓ | 5 | ✓ | ✓ |
| Lactose | | | 5 | ✓ | ✓ |
| Lactose | | ✓ | 0 | ✗ | ✗ |
| Lactose | | | 0 | ✗ | ✗ |

*% of recommended concentration, as detailed in Appendix B

Table 4. Re-introduction of Nitrogen to Modified GC Agar

| Glucose/ Lactose | Nitrogen Source | Growth | |
|---|---|---|---|
| | | WT | Δ *lacZ* |
| Lactose | $NH_4Cl$ (1 g $L^{-1}$) | ✗ | ✗ |
| Glucose | $NH_4Cl$ (1 g $L^{-1}$) | ✗ | ✗ |
| Lactose | Biotin (0.03 g $L^{-1}$) | ✗ | ✗ |
| Lactose | Biotin (0.003 g $L^{-1}$) | ✗ | ✗ |
| Glucose | Biotin (0.003 g $L^{-1}$) | ✗ | ✗ |

Complete GC agar was shown to support growth of Nlac WT and *ΔlacZ,* in both the presence and absence of IPTG. The glucose was then replaced with an equal concentration of lactose and the agar continued to support the growth of both strains. Starch was then removed from the recipe, however plates continued to support the growth of both strains. A series of reductions in the concentration of proteose peptone were then performed. Growth was supported in both strains at a minimum of 5% of the recommended proteose peptone concentration, however colony size after overnight incubation appeared to decrease with the concentration of proteose peptone. When no proteose peptone was added, growth of neither strain was supported, regardless of whether IPTG was present.

No effect of IPTG on the growth of either strain was observed under any of the conditions, however it appears that a small amount of proteose peptone is essential for growth. It was noted that in the absence of proteose peptone, there is no nitrogen source in the media, which is a necessary requirement for bacterial growth. Nitrogen was re-introduced in the form of ammonium chloride or Biotin, however the media was still unable to support the growth of either strain (Table 4).

## 3.8 Discussion

### 3.8.1 Cytosine Hypermethylation and Restriction Modification Systems

Cytosine hypermethylation was shown to protect against the restriction endonuclease activity of NlaIII and NlaIV *in vitro* (Figure 7) and it was therefore hypothesised that exposing *N. lactamica* to hmPCR products would increase the rate of transformation when compared to DNA produced through standard PCR. *N. lactamica* was exposed to DNA encoding kanamycin-resistance gene *aphA3.* A significantly greater proportion of bacteria gained kanamycin resistance when the donor material was produced by hmPCR compared to standard PCR (Figure 8). It is likely that the increased rate of transformation is due to the protective effect of cytosine hypermethylation against restriction endonucleases such as NlaIII and NlaIV, which may otherwise have digested the donor DNA before it could be incorporated into the *N. lactamica* chromosome. These two endonucleases are of particular interest because they require a short recognition sequence of only four base pairs to digest DNA. Assuming equal distribution of bases, these enzymes would cut at a higher frequency than those with a larger recognition sequence. The *nlaIII* chromosomal locus was chosen for targeting transformation in these early experiments as it is naturally devoid of NlaIII recognition sites, and so it was considered that the unmethylated DNA constructs would have a greater chance of reaching chromosomal integration. The *aphA3* gene was codon

optimised to also be devoid of NlaIII recognition sites, whilst retaining the cognate amino acid sequence. For this reason, the increase in transformation efficiency observed is unlikely to involve NlaIII activity, and is more likely caused by the protective effect of cytosine hypermethylation against NlaIV and other restriction endonucleases. *N. lactamica* is known to express a variety of other endonucleases, some, but not all of which are likely to be hindered by cytosine hypermethylation. Therefore, additional DNA modifications may further enhance transformation.

The addition of the *CLOVER* gene to donor DNA meant that the construct contained up to two NlaIII recognition sites. When transforming with standard PCR products into the *nlaIII* locus, a dose-dependent decrease in the transformation efficiency was observed with increasing NlaIII recognition sites (Figure 9b). As expected, this effect was no longer observed when donor material was hypermethylated, suggesting that digestion by NlaIII was prevented. As before, hypermethylation resulted in a transformation efficiency significantly higher than that achieved with standard PCR, even when constructs contained no NlaIII sites. This increase is presumably due to blocking the function of other DNA-degrading enzymes.

Contrary to our observations in the *nlaIII* locus, when the *CLOVER-aphA3* cassette was targeted to NHCIS1, the presence of NlaIII recognition sites in *CLOVER* did not appear to affect transformation efficiency with unmethylated DNA (Figure 9c). Inactivation of NlaIII also did not result in an increase in transformation. In line with prior experiments, cytosine hypermethylation did still significantly increase the rate of transformation. The difference in results between the two loci may be caused by a variety of factors. Importantly, whilst the heterologous inserts were conserved between the two experiments, the homologous flanking regions were not. The *nlaIII* locus is naturally devoid of CATG sequences, however the NHCIS1 homologous region contains four NlaIII recognition sites, as shown in Figure 9c. Digestion of the transforming DNA at these sites may have confounded results. Furthermore, the transforming DNA used in the *nlaIII* locus contained 600 bp homologous DNA either side of approximately a 1700 bp heterologous insert (41% homology), whereas the transforming DNA used in the NHCIS1 locus contained approximately 1000 bp homologous DNA either side of the same insert (54% homology). It has been shown previously that the effect of NlaIV on transformation efficiency was only apparent with heterologous DNA (Ambur et al., 2012). It is possible that the same is true for NlaIII activity, and as the transformations into the NHCIS1 locus involved a greater proportion of homology than those into the *nlaIII* locus, this may explain the disparity. It is possible that with greater homology, chromosomal integration occurs before endonucleases are able to interfere. This would allow genetic exchange to support DNA repair with homologous DNA whilst preventing the incorporation of deleterious heterologous DNA fragments.

Considering the consistent increase in transformation efficiency when donor DNA contains hypermethylated cytosine residues, it is evident that hmPCR amplification of donor DNA overcomes a major barrier to transformation in *N. lactamica.*

### 3.8.2 Homologous Flanking Lengths, Donor DNA Concentration and DUSs

Other than cytosine hypermethylation and the involvement of RMSs, it was considered that various other factors are likely to affect transformation in *N. lactamica.* Based on previous studies, it was hypothesised that transformation efficiency would increase with the homology between Nlac chromosomal DNA and the donor DNA; with the concentration of donor DNA and with the density of DUSs present in donor DNA.

When altering the length of DNA homologous to Nlac positioned either side of the heterologous insert (*aphA3*), a strong positive correlation was observed between the length of homologous DNA and transformation efficiency (Figure 10a). Interestingly, the variation in results also increased with the length of homology. The maximum flanking length used in this experiment was 1200 bp either side of the approximately 900 bp heterologous insert. Considering this exceeds the length at which no further increase in transformation efficiency was observed with *X. fastidiosa* (Kung et al., 2013), we may have also expected to reach a plateau in transformation efficiency, where increasing homology no longer has any effect on the rate of transformation. This was not observed in *N. lactamica* and so greater lengths of homology are required to determine where that point may lie. The extent of homology evidently has a substantial effect on the ability of DNA to transform *N. lactamica.* It is likely that this effect occurs during homologous recombination, where DNA homology is required for RecA to align and incorporate transforming DNA. Homologous transforming DNA will have a greater affinity for chromosomal DNA and will more likely be localised in a manner that permits homologous recombination. DNA homology may also reduce the time required for recombination to occur, thereby reducing the opportunity for enzymatic digestion of transforming DNA.

The concentration of donor DNA was varied from 0.06 to 1.06 pMol to determine the optimum concentration for transformation (Figure 10b). No effect of DNA concentration was observed, suggesting that the range used was insufficient to detect any effect. It is possible that 0.06 pMol is saturating in the protocol used for these experiments. A large number of PCR reactions are required to generate 1.06 pMol, therefore it is impractical to attempt this experiment with

greater concentrations, however it is feasible to perform further dilutions to reduce the minimum concentration. A 10-fold dilution series may have produced a more appropriate scale for the titration of DNA concentration in this experiment.

To examine the effect of DUSs on transformation of *N. lactamica,* hypermethylated donor DNA of various sizes was produced to contain either one DUS and one scrambled DUS, or two DUSs. As hypothesised, when *N. lactamica* was exposed to these hmPCR products, transformation was consistently higher with two DUSs compared to one (Figure 10c). These findings are consistent with previous studies in *N. meningitidis* (Ambur et al., 2012; Berry et al., 2013) and *N. gonorrhoeae* (Goodman and Scocca, 1991), where it has been shown that the density of DUSs positively influence the affinity between DNA and bacteria (Duffin and Seifert, 2010). This data strongly supports the theory that, like in other *Neisseria,* the presence of DUSs on extracellular DNA mediates binding to ComP on *N. lactamica* pili, thereby ensuring the preferential uptake of homotypic DNA.

### 3.8.3 The Role of Type IV Pili in *N. lactamica* Transformation

Considering transformation in *N. lactamica* was found to be enhanced by the presence of DUSs in donor DNA, it was considered that competency in this species is likely mediated by the binding and internalisation of DNA by Type 4 pili. In an apiliated strain, produced through insertional inactivation of *pilE*, transformation was entirely ablated (Figure 11). These findings suggest that pili are required for efficient transformation of *N. lactamica* and resemble previous results from both pathogenic and commensal *Neisseria* species (Chen and Dubnau, 2004; Higashi et al., 2011; Seifert et al., 1990; Tang et al., 2016). To confirm this finding, it is conventional to re-introduce the knocked-out gene and complement the activity. However, it was shown that Nlac *ΔpilE* could not be transformed and it is therefore not possible to perform complementation in this case.

In the absence of pili, there is presumably no facility for the binding and internalising of extracellular DNA. It would be interesting to examine whether in the absence of pili, the density of DUSs still has any affect on the affinity of DNA for *Neisseria* cells.

### 3.8.4 Accuracy of Targeting the Recombination Locus

With the goal of using GM *N. lactamica* for molecular studies or for therapeutic ends, it is imperative that any modifications to the genome can be accurately targeted to a particular region

of interest. So far, heterologous genes (*aphA3, CLOVER* and *aadA1*) have been targeted to specific chromosomal loci by enclosing them within sections of DNA that are homologous to the desired site of insertion. Transformation was signified by the acquisition of antibiotic resistance, but it is important to confirm that the antibiotic resistance cassette was not randomly inserted elsewhere in the chromosome. By PCR amplification of the region of interest from the gDNA of putative transformants, we were able to demonstrate an insertion at the targeted locus in all transformed samples, relative to WT (Figure 12). If the resistance gene had been non-specifically inserted elsewhere in the genome instead, a band consistent with that of WT would be expected. This confirms that our protocol yields consistent and accurate targeting of genetic modifications to the desired locus and bodes well for both molecular biology studies and the production of therapeutic GM strains.

### 3.8.5 Genetic Exchange between *Neisseria* Species

One of the major differences between *N. lactamica* and *N. meningitidis* is the ability of *N. meningitidis* to express capsule. Capsule expression is a major virulence factor, promoting survival of the bacteria in the blood, where it is harmful to the host. One of the concerns that may arise with the proposal of using GM *N. lactamica* in humans is that it may acquire additional virulence factors and become pathogenic. The data so far in this chapter has supported the idea that *N. lactamica* is capable of genetic exchange and that its competence mechanisms align with those of other *Neisseria* species. Considering that *N. meningitidis* is a closely related potential pathogen that *N. lactamica* may encounter in the nasopharynx, we considered that genetic exchange may be likely between these species. As previous data has shown that genetic exchange between bacteria is enhanced when the bacteria are present within biofilms, spectinomycin-resistant Nlac and kanamycin-resistant Nmen were co-cultured in biofilms to assess for genetic exchange (Figure 13).

In monoculture biofilms, all isolates retained their pre-existing antibiotic resistance profile. When Nlac and Nmen were grown in co-culture biofilms, a small proportion of isolates became resistant to both spectinomycin and kanamycin, which is indicative of genetic exchange between the two species. Interestingly, all colonies were white on X-Gal-containing agar, suggesting that they were all meningococcal isolates that gained spectinomycin resistance from Nlac. It is possible that the population of double-resistant bacteria may include isolates that have gained resistance to an antibiotic by a method other than genetic exchange, or Nlac isolates that have lost the ability to

metabolise X-Gal, but as neither of these events were ever encountered in monoculture biofilms, it seems highly unlikely.

The unidirectional exchange of genetic material from Nlac to Nmen could be due to a variety of reasons and requires additional research. Whilst it has been posed that Nlac is a likely source of genetic material naturally acquired by *N. meningitidis* (Bowler et al., 1994; Fermer et al., 1995; Qvarnstrom and Swedberg, 2006), recombination into *N. lactamica* Y92-1009 was not observed when it co-colonised with *N. meningitidis in vivo* (Anish Pandey, awaiting publication). Considering the effect of RMSs in Nlac, observed in Section 3.2, it is possible that meningococcal DNA was internalised by *N. lactamica* in co-culture biofilms, but was enzymatically digested rather than incorporated into the genome. It has been reported than restriction endonucleases are most active on heterologous DNA (Ambur et al. 2012; Figure 3), which is applicable in this research as both kanamycin and spectinomycin resistance cassettes were positioned within chromosomal loci unique to Nmen and Nlac, respectively. Of note, the kanamycin resistance cassette used in this experiment was inserted into the meningococcal locus of most concern – the capsule locus – and no genetic exchange from this locus to *N. lactamica* was observed.

There is a requirement for extensive biosafety analysis of any GM strain that may get produced for use in humans, however it is reassuring that *N. lactamica* Y92-1009 appears particularly refractory to the acquisition of heterologous genes. This finding suggests that whilst the production of such strains may be difficult, the stability of the genome reduces the risk of adverse events, such as the acquisition of virulence factors.

### 3.8.6 Screening or Selection to Identify Transformants

WT *N. lactamica* is acutely sensitive to cefotaxime and ceftriaxone, and overall antibiotic resistance is low in this species (Arreaza et al., 2002). It is unacceptable for our GM bacterial products to contribute to the spread of antibiotic resistance, and we therefore opted against the use of antibiotic selection in transforming strains for environmental release or human use. Instead we developed a screening protocol, whereby the removal and re-introduction of β-gal activity produces either white or blue colonies on X-Gal-containing agar (Figure 14). Notably, the expression of β-gal in WT *N. lactamica*, but not *N. meningitidis,* has historically provided a valuable means of differentiating between these species in clinical and environmental samples (Corbett and Catlin, 1968; Hollis et al., 1969).

This method of transformation allows the antibiotic-resistance free introduction of heterologous genetic material into *N. lactamica.* However, it is laborious and time-consuming, as it requires broad screening of thousands of colonies in order to identify a single, transformed isolate. To optimise the process, we attempted to develop a modified agar formulation, based on GC agar, for the selective growth of β-gal-expressing strains (Table 3 and Table 4). Unfortunately all formulations supported the growth of both β-gal-expressing (WT) and β-gal-deficient (*ΔlacZ) N. lactamica* or did not support growth of either strain. Whilst β-gal activity was apparent, through the blue appearance of colonies on X-Gal-containing media, replacing glucose sources with lactose did not appear to provide any advantage for Nlac WT. GC agar is not entirely defined and it is likely that proteose peptone provides both carbon and nitrogen sources for bacterial growth. It was necessary to omit proteose peptone in order to prevent growth of Nlac *ΔlacZ,* however this also prevented the growth of the WT organism, presumably due to the absence of an alternative nitrogen source. Unfortunately the addition of ammonium chloride or biotin was insufficient to replace proteose peptone, regardless of whether glucose or lactose was present.

The presence of IPTG had no effect on the growth of either strain but this is not entirely surprising. An early study described constitutive expression of the enzyme responsible for lactose metabolism in *N. lactamica* (Corbett and Catlin, 1968), which is now known to be β-gal, encoded by *lacZ.* This gene is best characterised in *E. coli*, where it exists in an operon alongside *lacY* and *lacA,* as shown in Figure 15. The enzyme β-gal cleaves lactose into glucose and galactose. Lactose permease, encoded by *lacY,* enables the transport of lactose through the cytoplasmic membrane into the cell. The function of galactoside O-acetyltransferase, encoded by *lacA* is not fully understood (Wang et al., 2002). Located upstream of the *lac* operon in *E. coli* is *lacI,* which encodes a repressor. In *E. coli,* the *lac* operon repressor regulates expression of the *lac* operon so that transcription only occurs in the presence of lactose and the absence of glucose. In this system, IPTG can be used to mimic lactose and induce transcription. However, whilst *N. lactamica* possesses a homologue to the *E. coli lac* operon, consisting of *lacZ, lacY* and *lacA,* there is no homologue for *lacI*, which explains why β-gal is constitutively active in this species.

Unfortunately, attempts to develop a modified GC agar that is selective for β-gal-expressing *N. lactamica* were unsuccessful, however it remains possible to use β-gal activity to screen for transformants, in an antibiotic resistance-free manner, on TSB X-Gal.

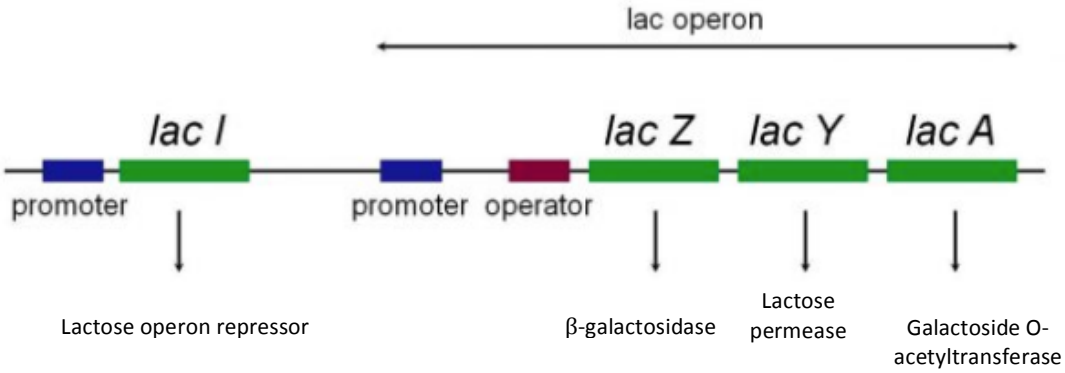



Figure 15. *E. coli lac* Operon

*N. lactamica* expresses the same lac operon as *E. coli*, but is lacking the repressor, which controls transcription. Therefore the operon is constitutively active in *N. lactamica*. Figure adapted from https://www.golifescience.com/lac-operon-gene-expression-in-prokaryotes/

## 3.9 Conclusions

The experiments in this chapter have helped to characterise the process of transformation in *N. lactamica* and have shed light on the mechanisms of natural competency in this species.

Incorporating hypermethylated cytosine into the dNTP mix of PCR reactions generated a PCR product that is resistant to restriction by NlaIII and NlaIV *in vitro*. The use of hmPCR products as donor DNA consistently increased the rate of transformation of *N. lactamica* compared to standard PCR products. This may be due to the increased resistance of hmPCR products to endonuclease activity. Whilst the presence of NlaIII recognition sites affected transformation efficiency in the *nlaIII* locus, this finding was not reflected in the NHCIS1 locus. The effect of restriction on transformation may be influenced by other factors, such as the chromosomal insertion locus and the extent of homology between transforming and chromosomal DNA. Insertional inactivation of NlaIII did not affect transformation efficiency in the NHCIS1 locus, however small changes in the level of transformation may have been obscured by the activity of other RMSs. RMSs present a significant barrier to transformation of *N. lactamica.* This barrier is partially overcome by cytosine hypermethylation but transformation may be increased further by additional DNA modifications.

We have shown a correlation between the length of homologous DNA flanking the heterologous insert and the efficiency of transformation. The flanking length continues to influence the transformation efficiency up to at least 1200 bp homology either side of the heterologous insert (approximately 900 bp). The effect of DNA concentration added to bacteria during transformation was examined, but no effect was observed between 0.06 to 1.06 pMol DNA. It is likely that DNA concentration does affect transformation, however the range used in this experiment may have been insufficient to detect any effect. In accordance with findings in other *Neisseria* species, transformation efficiency is significantly enhanced when donor DNA contains 2 DUSs, compared to 1. Transformation of *N. lactamica* was also found to be entirely pilus dependent. These data reflect findings in other Neisserial species and support the theory that competence in the commensal *Neisseria* utilises the same mechanisms and requires the same intracellular machinery as the pathogenic species.

Despite sharing competence methods, we were unable to observe genetic exchange from *N. meningitidis* to *N. lactamica* in biofilms, however we did see exchange occurring in the opposite direction. The unidirectional transfer of genetic material in these species may be due to differing

armouries of RMSs, but it is also possible that the Nlac chromosome is more stable for other, unknown reasons.

It was not possible to utilise β-gal activity to select for transformants, as an appropriate media could not be identified. However, it remains possible to screen for β-gal-expressing strains on X-Gal-containing media. It is important to utilise the data generated in this chapter to maximise the number of transformation events occurring. This will increase the probability of recovering a transformant, while still using a relatively low-efficiency screening method.

# Chapter 4 Type 4 Pili and Adherence

*Note: Support with microscopy was provided by the University of Southampton Biomedical Imaging Unit. Technical support for adherence assays was provided by Graham Berreen and Konstantinos Belogiannis.*

## 4.1 Introduction

The production of a *pilE*-deficient strain of *N. lactamica* in Chapter 3 presents an opportunity to further examine the role of pili in this species. *Neisseria* species express T4P, as do *Pseudomonas, Vibrio, Salmonella, Escherichia* and *Legionella* species (Fronzes et al., 2008; Pelicic, 2008). In *Neisseria,* these protein appendages are primarily composed of multiple PilE subunits (Pelicic, 2008), known as the major pilin, which are transported across the outer membrane through the PilQ pore (Figure 16). The energy required for pilus assembly is provided by PilF, an ATPase that is located in the cytoplasm. PilM, PilN and PilO are essential for pilus biogenesis and anchor the pilus and PilF to the inner membrane (Carbonnelle et al., 2006). T4P are also comprised of a variety of minor pilins and binding proteins, such as PilV, PilX, ComP, and PilC2, which are non-essential for pilin biogenesis but are thought to contribute to the variety of roles these pili fulfil (Berry et al., 2013; Helaine et al., 2007; Imhaus and Duménil, 2014; Mikaty et al., 2009; Virji et al., 1995b).

As shown in Section 3.8.3, T4P are involved in genetic competence (Berry et al., 2013; Cehovin et al., 2013; Redfield et al., 2006; Seifert et al., 1990; Tang et al., 2016). Further to their role in the binding and internalisation of extracellular DNA, T4P are considered the primary adhesin in the pathogenic *Neisseria,* and have been shown to be critical for adhesion to epithelial cells (Deghmane et al., 2000; Imhaus and Duménil, 2014; Tang et al., 2016) and auto-aggregation (Taktikos et al., 2015). T4P facilitate twitching motility due to their ability to partially retract whilst the tip remains bound to an extracellular surface (Biais et al., 2008). They are also fundamental players in biofilm formation (Chiang and Burrows, 2003), required for initial adherence to a surface, association with other bacterial cells and detachment of cells to maintain biofilm morphology. Pilus inactivation can have profound effects on the structure of microcolonies (Klausen et al., 2003), which represent an early stage in biofilm formation. When modulating pilus expression in *N. meningitis*, genetic competence could be observed at 20%, aggregation at 40% and endothelial plasma cell membrane reorganisation at 70% of WT expression levels, which led to the suggestion that various roles of T4P require different ranges of pilus density (Imhaus and Duménil, 2014).

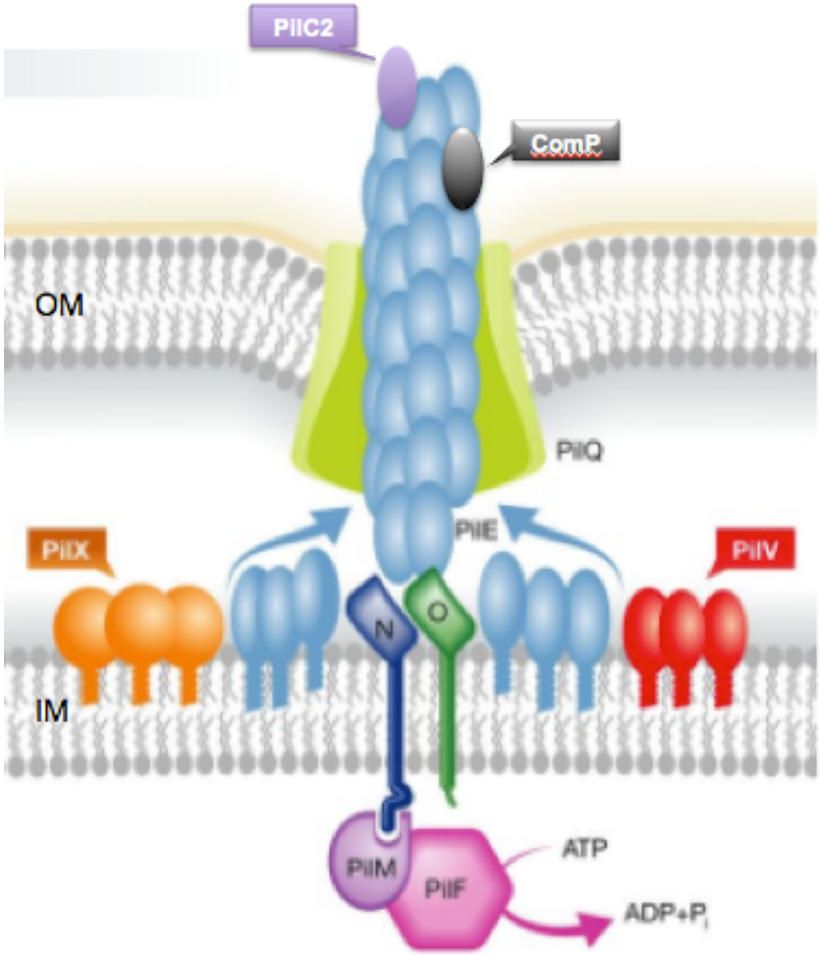



Figure 16. The Structure of Type 4 Pili in *Neisseria*

*Neisseria* T4P are comprised of multiple PilE subunits, assembled using energy from the PilF ATPase. The completed structure also contains a number of less abundant proteins, such as PilV, PilX, PilC2 and ComP, which are exported through the outer membrane (OM) via the PilQ pore, along with PilE. T4P are anchored to the inner membrane (IM) by a complex of PilM, PilN and PilO, which also facilitates interaction with PilF on the cytosolic side of the IM. Image adapted from http://emboj.embopress.org/content/early/2014/06/25/embj.201489096

### 4.1.1 Type IV Pili and Host Cell Interactions

Early studies identified CD46 as the T4P receptor on epithelial cells (Kallstrom et al., 1998; Källström et al., 1997), however this has been disputed by Kirchner et al. (2005), who showed CD46-independent binding of piliated *N. gonorrhoeae* and the purified pilus protein PilC2 to epithelial cells. CD147 has been identified as a host cell receptor for meningococcal PilV and PilE in endothelial cells (Bernard et al., 2014). Binding of pathogenic *Neisseria* to the epithelial cell surface induces surface tension, which triggers cytosolic calcium flux and cortical plaque formation, in a pilin-dependent manner (Merz et al., 1999). Pilus-mediated binding of meningococci to epithelial cells requires contact-dependent upregulation of *pilC1* (Taha et al., 1998). Induction of the transcription of this gene occurs in response to contact with epithelial cells, and involves the promoter element Contact Regulatory Element of *Neisseria* (CREN) (Taha et al., 1998). This promoter element has been identified upstream of various genes in multiple *Neisseria* species and allows transient gene induction in response to cell contact (Deghmane et al., 2000).

### 4.1.2 Class 1 and Class 2 Type IV Pili

Neisserial T4P can be categorised into 2 distinct classes (Diaz et al., 1984). Class 1 pili were the first to be identified. They are found on gonococcal and some meningococcal strains and are identified by their interaction with the antibody SM1 (Diaz et al., 1984). Class 2 T4P are not bound by SM1. They are present on some strains of *N. meningitidis* and can be co-expressed with class 1 pili in this species (Wörmann et al., 2014). Class 2 pili were identified when some meningococcal strains that did not react with SM1 were found to be piliated by microscopy (Diaz et al., 1984). The gene *pilE*, which codes for the major pilin subunit in both T4P classes of *Neisseria*, has different genetic loci depending on the class of pilus (Aho et al., 1997). Class 2 PilE has a lower molecular mass than class 1 and is missing a hypervariable region involved in antigenic variation (Aho et al., 1997). Gonococcal (class 1) PilE has a conserved region, which has been shown to have erythrocyte receptor activity, however antibodies directed against this conserved region do not react with class 2 pili (Diaz et al., 1984). From this we can infer that at least part of the conserved region is missing or altered. Finally, class 2 *pilE* are missing a downstream Sma/Cla repeat that is present in class 1 (Aho et al., 1997). This repeat promotes recombination with *pilS,* a silent version of *pilE* that donates genetic information for further antigenic variation (Wainwright et al., 1997).

The T4P of commensal species *N. cinerea* and *N. lactamica* are both class 2 (Aho et al., 2000; Hart and Rogers, 1993) and horizontal gene transfer has been suggested between *N. lactamica* and class 2 T4P-expressing strains of *N. meningitidis* (Wörmann et al., 2014). Whilst no mechanisms of

antigenic variation have been identified for class 2 T4P (Wörmann et al., 2014), both class 1- and class 2-expressing meningococcal strains have been implicated in invasive disease (Aho et al., 2000). One study by Gault et al. (2015) identified multisite glycosylation of T4P, specific to class 2-expressing strains, which appears to provide a mechanism of immune escape in these bacteria.

A recent study of commensal species *N. cinerea* (Tang et al., 2016) identified that whilst insertional inactivation of *pilE* abrogated DNA competence (as has been observed in other *Neisseria* (Seifert et al. 1990; Redfield et al. 2006; Berry et al. 2013; Cehovin et al. 2013)), it did not reduce adhesion to Detroit or A549 epithelial cell lines. This was in contrast to *N. meningitidis,* which displayed significantly reduced adhesion to epithelial cells in the *pilE*-null strain. *N. cinerea* express class 2 T4P and the meningococcal strain used in these experiments (8013) expresses class 1. Prior to this publication by Tang et al., pili were considered the primary adhesin of *Neisseria* species, however these results raise questions regarding the role of class 2 T4P in *Neisseria.* Considering adhesion to epithelial cells is necessary for colonisation of the nasopharynx, a better understanding of the mechanisms involved in this process could provide valuable insights for increasing colonisation with *N. lactamica* and thereby maximise the potential for this species to displace resident meningococci.

## 4.2 Observing Piliation by Microscopy

In contrast to *N. meningitidis* and *N. cinerea, N. lactamica* (strain NRL 36016) pili could not be observed by electron microscopy by Aho et al. (2000), despite the presence of the relevant *pil* genes in the *N. lactamica* genome. Pilus expression in *N. lactamica* Y92-1009 was assumed due to the change in competence phenotype that was observed when *pilE* was inactivated (Figure 11), however, initial attempts to visualise pili were unsuccessful by both Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) (Figure 17).

It was hypothesised that the inability to visualise pili using the original methods may have been because the forces generated during centrifugation removed the pili from the cell surface. Later, TEM attempts used a method adapted from those previously used to image *N. cinerea* (Tang et al., 2016), and did not require centrifugation of samples. These methods (see Section 2.27) resulted in images of piliated Nlac WT (Figure 18a). Using the same methods, the putative pilus structures were absent in images of Nlac Δ*pilE* (Figure 18b). Imaging was performed on three separate occasions and representative examples are shown.

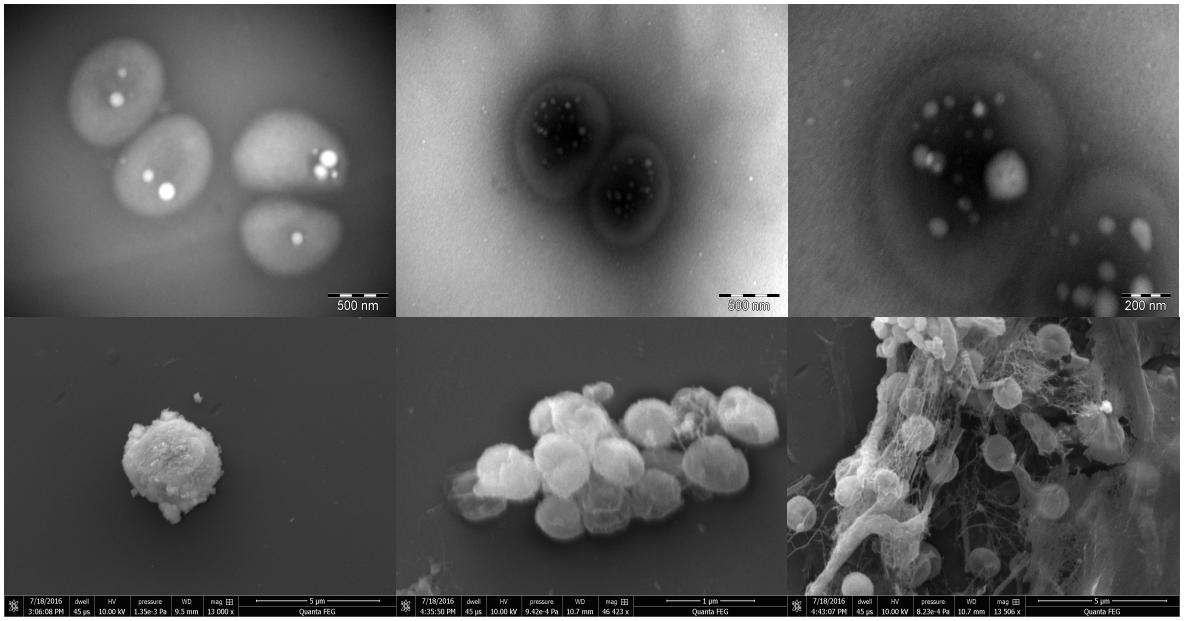



Figure 17. Electron Microscopy Images of *N. lactamica*

Initial attempts to visualise WT *N. lactamica* using both TEM (top) and SEM (bottom). TEM staining involved pelleting bacteria by centrifugation and washing in PBS, prior to staining with 5% ammonium molybdate plus 0.5% trehelose(aq) for 10 secs on Formvar-coated grids and imaging on a Hitachi H7000 TEM. SEM images were generated by resuspending pelleted cells in fixative (3% glutaraldehyde + 0.15% alcian blue in 0.1M cacodylate buffer) and dropping onto a glass cover slip, then undergoing an ethanol dilution series and critical point drying prior to imaging on a FEI Quanta 200 SEM.

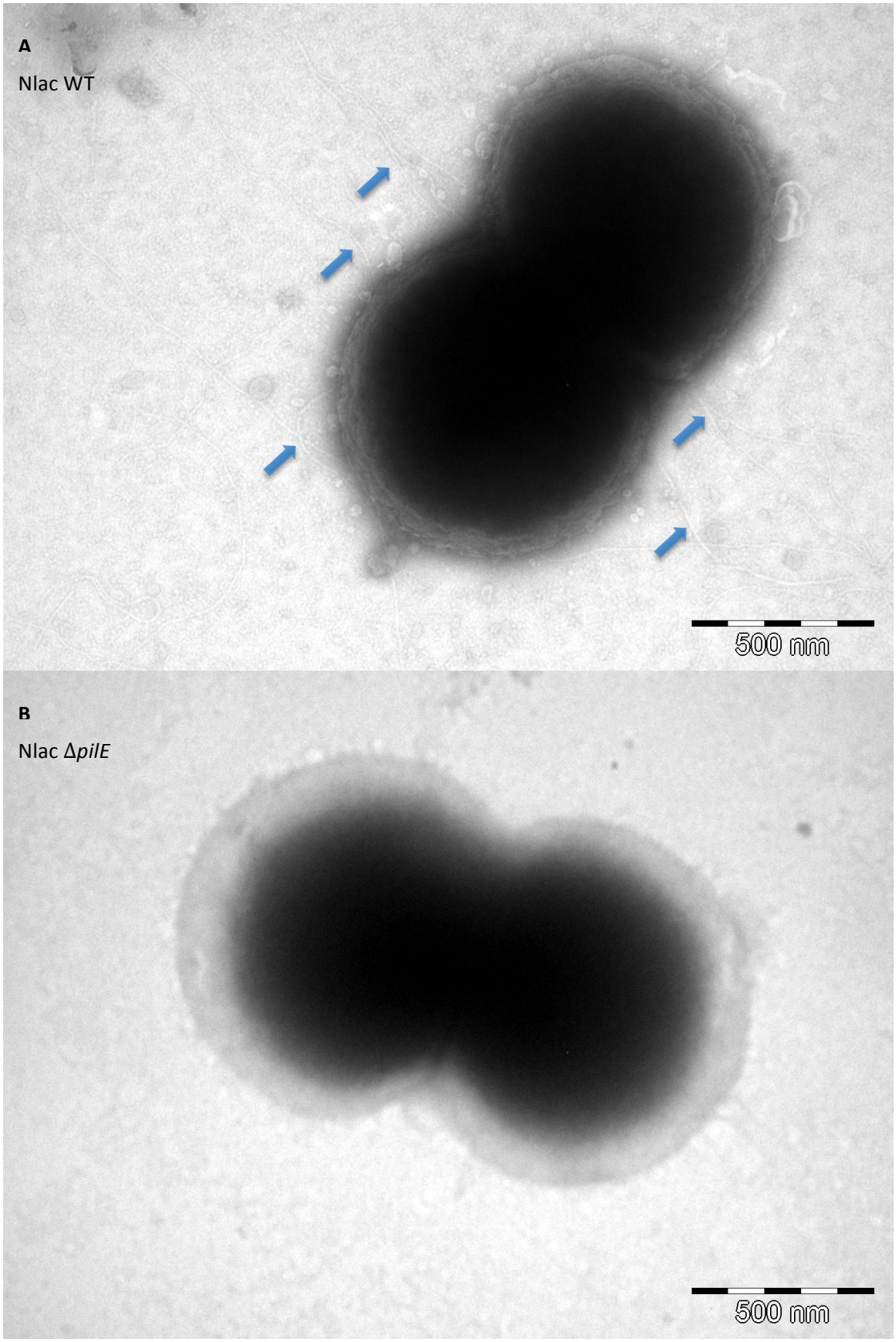



Figure 18. TEM of Piliated and Non-piliated *N. lactamica*

TEM of Nlac WT (A) and Nlac Δ*pilE* (B). Pili are identified by blue arrows in (a). OMVs can also be seen. A and B) Bacteria were grown overnight on TSB at 37°C, 5 % $CO_2$. Formvar-coated grids were touched onto individual colonies prior to washing 3 x 1 min in 10 µl $H_2O$ and staining for 10 s with 5% ammonium molybdate plus 0.5 % trehelose (aq). Images were taken on a Hitachi H7000 TEM.

## 4.3 Role of T4P in Adherence

The unexpected observation that adherence to epithelial cells is pilus-independent in *N. cinerea* (Tang et al., 2016) calls into question the role of these protein structures in other *Neisseria*. Fortunately, a novel opportunity to study T4P in *N. lactamica* has been afforded by the production of a pili-deficient strain.

For the purpose of comparing between species, WT and PilE-deficient strains of *N. meningitidis* and *N. cinerea* were acquired or derived. *N. meningitidis* 8013 (Nmen WT) and its Δ*pilE* derivative (Nmen Δ*pilE*) were generously provided by Professor Tang and Dr Exley at the University of Oxford. *N. cinerea* 346T (Ncin WT) was purchased from the culture collection of the University of Göteborg (CCUG). Genomic DNA from *N. cinerea* Δ*pilE1/2* (Tang et al., 2016) was kindly donated by Professor Tang and Dr Exley at the University of Oxford, and used to transform Ncin as described in Section 2.16, to derive strain Ncin Δ*pilE*. Insertion of the kanamycin resistance gene into the *pilE* locus of *N. cinerea* was confirmed by PCR amplification of gDNA using primers 29 & 30, targeted at the kanamycin resistance gene and the *pilE* locus, respectively, followed by DNA gel electrophoresis (data not shown).

As the adherence of these 6 strains would be assessed on epithelial cell lines and abiotic surfaces in cell culture media, their growth was first compared in DMEM + 10% FCS (Figure 19). The growth dynamics were surprisingly different between *N. lactamica, N. cinerea* and *N. meningitidis* but similar between any two strains of the same species. The turbidity of all six cultures increased over time but this was noticeably less in the *N. cinerea* strains that the other species (Figure 19a). The viable count data showed a decrease in viability of Ncin and *ΔpilE* over the course of 5 h. A modest increase was observed in the viability of Nmen and Nmen *ΔpilE*, whilst a more substantial increase occurred in the Nlac strains (Figure 19b). Area under the curve analysis was performed on each of the three repeats of the viable count data for each strain. One-way ANOVA with Sidak's multiple comparisons was used to compare the areas under the curve between the WT and *ΔpilE* strain of each species. Within each species, the growth curves were not found to be significantly different (Figure 19c).

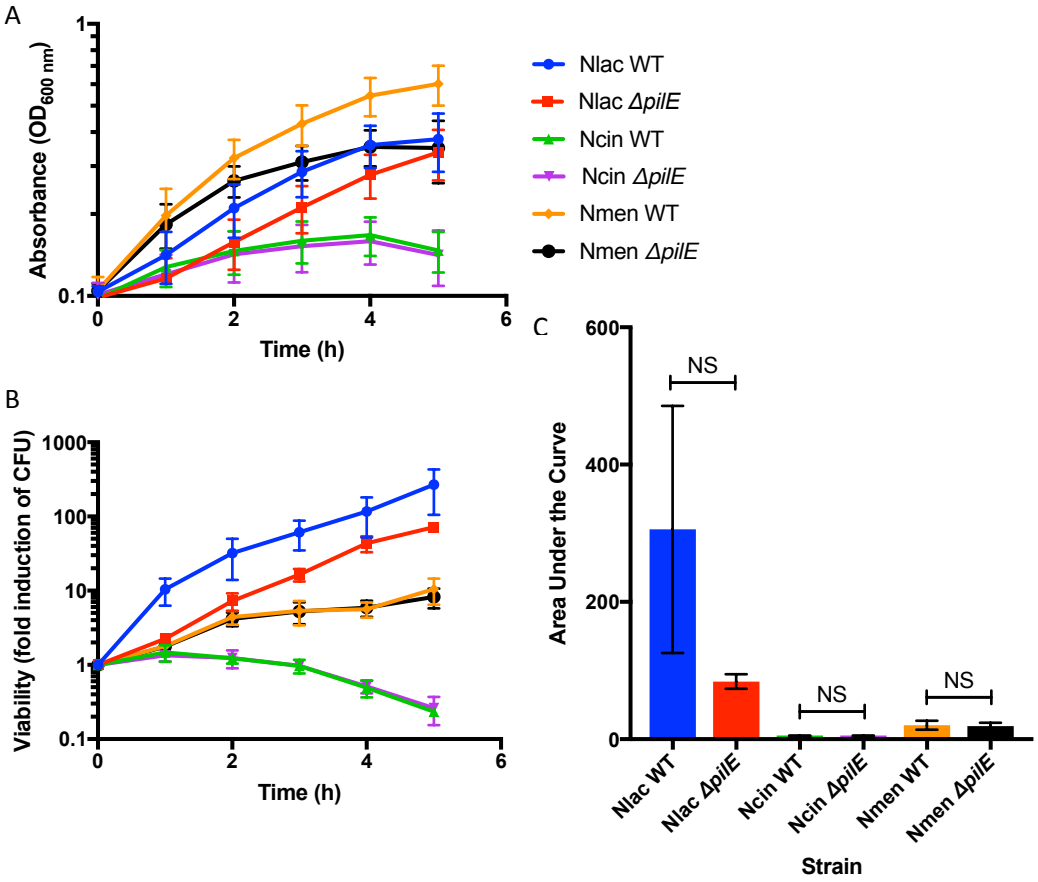



Figure 19. Growth of *Neisseria* strains in Cell Culture Media

A) Nlac, Ncin and Nmen WT and their Δ*pilE* derivatives were grown in DMEM +10% FCS at 37°C, 320 RPM. Hourly measurements of absorbance at $OD_{600nm}$ were taken. Mean ±SEM, n=3. B) Alongside measuring culture absorbance, serial dilutions were plated on CBA to obtain a viable count, which was normalised to fold induction from t0. Mean ±SEM, n=3. C) Data from (B) were used to calculate the area under the curve for each repeat. A one-way ANOVA with Sidak's multiple comparisons was performed to compare between the two strains of each species. Mean ±SEM, n=3.

HEp2 or Detroit cells were grown to confluence in a 24-well plate and a representative well was counted immediately prior to infection with log-phase bacterial culture, adjusted to a concentration of 30 CFU/cell. In parallel, empty plastic wells or glass coverslips were infected with bacteria at the same concentration. Infections were performed in duplicate over 0.5, 1.5 and 3 hours and wells were visually inspected after 3 hours to confirm that cell monolayers were maintained. Wells were saponised before performing viable counts to disrupt cells and release adhered or internalised bacteria. In order to account for the difference in growth dynamics between species and to allow meaningful comparisons between species, data were transformed to provide the relative adherence of each Δ*pilE* strain as a percentage of its respective WT strain (Figure 20). Untransformed data is shown in Appendix C.

All six strains displayed low level adherence to both plastic and glass, which increased over time. Adherence of all strains to plastic wells was approximately 2-fold greater than to glass coverslips and of the three species, adherence to both abiotic surfaces was highest in *N. meningitidis*. According to multiple t-test analysis (Appendix C), adherence to plastic was not significantly different between any WT strain and its Δ*pilE* derivative, however the adherence of Ncin Δ*pilE* to glass coverslips was significantly higher than WT at both 1.5 and 3 hours post infection. Adherence to both epithelial cells lines was higher than that to abiotic surfaces for all strains, and adherence to HEp2 cells was generally greater than to Detroit cells. There was a significant reduction in adherence to HEp2 cells by Nlac Δ*pilE* relative to WT at both 1.5 and 3 hours post infection. After 3 hours of infection, both Nlac Δ*pilE* and Nmen Δ*pilE* adhered significantly less to Detroit cells than their respective WTs, whereas Ncin Δ*pilE* adhered significantly more than Ncin WT. Notably, increased bacterial fitness in cell culture media did not correspond with increased adherence to either biotic or abiotic surfaces.

Figure 20 demonstrates that knocking out *pilE* from *N. cinerea* generally resulted in increased adhesion to plastic, glass and Detroit cells. There was also an increase in adherence to HEp2 cells but only at the 0.5 h time point. There was little to no effect of *pilE* deletion on the adherence of either *N. lactamica* or *N. meningitidis* to plastic and glass. Conversely, PilE-deficient strains of *N. lactamica* and *N. meningitidis* were both less efficient at adhering to HEp2 or Detroit cells across all three time points.

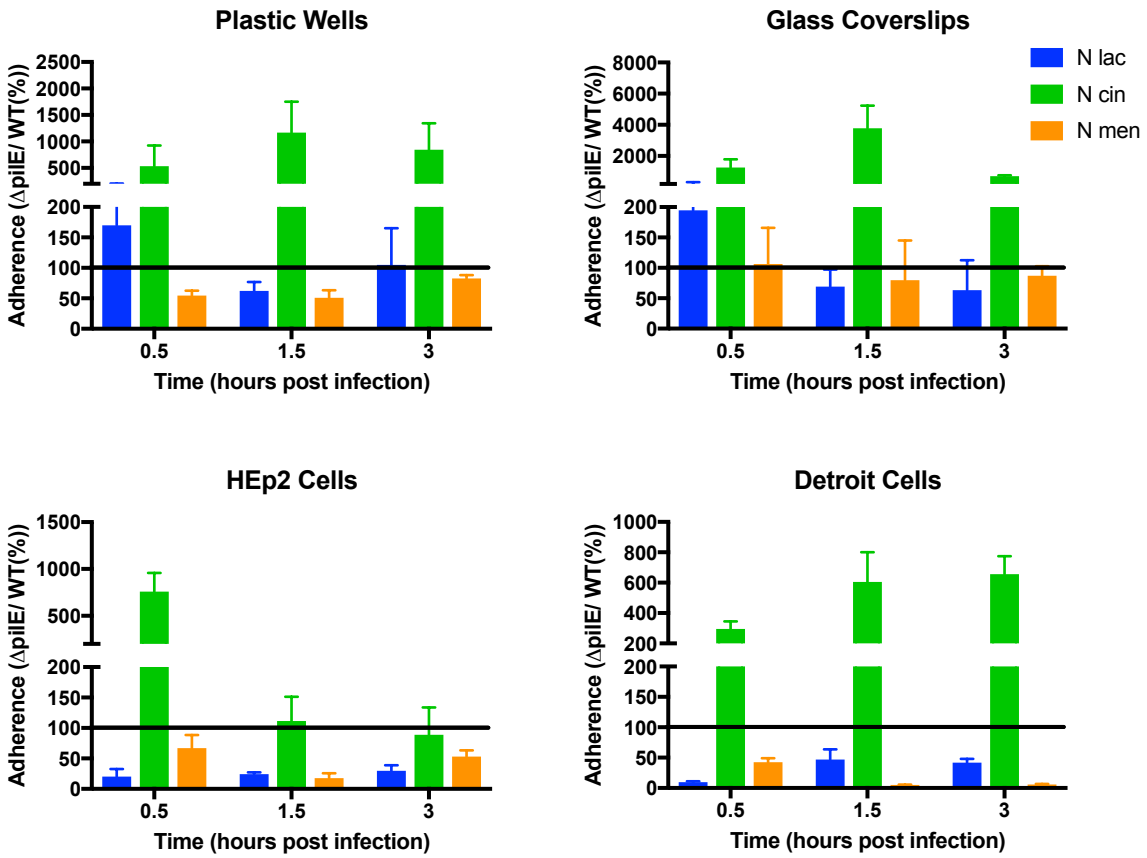



Figure 20. Effect of *pilE* Inactivation on Adherence to Surfaces

HEp2 or Detroit cells were grown to confluence in a 24-well plate and counted prior to infecting with 30 bacteria per cell in 0.5 ml cell culture media for 0.5, 1.5 or 3 hours at 37°C. Empty plastic wells or glass coverslips were concurrently exposed to the same bacterial concentration. After washing wells and treating with 1% saponin, the total number of remaining bacteria was quantified by plating serial dilutions on CBA. The resulting count for each Δ*pilE* strain was divided by that of its corresponding WT strain and expressed as a percentage. Mean ±SEM, n ≥3.

## 4.4 Discussion

Having previously knocked out *pilE* from *N. lactamica* by insertional inactivation with *aphA3* (Section 3.4), and observing ablation of natural transformation in the mutant strain (Figure 11), the opportunity was taken to further examine the role of PilE in the adherence phenotype of this species. A recent publication showed PilE-independent adherence to human epithelial cell lines in *N. cinerea* (Tang et al., 2016), which expresses class 2 T4P, as does *N. lactamica.* In *N. cinerea,* inactivation of *pilE* resulted in the loss of pili, which could be observed by electron microscopy. However, in the initial images of WT *N. lactamica,* produced using TEM or SEM, pili could not be observed. A previous publication was unable to identify pili in microscopy images of *N. lactamica,* despite successfully identifying these structures in *N. meningitidis* and *N. cinerea* (Aho et al., 2000). However, adapting the TEM methods from Tang et al. (2016) permitted the observation of structures resembling T4P in WT *N. lactamica.* As expected, these structures were not present in Nlac *ΔpilE*. Taken together with data in Figure 11 that Nlac *ΔpilE* is deficient in genetic competence, these findings suggest that insertion of *aphA3* into the *pilE* locus resulted in the loss of T4P.

The loss of PilE expression in *N. lactamica* had little or no effect on its adherence to abiotic surfaces (plastic and glass) over three hours but caused a reduction in adherence to both HEp2 and Detroit cell lines compared to WT, suggesting that *N. lactamica* T4P play a major role in the specific interaction between bacteria and cell surface receptors. It has been demonstrated previously that pilus-deficient variants of class 2 T4P-expressing meningococcal strains were not compromised in their ability to bind HEp2 cells, but pili expression was required for efficient adhesion in class 1-expressing strains (Virji et al., 1992). In contrast, we have demonstrated a role for the class 2 T4P of *N. lactamica* in adherence to HEp2 cells.

The results in *N. meningitidis* closely resembled those of *N. lactamica,* with the loss of pili causing a reduction in adherence to cell lines. In *N. meningitidis,* this effect was most pronounced with Detroit cells rather than HEp2 cells. HEp2 cells were selected for these experiments as they were readily available and were initially considered to be a laryngeal epithelial cell line. However, they have since been identified as a HeLa cell contaminant (Chen, 1988). Detroit cells are likely to be a more representative cell line for adherence experiments with these pharyngeal-colonising organisms, as they are nasopharyngeal epithelial cells. The dramatic reduction in adherence to Detroit cells with the mutation of *pilE* in *N. meningitidis* indicates that pili are extremely important adhesins in this species. A less substantial reduction in adherence to HEp2 cells may be indicative

of there being an important receptor for meningococcal pili binding that is present in Detroit cells and not in HEp2 cells.

WT *N. meningitidis* adhered most efficiently of the three species to both abiotic surfaces, but least efficiently to HEp2 cells. The most notable difference between *N. meningitidis* and the other two species in these experiments is the expression of capsule, which is likely to have promoted non-specific interactions with various surfaces, resulting in the increased adherence to plastic and glass. The presence of capsule also obscures a number of surface-expressed proteins present on the outer membrane of *Neisseria.* There are likely to have been multiple membrane proteins in *N. lactamica* and *N. cinerea* that interact with receptors on HEp2 cells, resulting in the greater adherence in these species. Capsule expression has previously been shown to reduce cell adhesion in *N. meningitidis* (Spinosa et al., 2007). Whilst *N. lactamica* and *N. meningitidis* exhibited reduced adherence to Detroit cells in the absence of pili, the reduction in *N. lactamica* was less pronounced. In *N. lactamica,* a greater interaction between the bacteria and cell surface was retained in the absence of pili, which is likely due to the lack of capsule allowing a number of bacterial surface proteins to engage with target receptors on the epithelial cell surface.

Most surprising were the results for the adherence of *N. cinerea* WT and Δ*pilE* to different surfaces. Inactivation of *pilE* resulted in significantly increased adherence of *N. cinerea* to plastic and glass, suggesting an increase in non-specific interactions. The adherence of *N. cinerea* strains to plastic and glass contradicts previous data, which showed equivalent binding in the WT and PilE-null strain (Tang et al., 2016). This is surprising considering that the same genetic modification was performed and the assay was extremely similar. The results observed in the empty wells cast some doubt over the adherence of the *N. cinerea* strains to cell lines. If there is increased binding of the Δ*pilE* to plastic in empty wells, there will also be increased binding of this strain to any exposed plastic in wells containing cells, so it is difficult to know if there is any effect of PilE mutation on adherence to the cells specifically. Cells were confluent prior to infection and remained confluent throughout the course of infection, however there was exposed plastic on the sides of the wells, providing a potential reservoir of non-specifically bound bacteria.

*N. cinerea* recovered from wells containing HEp2 cells suggested an increase (0.5 h) or no change (1.5 and 3 h) in adherence when *pilE* was inactivated. However, knowing that adherence to the empty wells was also increased confounds the data and makes it difficult to discern any conclusions regarding specific interactions between this species and the cell monolayer. In contrast to *N. lactamica* and *N. meningitidis,* Ncin Δ*pilE* also appeared to exhibit much greater adherence to Detroit cells than WT, however these observations are of limited value due to the

concurrent increase in non-specific interactions with abiotic surfaces. A previous study has observed direct interactions between Ncin Δ*pilE* and Detroit cells by microscopy (Tang et al., 2016). It is therefore tempting to conclude from the data in this chapter that the insertional inactivation of *pilE* in *N. cinerea* causes an increase in non-specific interactions with both abiotic (plastic and glass) and biotic (Detroit cell) surfaces. This is in contrast to the findings by Tang et al. (2016), who reported no effect of *pilE* inactivation on the adherence of *N. cinerea* to plastic, glass, A549 and Detroit cells, although a small but insignificant increase in adherence was observed with *pilE* inactivation. It is difficult to determine why results differ between the two studies when using very similar protocols, but may be attributed to normalising of the data, operator differences during interventions, or phase variation of surface structures affecting the behaviour of bacterial populations differently.

One possible explanation for an increase in adherence of *N. cinerea* to abiotic surfaces with the removal of PilE is that loss of PilE expression allowed increased production of alternative membrane proteins involved in adherence. It was also noted that during the construction of strain *N. cinerea* Δ*pilE1/2* in Oxford, from which the genomic DNA was donated for the construction of Ncin Δ*pilE* in this study, part of a gene neighbouring *pilE* (*yccS1*) contributed to the homologous overlap used to target the locus for mutation (Dr Rachel Exley, personal communication). This gene codes for protein YccS1, which is a membrane protein of unknown function. If this gene was disrupted during transformation, it may have altered the behaviour of Ncin Δ*pilE* in a manner that is independent of pilus activity. Primers 31&32 were used to amplify and sequence *yccS1* from gDNA of Ncin WT and Ncin Δ*pilE.* As no changes in the sequence of *yccS1* were detected in either strain, it was concluded that disruption to *yccS1* was not responsible for any changes in the adherence properties of *N. cinerea.*

It was considered that any differences in bacterial fitness or growth rate between the bacterial strains used in these experiments may act as a confounding factor when determining the different levels of adherence, however growth curves in cell culture media demonstrated that growth rates were not significantly different between strains of the same species and that strains with highest viability did not correspond with those returning the highest viable counts in adherence experiments. Therefore any differences in growth dynamics were unlikely to be responsible for observed differences in adherence to the various surfaces.

In all three species, re-introducing *pilE* and complementing any changes observed would confirm whether these changes were caused purely by the lack of PilE. Complementation is difficult in these strains as PilE-null strains are not genetically competent so would not take up the

complementary gene. Another limitation that has been identified in these experiments is that empty wells and glass cover slips were not incubated in cell culture media prior to infection and were only reflective of wells containing cell lines from the point of washing prior to infection. Coating of the wells with proteins present in the cell culture medium may have affected the ability of bacteria to adhere to these surfaces and should be taken into consideration when drawing comparisons between infected cells, glass and plastic.

## 4.5 Conclusions

We have confirmed that *N. lactamica* Y92-1009 express T4P, which can be observed by TEM. Encouragingly, we have failed to identify these cellular components in the *pilE*-deficient strain of this bacteria, produced in Section 3.4. It was already established that natural competence in *N. lactamica* is T4P-dependent (Figure 11). In this chapter we have further shown that whilst *N. lactamica* T4P are not required for adherence to abiotic surfaces (plastic or glass) they are required for efficient interactions with both HEp2 and Detroit cells.

Adherence assays have demonstrated remarkably different adherence properties of *N. lactamica, N. meningitidis* and *N. cinerea,* despite them being genetically similar and sharing the same environmental reservoir. Experiments have also confirmed that, like *N. meningitidis,* non-piliated *N. lactamica* are impaired in their ability to adhere to epithelial cell lines. This demonstrates that the unexpected 'pilin-independent binding' of *N. cinerea* to epithelial cells identified by Tang et al. (2016) is not shared by all class 2 T4P-expressing *Neisseria* species, despite being observed in the interaction between class 2-expressing meningococci and HEp2 cells (Virji et al., 1992).

Considering it has previously been demonstrated that *N. lactamica* can displace *N. meningitidis* from the nasopharynx *in vivo* (Deasy et al., 2015), future developments of this work may include enhancing PilE expression and examining the effect this has on epithelial cell binding. This model may also be developed to examine whether *N. lactamica* displaces *N. meningitidis* from Detroit cells *in vitro.* This could provide a platform for better understanding the interactions between these two species and may permit enhancing the ability of *N. lactamica* to displace *N. meningitidis in vivo*.

# *Chapter 5* Strategies and Challenges for Transforming *N. lactamica* with PorA

*Note: Data in* Figure 21 *were generated by Jay Laver and Alice Liu. SBA assays (*Figure 25*) were performed by Holly Humphries, PHE.*

## 5.1 Introduction

The overarching theme of this project was to assess the therapeutic potential of GM *N. lactamica*, either through mucosal delivery of antigens into the nasopharynx, or through competitively inhibiting growth and colonisation of this environmental niche by pathogenic microorganisms. The relationship between *N. lactamica* and *N. meningitidis* has been discussed in Chapter 1. Of note, nasopharyngeal colonisation with *N. lactamica* has been shown to displace and prevent colonisation by *N. meningitidis* (Deasy et al., 2015). This, combined with the close genetic relationship between these two species suggests that meningococcal meningitis could be a viable target for a *N. lactamica*-based therapeutic intervention.

Polysaccharide vaccines have shown remarkable efficacy at reducing disease and carriage rates of *N. meningitidis* serogroups A, C, Y and W (Kinlin et al., 2009; Pace et al., 2009), however they are not cross protective against other serogroups and cannot be generated for serogroup B (Lucidarme et al., 2013; Lo Passo et al., 2007), which is currently responsible for the majority of meningococcal meningitis cases in the UK. For this reason it is necessary to consider other immunogenic markers. Whilst the capsule defines meningococcal serogroups; serotype and serosubtype are based on outer membrane proteins PorB and PorA, respectively (Frasch et al., 1985). Of these, PorB is also expressed by *N. lactamica,* whereas PorA is not. Furthermore, PorA is highly immunogenic and has long been considered as a potential vaccine target (Findlow et al., 2015; van der Ley et al., 1995). PorA is considered to be the immunodominant subcapsular antigen and has been shown to induce production of SBA, which is currently considered the best correlate of protection against IMD (Genco and Wetzler, 2010). Despite a high degree of antigenic variation in the surface-exposed hypervariable regions VR1 and VR2, and variation in PorA expression levels between strains (van der Ende et al., 2000), PorA was considered a good candidate to demonstrate the proof of principle that meningococcal antigens can be expressed in *N. lactamica* in an immunologically relevant conformation.

This chapter will provide a commentary of the strategies used to achieve heterologous expression of PorA in *N. lactamica* and a discussion of any obstacles that were encountered in the process.

## *5.2* Expression of PorA P1.7,16 in *N. lactamica*

PorA P1.7,16 has been identified as the most prevalent serosubtype among meningococcal group B disease isolates monitored in the United States and Europe (Tondella et al., 2000). This PorA type is expressed in *N. meningitidis* H44/76. Prior to the start of this project, the gene encoding PorA P1.7,16 (*porA*), along with its promoter, was amplified from *N. meningitidis* H44/76 genomic DNA and assembled into a plasmid upstream of *lacZ*, under the control of the *lst* promoter. Dr Jay Laver transformed this cassette, encoding PorA P1.7,16 and β-gal, into the NHCIS1 locus of *N. lactamica ΔlacZ*, thereby generating strain Nlac 2Pp7a. Nlac 2Pp7a expresses PorA at the membrane but at a lower concentration than *N. meningitidis* H44/76, as determined by western blotting of crude membrane fractions (data not shown).

### 5.2.1 Identifying an Upstream Activating Sequence

In prokaryotic promoters, consensus sequences lay approximately 35 and 10 bp upstream of the transcription start site and initiate transcription by promoting the association and binding of RNA polymerase. In Nlac 2Pp7a, the *porA* promoter from *N. meningitidis,* along with an approximate and arbitrary 100 bp directly 5' of the promoter, were included upstream of *porA* in the NHCIS1 locus. It was since noted that the -35 promoter sequence has only 2 out of 6 bp homology to the *E. coli* consensus sequence. Whilst some variation in the -35 sequence is common, a poorly defined -35 domain can be indicative of a binding site upstream of the -10 domain, where a protein is able to associate and promote RNA polymerase binding (deHaseth and Helmann, 1995). These protein-binding regions are known as upstream activation sequences (UASs).

To determine whether there is indeed a UAS upstream of the *porA* promoter, Dr Jay Laver and Alice Liu produced plasmids containing DNA homologous to the NHCIS1 *N. lactamica* locus. The gene encoding β-gal, under the control of the *lst* promoter, was inserted into the NHCIS1 locus. Upstream of the promoter were various lengths of DNA taken from the putative UAS (the sequence directly 5' of the *porA* promoter in *N. meningitidis* H44/76). The constructs, shown in Figure 21, were transformed into *N. lactamica,* where β-gal activity was measured and any effect of the additional DNA on β-gal activity was observed.

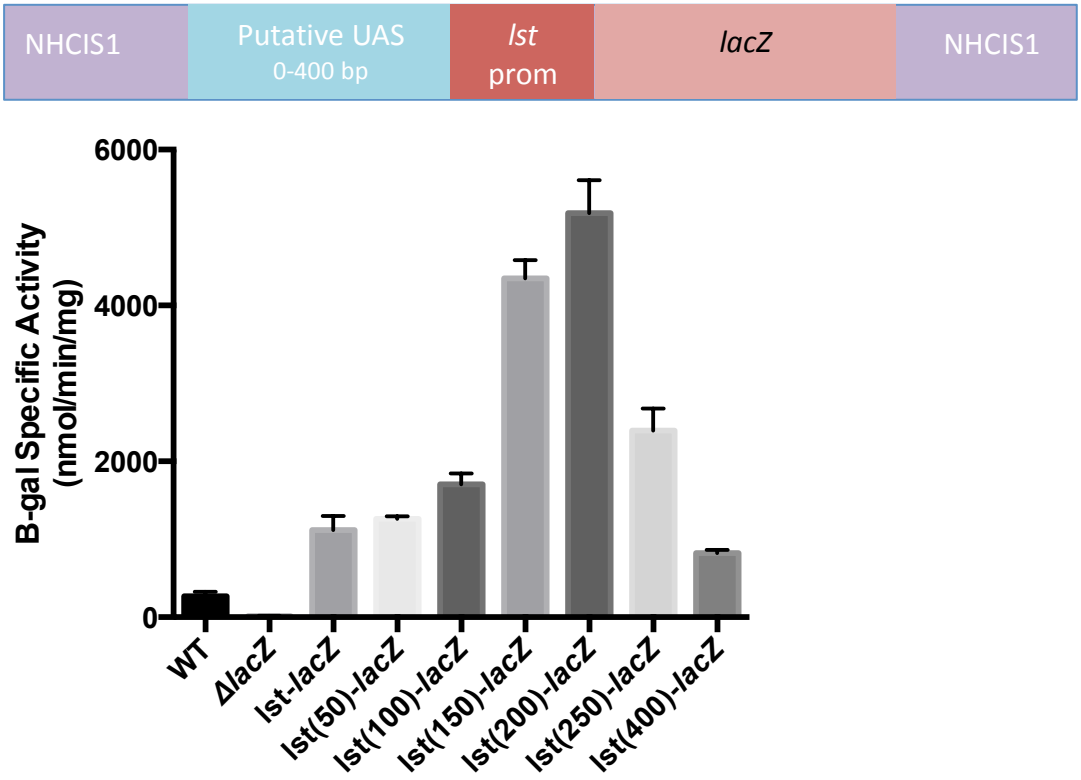



Figure 21. Identification of a Meningococcal *porA* Enhancer Sequence

Constructs were produced to encode *lacZ* under the control of the *lst* promoter, preceded by lengths of the putative PorA enhancer sequence from 0 to 400 bp. These constructs were flanked by DNA homologous to the Nlac NHCIS1 locus and transformed into *N. lactamica*. β-gal activity in these strains, as well as in in Nlac WT and *ΔlacZ* is shown.

In this model, it was demonstrated that the DNA sequence upstream of the *porA* promoter, when inserted upstream of the *lst* promoter, could enhance protein activity in *N. lactamica* (Figure 21). There was an increase in SA when between 100 and 250 bp of the sequence was included in the construct. Optimal β-gal activity was observed when the 200 bp immediately preceding the *porA* promoter were inserted upstream of the *lst* promoter, in the context of the *N. lactamica* NHCIS1 locus. This implies that this DNA region is involved in enhancing gene expression by a mechanism conserved between *N. lactamica* and *N. meningitidis*.

The inclusion of approximately 100 bp only of the putative UAS upstream of the *porA* promoter in Nlac 2Pp7a may contribute to the reduced PorA expression relative to *N. meningitidis*. Furthermore, the *porA* promoter contains a phase variable polymeric Guanidine (poly-G) tract between the -10 and -35 consensus sequences. Slip-strand mispairing at this site during DNA replication has been shown to alter PorA expression (van der Ende et al., 2000; Tauseef et al., 2013). Replacing some of the bases in the polymeric tract, thereby preventing phase variation at this site, may also enhance PorA expression in *N. lactamica.*

### 5.2.2 Optimising PorA Expression in *N. lactamica*

A construct was designed to generate a *N. lactamica* strain with increased PorA expression relative to Nlac 2Pp7a. A gBlock was obtained (*Integrated DNA Technologies*), encoding *porA* with its promoter and 200 bp of the putative UAS. The 17 bp between the -10 and -35 sites were replaced with those of the *porB* promoter, which is not phase-variable. The pSC101 plasmid, NHCIS1 locus, *lst* promoter and *lacZ* were PCR amplified from a pre-existing plasmid using primers 22 & 23. The gBlock was designed to contain 30 bp of complimentary sequence to the vector at each end, allowing the two components to be combined by isothermal assembly. The assembled plasmid, named pSC101 NHCIS1:*porA*(P1.7,16)-*lacZ* (Figure 22), was cloned into *E. coli* and selected for with ampicillin. Plasmids were extracted from *E. coli* and the presence of *porA* was confirmed by PCR amplification with primers 24 & 25, followed by gel electrophoresis (Figure 23a). The *porA* gene and promoter were sequenced to ensure fidelity. The cassette containing *porA* and *lacZ* flanked by NHCIS1 DNA was amplified by hmPCR using primers 16 & 17 and was transformed into Nlac Δ*lacZ*. Transformants were identified as blue colonies on X-Gal-containing agar. Genomic DNA was extracted and the insert amplified using primers 24 & 25 to check the size by DNA gel electrophoresis (Figure 23b), prior to sequencing. The resulting strain, Nlac 4PA1, contained the desired UAS and promoter sequence with the cognate PorA amino acid sequence of *N. meningitidis* H44/76.

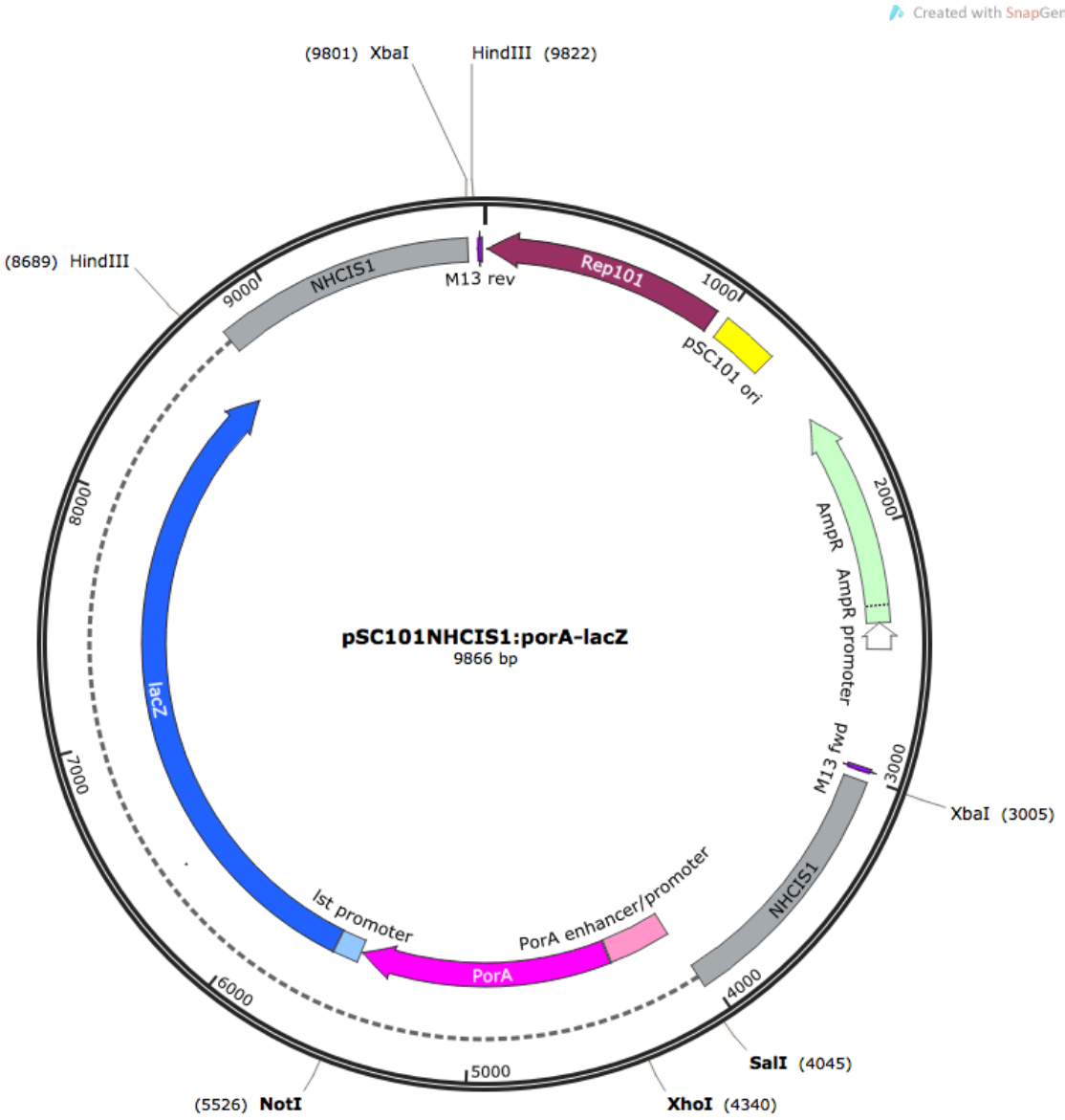



Figure 22. Plasmid map of pSC101 NHCIS1:*porA*(P1.7,16)-*lacZ*

The *porA*(P1.7,16) gene, under the control of its enhancer and a constitutively active promoter, was encoded in a gBlock. This fragment was combined with a PCR product amplified from a pre-existing pSC101 plasmid by isothermal assembly. The resulting plasmid, pSC101 NHCIS1:*porA-lacZ*, encodes PorA and LacZ, flanked by DNA homologous to the NHCIS1 locus in *N. lactamica* and confers ampicillin resistance. Genes, promoters, enhancers and relevant restriction sites are shown. Created using SnapGene.

### 5.2.3 Fitness and PorA Expression

Nlac 4PA1 demonstrated a slightly reduced growth rate in TSB when compared to WT or the parent strain: Nlac Δ*lacZ* (Figure 23c), and reached the same maximum cell density approximately 2-3 h later. This is likely to be a result of the increased metabolic burden caused by expressing an additional protein.

Flow cytometry was used to confirm surface expression of PorA. PorA was labelled with a primary mouse IgG anti-PorA (P1.7) antibody (NIBSC) and secondary rabbit anti-mouse IgG alexafluor-488 conjugate. The gating strategy, as shown in Figure 24a, ensured inclusion of both *N. lactamica* and *N. meningitidis* H44/76, which have slightly different forward and side scatter profiles. The primary antibody had been titrated previously (data not shown) and allowed excellent distinction between the negative (*N. lactamica*) and positive (*N. meningitidis* H44/76) controls (Figure 24c). The latter was used alongside a capsule-deficient strain of H44/76 (H44/76 *ΔsiaD*) to control for any effects of capsule on antibody binding. The lack of capsule appeared to reduce the variation between samples and significantly increased the MFI ($p=0.0002$), suggesting that the presence of meningococcal capsule obscures PorA from antibody recognition. Two negative controls were used, Nlac WT and 4YB2: a GM control, which contains the same construct as Nlac 2Pp7a and 4PA1 in the same Nlac Δ*lacZ* background, but lacking a *porA* open reading frame. No differences were identified between the two negative controls (Figure 24b).

PorA expression was observed in both Nlac 2Pp7A and 4PA1. Binding of antibody to the bacterial cells infers that at least the relevant PorA epitope is expressed in these strains and is exposed on the cell surface. Due to the low number of repeats, the level of expression in 2Pp7a is not significantly different to Nlac WT, however PorA expression in Nlac 4PA1 was significantly higher than both WT ($p=0.0034$) and 2Pp7a ($p=0.0163$). This demonstrates that modifications made to the promoter and/or UAS successfully increased PorA expression. PorA expression in Nlac 4PA1 was still significantly less than in Nmen H44/76 ($p<0.0001$) and H44/76 Δ*siaD* ($p<0.0001$).

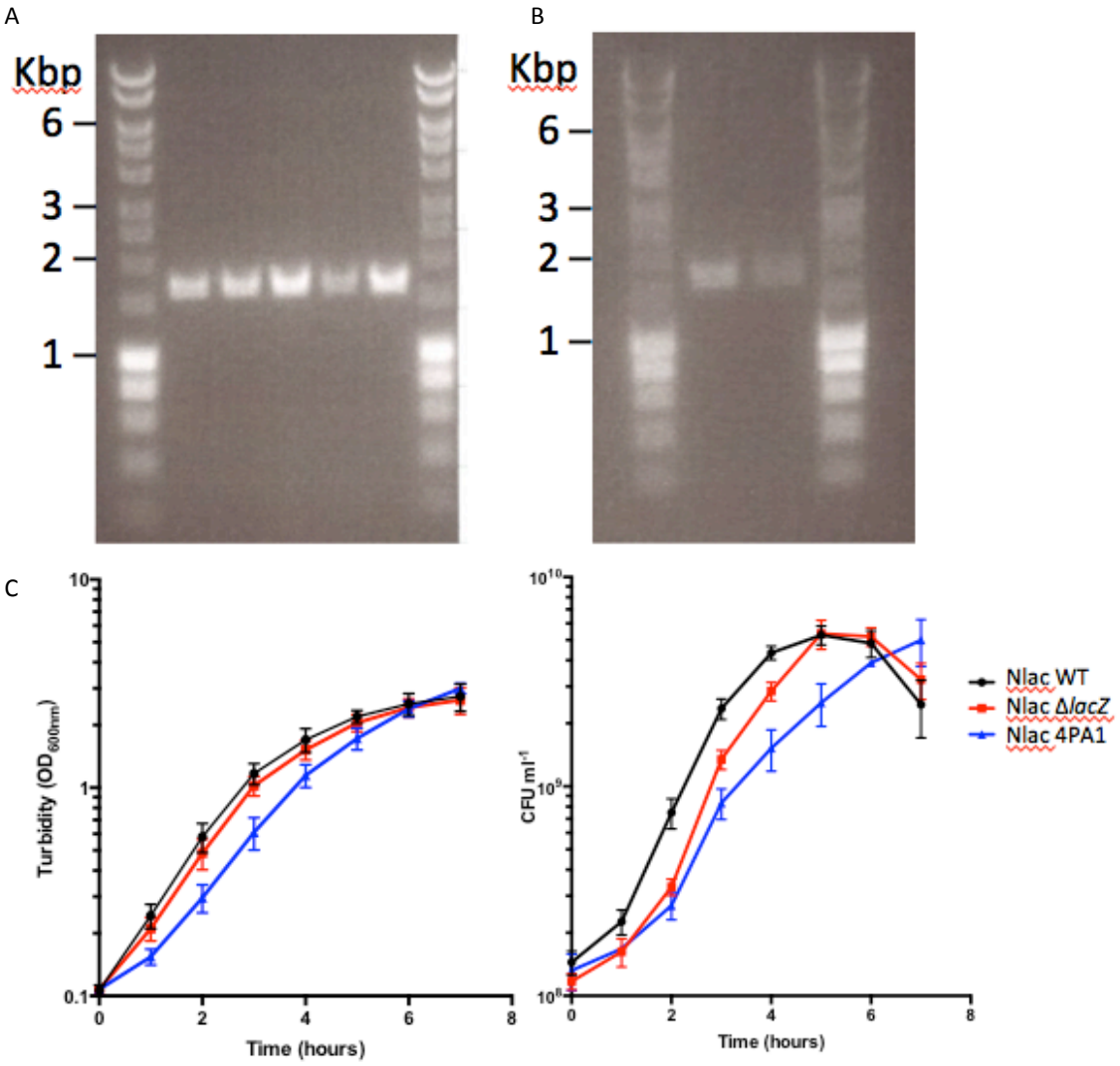



Figure 23. Synthesising Nlac Strain 4PA1.

A) Plasmids extracted from *E. coli* DH5α pSC101NHCIS1:*porA*(p1.7,16)-*lacZ* isolates A-E were digested with Xba1, prior to PCR amplification with primers 24&25. B) Genomic DNA extracted from Nlac Δ*lacZ* NHCIS1:*porA-lacZ* isolates 1 and 2 was PCR amplified with primers 24&25. A and B) PCR products were run on 0.7% agarose and all produced bands in agreement with the predicted product size of 1600 bp. C) Comparative growth curves of Nlac WT, Nlac Δ*lacZ* and Nlac 4PA1, showing both turbidity (left) and viable counts (right). Mean±SD (n=3).

Table 5. PorA Expression in Nlac and Nmen Strains

| Strain | PorA expression (MFI) | | | Mean MFI |
|---|---|---|---|---|
| Nlac WT | 3.17 | 5.1 | 2.61 | 3.63 |
| Nlac 4YB2 | 3.75 | 6.74 | 2.32 | 4.27 |
| Nlac 2Pp7A | 155 | 166 | 161 | 160.67 |
| Nlac 4PA1 | 785 | 809 | 880 | 824.67 |
| H44/76 WT | 1891 | 2700 | 2732 | 2441 |
| H44/76 Δ*siaD* | 3554 | 3734 | 3494 | 3594 |

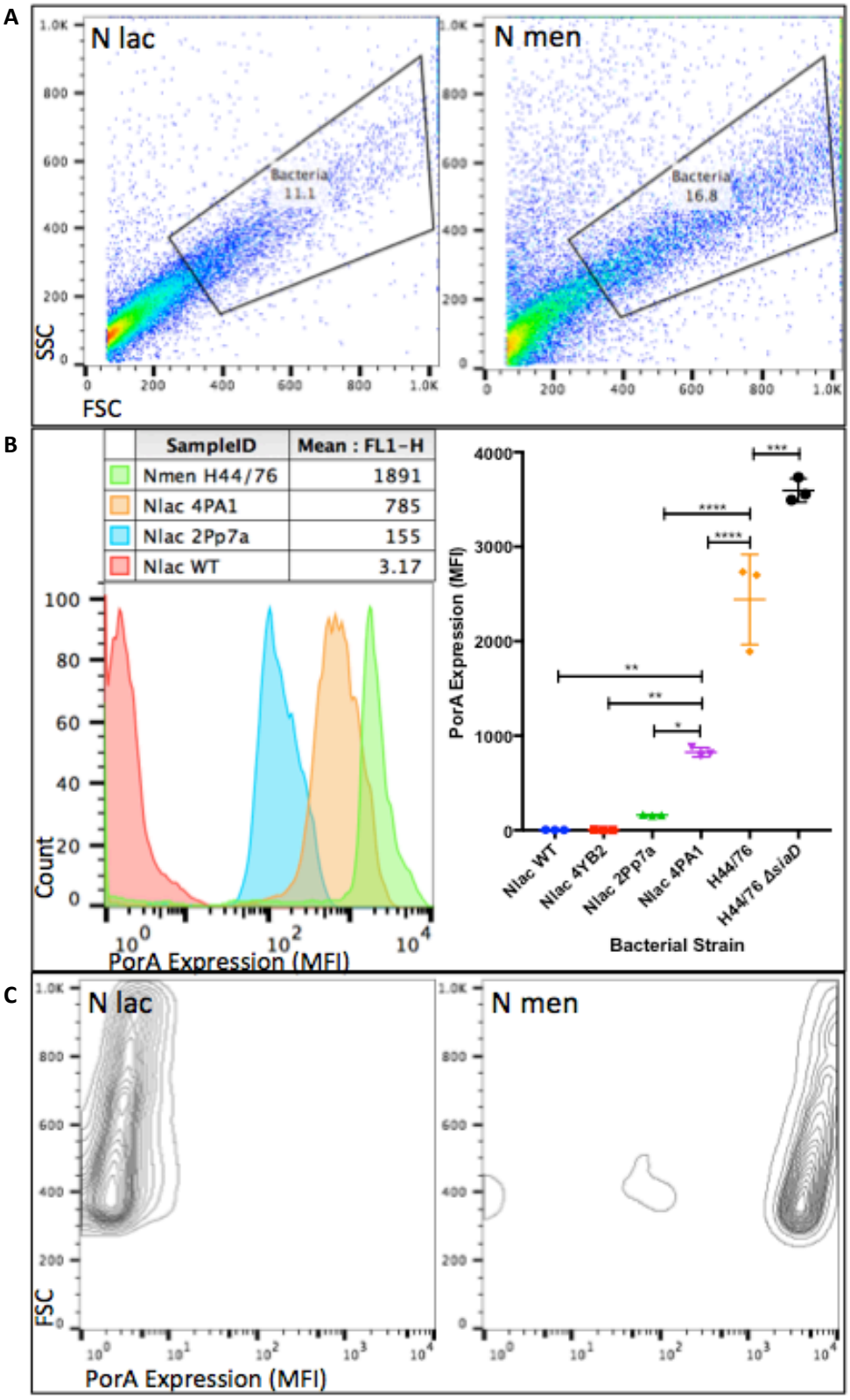



Figure 24. Flow Cytometric Analysis of PorA Expression in Nlac and Nmen Strains

Bacteria were grown in liquid culture to an absorbance ($OD_{600nm}$) of 0.3, then stained with mouse anti-PorA IgG, followed by anti-mouse IgG-A488. A) Scatter plots show the gating strategy, set to include the different forward and side scatter profiles of both *N. lactamica* Y92-1009 (left) and *N. meningitidis* H44/76 (right). B) Summary data of PorA expression (MFI) in Nlac WT, 4YB2, 2Pp7A and 4PA1 and Nmen H44/76 and H44/76 Δ*siaD* (right) with representative plot of Nlac WT, 2Pp7A and 4PA1 and Nmen H44/76 (left). Mean ±SEM, one-way ANOVA with Tukey's multiple comparison, n=3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. C) Density plots showing PorA expression in Nlac WT and Nmen H44/76.

## 5.3 4PA1 Outer Membrane Vesicles

OMVs were derived from Nlac 4PA1. These were run on SDS-PAGE, alongside GM control Nlac 4YB2. A silver stain was performed, which revealed multiple bands, demonstrating a variety of proteins present in OMVs from both strains (Figure 25a). There was no obvious additional band in 4PA1 that could be attributed to PorA, probably due to the region being obscured by other proteins of similar molecular weight. A western blot was performed to specifically probe for PorA. As can be seen in Figure 25b, there is a clear band present between 30 to 40 kDa in Nlac 4PA1 and not in 4YB2, which coincides with the molecular weight of PorA observed in previous publications (Martin et al., 2006) and infers PorA expression in Nlac 4PA1-derived OMVs.

OMVs were then sent to Public Health England (Porton Down), where SBA assays were performed by Dr Holly Humphries. Groups of 10 mice were immunised intraperitoneally with OMVs derived from Nlac WT, Nlac 4PA1 and Nmen H44/76. Sera were diluted and mixed with human complement and either Nmen H44/76 or Nmen MC58, to determine SBA activity of the sera against these meningococcal strains. Titres are shown as the reciprocal dilution which gave >50% killing compared with colonies at t=0 (Figure 25c and d). OMVs derived from Nlac WT elicited no SBA activity, however those derived from Nlac 4PA1 elicited an SBA response against both Nmen H44/76 (Figure 25c) and MC58 (Figure 25d).

In Figure 25c, the strongest SBA activity against Nmen H44/76 was unsurprisingly observed in mice immunised with H44/76-derived OMVs. The PorA type expressed in Nlac 4PA1 (P1.7,16) is identical in amino acid sequence to that in Nmen H44/76. It is therefore not surprising that anti-H44/76 SBA activity is high in mice immunised with Nlac 4PA1 OMVs. The increased SBA activity generated by Nmen H44/76-derived OMVs compared to Nlac 4PA1 OMVs is likely to be due to the presence of other H44/76-specific meningococcal antigens. Many other meningococcal antigens have previously been shown to induce SBA (Comanducci et al., 2002; Giuliani et al., 2006; Masignani et al., 2003). However, the dramatic increase in SBA activity resulting from immunisation with Nlac 4PA1 OMVs compared to Nlac WT OMVs confirms that PorA is highly immunogenic in the context of *N. lactamica* OMVs in mice.

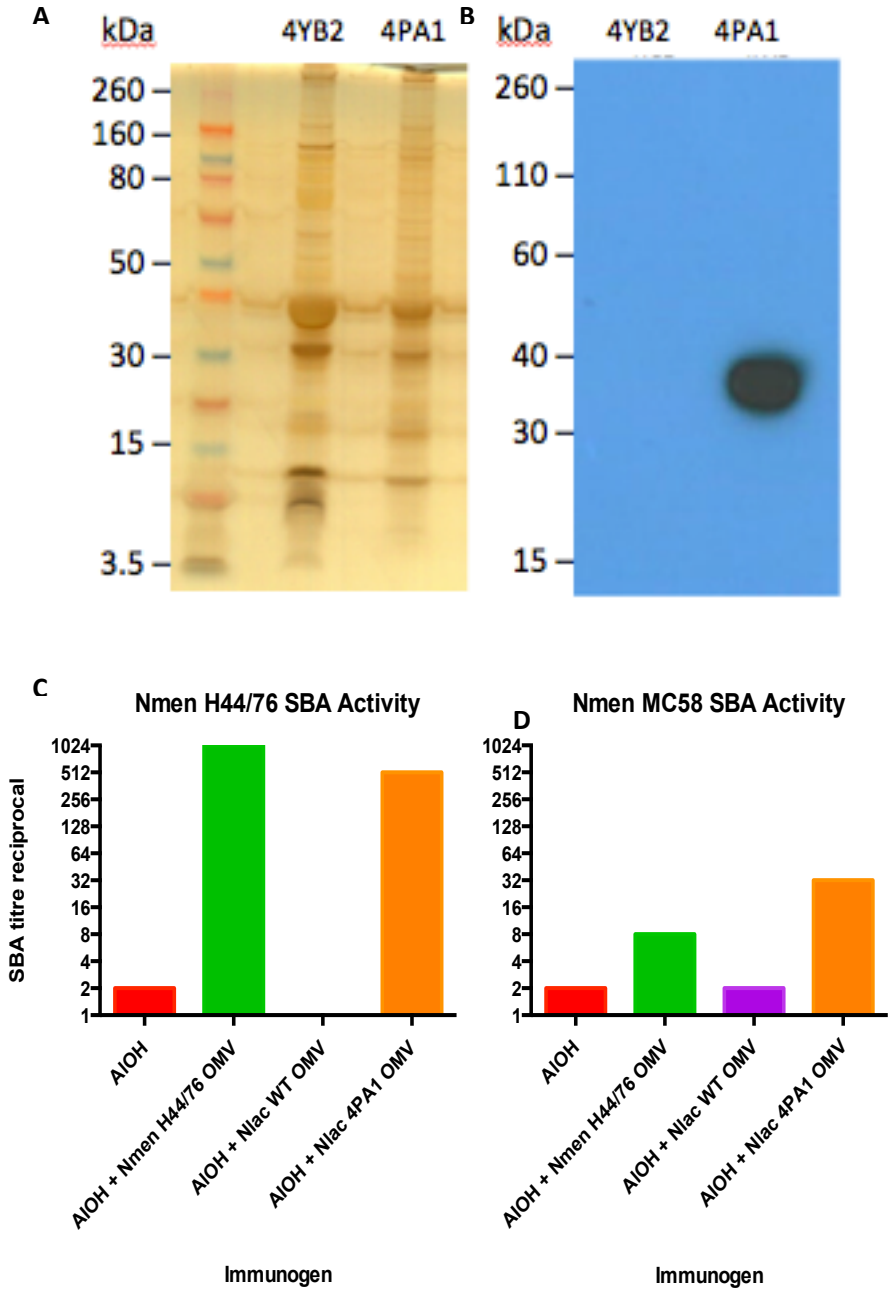



Figure 25. PorA Expression and SBA Activity of 4PA1 OMVs

OMVs were derived from Nlac 4YB2 and 4PA1. A and B) OMV samples (2.5 µg total protein) were separated by SDS-PAGE. A) The resulting bands were visualised by silver stain, which revealed multiple bands in both samples. B) A western blot was performed to probe for PorA, which was detected in Nlac 4PA1 only. C and D) Groups of 10 mice were immunised with either AlOH alone, or AlOH combined with 10µg OMV derived from Nmen H44/76, Nlac WT or Nlac 4PA1 on 3 occasions. Serum from immunised mice was pooled and tested for SBA activity against Nmen H44/76 (C) and MC58 (D) (n=1). Note: SBA activity was observed against Nmen H44/76 in serum from mice immunised with Nmen H44/76 OMVs at the maximum dilution tested (1/1024).

In Figure 25d, SBA activity against Nmen MC58 was higher following immunisation with Nlac 4PA1 OMVs than Nmen H44/76 OMVs, despite both OMVs containing the same PorA type. As this experiment was only performed once the difference cannot be assessed statistically and may be a result of sample variance. However, previous human studies have shown that vaccination with Nlac OMVs elicits a cross-reactive IgG response that is less than that elicited by Nmen OMVs against the homologous meningococcal strain (H44/76), whereas the cross-reactive IgG responses to a panel of heterologous serogroup B meningococcal strains was similar with WT Nlac or Nmen-derived OMVs (Findlow et al., 2006; Gorringe et al., 2009). It should be noted that the PorA type expressed by Nmen MC58 (P1.7,16b) compared to that expressed by Nmen H44/76 and Nlac 4PA1 (P1.7,16) only differs by a single amino acid. The increased response in the Nlac background is unlikely to be caused by Nlac-derived components, as immunisation with Nlac WT OMVs did not induce SBA activity. This suggests that the adjuvant properties of Nlac OMVs allow PorA P1.7,16 to induce immunity that is equally or more cross-protective against different meningococcal serosubtypes than non-homologous meningococcal OMVs.

Whilst the OMVs derived from both *N. lactamica* and *N. meningitidis* will have adjuvant properties, in that they alter the phenotype and/or amplitude of the immune response generated, it may be that the adjuvant properties of *N. lactamica*-derived OMVs cause PorA to induce a broader immunity that is protective against a wider range of meningococcal strains. It has previously been noted that a broad anti-meningococcal response is generated following vaccination with Nlac WT-derived OMVs (Gorringe et al., 2009). It is possible that in the context of meningococcal OMVs, the high concentration of PorA results in a PorA-dominated immune response. This may have been evolutionarily selected for in the meningococcus, as following the induction of an anti-PorA immune response, the bacteria can down-regulate PorA by phase variation, allowing immune escape. In the context of Nlac 4PA1-derived OMVs, the lower PorA concentration relative to Nmen OMVs could permit the induction of a cross-reactive immune response targeting other, potentially more essential surface structures that are not down-regulated under selective pressure.

A number of non-bactericidal antibodies are produced during exposure to or vaccination for *N. meningitidis.* Whilst a subset of these non-bactericidal antibodies can cooperate with bactericidal antibodies to enhance bactericidal activity (Vu et al., 2011), there have also been multiple examples of antibody production that blocks bactericidal activity against this species (Griffiss and Goroff, 1983; Jarvis and Griffiss, 1991; Ray et al., 2011). Blocking antibodies elicited by immunisation with Nmen-derived OMVs and not Nlac-derived OMVs may be dampening the bactericidal response.

In mice, vaccination with meningococcal OMVs has been shown to elicit SBA production, whereas OMVs derived from WT *N. lactamica* are protective against lethal challenge with *N. meningitidis,* without inducing the production of SBA (Oliver et al., 2002). It is possible that the different species result in the induction of a qualitatively different immune response. IgG antibodies can be broken down into 4 subgroups. Whilst subgroups 1 and 3 effectively bind C1q and activate complement, which would lead to bacterial lysis, subgroups 2 and 4 generally do not (Rispens and Vidarsson, 2014). Furthermore, Nmen H44/76 expresses capsule, and meningococcal capsules have previously been observed on OMVs (Fisseha et al., 2005). This may dramatically alter the immune response to OMVs. For example, IgG2, which ordinarily does not activate complement, has been shown to efficiently activate complement when at high densities, such as in the presence of polysaccharide capsules (Barrett and Ayoub, 1986; Siber et al., 1980; Usinger and Lucas, 1999). In fact, the antibody production in response to *S. pneumoniae* polysaccharide capsule is exclusively in the form of IgG2 (Barrett and Ayoub, 1986; Siber et al., 1980). Monoclonal antibodies of different mouse IgG isotypes directed against PorA (P1.16) had dramatically different SBA activity in the order IgG3 > IgG2b > IgG2a > IgG1 (Michaelsen et al., 2004). Conversely, a Nmen-derived OMV vaccine mainly induced IgG1 and IgG3 antibodies in humans (Næss et al., 1996). Unfortunately, the only study that involved vaccination of human volunteers with an *N. lactamica*-derived OMV did not quantify the IgG subclasses in the induced immune response (Gorringe et al., 2009).

It is important to note that this data is severely limited in that there is only one data point for each group. Additionally, it has previously been observed that SBA assays for the meningococci using non-human sera do not correspond with those performed with human serum. This is because the meningococci express fHbp, which binds to human factor H, a protein of the alternative complement pathway (Madico et al., 2006). Binding to factor H enhances the ability of meningococci to resist complement-mediated killing (Madico et al., 2006). As fHbp binds specifically to human factor H, killing occurs more readily in non-human sera (Granoff et al., 2009). Whilst a titer of 1:4 is considered protective in SBA assays using human sera, a titer of 1:128 is necessary to give equivalent protection when using rabbit sera, for example (Borrow et al., 2001; Santos et al., 2001).

## *5.4* Expression of PorA P1.7-2,4 in *N. lactamica*

Previous challenge studies have demonstrated that nasopharyngeal inoculation with live WT *N. lactamica* is safe and that active colonisation with these bacteria is protective against colonisation with *N. meningitidis* (Deasy et al., 2015; Evans et al., 2011). It is also known that mucosal vaccination elicits a stronger mucosal immune response than systemic vaccination (Rudin et al., 1998) and that PorA is immunogenic in mice in the context of *N. lactamica*-derived OMVs. Furthermore, *N. lactamica* can stably colonise individuals over multiple months (Deasy et al., 2015), and therefore live GM *N. lactamica* could become a platform for sustained delivery of antigen to the mucosal surface.

Nasopharyngeal inoculation with 'knock-in' GM bacteria has not been performed previously. As a result, extensive safety precautions are necessary. It is crucial that challenge strains are antibiotic susceptible, and as an additional safety measure, it is desirable to be able to vaccinate close contacts of colonised individuals against acquisition of the recombinant strain, if against all expectation the organism displays pathogenicity *in vivo*. Bexsero is the vaccine currently recommended for protection against serogroup B meningococcal disease in England, and was introduced into the national immunisation programme in September 2015. Preliminary data since the introduction of this vaccine suggests a reduction in serogroup B meningococcal disease (Ladhani and Public Health England, 2016; Parikh et al., 2016). The vaccine includes OMVs derived from Nmen NZ98/254, which contain PorA P1.7-2,4. Given the seemingly exquisite immune response to PorA identified in murine immunisation experiments (Section 5.3), for our first-in-man challenge experiment it was deemed appropriate to use a recombinant *N. lactamica* strain expressing the PorA serosubtype cognate to that present in the Bexsero vaccine. As such, constructs were designed in order to transform *N. lactamica* with DNA encoding *porA* (P1.7-2,4).

### 5.4.1 Constructing the Strain

A gBlock encoding a *N. lactamica* codon-optimised version of the *porA* (P1.7-2,4) gene was combined by isothermal assembly with the remainder of the plasmid, amplified from pSC101 NHCIS1:*porA*(P1.7,16)-*lacZ* with primers 26 & 27. The assembled plasmid was cloned into *E. coli,* and successful transformants were selected on LB agar as blue, ampicillin-resistant colonies. Following plasmid extraction, the size of the DNA insert was confirmed by PCR amplification with primers 24 & 25, followed by gel electrophoresis. Fidelity of the *porA* gene and promoter was confirmed by sequencing. The resulting plasmid was named pSC101 NHCIS1:HAEC4:*porA*(P1.7,2-4)-*lacZ* (Figure 26).

The NHCIS1:*porA*(P1.7-2,4)-*lacZ* cassette was amplified from pSC101 NHCIS1:HAEC4:*porA*(P1.7,2-4)-*lacZ* by hmPCR with primers 16 & 17. The product was used to transform *N. lactamica.* In three separate transformation attempts, only 1 blue Nlac colony was recovered, in which the *porA* sequence was incorrect. The substitutions were such that the amino acid sequence of the protein was altered (mis-sense). To improve the efficiency of transformation, an additional DUS was added to the forward primer, generating primer 28. Primers 28 & 17 were used to amplify the NHCIS1:HAEC4:*porA*(P1.7,2-4)-*lacZ* cassette by hmPCR and a further three transformation attempts were made. These attempts yielded a total of three blue *N. lactamica* colonies, each of which contained missense nucleotide substitutions in the PorA coding sequence. One isolate contained multiple errors and was therefore discarded. The remaining two isolates, 4PnzG4 and 4PnzG5, contained a change in only one amino acid each. These changes were in a transmembrane region and in VR1 respectively, as shown in Figure 28.

It is possible that mutations occurred in all recovered isolates because the native PorA P1.7-2,4 is toxic to *N. lactamica.* This could be due to intolerably high expression levels, erroneous substance transfer across the membrane, or could involve its interaction with PorB, which is known to form trimers in *N. meningitidis* (Stefanelli et al., 2016).

It was hypothesised that the change in amino acid sequence in VR1 in Nlac 4PnzG5 may affect antibody binding and that the change in the transmembrane region in 4PnzG4 may affect membrane stability and therefore prevent expression of the protein on the cell surface. Flow cytometry was used to test these hypotheses.

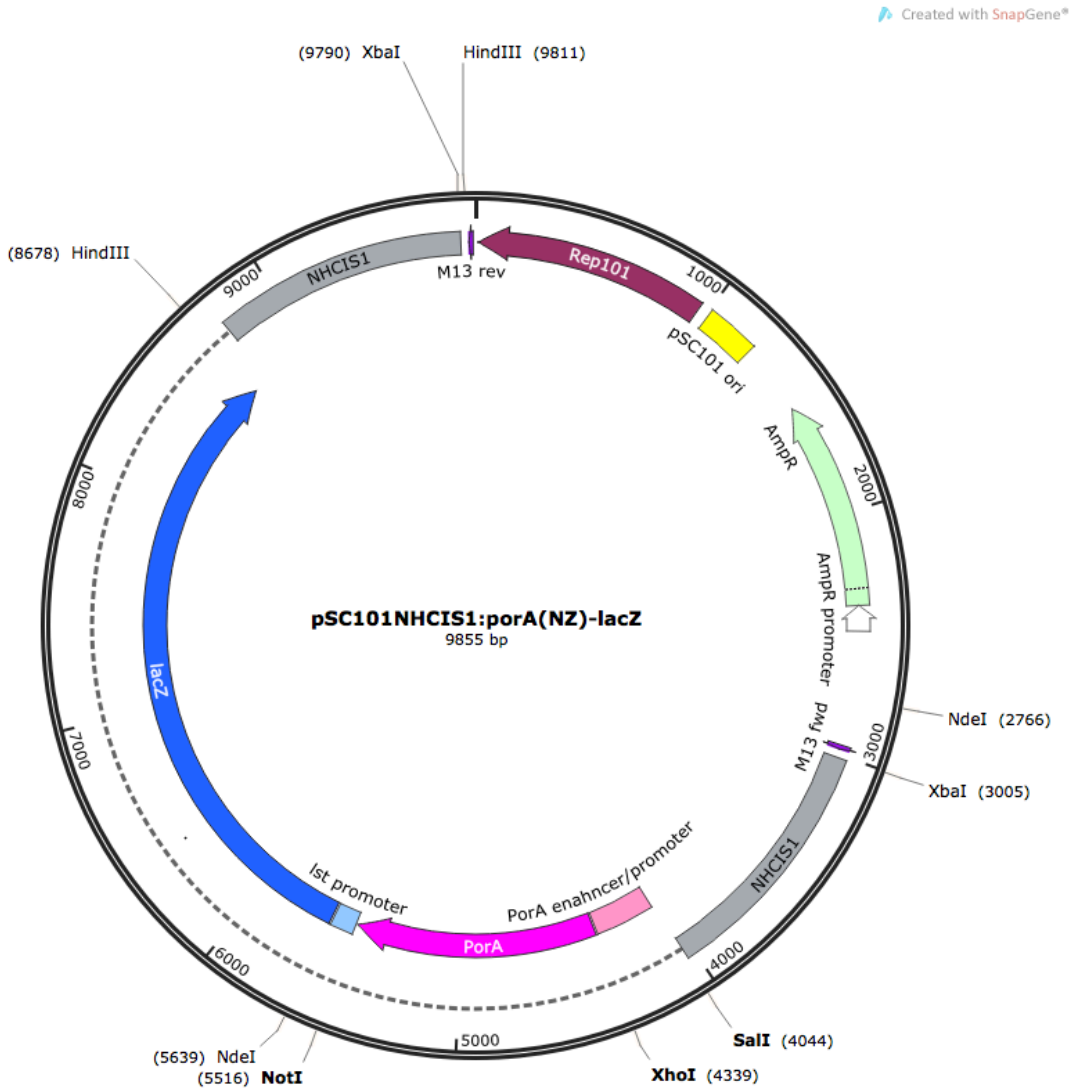



Figure 26. Plasmid Map of pSC101 NHCIS1:*porA*(NZ)-*lacZ*

A gBlock encoding *porA*(P1.7-2,4) was combined with a PCR product amplified from pSC101 NHCIS1:*porA-lacZ* by isothermal assembly. The resulting plasmid, pSC101 NHCIS1:*porA*(NZ)-*lacZ*, encodes PorA P1.7-2,4 and β-gal, flanked by DNA homologous to the NHCIS1 locus in *N. lactamica* and confers ampicillin resistance.
Genes, promoters, enhancers and relevant restriction sites are shown. Created using SnapGene.

### 5.4.2 Surface Expression of PorA

The gating strategy was set to include the different forward and side scatter profiles of both *N. lactamica* WT and *N. meningitidis* NZ98/254 (Figure 27a). These were used as negative and positive controls for PorA expression respectively. The mouse IgG anti-PorA (P1.4) primary antibody was titrated to maximally separate the peaks of these two controls, as demonstrated in Figure 27b. Figure 27c shows the relative PorA expression (MFI) of Nlac WT, 4YB2, 4PnzG4, 4PnzG5 and Nmen NZ98/254. No significant differences were observed by one-way ANOVA analysis. Whilst both 4PnzG4 and 4PnzG5 displayed PorA expression levels lower than that of Nmen NZ98/254, the MFIs for both strains were consistently higher than Nlac WT. This shows that in both strains, PorA was recognised and bound by the antibody and was expressed on the cell surface.

It is unclear why it was possible to express PorA P1.7,16 in *N. lactamica* but not PorA P1.7-2,4 from an otherwise identical construct in the same bacteria. The amino acid residues that became mutated in attempts to express PorA P1.7-2,4 (Nlac 4PnzG4 and 4PnzG5) are conserved between the native P1.7-2,4 AA sequence and PorA P1.7,16 (see alignment in Figure 29). Mutations across the isolates obtained from different transformation events were not consistent, which suggests that these changes were unlikely to be the result of selective pressure(s) against a particular region of the PorA protein. In Nlac 4PnzG4 and 4PnzG5 the amino acid changes were at opposite ends of the protein and one is an extracellular AA while the other sits within the membrane (see Figure 28). The fact that these substituted residues are ordinarily highly conserved across different PorA types suggests that they serve an important role. Perhaps the loss of this/these function(s) is what allows expression of the slightly altered protein in *N. lactamica*.

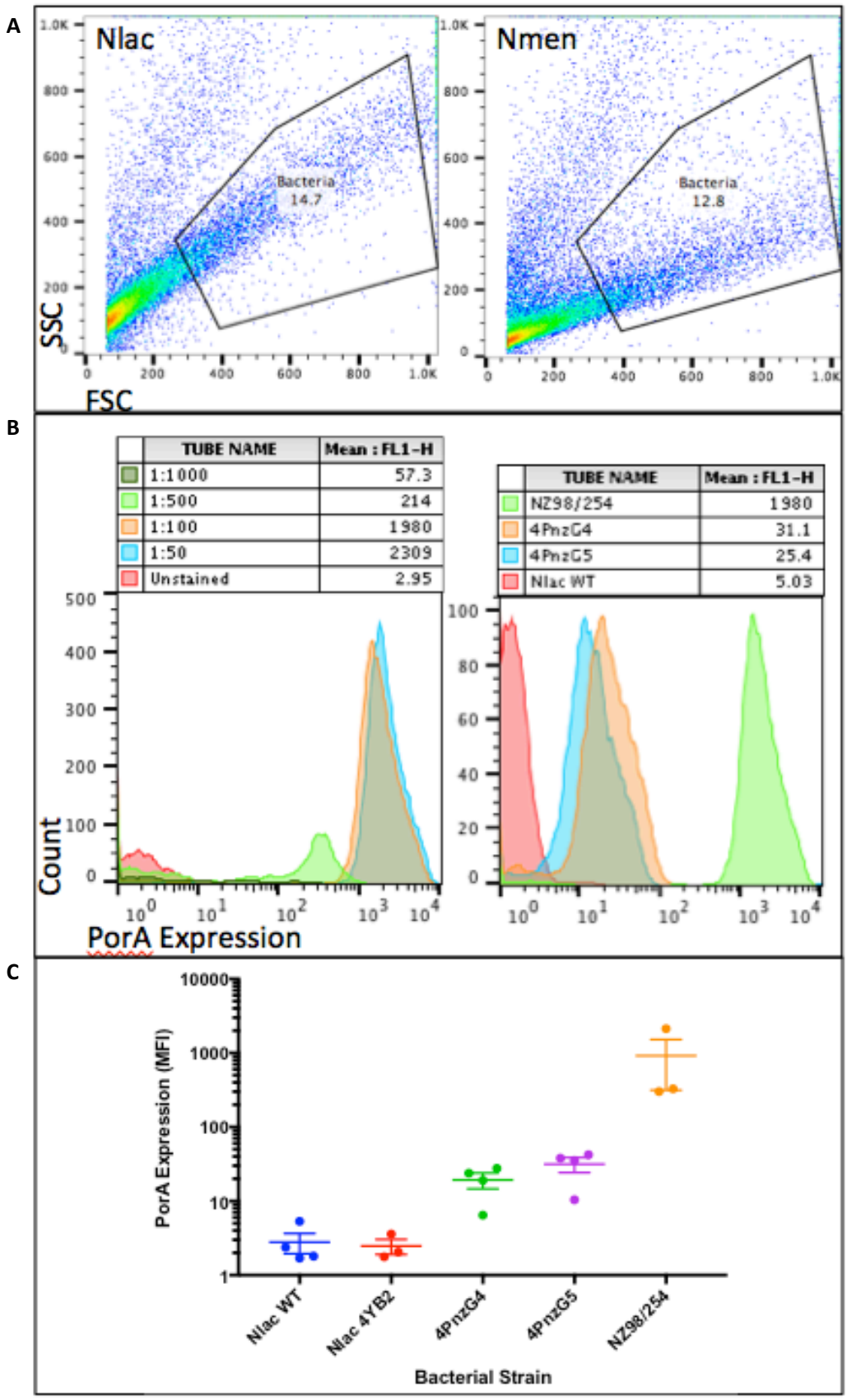



Figure 27. Flow Cytometric Analysis of PorA Expression in Nlac and Nmen Strains

A) Scatter plots showing gating strategy, set to include both *N. lactamica* Y92-1009 (left) and *N. meningitidis* NZ98/254 (right), which have different forward and side scatter profiles. B) Titration of primary antibody from 1:50 to 1:1000 in Nmen NZ98/254 (left) with representative plot of PorA expression in Nlac WT, 4PnzG4 and 4PnzG5 and Nmen NZ98/254 (right). C) PorA expression in bacterial strains by flow cytometry. No significant differences resulted from one-way ANOVA analysis. Mean ±SEM, n≥3.

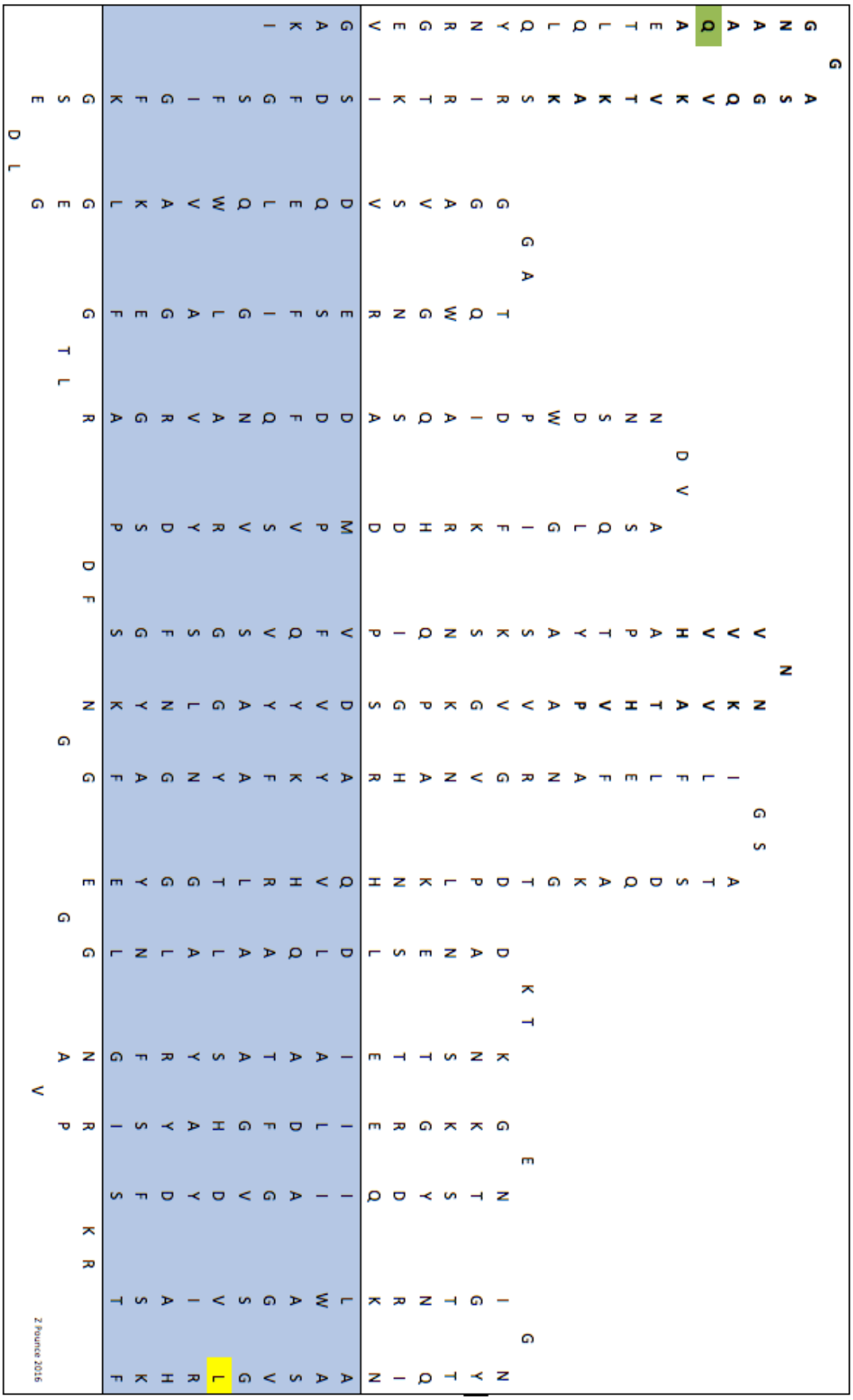


Figure 28. Model of Amino Acid Sequence in PorA P1.7-2,4

VR1 and VR2 are shown in bold and cell membrane in blue. In Nlac 4PnzG4, the Leucine highlighted in yellow is replaced with Methionine. In 4PnzG5, the Glutamine highlighted in green is replaced with Arginine. Model based on (van der Ley et al., 1991).

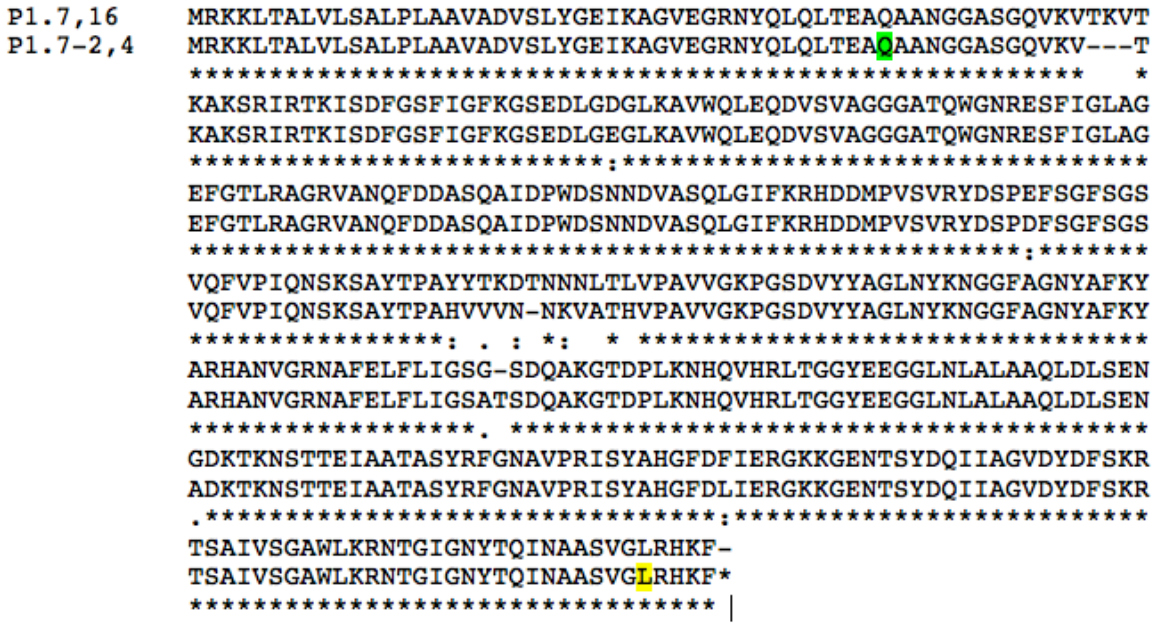



Figure 29. Amino Acid Sequence Alignment of PorA Serosubtypes P1.7-2,4 and P1.7,16

The amino acid sequences for PorA P1.7,16 and P1.7-2,4 were aligned using Clustal Omega. The amino acid residues that were changed in Nlac 4PnzG4 and 4PnzG5 are highlighted in yellow and green respectively. (*) Fully conserved residue. (:) Residues have strongly similar properties. (.) Residues have weakly similar properties. No symbol = residues are dissimilar. (-) Residue is not present.

Table 6. Mutations acquired through expressing PorA P1.7-2,4 in *N. lactamica*

| AA Position | PorA P1.7-2,4 | 4PnzG4 | 4PnzG5 | 4PnzG4.2 | 4PnzG5.2 |
|---|---|---|---|---|---|
| 44 | Q | Q | R | Q | R |
| 305 | T | T | T | T | M |
| 316 | G | G | G | G | S |
| 384 | G | G | G | G | S |
| 385 | L | M | L | M | L |
| 390 | Stop | Stop | Stop | K | K |

### 5.4.3 Mutations in PorA do not allow it to be tolerated in *N. lactamica*

To determine whether the mutations that were observed in Nlac 4PnzG4 and 4PnzG5 allowed PorA P1.7-2,4 to be tolerated in *N. lactamica,* the NHCIS1 loci containing the altered PorA sequences and *lacZ* were hmPCR amplified from the gDNA of 4PnzG4 and 4PnzG5 using primers 28 & 17. The hmPCR products were then transformed into Nlac *ΔlacZ* and transformants were identified as blue colonies on TSB X-Gal. One blue colony (named 4PnzG4.2) was identified amongst bacteria exposed to the 4PnzG4 *porA* sequence and one blue colony (named 4PnzG5.2) amongst bacteria exposed to the 4PnzG5 *porA* sequence. The two colonies were isolated and the *porA* genes sequenced with primers 24 & 25. Any changes from the cognate PorA P1.7-2,4 amino acid sequence are shown in Table 6.

The mutations that were present in the original 4PnzG4 and 4PnzG5 strains persisted in the next generation, however the coding sequence of both versions of the protein also acquired additional missense mutations. In both 4PnzG4.2 and 4PnzG5.2, the stop codon was replaced with lysine. In 4PnzG5.2 there were a further three missense SNPs that were not present in 4PnzG5.

The development of additional mutations in PorA when the sequences from 4PnzG4 and 4PnzG5 were used as donor DNA suggests that the original mutations in these strains did not make PorA tolerable for *N. lactamica.* Instead, they infer that expression of this protein places too high a burden on *N. lactamica.* Probability states that the majority of random DNA mutations provide no evolutionary advantage. They are more likely to be neutral or deleterious. The accumulation of mutations in PorA is likely to exert a progressively detrimental effect on its function as a porin, therefore reducing its impact on the normal functioning of the cell.

### 5.4.4 Use of 'Mutated' PorA in Human Challenge

Due to the changes in PorA amino acid sequence in Nlac 4PnzG4 and 4PnzG5, they contain unrecognised proteins that have not previously been reported in nature. This comes with both advantages and disadvantages regarding use of these strains in human challenge. It appears that the cognate AA sequence of PorA P1.7-2,4 is not tolerated in *N. lactamica*, and it is likely that the mutations have altered the function of this protein. In both 4PnzG4 and 4PnzG5, the protein is expressed and recognised by the mouse anti-porA monoclonal antibody, which means it is also likely to be recognised by the human immune system and it is possible that Bexsero vaccination would elicit an effective response against these strains. This could be tested by comparing the SBA activity of Bexsero-vaccinated human sera and non-immunised human sera against Nlac

4PnzG4 and 4PnzG5. PorA was the protein chosen for proof of principle that a heterologous protein could be expressed in Nlac due to its immunogenic properties. Its function as a porin is not important for our studies. Therefore an immunogenic but non-functional variant may be a better alternative to the cognate PorA AA sequence for our purposes. However, a difference of just one amino acid in the PorA sequence of meningococcal strains MC58 and H44/76 made a large difference to the SBA activity elicited by mice immunised with H44/76-derived OMVs in Figure 25. Perhaps the single amino acid changes in 4PnzG4 and 4PnzG5 would have similarly detrimental effects on immune activation. This is particularly likely in 4PnzG5, where the AA substitution occurred in an extracellular loop of the protein.

Despite being recognised by a mouse anti-PorA antibody, we currently have little data regarding the effect of these AA substitutions on the immunogenicity of PorA in humans or on its functionality as a porin in *N. lactamica.* We have also not examined the effect of the genetic modification on the fitness of strains 4PnzG4 and 4PnzG5. Being able to vaccinate against GM *N. lactamica* for human challenge is not essential as there is no expectation that the addition of PorA to *N. lactamica* should cause any danger to human health. Furthermore, any strains would be tested for antibiotic sensitivity prior to use, confirming our ability to treat and clear carriage in all volunteers in case of adverse events. We have effectively produced unknown proteins in Nlac 4PnzG4 and 4PnzG5. Furthermore, the expression of these proteins was very low, which would likely affect their ability to elicit an immune response. Therefore strains 4PnzG4 and 4PnzG5 were deemed unsuitable to be progressed for use in humans. The successful transformation of *N. lactamica* with PorA P1.7,16 (Section 5.2) presents a preferable option to progress for human challenge.

## *5.5* Expression of PorA P1.5,2 in *N. lactamica*

As there is currently no explanation for why *N. lactamica* was readily transformed with PorA P1.7,16 but not the cognate AA sequence of PorA P1.7-2,4, a construct containing a third PorA type (P1.5,2) was produced. The aim was to transform *N. lactamica* with the additional PorA to determine whether PorA is generally tolerated in *N. lactamica* and whether the successful transformation with PorA P1.7,16 or the unsuccessful transformation with PorA P1.7-2,4 was the exception. PorA P1.5,2 was selected as it is one of the most commonly occurring serosubtypes among MenB disease isolates (Tondella et al., 2000; Urwin et al., 2004).

The construct (NHCIS1:*porA*(P1.5,2)-*lacZ*) was produced in the same manner as NHCIS1:*porA*(P1.7-2,4)-*lacZ* (see Section 5.4) and was identical in every way other than the PorA

sequence. The construct was amplified from linearised plasmid by hmPCR before transforming it into Nlac Δ*lacZ,* using the same protocol as in Sections 5.2 and 5.4. No blue isolates were recovered following selection on TSB X-Gal.

The fact that *N. lactamica* was not readily transformed with PorA P1.7-2,4 or P1.5,2 suggests that this species does not generally tolerate PorA expression. The choice of PorA P1.7,16 in the initial attempts to express this protein appears to have been fortunate, in that for some reason this type is better tolerated than others. It is possible that expression of all PorA types is achievable in *N. lactamica,* but that the frequency of transformation events is below the level of detection for some types, using our protocol. This problem could be overcome by simultaneously transforming *N. lactamica* with both PorA and an antibiotic-resistance cassette, however the requirement to produce strains appropriate for human challenge precludes the use of antibiotic selection.

## 5.6 Conclusions

The data in this chapter has confirmed that *N. lactamica* can be transformed, using our protocol, with heterologous DNA and without the addition of antibiotic-resistance markers. Heterologous antigen can be expressed in *N. lactamica,* though the expression and number of transformation events appears to be protein-specific. We have identified an upstream activation sequence from *N. meningitidis*, which has been included upstream of the *porA* promoter to augment gene expression levels in *N. lactamica*. PorA was presented on the cell surface and in OMVs in an immunologically relevant conformation, as demonstrated with meningococcal PorA (P1.7,16). The protein was recognised by serosubtyping antibody in flow cytometric analysis and induced SBA activity in mice following vaccination with *N. lactamica*-derived OMVs. We have shown that SBA induced by intraperitoneal immunisation of mice with OMVs derived from PorA (P1.7,16)-expressing *N. lactamica* is not only active against *N. meningitidis* with cognate PorA but may also have some cross-protective activity against strains expressing other PorA types.

It has not been possible to generate a strain of *N. lactamica* that expresses PorA P1.7-2,4 due to the occurrence of inconsistent nucleotide substitutions in all viable transformants, or PorA P1.5,2. This may be because the frequency of transformation events was below the level of detection or because these PorA types are not well tolerated in *N. lactamica*.

# Chapter 6 Phase Variable PorA

*Note: Nlac strains ON(100), PV(100)A and PV(100)B were constructed by Graham Berreen. Technical assistance for the Gentamicin Protection Assay was provided by Graham Berreen and Seth James. Antibiotic sensitivity testing was performed by Muktar Ibrahim, PHE. SBA assays were performed by Holly Humphries, PHE. Genome annotations of ON(100), PV(100)A and PV(100)B were provided by Anish Pandey.*

## 6.1 Introduction

Following the production of a PorA-expressing strain of *N. lactamica* (Section 5.2), one of the group's research aims was to investigate the effect of antigen phase variation on colonisation of the nasopharynx. In *N. meningitidis,* PorA expression is regulated by a phase variable (PV) promoter, consisting of a polymeric guanidine (poly-G) tract between the -10 and -35 conserved regions (van der Ende et al., 2000). Whilst other methods of varying the expression of this protein exist, changes to the poly-G tract occur most frequently (van der Ende et al., 2000)). *In vitro* exposure of *N. meningitidis* to serum and anti-PorA antibody resulted in a shift from 11 to 10 Gs in this tract, with a concurrent 2-fold reduction in PorA expression and apparent immune escape (Tauseef et al., 2013). Changes in tract length have also been observed in this promoter during *in vivo* meningococcal carriage, however initial tract lengths varied between carriers and it is unclear whether changes in tract length correlated with a reduction in PorA expression (Alamro et al., 2014).

The ability of *N. meningitidis* to vary expression of surface antigens is thought to be a mechanism of immune escape for enhancing persistence within the nasopharynx. It was hypothesised that bacteria expressing the immunogen PorA would be more likely to persist if expression were phase variable, as a proportion of the bacteria would have reduced PorA expression and would therefore be less susceptible to recognition by anti-PorA antibodies. As a commensal organism, *N. lactamica* was considered a safer alternative to *N. meningitidis* for testing this hypothesis *in vivo.* It was therefore necessary to produce a strain of *N. lactamica* expressing PorA under the control of a PV promoter, for comparison to the constitutive expression of Nlac 4PA1 (Section 5.2).

Initial attempts involved trying to produce a Nlac strain identical to 4PA1, including the 200 bp UAS, but using the original PorA promoter sequence from Nmen H44/76. The promoter was slightly modified to contain a poly-G tract of 11 bp rather than the 12 bp shown in the published

H44/76 genome sequence. This made the promoter the same size as in Nlac 4PA1 and is the length for optimal protein expression (van der Ende et al., 2000). Unfortunately, attempts to produce this strain were unsuccessful and all transformants contained missense nucleotide polymorphisms.

Importantly, construct insertion and PorA expression were confirmed in Nlac 2Pp7a (Section 5.2), which used the cognate PV *porA* promoter but without the enhanced expression afforded by the UAS. It was therefore hypothesised that PorA could not be tolerated in *N. lactamica* under the optimised conditions of its own promoter and full UAS. The UAS was therefore truncated to reduce expression to a level where both constitutive and phase variable promoters were tolerated (100 bp. See Figure 21). Two PV *N. lactamica* isolates were recovered: PV(100)A and PV(100)B, containing 11 Gs and 9 Gs respectively in the poly-G tract of the PorA promoter. A matching non-PV strain containing 100 bp UAS and the same fixed on promoter as 4PA1 was also produced (ON(100)). The three strains are depicted in Figure 30. Previous work with disease and carrier isolates of *N. meningitidis* suggests that PorA expression is substantially reduced when the promoter contains 9 Gs, rather than 11 (van der Ende et al., 2000).

As these constitutive and PV PorA-expressing strains were constructed in order to compare their ability to colonise and persist in the nasopharynx of human volunteers, it is necessary to (i) determine whether the strains are comparable (ii) examine the biosafety profiles of the strains and (iii) confirm that the PV strains are capable of varying PorA expression.

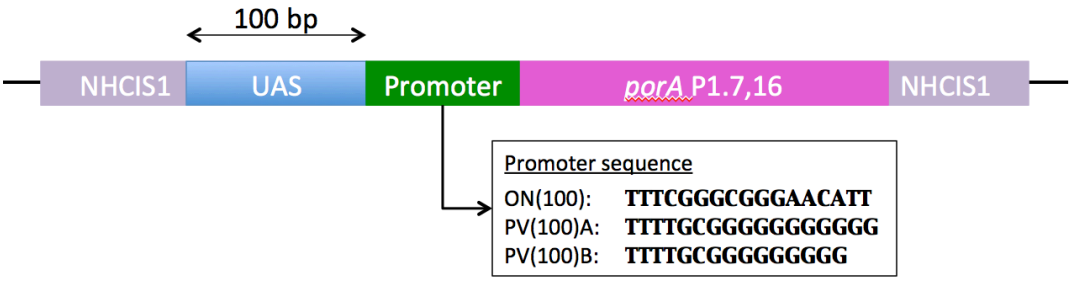



Figure 30. Fixed on and PV PorA-expressing Strains of *N. lactamica*

In strains Nlac ON(100), PV(100)A and PV(100)B, *porA* (P1.7,16), along with its promoter and upstream activation sequence (UAS) were inserted into NHCIS1. The UAS has been truncated from 200 to 100 bp in all three stains to modulate PorA expression. Strains vary by the spacer sequence between the -10 and -35 boxes of the PorA promoter, as shown.

## *6.2* Comparing PorA-expressing Strains of *N. lactamica*

### 6.2.1 Genomic Strain Comparison

To identify genomic differences between the three porA-expressing strains and WT *N. lactamica,* Nlac ON(100), PV(100)A and PV(100)B were sent to MicrobesNG for whole genome sequencing. The Fastq files, containing sequencing reads, were mapped to the published WT *N. lactamica* Y92-1009 genome sequence (Pandey et al., 2017), and any insertions (ins), deletions (del), recombination events or SNPs were identified (Table 7). The raw snippy output data can be found in Appendix D. Limitations of this method meant that a large insertion, such as the introduction of *porA*, would not be detected. For this reason the NHCIS1 loci of Nlac ON(100), PV(100)A and PV(100)B were sequenced separately with primers 24 & 25, and this confirmed that there were no changes to the intended DNA sequence of the *porA* gene or promoter.

Of 13 mutations detected in the genomes of PorA-expressing strains, 9 were deletions in a phase variable, polymeric tract. The remaining 4 mutations were SNPs, of which only one changed the amino acid sequence of a protein. This change was present in all PorA-expressing strains and caused a change from glycine to glutamic acid in the AA sequence of a substrate-binding protein for an iron ABC transporter. The majority of mutations observed were present in all strains, suggesting they were also present in the Nlac *ΔlacZ* parent strain. One mutation was observed in PV(100)A and PV(100)B only. This deletion in a poly-T tract caused a frameshift mutation in 5-(carboxyamino)imidazole ribonucleotide synthase. There were a further four mutations that were exclusive to PV(100)B. These were a deletion in an intergenic poly-A tract and three synonymous SNPs.

To observe the relative genetic relatedness of Nlac WT, ON(100), PV(100)A and PV(100)B, the data generated in Table 7 were combined to produce one core genome. The divergence of each strain from the core genome was used to create a phylogenetic tree with phyML, which was rooted on the WT and visualised in FigTree. All four strains are extremely similar, with PV(100)B being the most divergent strain. This tree does not account for the additional DNA in the NHCIS1 locus, as this large insertion could not be captured during the initial snippy analysis.

Table 7. Mutations in PorA-expressing *N. lactamica,* relative to WT

| Strains | Type | Location | Effect | Gene Product |
|---|---|---|---|---|
| All | del (poly-A tract) | CDS | Frameshift | AmpG family muropeptide MFS transporter |
| PV(100)B | del (poly-A tract) | Intergenic | | |
| All | del (poly-T tract) | CDS | Frameshift | sodium/glutamate symporter |
| PV(100)B | SNP (C > G) | CDS | Sense (L>L) | LPS biosynthesis protein |
| All | SNP (G > A) | CDS | Missense (G>E) | iron ABC transporter substrate-binding protein |
| All | del (poly-A tract) | Promoter | | restriction endonuclease accession ARB04129 |
| PV(100)A PV(100)B | del (poly-T tract) | CDS | Frameshift | 5-(carboxyamino)imidazole ribonucleotide synthase |
| All | del (poly-A tract) | Promoter | | tRNA lysidine synthetase TilS |
| All | del (poly-A tract) | CDS | Frameshift | hypothetical protein* |
| All | del (poly-T tract) | CDS | Frameshift | hypothetical protein* |
| PV(100)B | SNP (C > T) | CDS | Sense (C>C) | beta-galactosidase |
| PV(100)B | SNP (T > C) | CDS | Sense (P>P) | beta-galactosidase |
| All | del (poly-A tract) | CDS | Frameshift | polyamine ABC transporter substrate-binding protein |

*These proteins are out of frame in the reference genome, both containing an early stop codon.

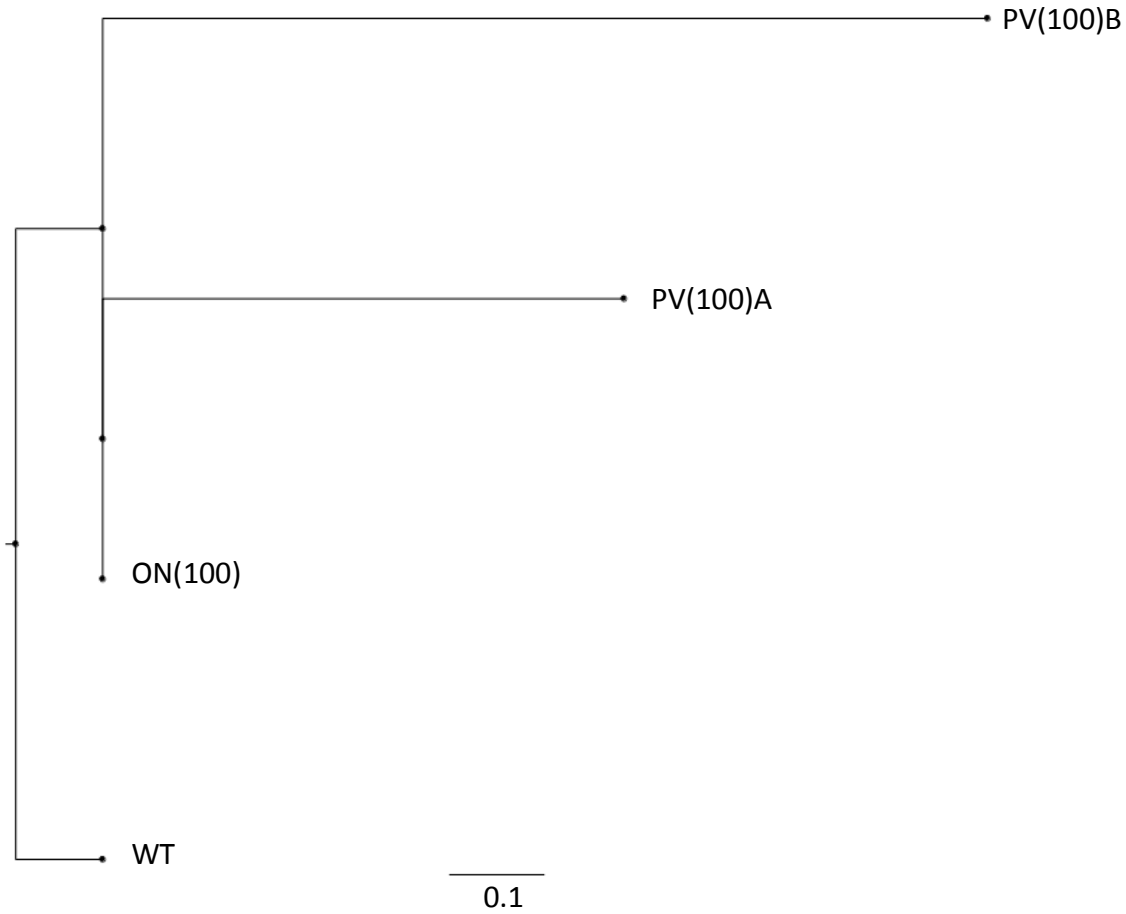



Figure 31. Phylogenetic Tree of WT and PorA-expressing *N. lactamica*

Strains ON(100), PV(100)A and PV(100)B were compared to the WT reference genome and a core genome for all four strains was produced using snippy. PhyML was used to produce a phylogenetic tree based on divergence from the core genome, which was rooted on the WT and viewed using FigTree. Scale bar = substitutions per variant site, where there are 13 variant sites identified.

### 6.2.2 Growth Dynamics

Growth curves were performed on each of the three PorA-expressing strains (ON(100), PV(100)A and PV(100)B) and WT *N. lactamica* in both nutrient rich (TSB) and nutrient poor (Frantz) media to compare their fitness under these conditions (Figure 32). By both $OD_{600nm}$ and viable counts, the growth of all strains over time was highly similar in nutrient rich broth (Figure 32a). Growth of PV(100)B appeared to be slightly slower than other strains by viable counting but the difference was negligible. In Frantz media, there was little growth across all strains (Figure 32b). However, Nlac WT appeared the fittest under these conditions in that WT cultures displayed a slight increase in turbidity and viability over time. The turbidity of all PorA-expressing cultures increased slowly over time with a concurrent overall reduction in viability. PV(100)B suffered the greatest reduction in viability in Frantz media, while ON(100) and PV(100)A were comparable.

### 6.2.3 PorA Expression

Flow cytometry was used to assess levels of PorA expression in multiple strains of *Neisseria* (Figure 33). Nlac WT and the GM control 4YB2 do not express PorA. Nlac 2Pp7a is the original PorA-expressing strain of *N. lactamica,* which was modified to optimise expression in Section 5.2, resulting in strain 4PA1. The UAS was then truncated, resulting in PV strains PV(100)A and PV(100)B, and non-PV strain ON(100). Two strains of *N. meningitidis* are also shown: H44/76, which expresses the same PorA type as the GM *N. lactamica* strains (P1.7,16), and H44/76 *ΔsiaD,* an acapsulate derivative to control for any interference of capsule in detecting PorA expression.

Following staining for PorA, the mean fluorescence intensity (MFI) was greatest in the meningococci and was significantly greater in the acapsulate strain than in WT. Of all *N. lactamica* strains, the MFI was highest in 4PA1. Truncating the UAS resulted in a reduced MFI. When strains had the same UAS, expression was higher with a phase variable promoter than a constitutive one, demonstrated by PV(100)A and ON(100), respectively. Strain PV(100)B, which had 9 Gs in the poly-G tract rather than 11, displayed a lower MFI than PV(100)A, though the difference was not significant. The MFI in PV(100)B was slightly lower than that in ON(100).

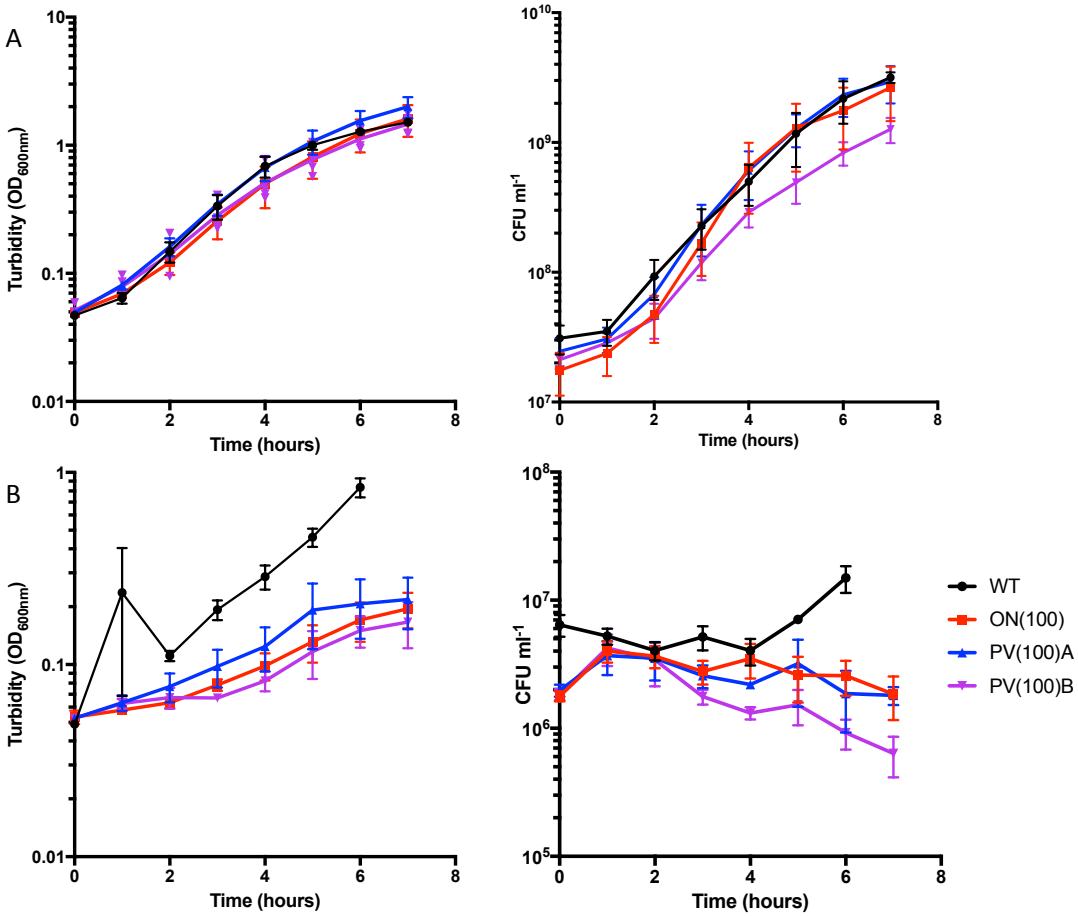



Figure 32. Growth of PorA-expressing *N. lactamica* Strains, Compared to WT, in Nutrient Rich and Poor Media

Nlac WT, ON(100), PV(100)A and PV(100)B were grown in TSB (A) or Frantz (B) media at 37 °C, 5 % $CO_2$, 320 RPM from a starting absorbance of 0.05 $OD_{600nm}$. At hourly intervals, absorbance readings were taken at 600 nm (left) and serial dilutions were performed on CBA. CBA plates were incubated overnight at 37 °C, 5 % $CO_2$ and viable counts were calculated (right).

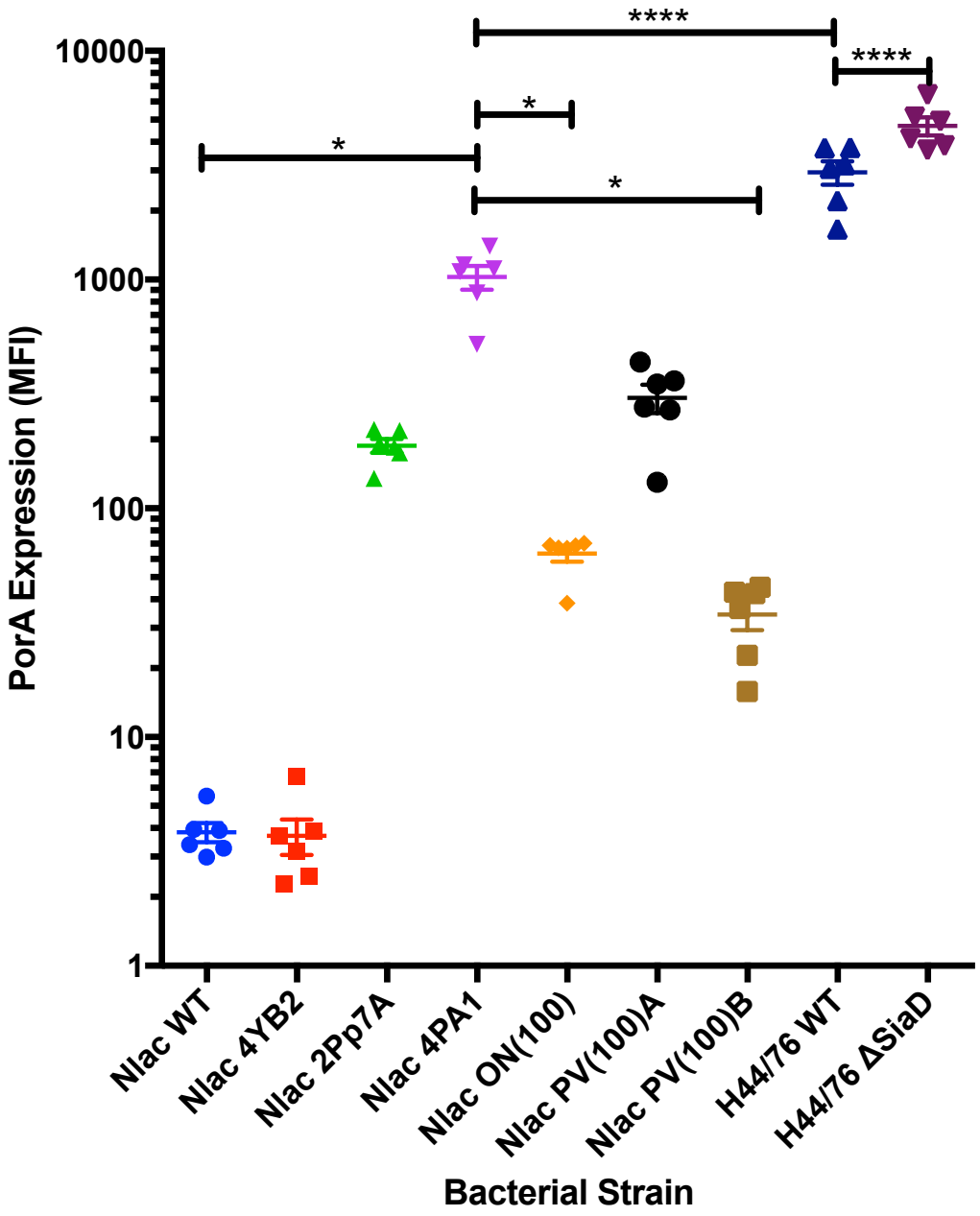



Figure 33. PorA Expression Analysis

Bacteria were grown in TSB to an absorbance (OD600nm) of 0.3, then stained with mouse anti-PorA IgG, followed by anti-mouse IgG-A488. PorA expression (MFI) was measured by flow cytometry on FACS Aria. Mean ±SEM, one-way ANOVA with Tukey's multiple comparisons, n=6. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

### 6.2.4 Interaction with Epithelial Cells

The Gentamicin Protection Assay was used to compare the ability of *N. lactamica* strains to bind to and invade the Detroit 562 nasopharyngeal epithelial cell line (Figure 34). Cells were grown to confluence and exposed to each bacterial strain for 2, 4 or 6 hours before washing to remove non-adhered bacteria. Cells were either lysed with saponin and disrupted to perform a viable count of total cell-associated bacteria, or treated with gentamicin and penicillin to kill extracellular bacteria before lysing cells and counting exclusively internalised bacteria. Viable counts of the cell supernatant were also performed at each time point.

The total number of CFU recovered from lysates of Detroit cells infected with either Nlac WT or 4YB2 tended to increase over the course of the experiment (Figure 34). As the number of cell-associated bacteria increased in each sample, so did the number of CFU that were internalised by the Detroit cells (Figure 34a). These trends were broadly reflected in lysates of Detroit cells infected with either PV(100)A or ON(100). Viable counts of each supernatant at a given time-point showed similar numbers of CFU, except for wells infected with PV(100)B, from which fewer bacteria were recovered. However, the reduced bacterial density in these wells did not appreciably affect their interaction with the Detroit cells; in fact there were more internalised bacteria recovered from PV(100)B-infected lysates than from any other lysate at each time point.

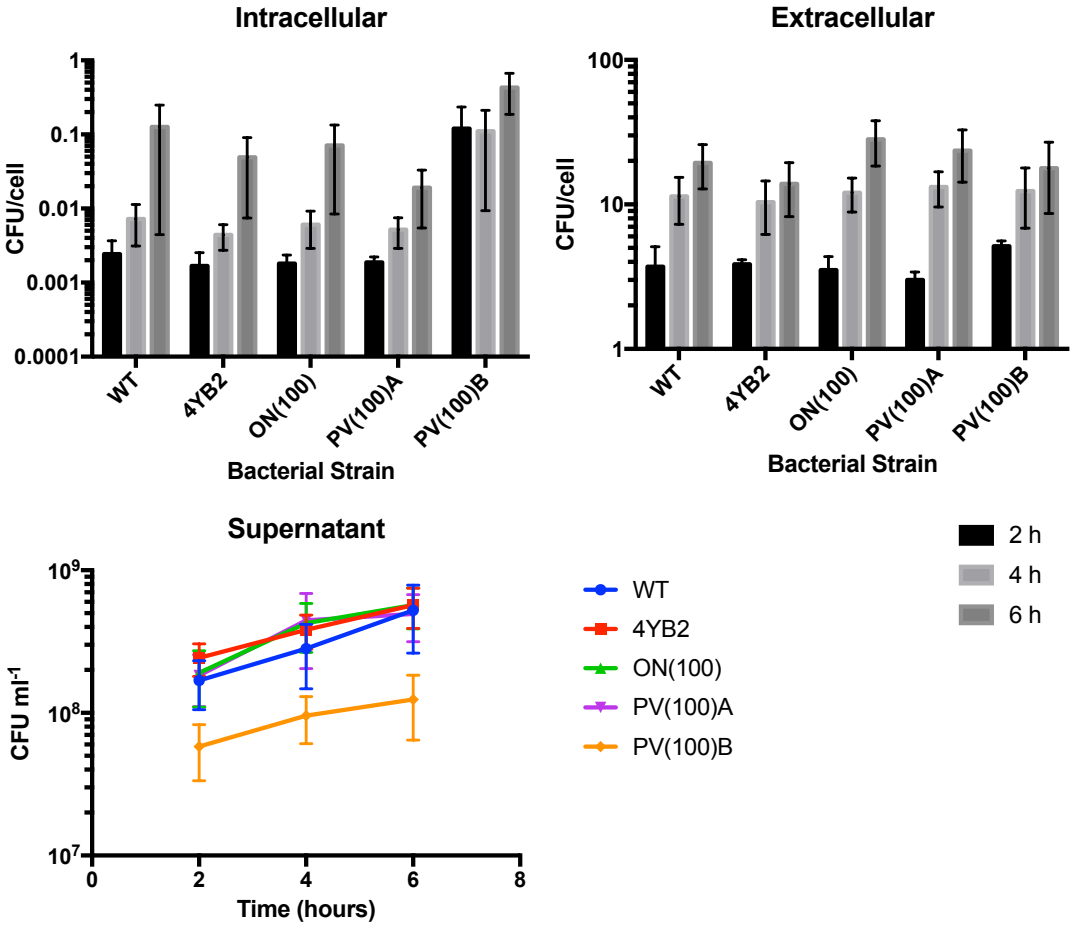



Figure 34. Binding and Internalisation of *N. lactamica* in Detroit Cells

Detroit cells were grown to confluence in MEM +10% FCS in a 24-well plate, then washed prior to infecting with bacteria at a MOI of 100 for 2, 4 or 6 hours, in duplicate. Cells were either lysed with saponin and disrupted to perform a viable count of total cell-associated bacteria, or treated with gentamycin and penicillin to kill extracellular bacteria before lysing cells and counting exclusively internalised bacteria. Viable counts of the cell supernatant were also performed at each time point. All viable counts were calculated by plating tenfold serial dilutions on CBA and incubating overnight at 37 °C, 5% $CO_2$. Mean ±SEM, n=3.

## 6.3 Biosafety Analysis

### 6.3.1 Antibiotic Sensitivity

To confirm that the three PorA-expressing strains of *N. lactamica* (ON(100), PV(100)A and PV(100)B) are safe for human challenge experiments, it was necessary to characterise their biosafety profiles. To confirm that the GM strains remained susceptible to killing by relevant antibiotics, E-test analyses were performed for ciprofloxacin, ceftriaxone and rifampicin. Testing was performed by a PHE biomedical scientist, according to EUCAST guidelines (EUCAST, 2018).

As shown in Table 8, all GM strains were acutely sensitive to each of the three antibiotics tested, and demonstrated a similar antibiotic susceptibility profile to Nlac WT. The MIC of rifampicin for WT (0.38) was higher than that for the three GM strains (0.25). EU standards state that *N. meningitidis* should be considered sensitive to rifampicin if the MIC is less than or equal to 0.25 (EUCAST, 2018). However, these guidelines vary by species and there are no guidelines available for *N. lactamica.*

### 6.3.2 Genome stability

To test the genomic stability over time of *N. lactamica* ON(100), PV(100)A and PV(100)B, each strain was serially passaged daily on CBA for 28 days and maintained at 37 °C. Day 0 and day 28 isolates were sent to MicrobesNG for whole genome sequencing. The unassembled sequencing reads from day 28 isolates of each strain were scaffolded to the assembled genomes of their respective day 0 isolates. Snippy was used to identify any insertions, deletions, recombination events or SNPs that occurred during the 28-day serial passage (for raw data, see Appendix D). The manually screened microevolution of Nlac ON(100), PV(100)A and PV(100)B is shown in Table 9.

There were minimal changes to all genomes over the course of this experiment. In ON(100), one insertion of a single bp was introduced, causing a frameshift mutation in an ABC transporter permease and there was one SNP in an intergenic locus. In PV(100)A, two missense SNPs were found to affect an Opa protein and a hypothetical protein that is conserved among various *Neisseria* species but is of unknown function. There was a missense SNP in an intergenic locus and one same sense SNP in a hypothetical protein. In PV(100)B, one same sense SNP and three missense SNPs occurred. Missense SNPs affected an ABC transporter permease involved in LPS export, a putative dimethyladenosine transferase and a substrate binding protein for an iron ABC transporter. No changes were observed to the *porA* sequence or its promoter in any strain.

Table 8. Antibiotic Sensitivity

| Antibiotic | Strain | MIC (mg/L) |
|---|---|---|
| Ciprofloxacin | WT | 0.003 |
| | ON(100) | 0.004 |
| | PV(100)A | 0.004 |
| | PV(100)B | 0.004 |
| Ceftriaxone | WT | <0.002 |
| | ON(100) | <0.002 |
| | PV(100)A | <0.002 |
| | PV(100)B | <0.002 |
| Rifampicin | WT | 0.38 |
| | ON(100) | 0.25 |
| | PV(100)A | 0.25 |
| | PV(100)B | 0.25 |

Table 9. Microevolution of PorA-expressing *N. lactamica* during 28 day *in vitro* passage

| **Strain** | **Type** | **Ref** | **Alt** | **Location** | **Effect** | **Gene Product** |
|---|---|---|---|---|---|---|
| ON(100) | ins | TAA | TAAA | Coding | Frameshift | ABC transporter permease |
| ON(100) | SNP | A | C | Intergenic | | |
| PV(100)A | SNP | T | G | Coding | Missense (S>A) | hypothetical protein - *Neisseria* specific |
| PV(100)A | SNP | T | C | Coding | Missense (N>D) | hypothetical protein - conserved among *Neisseria* |
| PV(100)A | SNP | G | A | Coding | Sense (Stop) | small hypothetical protein also in *N. polysaccharea* |
| PV(100)A | SNP | A | G | Coding | Missense (I>V) | Opa protein |
| PV(100)A | SNP | T | G | Intergenic | | |
| PV(100)B | SNP | C | T | Coding | Missense (A>T) | LPS export ABC transporter permease LptF |
| PV(100)B | SNP | T | G | Coding | Missense (D>E) | putative dimethyladenosine transferase |
| PV(100)B | SNP | G | C | Coding | Sense (L>L) | LPS-assembly protein LptD |
| PV(100)B | SNP | T | C | Coding | Missense (F>S) | iron ABC transporter substrate-binding protein |

### 6.3.3 Immunogenicity

OMVs were derived from Nlac ON(100), PV(100)A and PV(100)B. Western blots were performed on OMVs to confirm the presence of PorA and are shown in Figure 35. As expected, PorA was detected in OMVs derived from Nlac ON(100), PV(100)A and PV(100)B, but not 4YB2. PorA concentration was greatest in OMVs derived from strain PV(100)A and a long exposure time of 1 h was required to observe bands in OMVs derived from the other two PorA-expressing strains.

OMVs were sent to PHE, Porton Down, for SBA assays to be carried out, as in Section 5.3. Mice immunised with OMVs derived from Nmen H44/76 or Nlac 4PA1 generated strong serum bactericidal activity against H44/76. In contrast, immunising mice with Nlac ON(100), PV(100)A or PV(100)B did not elicit a SBA response (Figure 35).

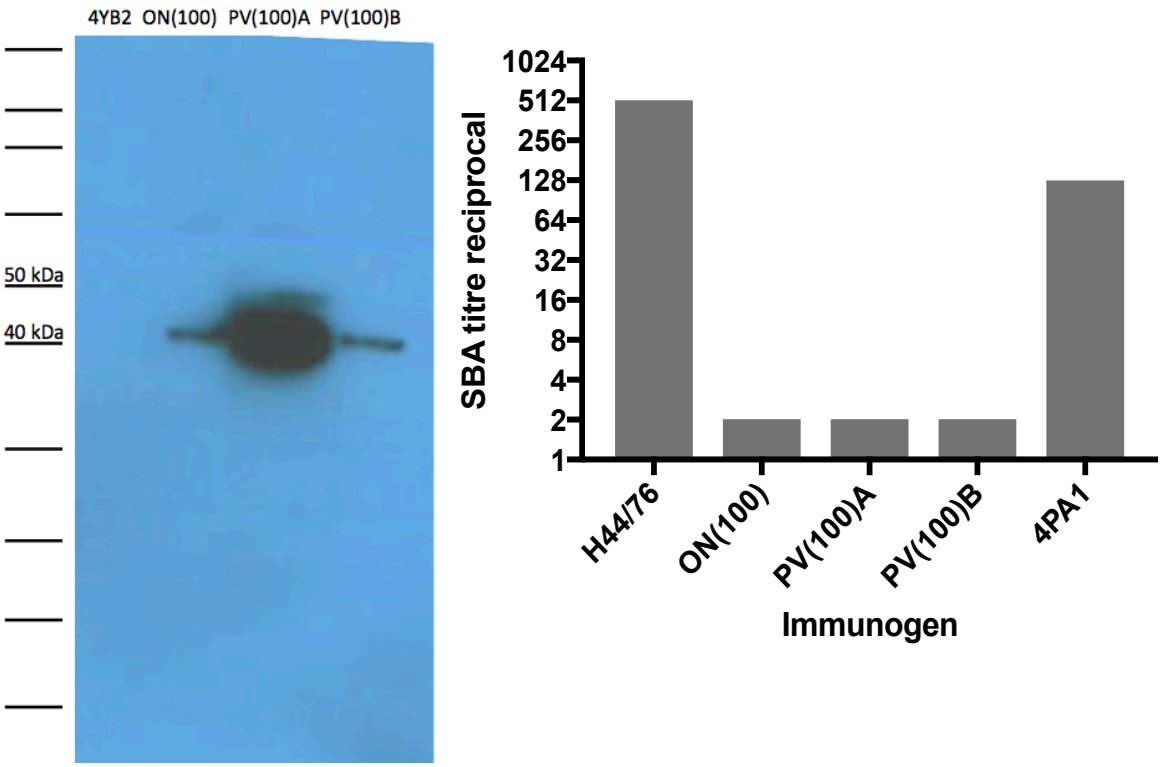



Figure 35. SBA Activity in Mice following Immunisations with OMVs derived from PorA-expressing *N. lactamica*

(Left) Western blot for PorA in Nlac WT, ON(100), PV(100)A and PV(100)B, exposed for 1 hour. (Right) Groups of 10 mice were immunised with or AlOH combined with 10µg OMV derived from Nmen H44/76, Nlac WT, ON(100), PV(100)A or PV(100)B on 3 occasions. Serum from immunised mice was pooled and tested for SBA activity against Nmen H44/76.

## 6.4 Phase Variation of PorA

### 6.4.1 Ab-Directed Serum Killing to Enrich for Phase Variants

To observe phase variation of PorA expression in *N. lactamica,* methods were adapted from those by Tauseef et al. (2013). Aliquots of $10^6$ bacteria were exposed to pooled human sera, as a source of complement, and a bactericidal anti-PorA antibody to direct killing of bacteria expressing high levels of PorA, therefore enriching for those with reduced expression.

In contrast to previous studies with *N. meningitidis,* these experiments were not performed in PBS, as we observed a rapid loss of Nlac viability in this buffer (data not shown). Frantz media was used instead, in an attempt to prevent large fluctuations in viability. Whilst Frantz media was previously found to sustain the viability of Nlac cultures at low bacterial concentrations (Figure 32), there was a loss in Nlac viability over the course of this experiment, possibly due to the greater bacterial concentrations used (Figure 36). In contrast, the viability of meningococcal cultures in Frantz media was sustained over 4 h. In this species, the addition of serum allowed a slight growth in bacterial numbers throughout the experiment. Frantz media was selected for these experiments because it limits bacterial growth and in contrast to PBS, sufficient bacteria survived the course of the experiment to analyse the data. In order to better observe the specific effect of antibody-mediated killing on culture viability, survival in the presence of serum and Ab was normalised relative to survival in the presence of serum alone (Figure 37).

Initially, *N. lactamica* were exposed to varying concentrations of serum in Frantz media, in the absence of antibody, to titrate the serum concentration to the maximum level where non-specific serum killing was prevented (Figure 37a). Data were normalised to show the viability of bacteria in the presence of serum relative to that in the same concentration of heat-inactivated serum. No bacterial growth occurred under these conditions, however a dose responsive killing of *N. lactamica* was observed with increasing serum concentrations. At 10 % serum, there was a minimal loss of viability over two serial 1-hour passages.

WT *N. lactamica* were then inoculated into Frantz media containing 10 % serum and varying concentrations of anti-PorA monoclonal antibody (NIBSC), to check for any effect of Ab on the viability of this strain (Figure 37b). Nlac WT does not express PorA so no effect is expected. Only strains expressing PorA should bind the Ab and become targeted for complement-mediated killing. Bacteria were incubated at 37°C for 3 h with viable counts performed at hourly intervals. At various dilutions, no effect of Ab was observed.

Various bacterial strains were passaged hourly in Frantz media with 10 % serum in the presence or absence of 1:100 dilution of Ab (Figure 36 and Figure 37c). Viable counts were performed after each passage. Half of the culture was transferred to a new well containing fresh serum and Ab in Frantz media and then re-incubated. After the first passage with serum and Ab, there was reduced viability in Nlac WT and 4YB2, relative to serum alone. These strains do not express PorA. A reduction in viability was also observed in Nmen H44/76 WT and it's acapsulate derivative (*ΔsiaD*) when Ab was present. These strains both express the protein at high levels. The remaining strains showed no change in viability with Ab and serum relative to serum alone. Addition of Ab caused the greatest reduction in viability of H44/76 *ΔsiaD* cultures.

Following two passages, the presence of antibody resulted in reduced viability in Nlac WT, PV(100)B and, more noticeably, in H44/76. After 2 passages with antibody, no live bacteria were recovered from H44/76 *ΔsiaD* cultures, despite this strain remaining viable in 10% serum alone (Figure 36).

After 3 and 4 passages there were no further reductions in the viability of cultures resulting from the presence of antibody. The only exception is Nlac PV(100)B, which appeared to benefit from the presence of antibody after 3 passages but was negatively impacted by the presence of antibody after 4.

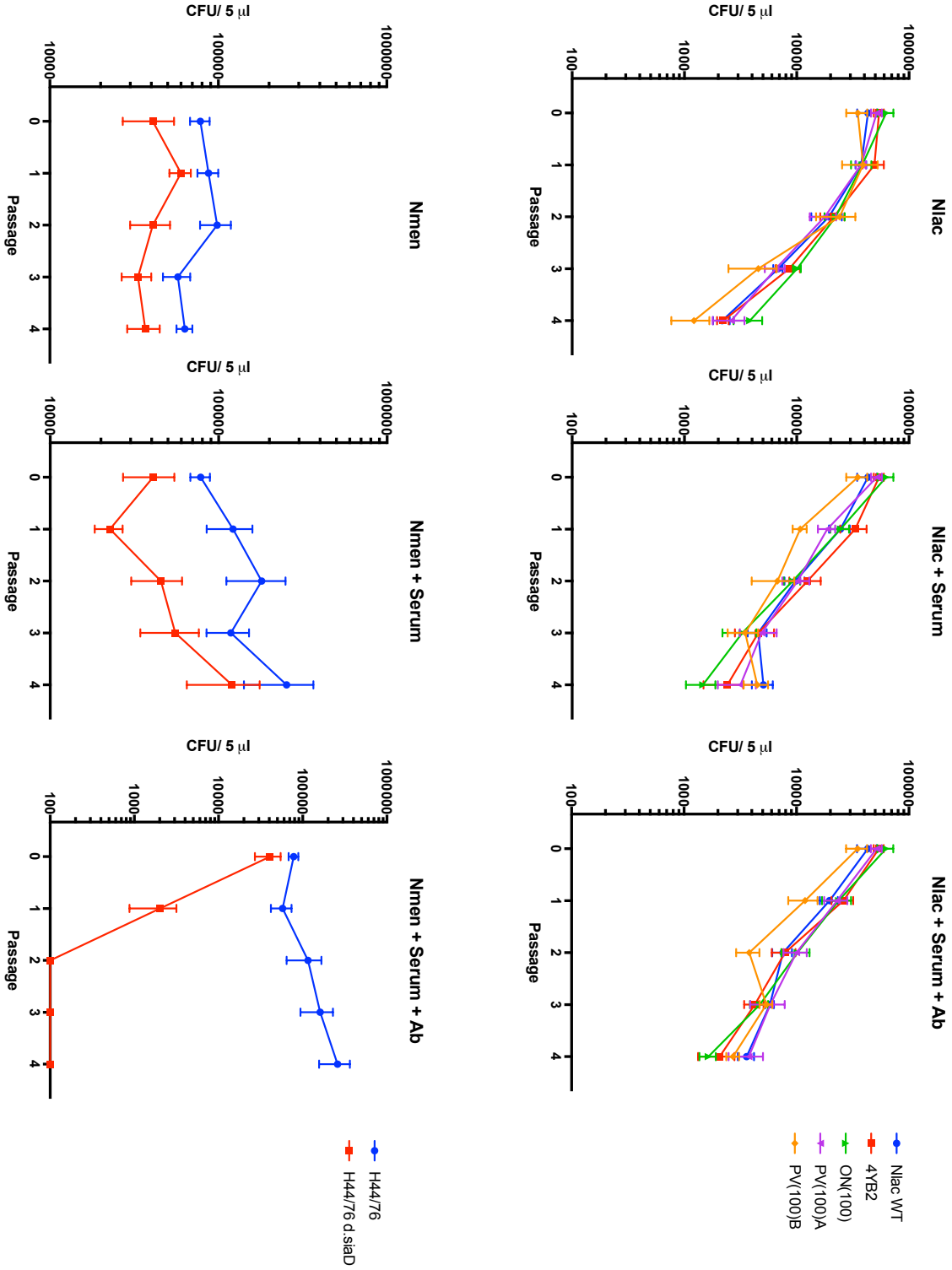



Figure 36. Antibody-Directed Serum Killing: Raw Data

Strains of *N. lactamica* (top) and *N. meningitidis* (bottom) were incubated in Frantz media at 37°C, 5 % $CO_2$, 320 RPM. Where relevant, 10% serum and/or 1:100 dilution of anti-PorA antibody were added. After 1 hour, 50 % of the culture was transferred to fresh Frantz media with replenished serum and antibody. Bacteria were passaged in this manner 4 times and viable counts were performed at each passage. Mean ±SEM, n= 5.

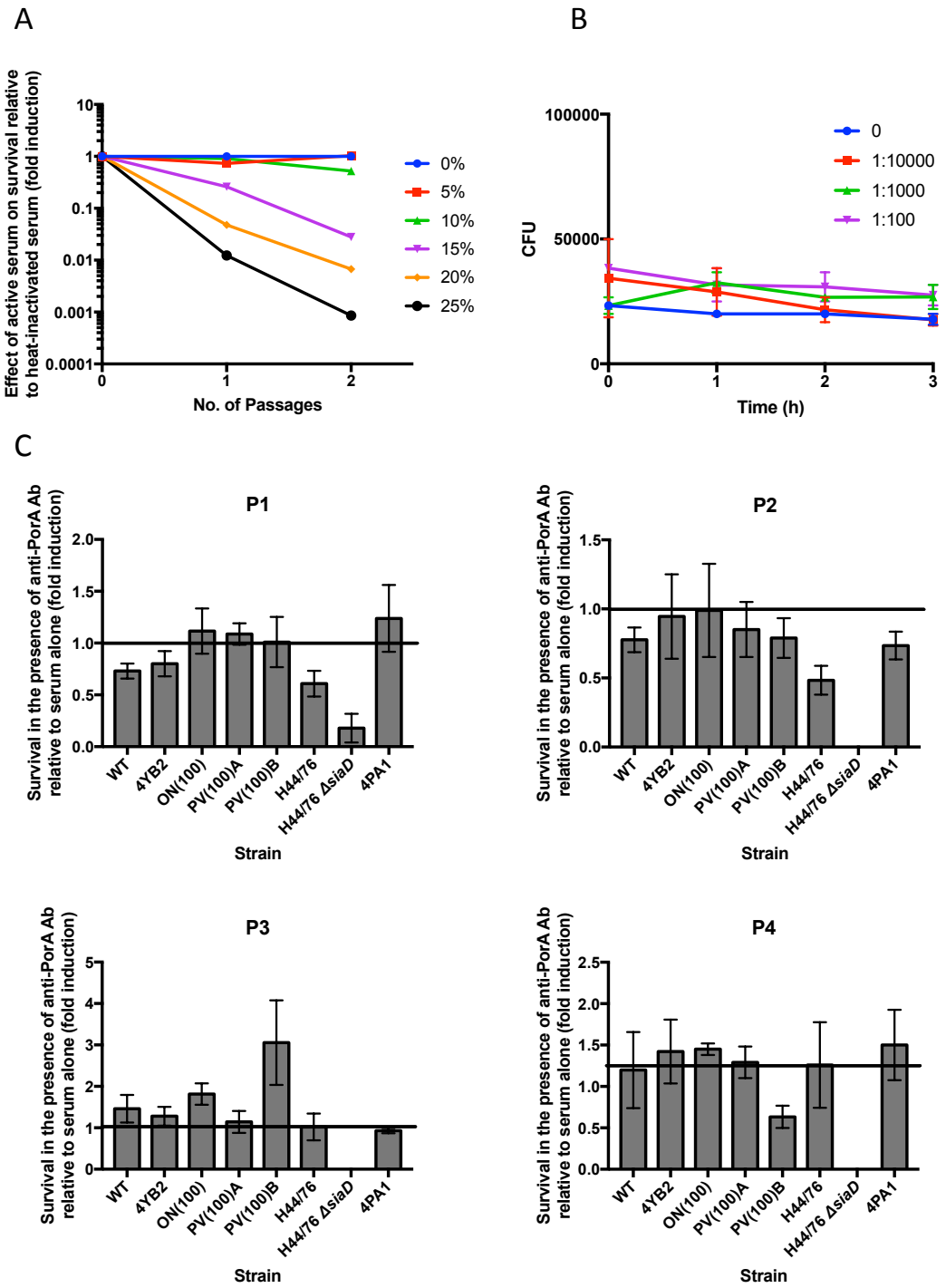



Figure 37. Antibody-Directed Serum Killing: Titrations and Normalised Data

A) Nlac were suspended in Frantz media with varying concentrations of human serum and incubated at 37 °C, 5 % $CO_2$, 320 RPM. Viable counts were performed at hourly intervals. The effect of serum, relative to heat-inactivated serum, on bacterial viability is shown (n=1). B) Nlac were incubated in Frantz media with 10% serum and varying concentrations of anti-PorA antibody at 37 °C, 5 % $CO_2$, 320 RPM. Viable counts were performed at hourly intervals. Mean ±SEM, n=2. C) Bacteria were incubated in Frantz media with 10 % serum and 1:100 dilution of anti-PorA antibody at 37 °C, 5 % $CO_2$, 320 RPM. After 1 hour, 50% of the culture was transferred to fresh Frantz media with replenished serum and antibody. Bacteria were passaged in this manner 4 times and viable counts were performed at each passage. Data is shown as fold induction of viable bacteria when exposed to both serum and antibody, relative to serum alone. Mean ±SEM, n= 5.

### 6.4.2 Detecting PorA Phase Variation in *N. lactamica*

In an attempt to identify isolates of Nlac PV(100)A that have an altered poly-G tract length, resulting in reduced PorA expression, colony immunoblots were performed on bacteria pre- and post- serum selection (Figure 38). As was encountered previously when comparing relative PorA expression levels in *N. meningitidis* (Tauseef et al., 2013), this technique was not sufficiently sensitive to discriminate between various levels of expression in *N. lactamica*. Whilst the difference in PorA expression could be observed between negative controls and PorA-expressing *N. lactamica,* and also between *N. lactamica* and meningococcal strains, it was not possible to differentiate between Nlac ON(100), PV(100)A and PV(100)B, which were previously shown by flow cytometry to express PorA at different levels (Figure 33). Densitometry analysis of the colony immunoblots using ImageJ was also unable to distinguish between these strains. It was therefore not possible to use colony immunoblots as a high-throughput screening method to identify potential colonies of interest for further investigation.

In the study by Tauseef et al. (2013), exposure of *N. meningitidis* 8047 to human serum and an anti-PorA Ab over 4 passages led to phase variation of the poly-G tract from 11 bp to 10 bp in 100 % of examined isolates. Whilst this genetic change could not be observed at the expression level by colony immunoblots (Tauseef et al., 2013), we have previously demonstrated a greater sensitivity using flow cytometry (Figure 33). PorA expression levels were therefore analysed in multiple Nlac PV(100)A isolates after 0, 2 and 4 passages by flow cytometry (Figure 39). No change in PorA expression was observed in these isolates.

To check whether there was a widespread genetic change in the PorA promoter of Nlac PV(100)A during exposure to serum and Ab, selected colonies from three separate serum killing experiments were analysed by GeneScan, to identify any changes in the length of the PorA promoter (Table 10). PCR amplification of the promoter region from each isolate using primers 37 & 39 resulted in PCR products that were measured by capillary electrophoresis, which gave an overall length of the amplified region. Representative samples were sequenced to correlate the PCR product size to the length of the poly-G tract, and results were extrapolated to other isolates with the same sized PorA promoter region. From each experiment, ten PV(100)A isolates after 0, 2 and 4 passages of serum selection were processed for GeneScan analysis, along with one PV(100)B and one ON(100) isolate that had not undergone serum selection. The number of those isolates that produced GeneScan results meeting the quality acceptance criteria is shown in brackets (Table 10). Results from other isolates were inconclusive and therefore discarded.

In isolates that had not been passaged, the PV(100)A PorA promoter contained a poly-G tract of 11 bp. In the same locus, ON(100) contained 11 bp of Cs, As, Gs and Ts (as shown in Figure 30). The PorA promoter in PV(100)B was shorter by 2 bp and contained a poly-G tract of 9 bp. Following 2 or 4 passages of serum selection, there were no changes in the length of the poly-G tract in PV(100)A. All isolates contained an 11 bp poly-G tract in the PorA promoter.

### 6.4.3 Detecting Phase Variation in *N. meningitidis*

It was hypothesised that phase variation was not being detected because the expression in *N. lactamica* is already low, and therefore the presence of serum and antibody provides no selective pressure. To confirm that the assay was capable of enriching bacterial populations for those expressing little or no PorA, meningococcal isolates were sequenced throughout 4 passages with serum and antibody (Table 11). Three isolates were sequenced at each passage using primers 37 and 38. Consistent with the published genome sequence for H44/76 (Budroni et al., 2011), the PorA promoter prior to exposure to serum and Ab contained a 12 bp poly-G tract, rather than the 11 bp that is considered optimal. Surprisingly, the poly-G tract remained at 12 bp in all isolates throughout the course of the experiment. Sequencing also captured a 7 bp poly-A tract that is present early in the PorA coding sequence. No changes were observed in this tract following serum selection (Table 11). The total spacer length between the -10 and -35 conserved regions of the promoter was also examined. There were 20 bp separating these regions before passaging and no changes were observed in any of the isolates through the course of the experiment (Table 11).

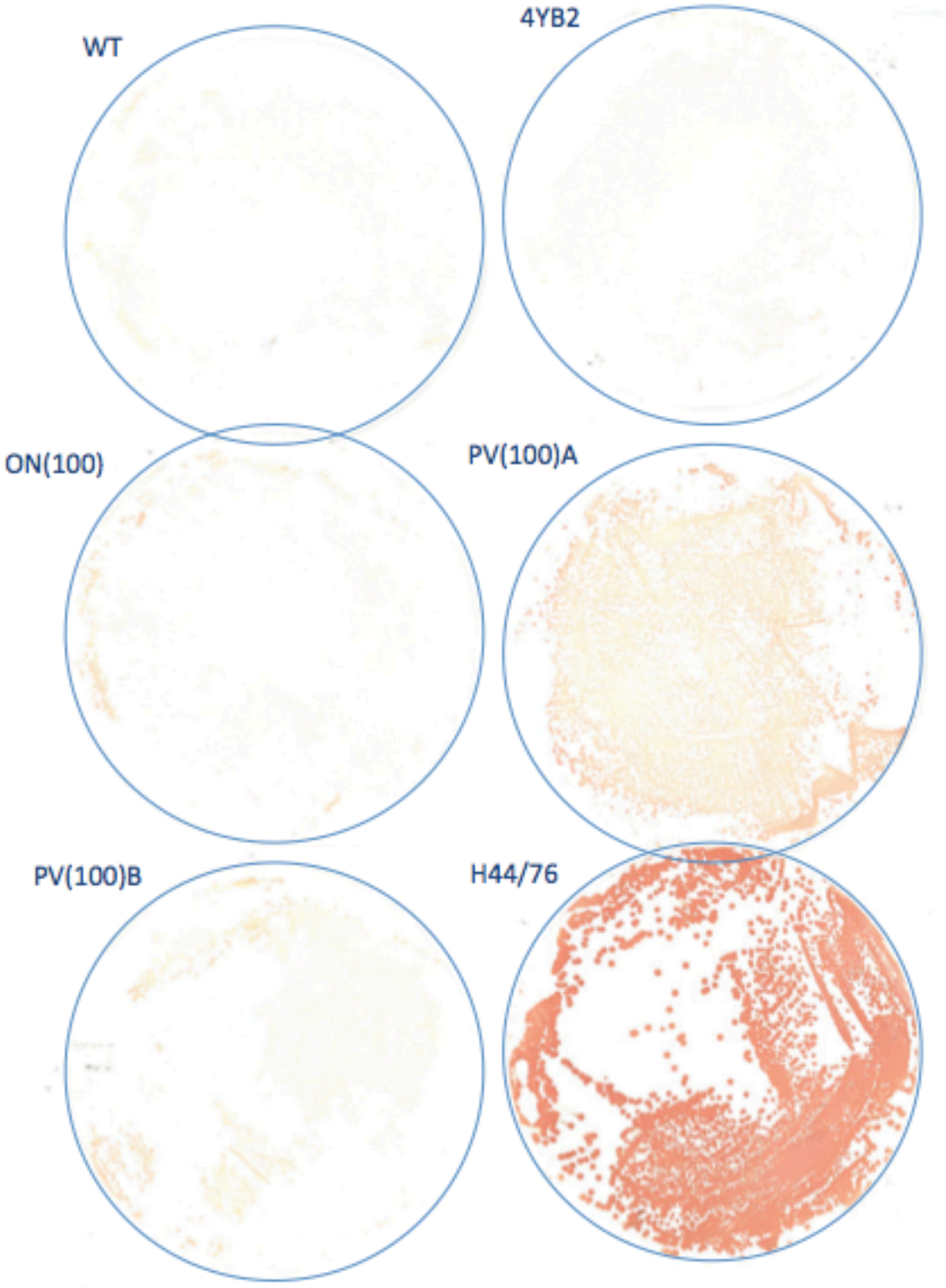



Figure 38. Colony Immunoblots for PorA Expression

Bacteria were spread on TSB agar and grown overnight at 37 °C, 5% $CO_2$. Colonies were transferred to a nitrocellulose membrane and washed prior to blocking in 5 % milk PBS. Membranes were probed with a mouse anti-PorA IgG antibody and then with anti-mouse IgG conjugated to HRP. The HRP substrate was added to produce red pigmentation and the reaction was stopped after 10 minutes by washing in $dH_2O$. Membranes were left to dry and then scanned.

| 0 | 2 | 4 |
|---|---|---|
| 134 | 153 | 170 |
| 130 | 141 | 151 |
| 152 | 178 | 181 |
| 145 | 150 | 141 |
| | 126 | 164 |
| | 160 | 148 |
| | 195 | 145 |
| | 117 | 204 |
| | 157 | 118 |
| | 144 | 129 |

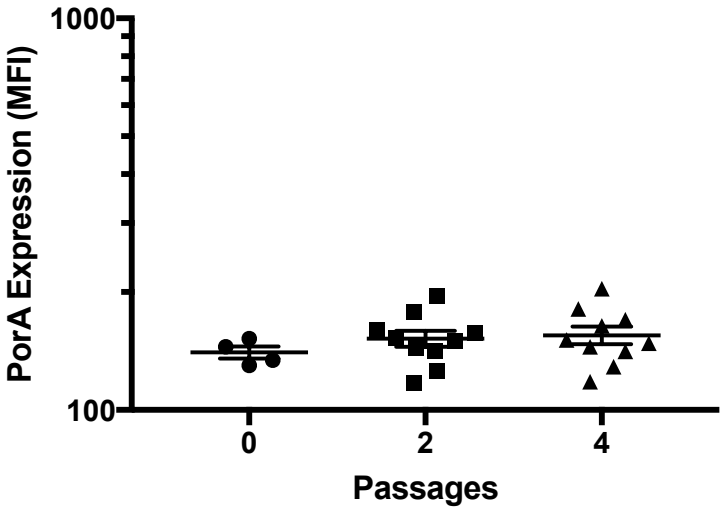



Figure 39. PorA Expression by Flow Cytometry during Serum Selection

Following exposure to 10% serum and 1% anti-PorA antibody in Frantz media for 0 (n=5), 2 (n=10) or 4 (n=10) passages, PV(100)A isolates were grown in TSB to log phase, before staining for PorA with an alexafluor-488-conjugated secondary antibody. PorA expression (MFI) was measured by flow cytometry on FACS aria. Mean ±SEM.

Table 10. GeneScan Analysis of the PorA Promoter in GM *N. lactamica*

| Strain | Passage | Length of promoter tract in bp (no. samples) | | |
|---|---|---|---|---|
| | | Exp 1 | Exp 2 | Exp 3 |
| ON(100) | 0 | 11 (1) | 11 (1) | 11 (1) |
| PV(100)B | 0 | 9 (1) | 9 (1) | 9 (1) |
| PV(100)A | 0 | 11 (9) | 11 (10) | 11 (9) |
| | 2 | 11 (4) | 11 (8) | 11 (9) |
| | 4 | 11 (6) | 11 (10) | 11 (9) |

Table 11. PorA Sequence Data from Serum Selection of *N. meningitidis* H44/76

| **Passage** | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| **Poly-G tract (bp)** | 12 | 12 | 12 | 12 | 12 |
| **Poly-A tract (bp)** | 7 | 7 | 7 | 7 | 7 |
| **Spacer (bp)** | 20 | 20 | 20 | 20 | 20 |

## 6.5 Discussion

### 6.5.1 Comparing PorA-expressing Strains of *N. lactamica*

Three PorA-expressing strains of *N. lactamica* were produced for development towards use in human challenge experiments. These strains were designed to be genetically identical with the exception of the PorA promoter sequence. Sequencing of the PorA protein and surrounding locus supported that the strains met their criteria. All strains contained 100 bp UAS and the cognate PorA coding sequence of *N. meningitidis* H44/76. The only difference was that Nlac PV(100)A contained an 11 bp poly-G tract in the PorA promoter, PV(100)B contained a 9 bp poly-G tract and ON(100) contained 11 assorted bases in place of the poly-G tract.

Whole genome sequencing revealed some additional differences to WT outside of the NHCIS1 locus. The majority of mutations were present in all three PorA-expressing strains. Considering these strains were produced through independent transformations of Nlac *ΔlacZ*, it is likely that these mutations were also present in the parent strain. It is difficult to determine the phenotypic effect these genetic alterations may have caused. There were two deletions in hypothetical proteins that both included frame shift mutations resulting in an early stop codon in the WT reference genome. As the deletions in PorA-expressing strains occurred after the stop codon, they are unlikely to have any effect. Same sense mutations are also unlikely to have much effect, but may change expression levels depending on the codon usage in *N. lactamica*. There were multiple changes in polymeric tracts, indicative of phase variation. As phase variation occurs frequently in *Neisseria,* and tracts can be both increased or decreased during replication (Snyder et al., 2001), these changes are not a concern and would likely be remedied if necessary under selective conditions. There was a missense SNP in an iron ABC transporter substrate-binding protein, which is likely to have affected protein function. It was present in all three PorA-expressing strains so they are still comparable, but it is not clear what affect this mutation may have on the fitness of these bacteria *in vivo.* There was no obvious phenotypic effect in the iron-rich TSB media but this mutation may explain the relative reduction in fitness compared to WT in the nutrient-poor, Frantz media. Furthermore, the addition of PorA likely introduced a metabolic burden, which in the absence of excess nutrients, diverts necessary energy from cell growth and division.

Interestingly, the viability of *N. lactamica* strains remained relatively constant in Frantz media over multiple hours. Survival of Nlac PV(100)B in Frantz media appeared slightly reduced compared to the other GM strains, which was unexpected because the 9 bp poly-G tract should

theoretically promote less PorA expression than in the other strains and therefore impart the smallest metabolic burden.

Indeed when comparing PorA expression by flow cytometry, we observed lowest expression in Nlac PV(100)B. When the promoter contained either 11 guanosine residues or an equivalent length of assorted bases, PorA expression was higher. This agrees with previous findings in *N. meningitidis,* that the 11 bp poly-G tract is optimal and that a reduction to 9 bp correlates with a decrease in PorA expression (van der Ende et al., 2000). Interestingly, PorA expression was higher under the control of the PV promoter than the constitutive one, which contradicts previous findings that disruption of the poly-G tract correlates with increased PorA expression in meningococcal strains (van der Ende et al., 2000). The effect of this tract on promoter activity may explain why PorA expression was achieved in *N. lactamica* with 200 bp UAS and a constitutive promoter but not with 200 bp UAS and a PV promoter. The additional activity of the PV promoter may have increased PorA expression beyond the tolerated limit in this species, though it is not known why activity would be higher with the PV promoter. Reducing the UAS from 200 bp in strain 4PA1 to 100 bp in strain ON(100) led to significantly reduced PorA expression, supporting our previous data that this DNA sequence enhances promoter activity (Figure 21). As observed previously (Section 5.2.3), we also saw a significantly higher MFI for *N. meningitidis* H44/76 *ΔsiaD* relative to WT H44/76. It is unlikely that PorA expression is higher in the acapsulate strain and the difference is probably due to the presence of capsule in the WT obscuring PorA and preventing antibody recognition.

The GPA was employed to determine whether genetic modifications to *N. lactamica* had altered its interaction with epithelial cells. We found that binding and internalisation of epithelial cells by WT and all GM strains was largely comparable. Nlac PV(100)B appeared to be internalised by cells slightly more readily than other strains, which was surprising considering that the supernatants of wells containing PV(100)B yielded less CFUs than other strains. It is possible that PV(100)B varies from the other strains so that auto-aggregation is increased, causing it to grow in 'clumps'. These would likely be broken up by the process of saponising and disrupting the cell monolayer, but not when measuring the viability of the cell supernatant, causing multiple bacteria to only appear as one CFU in the supernatant count. This might also explain why viability appeared lower in PV(100)B compared to other strains in Figure 32. Although sequencing data revealed no obvious explanation as to the phenotypic differences in PV(100)B, this strain did undergo more mutations than the other strains. The deletion in an intergenic polymeric tract in this strain may be relevant, as this does not fit the standard profile of phase variation occurring in surface expressed proteins

for immune escape. The phenotypic effects of genetic changes at this locus may be an avenue for further research.

Overall this series of experiments has demonstrated that Nlac ON(100) and PV(100)A would be suitable comparators. They behave similarly to WT in terms of their interaction with epithelial cells. Growth is similar in nutrient rich conditions but, unsurprisingly, expression of PorA, and potentially a small number of mutations common to both strains introduce a slight fitness cost in nutrient-deprived conditions. ON(100) and PV(100)A vary only in the make up of the DNA sequence between the -10 and -35 consensus sequences of the PorA promoter. PorA expression is slightly higher in PV(100)A than ON(100) but the difference is not significant. These experiments have also highlighted a possible difference in the growth phenotype between PV(100)B and the other two GM strains. PorA expression is also very low in PV(100)B and it may be less suitable for use in human challenge experiments.

### 6.5.2 Biosafety Analysis

Biosafety analysis was performed on these strains to determine their suitability for use in human challenge. Antibiotic resistance profiles were of particular interest because it is essential to be able to treat patients and prevent the spread of challenge strains in the unlikely case of adverse events. All three PorA-expressing strains retained a very similar antibiotic susceptibility profile to WT. They remained acutely sensitive to ceftriaxone and cefotaxime and slightly more sensitive to rifampicin. These antibiotics were chosen for our analysis because ceftriaxone and cefotaxime are used to treat meningococcal disease in adults and children, respectively. Rifampicin is used to clear nasopharyngeal carriage of *N. meningitidis* and for prophylactic treatment of the close contacts of patients. Ciprofloxacin can also be used to clear carriage. It is reassuring that these antibiotics can be used in conjunction to treat, clear and prevent spread of GM *N. lactamica* if necessary.

It is important to consider the genetic stability of potential challenge strains as there is a risk that these naturally competent bacteria may acquire virulence genes from other organisms encountered *in vivo* and become pathogenic. In Section 3.8.5, we observed transfer of an antibiotic resistance cassette from *N. lactamica* to *N. meningitidis* in biofilms, but no transfer in the opposite direction. Notably the antibiotic resistance cassette in *N. meningitidis* was placed in the locus of most concern for *N. lactamica* to acquire virulence – the capsule locus. Combined with observations from Pandey et al. (awaiting publication), that there was no recombination into

*N. lactamica* Y92-1009 during carriage *in vivo*, despite recombination into *N. meningitidis* and other strains of *N. lactamica,* it was inferred that Y92-1009 is particularly refractory to transformation with heterologous DNA.

To check whether the genome of GM strains remained stable over time, the microevolution of ON(100), PV(100)A and PV(100)B was observed following 28 days of *in vitro* serial passage. Very few, single nucleotide changes were observed over this time period: two in ON(100), five in PV(100)A (including one synonymous mutation) and four in PV(100)B (also including one synonymous mutation). Importantly, there were no changes to the GM locus, showing that the modification was stably inherited.

A previous study by our group using WT *N. lactamica* Y92-1009 concluded that substitution rate estimates were five times greater in isolates serially passaged *in vitro* compared to prolonged carriage of the same strain *in vivo*. However, a greater number of mutations in genes containing hypermutable, polymeric tracts, i.e. phase variable genes, were observed *in vivo* (Pandey et al., awaiting publication). It is likely that the majority of mutations that have occurred (in both WT and PorA-expressing *N. lactamica*) *in vitro* would have been selected against by the harsh and competitive conditions *in vivo*, which explains the increased substitutions *in vitro* relative to *in vivo.* Also, deleterious mutations that would be fatal in the nasopharynx may increase bacterial fitness in nutrient rich conditions, as they reduce the metabolic burden on the bacteria. The amount and extent of the mutations observed in ON(100), PV(100)A and PV(100)B during 28-day serial passage was comparable to that seen during *in vitro* passage of the WT organism, suggesting that these strains are no more likely than WT *N. lactamica* to acquire virulence genes that will make them pathogenic.

### 6.5.3 Phase Variation

Antibody-directed serum killing assays were performed in an attempt to detect phase varied isolates of PorA-expressing *N. lactamica.* The aim was to use an anti-PorA antibody to bind and target high expressers of PorA for complement-mediated killing, therefore enriching the population for bacteria with reduced PorA expression. Phase variation happens at the level of DNA replication and therefore a population of bacteria must already contain phase variants in order for phase variation to be an evolutionary/survival process. The presence of existing phase variants in the Nlac PV(100)A population is therefore necessary for the phase variability of this strain to be of relevance in a human challenge experiment. We did not expect application of a

selective pressure to reveal any variants in the Nlac PV(100)B population, as the 9 bp poly-G tract meant that the expression was already phased down.

Previous studies in *N. meningitidis* have shown that this method of antibody-directed completed-mediated killing causes a reduction in culture viability, followed by recovery and outgrowth of phased down or phased off isolates (Tauseef et al., 2013). A reduction from 11 to 10 bp was observed in the poly-G tract of the PorA promoter in all recovered meningococcal isolates. Whilst previous experiments were carried out in PBS, *N. lactamica* did not survive for extended periods in this buffer. Frantz media was used as an alternative because we previously observed that it sustained relatively constant *N. lactamica* viability (Figure 32). Unfortunately, we found that with the higher inoculum sizes used in this experiment, the viability of all *N. lactamica* strains decreased over time, whilst the viability of meningococcal strains remained constant. The addition of 10 % serum appeared to provide a nutrient source for meningococcal strains and slightly enhance growth in Frantz media, but had no effect on *N. lactamica* growth or survival. At higher serum concentrations there was a negative impact on *N. lactamica* survival, presumably due to the activity of antimicrobial peptides and the alternative pathway of the complement system, therefore 10 % serum was used. The antibody was titrated on Nlac WT, which should not bind the antibody, to ensure it didn't negatively impact growth. A high antibody concentration of 1:100 was then chosen to encourage a selective pressure against the relatively low PorA expression of Nlac PV(100)A.

The efficacy of the anti-PorA antibody to direct complement-mediated killing was exquisitely demonstrated by the acapsulate meningococcal strain, whose cultures did not survive more than one passage with antibody and serum, despite growth in serum alone. The lack of recovery in this strain suggests that either phased down variants were not present in the $10^6$ CFU inoculum at a sufficient concentration to repopulate the culture, or that even with reduced expression, the system was too aggressive for acapsulate meningococci to survive. WT *N. meningitidis* was more resistant than its acapsulate derivative to complement-mediated killing. The WT strain displayed a slight reduction in viability followed by recovery, indicative of the enrichment of phase variants. However, sequencing pre- and post- passaging revealed no changes to the PorA promoter. It is possible that antigenic variation within the hypervariable loops of PorA reduced antibody binding and provided a mechanism of immune escape in *N. meningitidis* but unfortunately these loops were not captured in the sequencing data. The presence of a 12 bp poly-G tract in the PorA promoter of H44/76, rather than the 11 bp considered optimal, may have meant that expression was already at a 'phased-down' level. Whilst this method induced a shift from 11 to 10 bp in *N. meningitidis* 8047, the use of a hyper-mutable strain was required in order to observe any further

changes to the promoter (Tauseef et al., 2013), therefore it may be unreasonable to expect variation in an already sub-optimal promoter.

If the PorA expression in H44/76 is sub-optimal, then the significantly reduced expression of all *N. lactamica* strains is unlikely to be sufficient for antibody and serum to exert any selective pressure. Furthermore, the non-specific death of Nlac strains in Frantz media may have resulted in the loss of phase-varied isolates. Data for the survival of each strain with serum and Ab was normalised to serum alone to compensate for the loss of viability, however we did not observe any antibody-mediated killing or subsequent recovery in any of the PorA-expressing strains of *N. lactamica*. Unsurprisingly, we were not able to detect phase varied isolates of Nlac PV(100)A by colony immunoblots, flow cytometry or GeneScan analysis.

Whilst phase variants likely exists amongst the PV(100)A population, sufficiently broad screening for these isolates without enriching for them would be expensive and highly impractical. Furthermore, phase variation of PorA was considered to be advantageous in GM strains because it would allow the bacteria to avoid immune recognition and killing after inducing an anti-PorA response in the human nasopharynx. The complete lack of SBA induction from ON(100), PV(100)A or PV(100)B OMV immunisations in mice suggests that the level of PorA expression in these strains may be insufficient to generate any PorA-specific immunological response in humans, therefore rendering the ability to phase down PorA expression irrelevant. It is important to note that SBA is not the only mechanism of protection against *N. meningitidis.* The production of opsonophagocytic antibodies is important for neutrophil killing of *N. meningitidis* (Ross et al., 1987) and was found to be effective even in complement-deficient individuals (Platonov et al., 2003). Similarly, the protection afforded by meningococcal vaccines has often been shown to be greater than that suggested by SBA assays (Milagres et al., 1994; de Moraes et al., 1992; Sandbu et al., 2007). Therefore it is not necessarily true that a lack of SBA means a lack of immunity, but SBA is currently the best correlate of protection available.

If a GM Nlac strain expressed PorA at a level that was sufficient to induce SBA, but insufficient to be targeted for killing by that SBA, it would provide ideal conditions for eliciting anti-meningococcal immunity whilst allowing *N. lactamica* to persist in the nasopharynx. If this level of expression could be achieved, phase variation of PorA would be unnecessary and the sustained expression of this protein at the mucosal membrane would allow continued antibody production and immune surveillance for *N. meningitidis.* Interestingly, OMVs derived from strain 4PA1, which constitutively expressed PorA at a level higher than that of PV(100)A, were found to induce SBA activity in mice (Figure 25). Therefore, a $10^6$ CFU inoculum of strain 4PA1 was exposed to 10%

serum and a 1:100 dilution of anti-PorA antibody in Frantz medium, as in Figure 36 and Figure 37, on three separate occasions. In all cases the presence of serum and antibody had no detrimental effect on 4PA1 viability relative to serum alone over 4 passages (data not shown). Although SBA induction in mice following intraperitoneal injection of OMVs derived from a bacterial strain does in no way guarantee that the same results would be achieved by nasopharyngeal inoculation of live bacteria of the same strain in humans, our data suggests that strain 4PA1 remains the preferable candidate for progression to human challenge.

## 6.6 Conclusions

The three strains analysed in this chapter were found to be genetically stable and antibiotic susceptible. PV(100)B appeared to have diverged slightly from the other strains and displayed a slightly altered growth phenotype for growth, but strains ON(100) and PV(100)A were deemed suitable comparators for determining the effect of PorA phase variation on colonisation, persistence and immunogenicity of *N. lactamica* in the human nasopharynx. However, due to the low expression levels of PorA; the lack of SBA induction from OMV immunisations in mice and our inability to observe phase variation of PorA in the 'phase-variable' strain, it was decided that these strains would not be progressed for use in human challenge. With further analysis, strain 4PA1 may prove to be a better candidate.

# Chapter 7 Final Discussion

## 7.1 Summary

The overall strategic aim of this project was to investigate the therapeutic potential for GM *N. lactamica.* The WT strain Y92-1009 has demonstrated a good safety profile in healthy adults when administered live via intranasal inoculation (Deasy et al., 2015; Evans et al., 2011) or as OMVs by intramuscular injection (Gorringe et al., 2009). Whilst this species has previously proved resistant to *in vitro* genetic manipulation (O'Dwyer et al., 2004), we have successfully performed multiple targeted mutations at various loci of the *N. lactamica* Y92-1009 chromosome. In Chapter 3, the process of transforming this species was extensively characterised. We identified a number of factors that influence the efficiency of transformation, therefore informing the protocols used to introduce heterologous genes into this organism. Our methods were shown to be exquisitely accurate in targeting insertions to the desired chromosomal locus. Interestingly, we observed unidirectional genetic exchange of antibiotic-resistance cassettes from *N. lactamica* to *N. meningitidis* when grown in co-culture biofilms, suggesting that the *N. lactamica* Y92-1009 chromosome is highly stable and is resistant to natural genetic insertions. This provides confidence that the bacteria are unlikely to acquire virulence factors *in vivo* that could render them pathogenic. Finally, we developed a method for antibiotic-resistance-free transformation of *N. lactamica,* knocking out and re-introducing β-gal as a marker of transformation on X-Gal-containing agar. Removing the requirement for antibiotic selection was an essential step in producing GM strains for use in humans, so that there can be no dissemination of antimicrobial resistance genes from our challenge strains.

The PilE-deficient strain of *N. lactamica* produced in Chapter 3 was exploited in Chapter 4 to better understand the interaction of this species with nasopharyngeal epithelial cells. Whilst T4P are generally considered to be the major subcapsular adhesin in *Neisseria*, pili-independent epithelial cell binding was recently reported in *N. cinerea* (Tang et al., 2016). Similarly, pili-deficient variants of class 2 T4P-expressing *N. meningitidis* were found to be uncompromised in their ability to bind HEp2 cells (Virji et al., 1992). Like *N. cinerea, N. lactamica* express class 2 T4P. It was therefore hypothesised that pili-independent cell binding may also occur in this species. On the contrary, we found that PilE-deficient variants of both class 1 T4P-expressing *N. meningitidis* and class 2 T4P-expressing *N. lactamica* were compromised in their ability to bind HEp2 and Detroit cells. We were unable to replicate the findings of Tang et al. (2016) in *N. cinerea,* as the PilE-deficient strain displayed increased binding to abiotic surfaces relative to WT. The work in Chapter 4 has provided a platform for understanding the interactions of *Neisseria* species with the

epithelial cells of the human nasopharynx. Considering that nasopharyngeal colonisation with *N. lactamica* inversely correlates with the carriage of *N. meningitidis* and the incidence of IMD (Cartwright et al., 1987; Deasy et al., 2015; Gold et al., 1978), this work may be developed to investigate whether increased pili expression in *N. lactamica* could increase its binding and colonisation potential, therefore improving its efficacy as an anti-meningococcal probiotic.

An alternative approach to increasing the colonisation potential of *N. lactamica* in order to achieve a therapeutic benefit may be to induce heterologous antigen expression. An antigen-expressing strain of *N. lactamica* could be administered live into the nasopharynx, where it would provide sustained mucosal delivery of the antigen for the induction of an immune response. Alternatively the GM bacteria could provide the template for the production of OMVs. These OMVs would contain the antigen in the context of a strong mucosal adjuvant (Gorringe et al., 2009; Sardiñas et al., 2006), which could be administered intramuscularly. As a proof of principle for both of these approaches, in Chapter 5, we transformed *N. lactamica* with the meningococcal immunodominant subcapsular antigen, PorA. We demonstrated the successful transformation of *N. lactamica* with PorA, and were able to enhance expression by introducing an UAS. PorA was introduced in an antibiotic-resistance-free manner and expressed on the surface of whole bacteria and OMVs. Intraperitoneal injection of PorA-containing OMVs induced SBA activity in mice against both *N. meningitidis* H44/76 and MC58. Whilst we successfully transformed *N. lactamica* to express PorA P1.7,16 in multiple isolates with varying levels of expression, we were unable to express PorA P1.7-2,4 or P1.5,2. The very low frequency of transformation with these alleles suggests that the encoded proteins may be toxic in this species.

In Chapter 6 we compared three PorA-expressing strains of *N. lactamica* for potential use in human challenge studies: two strains with a phase variable promoter (PV(100)A and PV(100)B) and one with a constitutive promoter (ON(100)). PorA was not tolerated in *N. lactamica* with a PV *porA* promoter and full UAS, therefore to produce a strain that was genetically capable of PorA phase variation, the UAS was truncated and PorA expression reduced. We examined their biosafety profiles and ability to vary PorA expression. Expression was higher in PV(100)A than ON(100) and was lowest in PV(100)B, which we attribute to the loss of 2 bp in the poly-G tract of the promoter. *N. lactamica* strains ON(100) and PV(100)A behaved similarly to WT and to each other, but PV(100)B displayed a slightly different phenotype in terms of growth in culture and interaction with epithelial cells. The reduced levels of PorA expression in these strains relative to 4PA1, used in Chapter 5, meant that OMVs derived from these strains did not induce SBA in mice. It is also likely that the low level of PorA expression is responsible for our inability to detect phase variation in PV(100)A. Whilst the strains appeared to be generally safe, antibiotic susceptible and

genetically stable, the low frequency or lack of phase variation and the apparent lack of immunogenicity in mice led us to conclude that the strains were unsuitable for answering our research questions in human challenge studies.

## 7.2 Further Work

This project has led to multiple avenues for further research. The discovery that pili are essential for efficient binding of *N. lactamica* to epithelial cells suggests that there may be potential to overexpress PilE and increase cell binding. Whilst gain of function experiments have previously proven highly controversial amongst scientists when performed on pathogens (Casadevall and Imperiale, 2014), a hyper-piliated strain may allow more widespread carriage of this commensal organism, potentially reducing the carriage of *N. meningitidis.* It is important to note that hyperpiliation may also increase transformation efficiency in *N. lactamica*, which could increase its risk of acquiring virulence factors and becoming pathogenic. If a strain of *N. lactamica* could be produced with increased colonisation potential and no increased risk, this could be of great benefit in reducing the spread of meningococcal carriage during epidemic outbreaks and could be cheaply manufactured and administered.

The work with PorA in this project demonstrated the potential for expressing heterologous proteins in *N. lactamica.* Although we encountered problems expressing some PorA subtypes and in achieving high expression levels with the native, PV *porA* promoter, there is potential to progress the relatively high expressing, non-PV strain 4PA1 for use in humans, following further biosafety testing and characterisation of the strain. Furthermore, the technology developed here can also be used to express alternative proteins. The research group has already produced strains of *N. lactamica* that express NadA (unpublished data). This protein is a meningococcal adhesin and an immunogen that was found to be tolerated in *N. lactamica* at levels similar to those found on meningococcal strains. NadA-expressing *N. lactamica* has been approved by DEFRA for environmental release, to be inoculated into the nasopharynx of human volunteers (DEFRA, 2017). This first-in-man challenge experiment will primarily examine the safety and immunogenicity of the strain, but in additional it is conceivable that NadA will enhance the colonisation potential of *N. lactamica.* The mutative mechanism for this would be by aiding adherence of the modified strain to epithelial cells of the nasopharynx. Delivery of NadA to the nasopharyngeal mucosa may also induce an anti-meningococcal immune response directed at NadA. This study will be a pathfinder for the future use of knock-in transformants of non-pathogenic organisms to observe the host response to specified bacterial antigens, and may lead to practical therapeutics and immunoprophylaxis.

# Appendix A List of Primers

| Primer No | Primer Name | Primer Sequence |
|---|---|---|
| 1 | F1 | GCCTTACAAGCAGAATGTCG |
| 2 | R4 | TCGACCAATTTGCCCAAGCC |
| 3 | nlaIIIaphA3-600FOR | GCATTGGCGTATGGCAATTG |
| 4 | nlaIIIaphA3-600REV | ACAAAGCAGGTTCGAGCGAC |
| 5 | nlaIIIaphA3-300FOR | CAGTCGATATCGACCGGACGACCGCCTTTATG |
| 6 | nlaIIIaphA3-300REV | GAAGTTCGCCCGCTTTAAGAATAC |
| 7 | nlaIIIaphA3-300DUSFOR | TTCAGACGGCATACCGGACGACCGCCTTTATG |
| 8 | nlaIIIaphA3-150FOR | CAGTCGATATCGCAAAAACAGAACTATTTTTAAGATTAG |
| 9 | nlaIIIaphA3-150REV | AAAATACTTTATCAGACAGCG |
| 10 | nlaIIIaphA3-150DUSFOR | TTCAGACGGCATCAAAAACAGAACTATTTTTAAGATTAG |
| 11 | nlaIIIaphA3-75FOR | CAGTCGATATCGTGCCGGGGAATATAAAGATTTG |
| 12 | nlaIIIaphA3-75REV | TGGAGCCGATATAATTCATCCG |
| 13 | nlaIIIaphA3-75DUSFOR | TTCAGACGGCATTGCCGGGGAATATAAAGATTTG |
| 14 | LacZ locus For | GGGTACAGTCAATCGGTTTCTTTG |
| 15 | LacZ locus Rev | GAAAGGGGGCGTGTGTTC |
| 16 | 5primeendNHCIS1FOR | CTCTAGAGTCCTGATACCGAGCTTTTCCCATG |
| 17 | 3PrimeendNHCIS1REV | CTTATTTATGAAGGTGATAGCTTCTTTCAGTCTAGAGAC |
| 18 | *ΔpilE:aadA1*vector FOR | GACCTGCAGGCATGCAAGCTTG |
| 19 | *ΔpilE:aadA1*vector_REV | GACTCTAGAGGATCCCCGGGTAC |
| 20 | PilE FOR | TGAAAGCAATCCAAAAAGGTTTCACC |
| 21 | PilE REV | TTATTTGGCGCGGCAGGAAG |
| 22 | pSC101porAVECTORfor | GTAAGTTTCTTGCGCATCTCGAGTTCCTTTTGTAAATTTGATAAAAACC |
| 23 | pSC101porAVECTORrev | CGCCACAAATTCTAAGCGGCCGCATATTCGGCAAC |
| 24 | HAEC1/2 FOR | CTTCGTCGGTCTTTTTTTTGTTG |
| 25 | HAEC2 REV | GAAACCCGGTGCGGAAAATG |
| 26 | ONporANZ98_vectorFOR | CGGCAACTGTCGGAATATCTG |
| 27 | ONporANZ98_vectorREV | CTCGAGTTCCTTTTGTAAATTTGATAAAAACC |
| 28 | DUSNHCIS1 FOR | TTCAGACGGCATCTCTAGAGTCCTGATACCGAGCTTTTCCCATG |
| 29 | KanRinternalFOR | GTTGATGCGCTGGCAGTGTTC |
| 30 | CinereaPilRev | AGCCTGCAACCCGGATTCAC |
| 31 | YccS1FOR | TTCGTTCCGAACGGGCGGAAAAC |
| 32 | YccS1REV | CATCATCCGCATCCGTGTGC |
| 33 | CLOVERcatg1FOR | ACCCGGATCATATGAAACAACACGAC |
| 34 | CLOVERcatg1REV | AGCGCGAGAAGCAGGCGA |
| 35 | CLOVERcatg2FOR | AACGCGACCA**T**ATGGTTCTGC |
| 36 | CLOVERcatg2REV | TTTCATTCGGATCTTTGGACAG |
| 37 | PorA P21 REV | GGCCGGCTTTGATTTCGCCGTACAG |
| 38 | Nmen PorA FOR | TGTGCACGCCGTCTGAGTCGAC |
| 39 | HAEC1/2FOR-FAM | [6FAM]CTTCGTCGGTCTTTTTTTTGTTG |

# Appendix B Media and Buffer Recipes

## B.1 MC.7 Media

| Reagent | Concentration (g $L^{-1}$) |
|---|---|
| NaCl | 5.8 |
| $K_2HPO_4$ | 4 |
| $NH_4Cl$ | 1 |
| $K_2SO_4$ | 1 |
| D-(+)-glucose | 10 |
| $MgCl_2.6H_20$ | 0.4 |
| $CaCl_2.2H_2O$ | 0.03 |
| Yeast Extract | 0.8 |
| HEPES | 5.96 |
| EDDA | 0.005 |
| L-glutamic acid | 3.9 |
| L-Cysteine.HCl | 0.1 |

**Buffer 1**

0.1 M Tris-HCl pH 8.6
10 mM EDTA
0.5% (W/V) Deoxycholic acid sodium salt

**Buffer 2**

50 mM Tris-HCl pH 8.6
2 mM EDTA
1.2% Deoxycholic acid sodium salt
20% Sucrose (W/V)

**Buffer 3**

0.2 M glycine buffer
3% Sucrose

## B.2 GC Agar

| **Reagent** | **Concentration (g $L^{-1}$)** |
|---|---|
| Proteose Peptone 3 (*BD Bacto)* | 10 |
| Bacteriological Agar | 12 |
| Starch | 1 |
| $K_2HPO_4.3H_2O$ | 5.24 |
| $KH_2PO_4$ | 1 |
| NaCl | 5 |

Autoclave and allow to cool prior to approximately 50°C prior to adding filter sterilised Supplement A (8 ml $L^{-1}$) and Supplement B (2 ml $L^{-1}$).

**Supplement A**

| **Reagent** | **Concentration (g/800ml)** |
|---|---|
| Glucose | 100 |
| L-glutamine | 10 |
| Para-amino-benzoic acid | 0.013 |
| B-nicotinamide adenine dinucleotide | 0.25 |
| Thiamine hydrochloride | 0.003 |
| Co-carboxylase | 0.1 |
| Cyanocobalamin | 0.01 |
| Ferric nitrate | 0.02 |

**Supplement B**

| **Reagent** | **Concentration (g/800ml)** |
|---|---|
| L-Cysteine hydrochloride | 104 |
| Adenine | 4 |
| Guanidine hydrochloride | 0.12 |
| Uracil | 3.2 |
| Hypoxanthine | 1.28 |

## B.3 Frantz Media

**Solution A**

| **Reagent** | **Concentration (g $L^{-1}$)** |
|---|---|
| L-glutamic acid | 1.6 |
| NaCl | 6 |
| $Na_2HPO_4$ | 2.5 |
| $NH_4Cl$ | 1.25 |
| KCl | 0.1 |
| D-cysteine hydrochloride | 0.025 |

Autoclave solution A, then add 50ml filter-sterilised Solution B per litre.

**Solution B**

| **Reagent** | **Concentration (g/50ml)** |
|---|---|
| MgSO4 | 0.6 |
| Yeast extract | 2 |
| Glucose | 5 |

# Appendix C Effect of *pilE* Deletion on Adherence to Biotic and Abiotic Surfaces

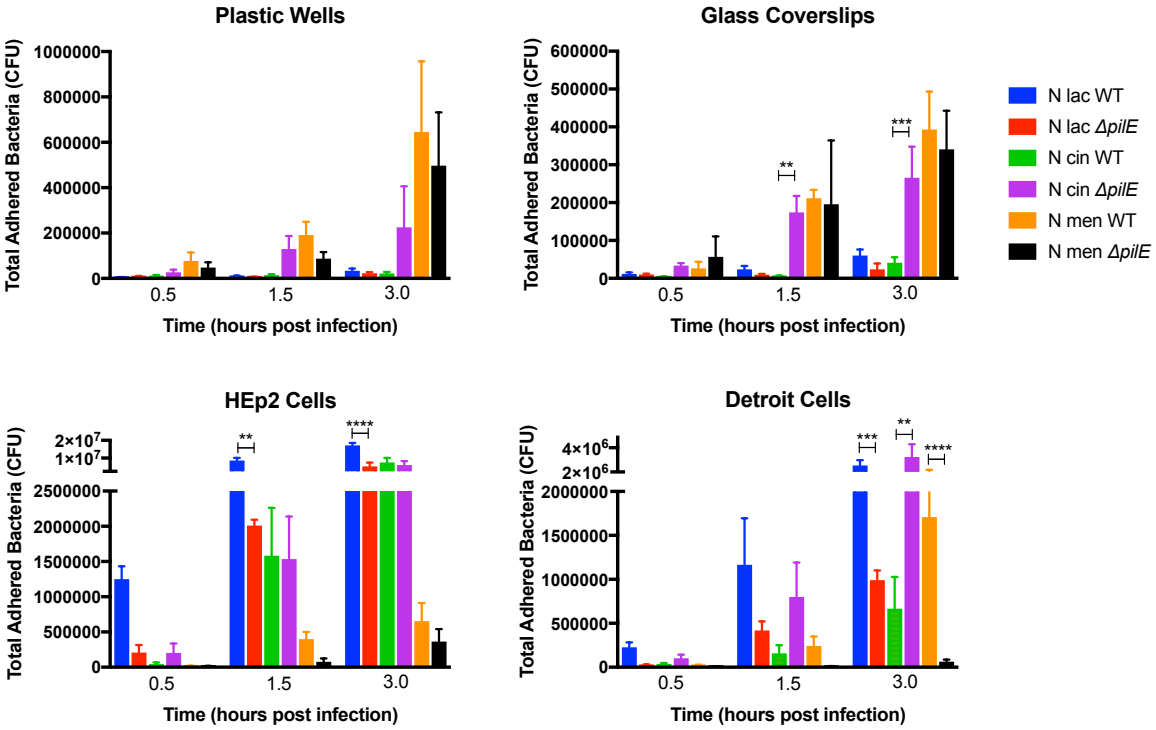



Figure 40. Adherence of Bacteria to Various Surfaces

HEp2 or Detroit cells were grown to confluence in a 24-well plate and counted prior to infecting with 30 bacteria per cell for 0.5, 1.5 or 3 hours at 37°C. Empty plastic wells or glass coverslips were concurrently exposed to the same bacterial concentration. After washing wells and treating with 1% saponin, the total number of remaining bacteria was quantified by plating serial dilutions on CBA. Mean ± SEM, n≥3, multiple t-tests, * ≤0.05, ** ≤0.01, *** ≤0.001, **** ≤0.0001.

# Appendix D Snippy Output Data

Comparing Nlac ON(100), PV(100)A and PV(100)B to WT

| CHROM | POS | TYPE | REF | ALT | EVIDENCE | FTYPE | STRAND | NT_POS | AA_POS | LOCUS_TAG | PRODUCT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **ON(100)** | | | | | | | | | | | |
| 894 | 71849 | del | GAAAAAAAAG | GAAAAAAAG | GAAAAAAAG:22 GAAAAAAAAG:0 | CDS | - | 372/1285 | 124/428 | B2G52_RS00380 | AmpG family muropeptide MFS transporter |
| 894 | 373153 | del | GTTTTTTC | GTTTTTC | GTTTTTC:36 GTTTTTTC:0 | CDS | - | 197/1216 | 66/405 | B2G52_RS02235 | sodium/glutamate symporter |
| 894 | 574728 | snp | G | A | A:43 G:0 | CDS | - | 248/966 | 83/322 | B2G52_RS03280 | iron ABC transporter substrate-binding protein |
| 894 | 676010 | del | GAAAAAAAC | GAAAAAAC | GAAAAAAAC:33 GAAAAAAAAC:0 | | | | | | |
| 894 | 1320603 | del | GAAAAAAAAAC | GAAAAAAAAC | GAAAAAAAAAC:31 GAAAAAAAAAAC:0 | | | | | | |
| 894 | 1369848 | del | CAAAAAAAC | CAAAAAAC | CAAAAAAAC:34 CAAAAAAAAC:0 | CDS | + | 174/481 | 58/160 | B2G52_RS07680 | hypothetical protein |
| 894 | 1632229 | del | GTTTTTTTTA | GTTTTTTTA | GTTTTTTTTA:11 GTTTTTTTTTA:0 | CDS | + | 138/206 | 46/68 | B2G52_RS08975 | hypothetical protein |
| 894 | 2119604 | del | GAAAAAAAC | GAAAAAAC | GAAAAAAAC:26 GAAAAAAAAC:0 | CDS | + | 61/1144 | 20/381 | B2G52_RS11295 | polyamine ABC transporter substrate-binding protein |
| **PV(100)A** | | | | | | | | | | | |
| 894 | 71849 | del | GAAAAAAAAG | GAAAAAAAG | GAAAAAAAG:73 GAAAAAAAAG:0 | CDS | - | 372/1285 | 124/428 | B2G52_RS00380 | AmpG family muropeptide MFS transporter |
| 894 | 373153 | del | GTTTTTTC | GTTTTTC | GTTTTTC:87 GTTTTTTC:1 | CDS | - | 197/1216 | 66/405 | B2G52_RS02235 | sodium/glutamate symporter |
| 894 | 574728 | snp | G | A | A:106 G:0 | CDS | - | 248/966 | 83/322 | B2G52_RS03280 | iron ABC transporter substrate-binding protein |
| 894 | 676010 | del | GAAAAAAAC | GAAAAAAC | GAAAAAAAC:88 GAAAAAAAAC:1 | | | | | | |
| 894 | 1237988 | snp | A | C | C:46 A:0 | | | | | | |
| 894 | 1278727 | del | GTTTTTTTTG | GTTTTTTTG | GTTTTTTTTG:51 GTTTTTTTTTG:0 | CDS | - | 1041/1138 | 347/379 | B2G52_RS07195 | 5-(carboxyamino)imidazole ribonucleotide synthase |
| 894 | 1320603 | del | GAAAAAAAAAAC | GAAAAAAAAAC | GAAAAAAAAAC:58 GAAAAAAAAAAC:1 | | | | | | |
| 894 | 1369848 | del | CAAAAAAAC | CAAAAAAC | CAAAAAAC:93 CAAAAAAAC:0 | CDS | + | 174/481 | 58/160 | B2G52_RS07680 | hypothetical protein |
| 894 | 1632229 | del | GTTTTTTTTA | GTTTTTTTA | GTTTTTTTA:29 GTTTTTTTTA:0 | CDS | + | 138/206 | 46/68 | B2G52_RS08975 | hypothetical protein |
| 894 | 1957211 | snp | G | A | A:83 G:0 | CDS | - | 1006/3039 | 335/1013 | B2G52_RS10510 | beta-galactosidase |
| 894 | 2119604 | del | GAAAAAAAC | GAAAAAAC | GAAAAAAAC:60 GAAAAAAAAC:0 | CDS | + | 61/1144 | 20/381 | B2G52_RS11295 | polyamine ABC transporter substrate-binding protein |

Appendix D

Comparing Nlac ON(100), PV(100)A and PV(100)B to WT

| CHROM | POS | TYPE | REF | ALT | EVIDENCE | FTYPE | STRAND | NT_POS | AA_POS | LOCUS_TAG | PRODUCT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PV(100)B | | | | | | | | | | | |
| 894 | 71849 | del | GAAAAAAAAG | GAAAAAAAG | GAAAAAAAAG:76 GAAAAAAAAG:0 | CDS | - | 372/1285 | 124/428 | B2G52_RS00380 | AmpG family muropeptide MFS transporter |
| 894 | 273387 | del | CAAAAAAAAAAC | CAAAAAAAAAC | CAAAAAAAAAAC:38 CAAAAAAAAAAC:2 | | | | | | |
| 894 | 373153 | del | GTTTTTTC | GTTTTTC | GTTTTTC:59 GTTTTTTC:0 | CDS | - | 197/1216 | 66/405 | B2G52_RS02235 | sodium/glutamate symporter |
| 894 | 511128 | snp | C | G | G:66 C:2 | CDS | + | 21/2406 | 7/802 | B2G52_RS02975 | LPS biosynthesis protein |
| 894 | 574728 | snp | G | A | A:91 G:2 | CDS | - | 248/966 | 83/322 | B2G52_RS03280 | iron ABC transporter substrate-binding protein |
| 894 | 676010 | del | GAAAAAAAAC | GAAAAAAAC | GAAAAAAAAC:67 GAAAAAAAAC:0 | | | | | | |
| 894 | 1278727 | del | GTTTTTTTTG | GTTTTTTTG | GTTTTTTTTG:0 GTTTTTTTG:46 | CDS | - | 1041/1138 | 347/379 | B2G52_RS07195 | 5-(carboxyaminoimidazole ribonucleotide synthase |
| 894 | 1320603 | del | GAAAAAAAAAAC | GAAAAAAAAAC | GAAAAAAAAAAC:57 GAAAAAAAAAAC:0 | | | | | | |
| 894 | 1369848 | del | CAAAAAAAAC | CAAAAAAAC | CAAAAAAAAC:72 CAAAAAAAAC:0 | CDS | + | 174/481 | 58/160 | B2G52_RS07680 | hypothetical protein |
| 894 | 1632229 | del | GTTTTTTTTA | GTTTTTTTA | GTTTTTTTTA:31 GTTTTTTTTA:0 | CDS | + | 138/206 | 46/68 | B2G52_RS08975 | hypothetical protein |
| 894 | 1955355 | snp | C | T | T:56 C:0 | CDS | - | 2862/3039 | 954/1013 | B2G52_RS10510 | beta-galactosidase |
| 894 | 1957818 | snp | T | C | C:49 T:0 | CDS | - | 399/3039 | 133/1013 | B2G52_RS10510 | beta-galactosidase |
| 894 | 2119604 | del | GAAAAAAAAC | GAAAAAAAC | GAAAAAAAAC:40 GAAAAAAAAC:0 | CDS | + | 61/1144 | 20/381 | B2G52_RS11295 | polyamine ABC transporter substrate-binding protein |

Comparing 28 day serial passaged isolates to day 0 isolates (microevolution)

| CHROM | POS | TYPE | REF | ALT | EVIDENCE | FTYPE | STRAND | NT_POS | AA_POS | LOCUS_TAG | PRODUCT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **ON(100)** | | | | | | | | | | | |
| ON0_3 | 100341 | ins | TAA | TAAA | TAAA:10 TAA:1 | CDS | - | 144/192 | 48/64 | ON0_00574 | ABC transporter permease |
| ON0_30 | 11347 | snp | A | C | C:15 A:0 | | | | | | |
| **PV(100)A** | | | | | | | | | | | |
| PVA0_1 | 190401 | snp | T | G | G:35 T:0 | | | | | | |
| PVA0_2 | 48697 | snp | T | C | C:102 T:10 | CDS | - | 16/492 | 5/164 | PVA0_00417 | hypothetical protein |
| PVA0_2 | 48718 | snp | G | A | A:109 G:3 | | | | | | |
| PVA0_2 | 95301 | snp | A | G | G:18 A:0 | CDS | + | 109/630 | 36/210 | PVA0_00461 | Opa protein |
| PVA0_2 | 96001 | snp | T | G | G:100 T:0 | | | | | | |
| **PV(100)B** | | | | | | | | | | | |
| PVB0_2 | 81779 | snp | C | T | T:89 C:0 | CDS | - | 43/1116 | 14/372 | PVB0_00442 | LPS export ABC transporter permease LptF |
| PVB0_3 | 79402 | snp | T | G | G:72 T:0 | CDS | + | 102/780 | 34/260 | PVB0_00677 | putative dimethyladenosine transferase [Neisseria lactamica 020-06] |
| PVB0_9 | 63231 | snp | G | C | C:111 G:0 | CDS | + | 21/2406 | 7/802 | PVB0_01479 | LPS-assembly protein LptD |
| PVB0_12 | 41877 | snp | T | C | C:97 T:0 | CDS | + | 248/966 | 83/322 | PVB0_01677 | iron ABC transporter substrate-binding protein |

# List of References

Abio, A., Neal, K.R., and Beck, C.R. (2013). An epidemiological review of changes in meningococcal biology during the last 100 years. Pathog. Glob. Health *107*, 373–380.

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

Aho, E.L., Keating, A.M., and McGillivray, S.M. (2000). A comparative analysis of pilin genes from pathogenic and nonpathogenic *Neisseria* species. Microb. Pathog. *28*, 81–88.

Alamro, M., Bidmos, F.A., Chan, H., Oldfield, N.J., Newton, E., Bai, X., Aidley, J., Care, R., Mattick, C., Turner, D.P.J., et al. (2014). Phase variation mediates reductions in expression of surface proteins during persistent meningococcal carriage. Infect. Immun. *82*, 2472–2484.

van Alen, T., Claus, H., Zahedi, R.P., Groh, J., Blazyca, H., Lappann, M., Sickmann, A., and Vogel, U. (2010). Comparative proteomic analysis of biofilm and planktonic cells of *Neisseria meningitidis*. Proteomics *10*, 4512–4521.

Ali, M.Y. (1965). Histology of the human nasopharyngeal mucosa. J. Anat. *99*, 657–672.

Ambur, O.H., Frye, S.A., Nilsen, M., Hovland, E., and Tønjum, T. (2012). Restriction and sequence alterations affect DNA uptake sequence-dependent transformation in *Neisseria meningitidis*. PLoS One *7*, e39742.

Andrews, S.M., and Pollard, A.J. (2014). A vaccine against serogroup B *Neisseria meningitidis*: dealing with uncertainty. Lancet Infect. Dis. *14*, 426–434.

Apicella, M.A., Shao, J., and Neil, R.B. (2012). Methods for studying *Neisseria meningitidis* biofilms. In Methods in Molecular Biology (Clifton, N.J.), pp. 169–184.

Arenas, J., Schipper, K., van Ulsen, P., van der Ende, A., and Tommassen, J. (2013). Domain exchange at the 3' end of the gene encoding the fratricide meningococcal two-partner secretion protein A. BMC Genomics *14*, 622.

Arenas, J., de Maat, V., Catón, L., Krekorian, M., Herrero, J.C., Ferrara, F., and Tommassen, J. (2015). Fratricide activity of MafB protein of *N. meningitidis* strain B16B6. BMC Microbiol. *15*, 156.

Arenas, J., Paganelli, F.L., Rodríguez-Castaño, P., Cano-Crespo, S., van der Ende, A., van Putten, J.P.M., and Tommassen, J. (2016). Expression of the gene for autotransporter AutB of *Neisseria meningitidis* affects biofilm formation and epithelial transmigration. Front. Cell. Infect. Microbiol. *6*, 162.

Arpin, C., de Bouteiller, O., Razanajaona, D., Fugier-Vivier, I., Brière, F., Banchereau, J., Lebecque, S., and Liu, Y.J. (1998). The normal counterpart of IgD myeloma cells in germinal center displays extensively mutated *IgVH* gene, Cmu-Cdelta switch, and lambda light chain expression. J. Exp. Med. *187*, 1169–1178.

Arreaza, L., Salcedo, C., Alcalá, B., and Vázquez, J.A. (2002). What about antibiotic resistance in *Neisseria lactamica*? J. Antimicrob. Chemother. *49*, 545–547.

Barrett, D.J., and Ayoub, E.M. (1986). IgG2 subclass restriction of antibody to pneumococcal polysaccharides. Clin. Exp. Immunol. *63*, 127–134.

Becker, J. (2016). Systems metabolic engineering of *Escherichia coli* for the heterologous production of high value molecules — a veteran at new shores. Curr. Opin. Biotechnol. *42*, 178–188.

Bernard, S.C., Simpson, N., Join-Lambert, O., Federici, C., Laran-Chich, M.-P., Maïssa, N., Bouzinba-Ségard, H., Morand, P.C., Chretien, F., Taouji, S., et al. (2014). Pathogenic *Neisseria meningitidis* utilizes CD147 for vascular colonization. Nat. Med. *20*, 725–731.

Berry, J.-L., Cehovin, A., McDowell, M.A., Lea, S.M., and Pelicic, V. (2013). Functional analysis of the interdependence between DNA Uptake Sequence and its cognate ComP receptor during natural transformation in *Neisseria* Species. PLoS Genet. *9*, e1004014.

Biais, N., Ladoux, B., Higashi, D., So, M., and Sheetz, M. (2008). Cooperative retraction of bundled type IV pili enables nanonewton force generation. PLoS Biol. *6*, e87.

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., van Gils, E., Bruin, J., Montijn, R., Bonten, M., and Sanders, E. (2011). Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS One *6*, e17035.

Borrow, R., Andrews, N., Goldblatt, D., and Miller, E. (2001). Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: Reevaluation of correlates of protection. Infect. Immun. *69*, 1568–1573.

Bowler, L.D., Zhang, Q.Y., Riou, J.Y., and Spratt, B.G. (1994). Interspecies recombination between the *penA* genes of *Neisseria meningitidis* and commensal *Neisseria* species during the emergence of penicillin resistance in *N. meningitidis*: natural events and laboratory simulation. J. Bacteriol. *176*, 333–337.

Braat, H., Rottiers, P., Hommes, D.W., Huyghebaert, N., Remaut, E., Remon, J., van Deventer, S.J.H., Neirynck, S., Peppelenbosch, M.P., and Steidler, L. (2006). A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin. Gastroenterol. Hepatol. *4*, 754–759.

Brookes, C., Kuisma, E., Alexander, F., Allen, L., Tipton, T., Ram, S., Gorringe, A., and Taylor, S. (2013). Development of a large scale human complement source for use in bacterial immunoassays. J. Immunol. Methods *391*, 39–49.

Budroni, S., Siena, E., Dunning Hotopp, J.C., Seib, K.L., Serruto, D., Nofroni, C., Comanducci, M., Riley, D.R., Daugherty, S.C., Angiuoli, S. V, et al. (2011). *Neisseria meningitidis* is structured in clades associated with restriction modification systems that modulate homologous recombination. Proc. Natl. Acad. Sci. U. S. A. *108*, 4494–4499.

Carbonnelle, E., Helaine, S., Nassif, X., and Pelicic, V. (2006). A systematic genetic analysis in

*Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. Mol. Microbiol. *61*, 1510–1522.

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

Cartwright, K.A., Stuart, J.M., Jones, D.M., and Noah, N.D. (1987). The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol. Infect. *99*, 591–601.

Casadevall, A., and Imperiale, M.J. (2014). Risks and benefits of gain-of-function experiments with pathogens of pandemic potential, such as influenza virus: a call for a science-based discussion. MBio *5*, e01730–14.

Cehovin, A., Simpson, P.J., McDowell, M.A., Brown, D.R., Noschese, R., Pallett, M., Brady, J., Baldwin, G.S., Lea, S.M., Matthews, S.J., et al. (2013). Specific DNA recognition mediated by a type IV pilin. Proc. Natl. Acad. Sci. *110*, 3065–3070.

Cesta, M.F. (2006). Normal structure, function, and histology of mucosa-associated lymphoid tissue. Toxicol. Pathol. *34*, 599–608.

Chen, T.R. (1988). Re-evaluation of HeLa, HeLa S3, and HEp-2 karyotypes. Cytogenet. Cell Genet. *48*, 19–24.

Chen, I., and Dubnau, D. (2004). DNA uptake during bacterial transformation. Nat. Rev. Microbiol. *2*, 241–249.

Chen, K., Xu, W., Wilson, M., He, B., Miller, N.W., Bengtén, E., Edholm, E.-S., Santini, P.A., Rath, P., Chiu, A., et al. (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. Nat. Immunol. *10*, 889–898.

Chiang, P., and Burrows, L.L. (2003). Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. J. Bacteriol. *185*, 2374–2378.

Claus, H., Frosch, M., and Vogel, U. (1998). Identification of a hotspot for transformation of *Neisseria meningitidis* by shuttle mutagenesis using signature-tagged transposons. Mol. Gen. Genet. *259*, 363–371.

Comanducci, M., Bambini, S., Brunelli, B., Adu-Bobie, J., Aricò, B., Capecchi, B., Giuliani, M.M., Masignani, V., Santini, L., Savino, S., et al. (2002). NadA, a novel vaccine candidate of *Neisseria meningitidis*. J. Exp. Med. *195*, 1445–1454.

Corbett, W.P., and Catlin, B.W. (1968). Galactosidase activity of lactose-positive *Neisseria*. J. Bacteriol. *95*, 52–57.

Cremers, A.J., Zomer, A.L., Gritzfeld, J.F., Ferwerda, G., van Hijum, S.A., Ferreira, D.M., Shak, J.R., Klugman, K.P., Boekhorst, J., Timmerman, H.M., et al. (2014). The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. Microbiome *2*, 44.

Davidsen, T., Rødland, E.A., Lagesen, K., Seeberg, E., Rognes, T., and Tønjum, T. (2004). Biased distribution of DNA uptake sequences towards genome maintenance genes. Nucleic Acids Res. *32*, 1050–1058.

Dawid, S., Roche, A.M., and Weiser, J.N. (2007). The *blp* bacteriocins of *Streptococcus pneumoniae* mediate intraspecies competition both *in vitro* and *in vivo*. Infect. Immun. *75*, 443–451.

Deasy, A.M., Guccione, E., Dale, A.P., Andrews, N., Evans, C.M., Bennett, J.S., Bratcher, H.B., Maiden, M.C.J., Gorringe, A.R., and Read, R.C. (2015). Nasal inoculation of the commensal *Neisseria lactamica* inhibits carriage of *Neisseria meningitidis* by young adults: A controlled human infection study. Clin. Infect. Dis. *60*, 1512–1520.

Deghmane, A.-E., Giorgini, D., Larribe, M., Alonso, J.-M., and Taha, M.-K. (2002). Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. Mol. Microbiol. *43*, 1555–1564.

Deghmane, A.E., Petit, S., Topilko, A., Pereira, Y., Giorgini, D., Larribe, M., and Taha, M.K. (2000). Intimate adhesion of *Neisseria meningitidis* to human epithelial cells is under the control of the *crgA* gene, a novel LysR-type transcriptional regulator. EMBO J. *19*, 1068–1078.

deHaseth, P.L., and Helmann, J.D. (1995). Open complex formation by *Escherichia coli* RNA polymerase: the mechanism of polymerase-induced strand separation of double helical DNA. Mol. Microbiol. *16*, 817–824.

Denning, D.W., and Gill, S.S. (1991). *Neisseria lactamica* meningitis following skull trauma. Rev. Infect. Dis. *13*, 216–218.

Department for Environment Food and Rural Affairs (2017). Genetically Modified Organisms: University of Southampton (17/R50/01).

Diallo, K., Trotter, C., Timbine, Y., Tamboura, B., Sow, S.O., Issaka, B., Dano, I.D., Collard, J.-M., Dieng, M., Diallo, A., et al. (2016). Pharyngeal carriage of *Neisseria* species in the African meningitis belt. J. Infect. *72,* 667-677.

Diaz, J.L., Virji, M., and Heckels, J.E. (1984). Structural and antigenic differences between two types of meningococcal pili. FEMS Microbiol. Lett. *21*, 181–184.

van den Dobbelsteen, G.P.J.M., van Dijken, H.H., Pillai, S., and van Alphen, L. (2007). Immunogenicity of a combination vaccine containing pneumococcal conjugates and meningococcal PorA OMVs. Vaccine *25*, 2491–2496.

Drijkoningen, J.J.C., and Rohde, G.G.U. (2014). Pneumococcal infection in adults: burden of disease. Clin. Microbiol. Infect. *20 Suppl 5*, 45–51.

Duffin, P.M., and Seifert, H.S. (2010). DNA uptake sequence-mediated enhancement of transformation in *Neisseria gonorrhoeae* is strain dependent. J. Bacteriol. *192*, 4436–4444.

van der Ende, A., Hopman, C.T., and Dankert, J. (2000). Multiple mechanisms of phase variation of PorA in *Neisseria meningitidis*. Infect. Immun. *68*, 6685–6690.

EUCAST (2018). The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 8.0.

Evans, C.M., Pratt, C.B., Matheson, M., Vaughan, T.E., Findlow, J., Borrow, R., Gorringe, A.R., and Read, R.C. (2011). Nasopharyngeal colonization by *Neisseria lactamica* and induction of protective immunity against *Neisseria meningitidis*. Clin. Infect. Dis. *52*, 70–77.

Fermer, C., Kristiansen, B.E., Sköld, O., and Swedberg, G. (1995). Sulfonamide resistance in *Neisseria meningitidis* as defined by site-directed mutagenesis could have its origin in other species. J. Bacteriol. *177*, 4669–4675.

Findlow, J., and Borrow, R. (2017). Does post-implementation vaccine effectiveness data support pre-implementation predictions of 4CMenB utility? Pathog. Dis. *75*, 510–515.

Findlow, H., Vogel, U., Mueller, J.E., Curry, A., Njanpop-Lafourcade, B., Claus, H., Gray, S.J., Yaro, S., Traoré, Y., Sangaré, L., et al. (2007). Three cases of invasive meningococcal disease caused by a capsule null locus strain circulating among healthy carriers in Burkina Faso. J. Infect. Dis. *195*, 1071–1077.

Findlow, J., Taylor, S., Aase, A., Horton, R., Heyderman, R., Southern, J., Andrews, N., Barchha, R., Harrison, E., Lowe, A., et al. (2006). Comparison and correlation of *Neisseria meningitidis* serogroup B immunologic assay results and human antibody responses following three doses of the Norwegian meningococcal outer membrane vesicle vaccine MenBvac. Infect. Immun. *74*, 4557–4565.

Findlow, J., Bai, X., Findlow, H., Newton, E., Kaczmarski, E., Miller, E., and Borrow, R. (2015). Safety and immunogenicity of a four-component meningococcal group B vaccine (4CMenB) and a quadrivalent meningococcal group ACWY conjugate vaccine administered concomitantly in healthy laboratory workers. Vaccine *33*, 3322–3330.

Finkelman, F.D., van Boxel, J.A., Asofsky, R., and Paul, W.E. (1976). Cell membrane IgD: demonstration of IgD on human lymphocytes by enzyme-catalyzed iodination and comparison with cell surface Ig of mouse, guinea pig, and rabbit. J. Immunol. *116*, 1173–1181.

Finkelman, F.D., Woods, V.L., Berning, A., and Scher, I. (1979). Demonstration of mouse serum IgD. J. Immunol. *123*, 1253–1259.

Fisseha, M., Chen, P., Brandt, B., Kijek, T., Moran, E., and Zollinger, W. (2005). Characterization of native outer membrane vesicles from *lpxL* mutant strains of *Neisseria meningitidis* for use in parenteral vaccination. Infect. Immun. *73*, 4070–4080.

Frasch, C.E., Zollinger, W.D., and Poolman, J.T. (1985). Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Rev. Infect. Dis. *7*, 504–510.

Fronzes, R., Remaut, H., Waksman, G., Aas, F., Wolfgang, M., Frye, S., Dunham, S., Lovold, C., Koomey, M., Akeda, Y., et al. (2008). Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. EMBO J. *27*, 2271–2280.

Frye, S.A., Nilsen, M., Tønjum, T., and Ambur, O.H. (2013). Dialects of the DNA uptake sequence in *Neisseriaceae*. PLoS Genet. *9*, e1003458.

Gault, J., Ferber, M., Machata, S., Imhaus, A.-F., Malosse, C., Charles-Orszag, A., Millien, C., Bouvier, G., Bardiaux, B., Péhau-Arnaudet, G., et al. (2015). *Neisseria meningitidis* type IV pili composed of sequence invariable pilins are masked by multisite glycosylation. PLoS Pathog. *11*, e1005162.

Genco, C.A., and Wetzler, L.M. (2010). *Neisseria* : molecular mechanisms of pathogenesis (Caister Academic Press, Poole, UK).

Giuliani, M.M., Adu-Bobie, J., Comanducci, M., Aricò, B., Savino, S., Santini, L., Brunelli, B., Bambini, S., Biolchi, A., Capecchi, B., et al. (2006). A universal vaccine for serogroup B meningococcus. Proc. Natl. Acad. Sci. U. S. A. *103*, 10834–10839.

Gold, R., Goldschneider, I., Lepow, M.L., Draper, T.F., and Randolph, M. (1978). Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in infants and children. J. Infect. Dis. *137*, 112–121.

Goldschneider, I., Gotschlich, E.C., and Artenstein, M.S. (1969). Human immunity to the meningococcus. I. The role of humoral antibodies. J. Exp. Med. *129*, 1307–1326.

Goodman, S.D., and Scocca, J.J. (1991). Factors influencing the specific interaction of *Neisseria gonorrhoeae* with transforming DNA. J. Bacteriol. *173*, 5921–5923.

Gorringe, A.R., Taylor, S., Brookes, C., Matheson, M., Finney, M., Kerr, M., Hudson, M., Findlow, J., Borrow, R., Andrews, N., et al. (2009). Phase I safety and immunogenicity study of a candidate meningococcal disease vaccine based on *Neisseria lactamica* outer membrane vesicles. Clin. Vaccine Immunol. *16*, 1113–1120.

Granoff, D.M., Welsch, J.A., and Ram, S. (2009). Binding of complement factor H (fH) to *Neisseria meningitidis* is specific for human fH and inhibits complement activation by rat and rabbit sera. Infect. Immun. *77*, 764–769.

Greenfield, S., Sheehe, P.R., and Feldman, H.A. (1971). Meningococcal carriage in a population of "normal" families. J. Infect. Dis. *123*, 67–73.

Griffiss, J.M., and Goroff, D.K. (1983). IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms. J. Immunol. *130*, 2882–2885.

Guibourdenche, M., Popoff, M.Y., and Riou, J.Y. Deoxyribonucleic acid relatedness among *Neisseria gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea* and *"Neisseria polysaccharea".* Ann. Inst. Pasteur. Microbiol. *137B*, 177–185.

Hadad, R., Jacobsson, S., Pizza, M., Rappuoli, R., Fredlund, H., Olcén, P., and Unemo, M. (2012). Novel meningococcal 4CMenB vaccine antigens - prevalence and polymorphisms of the encoding genes in *Neisseria gonorrhoeae*. APMIS *120*, 750–760.

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

Hansman, D. (1978). Meningitis caused by *Neisseria lactamica*. N. Engl. J. Med. *299*, 491.

Harrison, O.B., Robertson, B.D., Faust, S.N., Jepson, M.A., Goldin, R.D., Levin, M., and Heyderman, R.S. (2002). Analysis of pathogen-host cell interactions in purpura fulminans: expression of capsule, type IV pili, and PorA by *Neisseria meningitidis in vivo*. Infect. Immun. *70*, 5193–5201.

Hart, C.A., and Rogers, T.R.F. (1993). Meningococcal disease: -- A review based on a symposium held on 11 July 1992 at the Liverpool School of Tropical Medicine. J. Med. Microbiol. *39*, 3–25.

Helaine, S., Dyer, D.H., Nassif, X., Pelicic, V., and Forest, K.T. (2007). 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. Proc. Natl. Acad. Sci. U. S. A. *104*, 15888–15893.

Higashi, D.L., Biais, N., Weyand, N.J., Agellon, A., Sisko, J.L., Brown, L.M., So, M., Catlin, B., Chen, I., Christie, P., et al. (2011). *N. elongata* produces type IV pili that mediate interspecies gene transfer with *N. gonorrhoeae*. PLoS One *6*, e21373.

Hiroi, T., Iwatani, K., Iijima, H., Kodama, S., Yanagita, M., and Kiyono, H. (1998). Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. Eur. J. Immunol. *28*, 3346–3353.

Hoang, L.M.N., Thomas, E., Tyler, S., Pollard, A.J., Stephens, G., Gustafson, L., McNabb, A., Pocock, I., Tsang, R., and Tan, R. (2005). Rapid and fatal meningococcal disease due to a strain of *Neisseria meningitidis* containing the capsule null locus. Clin. Infect. Dis. *40*, e38–e42.

Hollis, D.G., Wiggins, G.L., and Weaver, R.E. (1969). *Neisseria lactamicus* sp. n., a lactose-fermenting species resembling *Neisseria meningitidis*. Appl. Microbiol. *17*, 71.

Imhaus, A.-F., and Duménil, G. (2014). The number of *Neisseria meningitidis* type IV pili determines host cell interaction. EMBO J. *33*, 1767–1783.

Izquierdo, G., Torres, J.P., Santolaya, M.E., Valenzuela, M.T., Vega, J., and Chomali, M. (2015). Cost-effectiveness analysis of a multicomponent meningococcal serogroup B vaccine in hypothetic epidemic situation in a middle-income country. Hum. Vaccin. Immunother. *11*, 875–883.

Jafri, R.Z., Ali, A., Messonnier, N.E., Tevi-Benissan, C., Durrheim, D., Eskola, J., Fermon, F., Klugman, K.P., Ramsay, M., Sow, S., et al. (2013). Global epidemiology of invasive meningococcal disease. Popul. Health Metr. *11*, 17.

Jamet, A., Jousset, A.B., Euphrasie, D., Mukorako, P., Boucharlat, A., Ducousso, A., Charbit, A., and Nassif, X. (2015). A new family of secreted toxins in pathogenic *Neisseria* species. PLoS Pathog. *11*, e1004592.

Jarvis, G.A., and Griffiss, J.M. (1991). Human IgA1 blockade of IgG-initiated lysis of *Neisseria meningitidis* is a function of antigen-binding fragment binding to the polysaccharide capsule. J. Immunol. *147*, 1962–1967.

Johnsson, E., Andersson, G., Lindahl, G., and Hedén, L.O. (1994). Identification of the IgA-binding region in streptococcal protein Arp. J. Immunol. *153*, 3557–3564.

Johnston, C., Martin, B., Fichant, G., Polard, P., and Claverys, J.-P. (2014). Bacterial transformation: distribution, shared mechanisms and divergent control. Nat. Rev. Microbiol. *12*, 181–196.

Johswich, K.O., Zhou, J., Law, D.K.S., St Michael, F., McCaw, S.E., Jamieson, F.B., Cox, A.D., Tsang, R.S.W., and Gray-Owen, S.D. (2012). Invasive potential of nonencapsulated disease isolates of *Neisseria meningitidis*. Infect. Immun. *80*, 2346–2353.

Kaaijk, P., van Straaten, I., van de Waterbeemd, B., Boot, E.P.J., Levels, L.M.A.R., van Dijken, H.H., and van den Dobbelsteen, G.P.J.M. (2013). Preclinical safety and immunogenicity evaluation of a nonavalent PorA native outer membrane vesicle vaccine against serogroup B meningococcal disease. Vaccine *31*, 1065–1071.

Kallstrom, H., Islam, M.S., Berggren, P.-O., and Jonsson, A.-B. (1998). Cell signaling by the type IV pili of pathogenic *Neisseria*. J. Biol. Chem. *273*, 21777–21782.

Källström, H., Liszewski, M.K., Atkinson, J.P., and Jonsson, A.B. (1997). Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. Mol. Microbiol. *25*, 639–647.

Kinlin, L.M., Jamieson, F., Brown, E.M., Brown, S., Rawte, P., Dolman, S., Drews, S.J., and Fisman, D.N. (2009). Rapid identification of herd effects with the introduction of serogroup C meningococcal conjugate vaccine in Ontario, Canada, 2000-2006. Vaccine *27*, 1735–1740.

Kirchner, M., Heuer, D., and Meyer, T.F. (2005). CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. Infect. Immun. *73*, 3072–3082.

Kirsch, E.A., Barton, R.P., Kitchen, L., and Giroir, B.P. (1996). Pathophysiology, treatment and outcome of meningococcemia: a review and recent experience. Pediatr. Infect. Dis. J. *15*, 967–978; quiz 979.

Kiyono, H., and Fukuyama, S. (2004). Nalt- versus peyer's-patch-mediated mucosal immunity. Nat. Rev. Immunol. *4*, 699–710.

Klausen, M., Aaes-Jørgensen, A., Molin, S., and Tolker-Nielsen, T. (2003). Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. Mol. Microbiol. *50*, 61–68.

Koelsch, K., Zheng, N.-Y., Zhang, Q., Duty, A., Helms, C., Mathias, M.D., Jared, M., Smith, K., Capra, J.D., and Wilson, P.C. (2007). Mature B cells class switched to IgD are autoreactive in healthy individuals. J. Clin. Invest. *117*, 1558–1565.

Kouzel, N., Oldewurtel, E.R., and Maier, B. (2015). Gene transfer efficiency in gonococcal biofilms: Role of biofilm age, architecture, and pilin antigenic variation. J. Bacteriol. *197*, 2422–2431.

Kung, S.H., Retchless, A.C., Kwan, J.Y., and Almeida, R.P.P. (2013). Effects of DNA size on transformation and recombination efficiencies in *Xylella fastidiosa*. Appl. Environ. Microbiol. *79*, 1712–1717.

Ladhani, S., and Public Health England (2016). Invasive meningococcal disease in infants following the introduction of Bexsero® into the national immunisation programme in England. Abstract 03.

In Internationl Pathogenic *Neisseria* Conference, Manchester, UK.

Ladhani, S.N., Waight, P.A., Ribeiro, S., and Ramsay, M.E. (2015). Invasive meningococcal disease in England: assessing disease burden through linkage of multiple national data sources. BMC Infect. Dis. *15*, 551.

Lappann, M., and Vogel, U. (2010). Biofilm formation by the human pathogen *Neisseria meningitidis*. Med. Microbiol. Immunol. *199*, 173–183.

Lappann, M., Haagensen, J.A.J., Claus, H., Vogel, U., and Molin, S. (2006). Meningococcal biofilm formation: structure, development and phenotypes in a standardized continuous flow system. Mol. Microbiol. *62*, 1292–1309.

Lauer, B.A., and Fisher, C.E. (1976). Neisseria lactamica meningitis. Am. J. Dis. Child. *130*, 198–199.

LeBlanc, J.G., Aubry, C., Cortes-Perez, N.G., de Moreno de LeBlanc, A., Vergnolle, N., Langella, P., Azevedo, V., Chatel, J.-M., Miyoshi, A., and Bermúdez-Humarán, L.G. (2013). Mucosal targeting of therapeutic molecules using genetically modified lactic acid bacteria: an update. FEMS Microbiol. Lett. *344*, 1–9.

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

van der Ley, P., van der Biezen, J., and Poolman, J.T. (1995). Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the *porA* gene for use in the production of a multivalent outer membrane vesicle vaccine. Vaccine *13*, 401–407.

Liu, X., Wetzler, L.M., and Massari, P. (2008). The PorB porin from commensal *Neisseria lactamica* induces Th1 and Th2 immune responses to ovalbumin in mice and is a potential immune adjuvant. Vaccine *26*, 786–796.

Liu, Y.J., de Bouteiller, O., Arpin, C., Brière, F., Galibert, L., Ho, S., Martinez-Valdez, H., Banchereau, J., and Lebecque, S. (1996). Normal human IgD+IgM- germinal center B cells can express up to 80 mutations in the variable region of their IgD transcripts. Immunity *4*, 603–613.

Loh, E., Kugelberg, E., Tracy, A., Zhang, Q., Gollan, B., Ewles, H., Chalmers, R., Pelicic, V., and Tang, C.M. (2013). Temperature triggers immune evasion by *Neisseria meningitidis*. Nature *502*, 237–240.

Lucidarme, J., Gilchrist, S., Newbold, L.S., Gray, S.J., Kaczmarski, E.B., Richardson, L., Bennett, J.S., Maiden, M.C.J., Findlow, J., and Borrow, R. (2013). Genetic distribution of noncapsular meningococcal group B vaccine antigens in *Neisseria lactamica*. Clin. Vaccine Immunol. *20*, 1360–1369.

Madico, G., Welsch, J.A., Lewis, L.A., McNaughton, A., Perlman, D.H., Costello, C.E., Ngampasutadol, J., Vogel, U., Granoff, D.M., and Ram, S. (2006). The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. J. Immunol. *177*, 501–510.

Maier, B., Potter, L., So, M., Long, C.D., Seifert, H.S., and Sheetz, M.P. (2002). Single pilus motor forces exceed 100 pN. Proc. Natl. Acad. Sci. U. S. A. *99*, 16012–16017.

Mairey, E., Genovesio, A., Donnadieu, E., Bernard, C., Jaubert, F., Pinard, E., Seylaz, J., Olivo-Marin, J.-C., Nassif, X., and Duménil, G. (2006). Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood-brain barrier. J. Exp. Med. *203*, 1939–1950.

Marks, L.R., Reddinger, R.M., and Hakansson, A.P. (2012). High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. MBio *3*, e00200–e00212 – e00200–e00212.

Marshall, H., Wang, B., Wesselingh, S., Snape, M., and Pollard, A.J. (2016). Control of invasive meningococcal disease. Int. J. Evid. Based. Healthc. *14*, 3–14.

Martin, D.R., Ruijne, N., McCallum, L., O'Hallahan, J., and Oster, P. (2006). The VR2 epitope on the PorA P1.7-2,4 protein is the major target for the immune response elicited by the strain-specific group B meningococcal vaccine MeNZB. Clin. Vaccine Immunol. *13*, 486–491.

Masignani, V., Comanducci, M., Giuliani, M.M., Bambini, S., Adu-Bobie, J., Arico, B., Brunelli, B., Pieri, A., Santini, L., Savino, S., et al. (2003). Vaccination against *Neisseria meningitidis* using three variants of the lipoprotein GNA1870. J. Exp. Med. *197*, 789–799.

Merz, A.J., Enns, C.A., and So, M. (1999). Type IV pili of pathogenic *Neisseriae* elicit cortical plaque formation in epithelial cells. Mol. Microbiol. *32*, 1316–1332.

Michaelsen, T.E., Kolberg, J., Aase, A., Herstad, T.K., and Hoiby, E.A. (2004). The four mouse IgG isotypes differ extensively in bactericidal and opsonophagocytic activity when reacting with the P1.16 epitope on the outer membrane PorA protein of *Neisseria meningitidis*. Scand. J. Immunol. *59*, 34–39.

Mikaty, G., Soyer, M., Mairey, E., Henry, N., Dyer, D., Forest, K.T., Morand, P., Guadagnini, S., Prévost, M.C., Nassif, X., et al. (2009). Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. PLoS Pathog. *5*, e1000314.

Milagres, L.G., Ramos, S.R., Sacchi, C.T., Melles, C.E., Vieira, V.S., Sato, H., Brito, G.S., Moraes, J.C., and Frasch, C.E. (1994). Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect. Immun. *62*, 4419–4424.

Miller, J.H. (1972). Experiments in molecular genetics (Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press).

Miller, F., Phan, G., Brissac, T., Bouchiat, C., Lioux, G., Nassif, X., and Coureuil, M. (2014). The hypervariable region of meningococcal major pilin PilE controls the host cell response via antigenic variation. MBio *5*, e01024–13.

Monasta, L., Ronfani, L., Marchetti, F., Montico, M., Vecchi Brumatti, L., Bavcar, A., Grasso, D.,

Barbiero, C., and Tamburlini, G. (2012). Burden of disease caused by otitis media: systematic review and global estimates. PLoS One *7*, e36226.

de Moraes, J.C., Perkins, B.A., Camargo, M.C., Hidalgo, N.T., Barbosa, H.A., Sacchi, C.T., Landgraf, I.M., Gattas, V.L., Vasconcelos, H. de G., and Gral, I.M. (1992). Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. Lancet *340*, 1074–1078.

Morelli, L., Cancogni, D., Tontini, M., Nilo, A., Filippini, S., Costantino, P., Romano, M.R., Berti, F., Adamo, R., and Lay, L. (2014). Synthesis and immunological evaluation of protein conjugates of *Neisseria meningitidis* X capsular polysaccharide fragments. Beilstein J. Org. Chem. *10*, 2367–2376.

Morrison, D.A., Khan, R., Junges, R., Åmdal, H.A., and Petersen, F.C. (2015). Genome editing by natural genetic transformation in *Streptococcus mutans*. J. Microbiol. Methods *119*, 134–141.

Mortier-Barrière, I., Velten, M., Dupaigne, P., Mirouze, N., Piétrement, O., McGovern, S., Fichant, G., Martin, B., Noirot, P., Le Cam, E., et al. (2007). A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell *130*, 824–836.

Mowday, A.M., Guise, C.P., Ackerley, D.F., Minton, N.P., Lambin, P., Dubois, L.J., Theys, J., Smaill, J.B., and Patterson, A. V (2016). Advancing *Clostridia* to clinical trial: Past lessons and recent progress. Cancers (Basel). *8*. 63

Mukhopadhyay, T.K., Halliwell, D., O'Dwyer, C., Shamlou, P.A., Levy, M.S., Allison, N., Gorringe, A., and Reddin, K.M. (2005). Rapid characterization of outer-membrane proteins in *Neisseria lactamica* by SELDI-TOF-MS (surface-enhanced laser desorption ionization-time-of-flight MS) for use in a meningococcal vaccine. Biotechnol. Appl. Biochem. *41*, 175–182.

Muzzi, A., Mora, M., Pizza, M., Rappuoli, R., and Donati, C. (2013). Conservation of meningococcal antigens in the genus *Neisseria*. MBio *4*, e00163–13.

Næss, L.M., Rosenqvist, E., Høiby, E.A., and Michaelsen, T.E. (1996). Quantitation of IgG subclass antibody responses after immunization with a group B meningococcal outer membrane vesicle vaccine, using monoclonal mouse-human chimeric antibodies as standards. J. Immunol. Methods *196*, 41–49.

Nassif, X., Beretti, J.L., Lowy, J., Stenberg, P., O'Gaora, P., Pfeifer, J., Normark, S., and So, M. (1994). Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. Proc. Natl. Acad. Sci. U.S.A. *91*, 3769–3773.

NEB (2016). Q5 Site-Directed Mutagenesis Kit.

NEB (2017). NEBase Changer.

Neil, R.B., Shao, J.Q., and Apicella, M.A. (2009). Biofilm formation on human airway epithelia by encapsulated *Neisseria meningitidis* serogroup B. Microbes Infect. *11*, 281–287.

NHS Choices (2016). Middle ear infection (otitis media) (Department of Health). https://www.nhsinform.scot/illnesses-and-conditions/ears-nose-and-throat/middle-ear-infection-

otitis-media

O'Dwyer, C.A., Reddin, K., Martin, D., Taylor, S.C., Gorringe, A.R., Hudson, M.J., Brodeur, B.R., Langford, P.R., and Kroll, J.S. (2004). Expression of heterologous antigens in commensal *Neisseria spp.*: preservation of conformational epitopes with vaccine potential. Infect. Immun. *72*, 6511–6518.

O'Hallahan, J., McNicholas, A., Galloway, Y., O'Leary, E., and Roseveare, C. (2009). Delivering a safe and effective strain-specific vaccine to control an epidemic of group B meningococcal disease. N. Z. Med. J. *122*, 48–59.

Oggioni, M.R., Trappetti, C., Kadioglu, A., Cassone, M., Iannelli, F., Ricci, S., Andrew, P.W., and Pozzi, G. (2006). Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol. Microbiol. *61*, 1196–1210.

Oliver, K.J., Reddin, K.M., Bracegirdle, P., Hudson, M.J., Borrow, R., Feavers, I.M., Robinson, A., Cartwright, K., and Gorringe, A.R. (2002). *Neisseria lactamica* protects against experimental meningococcal infection. Infect. Immun. *70*, 3621–3626.

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

Pace, D., and Pollard, A.J. (2012). Meningococcal disease: Clinical presentation and sequelae. Vaccine *30*, B3–B9.

Pace, D., Pollard, A.J., and Messonier, N.E. (2009). Quadrivalent meningococcal conjugate vaccines. Vaccine *27 Suppl 2*, B30–B41.

Pandey, A.K., Cleary, D.W., Laver, J.R., Maiden, M.C.J., Didelot, X., Gorringe, A., and Read, R.C. (2017). *Neisseria lactamica* Y92-1009 complete genome sequence. Stand. Genomic Sci. *12*, 41.

Parikh, S.R., Andrews, N.J., Beebeejaun, K., Campbell, H., Ribeiro, S., Ward, C., White, J.M., Borrow, R., Ramsay, M.E., and Ladhani, S.N. (2016). Effectiveness and impact of a reduced infant schedule of 4CMenB vaccine against group B meningococcal disease in England: a national observational cohort study. Lancet *388*, 2775–2782.

van Passel, M.W.J., Bart, A., Luyf, A.C.M., van Kampen, A.H.C., and van der Ende, A. (2006). Identification of acquired DNA in *Neisseria lactamica*. FEMS Microbiol. Lett. *262*, 77–84.

Lo Passo, C., Romeo, A., Pernice, I., Donato, P., Midiri, A., Mancuso, G., Arigo, M., Biondo, C., Galbo, R., Papasergi, S., et al. (2007). Peptide mimics of the group B meningococcal capsule induce bactericidal and protective antibodies after immunization. J. Immunol. *178*, 4417–4423.

Peeters, C.C., Rümke, H.C., Sundermann, L.C., Rouppe van der Voort, E.M., Meulenbelt, J., Schuller, M., Kuipers, A.J., van der Ley, P., and Poolman, J.T. (1996). Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine. Vaccine *14*, 1009–

1015.

Pelicic, V. (2008). Type IV pili: e pluribus unum? Mol. Microbiol. *68*, 827–837.

Pérez-Ortega, J., Rodríguez, A., Ribes, E., Tommassen, J., and Arenas, J. (2017). Interstrain cooperation in meningococcal biofilms: Role of autotransporters NalP and AutA. Front. Microbiol. *8*, 434.

Petousis-Harris, H. (2017). Commentary: Impact of meningococcal group B OMV vaccines, beyond their brief. Hum. Vaccin. Immunother. 1–6.

Petousis-Harris, H., Paynter, J., Morgan, J., Saxton, P., Sherwood, J., McArdle, B., and Goodyear-Smith, F. (2016). Effectiveness of a group B OMV meningococcal vaccine on gonorrhea in New Zealand – a case control study. In 20th International Pathogenic *Neisseria* Conference, p. 68.

Piet, J.R., Huis in 't Veld, R.A.G., van Schaik, B.D.C., van Kampen, A.H.C., Baas, F., van de Beek, D., Pannekoek, Y., and van der Ende, A. (2011). Genome sequence of *Neisseria meningitidis* serogroup B strain H44/76. J. Bacteriol. *193*, 2371–2372.

Pizza, M. (2000). Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science (80). *287*, 1816–1820.

Platonov, A.E., Vershinina, I. V., Käyhty, H., Fijen, C.A.P., Würzner, R., and Kuijper, E.J. (2003). Antibody-dependent killing of meningococci by human neutrophils in serum of late complement component-deficient patients. Int. Arch. Allergy Immunol. *130*, 314–321.

Post, D.M.B., Zhang, D., Eastvold, J.S., Teghanemt, A., Gibson, B.W., and Weiss, J.P. (2005). Biochemical and functional characterization of membrane blebs purified from *Neisseria meningitidis* serogroup B. J. Biol. Chem. *280*, 38383–38394.

Pron, B., Taha, M.K., Rambaud, C., Fournet, J.C., Pattey, N., Monnet, J.P., Musilek, M., Beretti, J.L., and Nassif, X. (1997). Interaction of *Neisseria meningitidis* with the components of the blood-brain barrier correlates with an increased expression of PilC. J. Infect. Dis. *176*, 1285–1292.

Pruthi, V., Al-Janabi, A., and Pereira, B.M.J. (2003). Characterization of biofilm formed on intrauterine devices. Indian J. Med. Microbiol. *21*, 161–165.

Public Health England (2015). Health Protection Report. Infect. Rep. *9*.

Putonti, C., Nowicki, B., Shaffer, M., Fofanov, Y., and Nowicki, S. (2013). Where does *Neisseria* acquire foreign DNA from: an examination of the source of genomic and pathogenic islands and the evolution of the *Neisseria* genus. BMC Evol. Biol. *13*, 184.

Puyet, A., Greenberg, B., and Lacks, S.A. (1990). Genetic and structural characterization of *endA*. J. Mol. Biol. *213*, 727–738.

Quillin, S.J., and Seifert, H.S. (2018). *Neisseria gonorrhoeae* host adaptation and pathogenesis. Nat. Rev. Microbiol. *16*, 226–240.

Qvarnstrom, Y., and Swedberg, G. (2006). Variations in gene organization and DNA uptake signal sequence in the *folP* region between commensal and pathogenic *Neisseria* species. BMC

Microbiol. *6*, 11.

Ray, T.D., Lewis, L.A., Gulati, S., Rice, P.A., and Ram, S. (2011). Novel blocking human IgG directed against the pentapeptide repeat motifs of *Neisseria meningitidis* Lip/H.8 and Laz lipoproteins. J. Immunol. *186*, 4881–4894.

Redfield, R.J., Findlay, W.A., Bossé, J., Kroll, J.S., Cameron, A.D.S., and Nash, J.H. (2006). Evolution of competence and DNA uptake specificity in the *Pasteurellaceae*. BMC Evol. Biol. *6*, 82.

Revai, K., Mamidi, D., and Chonmaitree, T. (2008). Association of nasopharyngeal bacterial colonization during upper respiratory tract infection and the development of acute otitis media. Clin. Infect. Dis. *46*, e34–e37.

Rispens, T., and Vidarsson, G. (2014). Chapter 9 – Human IgG Subclasses. In Antibody Fc, pp. 159–177.

Roberts, M.S., and Cohan, F.M. (1993). The effect of DNA sequence divergence on sexual isolation in *Bacillus*. Genetics *134*, 401–408.

Ross, S.C., Rosenthal, P.J., Berberich, H.M., and Densen, P. (1987). Killing of *Neisseria meningitidis* by human neutrophils: implications for normal and complement-deficient individuals. J. Infect. Dis. *155*, 1266–1275.

Rouppe van der Voort, E., van der Voort, E.R., Schuller, M., Holst, J., de Vries, P., van der Ley, P., van den Dobbelsteen, G., and Poolman, J. (2000). Immunogenicity studies with a genetically engineered hexavalent PorA and a wild-type meningococcal group B outer membrane vesicle vaccine in infant cynomolgus monkeys. Vaccine *18*, 1334–1343.

Rowe, D.S., and Fahey, J.L. (1965). A new class of human immunoglobulins. II. Normal serum IgD. J. Exp. Med. *121*, 185–199.

Rudin, A., Johansson, E.-L., Bergquist, C., and Holmgren, J. (1998). Differential kinetics and distribution of antibodies in serum and nasal and vaginal secretions after nasal and oral vaccination of humans. Infect. Immun. *66*, 3390–3396.

Ruhe, Z.C., Low, D.A., and Hayes, C.S. (2013). Bacterial contact-dependent growth inhibition. Trends Microbiol. *21*, 230–237.

Sacchi, C.T., Whitney, A.M., Popovic, T., Beall, D.S., Reeves, M.W., Plikaytis, B.D., Rosenstein, N.E., Perkins, B.A., Tondella, M.L.C., and Mayer, L.W. (1992). Diversity and prevalence of PorA Types in *Neisseria meningitidis* Serogroup B in the United States, 1992–1998. J. Infect. Dis. *182*, 1169–1176.

Sakwinska, O., Bastic Schmid, V., Berger, B., Bruttin, A., Keitel, K., Lepage, M., Moine, D., Ngom Bru, C., Brüssow, H., and Gervaix, A. (2014). Nasopharyngeal microbiota in healthy children and pneumonia patients. J. Clin. Microbiol. *52*, 1590–1594.

Salvadori, G., Junges, R., Morrison, D.A., and Petersen, F.C. (2016). Overcoming the barrier of low ffficiency during genetic transformation of *Streptococcus mitis*. Front. Microbiol. *7*, 1009.

Sandbu, S., Feiring, B., Oster, P., Helland, O.S., Bakke, H.S.W., Naess, L.M., Aase, A., Aaberge, I.S., Kristoffersen, A.-C., Rydland, K.M., et al. (2007). Immunogenicity and safety of a combination of two serogroup B meningococcal outer membrane vesicle vaccines. Clin. Vaccine Immunol. *14*, 1062–1069.

Santos, G.F., Deck, R.R., Donnelly, J., Blackwelder, W., and Granoff, D.M. (2001). Importance of complement source in measuring meningococcal bactericidal titers. Clin. Vaccine Immunol. *8*, 616–623.

Sardiñas, G., Reddin, K., Pajon, R., and Gorringe, A. (2006). Outer membrane vesicles of *Neisseria lactamica* as a potential mucosal adjuvant. Vaccine *24*, 206–214.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics *30*, 2068–2069.

Seemann, T. (2018). Snippy. https://github.com/tseemann/snippy

Seib, K.L., Jen, F.E.-C., Scott, A.L., Tan, A., and Jennings, M.P. (2017). Phase variation of DNA methyltransferases and the regulation of virulence and immune evasion in the pathogenic *Neisseria*. Pathog. Dis. *75*.

Seifert, H.S., Ajioka, R.S., Paruchuri, D., Heffron, F., and So, M. (1990). Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutations lower DNA transformation competence. J. Bacteriol. *172*, 40–46.

Shak, J.R., Vidal, J.E., and Klugman, K.P. (2013). Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol. *21*, 129–135.

Siber, G.R., Schur, P.H., Aisenberg, A.C., Weitzman, S.A., and Schiffman, G. (1980). Correlation between serum IgG-2 concentrations and the antibody response to bacterial polysaccharide antigens. N. Engl. J. Med. *303*, 178–182.

Sim, R.J., Harrison, M.M., Moxon, E.R., and Tang, C.M. (2000). Underestimation of meningococci in tonsillar tissue by nasopharyngeal swabbing. Lancet (London, England) *356*, 1653–1654.

Simoni, P., and Wiatrak, B.J. (2004). Microbiology of stents in laryngotracheal reconstruction. Laryngoscope *114*, 364–367.

Smith, N.H., Holmes, E.C., Donovan, G.M., Carpenter, G.A., and Spratt, B.G. (1999). Networks and groups within the genus *Neisseria*: analysis of *argF, recA, rho,* and 16S rRNA sequences from human *Neisseria* species. Mol. Biol. Evol. *16*, 773–783.

Snyder, L.A.S., and Saunders, N.J. (2006). The majority of genes in the pathogenic *Neisseria* species are present in non-pathogenic *Neisseria lactamica*, including those designated as "virulence genes". BMC Genomics *7*, 128.

Snyder, L.A.S., Saunders, N.J., and Butcher, S.A. (2001). Comparative whole-genome analyses reveal over 100 putative phase-variable genes in the pathogenic *Neisseria* spp. Microbiology *147*, 2321–2332.

Spinosa, M.R., Progida, C., Talà, A., Cogli, L., Alifano, P., and Bucci, C. (2007). The *Neisseria*

*meningitidis* capsule is important for intracellular survival in human cells. Infect. Immun. *75*, 3594–3603.

Stearns, J.C., Davidson, C.J., McKeon, S., Whelan, F.J., Fontes, M.E., Schryvers, A.B., Bowdish, D.M.E., Kellner, J.D., and Surette, M.G. (2015). Culture and molecular-based profiles show shifts in bacterial communities of the upper respiratory tract that occur with age. ISME J. *9*, 1246–1259.

Stefanelli, P., Neri, A., Tanabe, M., Fazio, C., and Massari, P. (2016). Typing and surface charges of the variable loop regions of PorB from *Neisseria meningitidis*. IUBMB Life *68*, 488–495.

Steichen, C.T., Shao, J.Q., Ketterer, M.R., and Apicella, M.A. (2008). Gonococcal cervicitis: A role for biofilm in pathogenesis. J. Infect. Dis. *198*, 1856–1861.

Stephens, D.S. (1999). Uncloaking the meningococcus: dynamics of carriage and disease. Lancet *353*, 941–942.

Su, E.L., and Snape, M.D. (2011). A combination recombinant protein and outer membrane vesicle vaccine against serogroup B meningococcal disease. Expert Rev. Vaccines *10*, 575–588.

Taha, M. (2002). The duality of virulence and transmissibility in *Neisseria meningitidis*. Trends Microbiol. *10*, 376–382.

Taha, M.K., Morand, P.C., Pereira, Y., Eugène, E., Giorgini, D., Larribe, M., and Nassif, X. (1998). Pilus-mediated adhesion of *Neisseria meningitidis*: the essential role of cell contact-dependent transcriptional upregulation of the PilC1 protein. Mol. Microbiol. *28*, 1153–1163.

Taktikos, J., Lin, Y.T., Stark, H., Biais, N., and Zaburdaev, V. (2015). Pili-induced clustering of *N. gonorrhoeae* bacteria. PLoS One *10*, e0137661.

Tang, C.M., Horien, C.L., Exley, R.M., Johnson, E., Wörmann, M.E., Liu, G., and Aho, E. (2016). *Neisseria cinerea* isolates can adhere to human epithelial cells by type IV pilus-independent mechanisms. Microbiology *162*, 487–502.

Tappero, J.W., Lagos, R., Ballesteros, A.M., Plikaytis, B., Williams, D., Dykes, J., Gheesling, L.L., Carlone, G.M., Høiby, E.A., Holst, J., et al. (1999). Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. JAMA *281*, 1520–1527.

Tauseef, I., Ali, Y.M., and Bayliss, C.D. (2013). Phase variation of PorA, a major outer membrane protein, mediates escape of bactericidal antibodies by *Neisseria meningitidis*. Infect. Immun. *81*, 1374–1380.

Teo, S.M., Mok, D., Pham, K., Kusel, M., Serralha, M., Troy, N., Holt, B.J., Hales, B.J., Walker, M.L., Hollams, E., et al. (2015). The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. Cell Host Microbe *17*, 704–715.

Tondella, M.L., Popovic, T., Rosenstein, N.E., Lake, D.B., Carlone, G.M., Mayer, L.W., Perkins, B.A., and Team (2000). Distribution of *Neisseria meningitidis* serogroup B serosubtypes and serotypes circulating in the United States. The Active Bacterial Core Surveillance Team. J. Clin. Microbiol. *38*,

3323–3328.

Torres, A., Bonanni, P., Hryniewicz, W., Moutschen, M., Reinert, R.R., and Welte, T. (2015). Pneumococcal vaccination: what have we learnt so far and what can we expect in the future? Eur. J. Clin. Microbiol. Infect. Dis. *34*, 19–31.

Toso, J.F., Gill, V.J., Hwu, P., Marincola, F.M., Restifo, N.P., Schwartzentruber, D.J., Sherry, R.M., Topalian, S.L., Yang, J.C., Stock, F., et al. (2002). Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. J. Clin. Oncol. *20*, 142–152.

Trappetti, C., Gualdi, L., Di Meola, L., Jain, P., Korir, C.C., Edmonds, P., Iannelli, F., Ricci, S., Pozzi, G., and Oggioni, M.R. (2011). The impact of the competence quorum sensing system on *Streptococcus pneumoniae* biofilms varies depending on the experimental model. BMC Microbiol. *11*, 75.

Treangen, T.J., Ambur, O.H., Tonjum, T., and Rocha, E.P.C. (2008). The impact of the neisserial DNA uptake sequences on genome evolution and stability. Genome Biol. *9*, R60.

Troncoso, G., Sanchez, S., Moreda, M., Criado, M.., and Ferreiras, C.. (2000). Antigenic cross-reactivity between outer membrane proteins of *Neisseria meningitidis* and commensal *Neisseria* species. FEMS Immunol. Med. Microbiol. *27*, 103–109.

Trotter, C.L., Chandra, M., Cano, R., Larrauri, A., Ramsay, M.E., Brehony, C., Jolley, K.A., Maiden, M.C.J., Heuberger, S., and Frosch, M. (2007). A surveillance network for meningococcal disease in Europe. FEMS Microbiol. Rev. *31*, 27–36.

Uria, M.J., Zhang, Q., Li, Y., Chan, A., Exley, R.M., Gollan, B., Chan, H., Feavers, I., Yarwood, A., Abad, R., et al. (2008). A generic mechanism in *Neisseria meningitidis* for enhanced resistance against bactericidal antibodies. J. Exp. Med. *205*, 1423–1434.

Urwin, R., Russell, J.E., Thompson, E.A.L., Holmes, E.C., Feavers, I.M., and Maiden, M.C.J. (2004). Distribution of surface protein variants among hyperinvasive meningococci: implications for vaccine design. Infect. Immun. *72*, 5955–5962.

Usinger, W.R., and Lucas, A.H. (1999). Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. Infect. Immun. *67*, 2366–2370.

Vasu, K., and Nagaraja, V. (2013). Diverse functions of restriction-modification systems in addition to cellular defense. Microbiol. Mol. Biol. Rev. *77*, 53–72.

Vaughan, A.T., Gorringe, A., Davenport, V., Williams, N.A., and Heyderman, R.S. (2009). Absence of mucosal immunity in the human upper respiratory tract to the commensal bacteria *Neisseria lactamica* but not pathogenic *Neisseria meningitidis* during the peak age of nasopharyngeal carriage. J. Immunol. *182*, 2231–2240.

Virji, M., Alexandrescu, C., Ferguson, D.J., Saunders, J.R., and Moxon, E.R. (1992). Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and

endothelial cells. Mol. Microbiol. *6*, 1271–1279.

Virji, M., Makepeace, K., Peak, I.R.A., Ferguson, D.J.P., Jennings, M.P., and Moxon, E.R. (1995a). Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. Mol. Microbiol. *18*, 741–754.

Virji, M., Makepeace, K., Peak, I., Payne, G., Saunders, J.R., Ferguson, D.J., and Moxon, E.R. (1995b). Functional implications of the expression of PilC proteins in meningococci. Mol. Microbiol. *16*, 1087–1097.

Vogel, U., Taha, M.-K., Vazquez, J.A., Findlow, J., Claus, H., Stefanelli, P., Caugant, D.A., Kriz, P., Abad, R., Bambini, S., et al. (2013). Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment. Lancet Infect. Dis. *13*, 416–425.

Vu, D.M., Wong, T.T., and Granoff, D.M. (2011). Cooperative serum bactericidal activity between human antibodies to meningococcal factor H binding protein and neisserial heparin binding antigen. Vaccine *29*, 1968–1973.

Wainwright, L.A., Frangipane, J. V, and Seifert, H.S. (1997). Analysis of protein binding to the Sma/Cla DNA repeat in pathogenic *Neisseriae*. Nucleic Acids Res. *25*, 1362–1368.

Wang, X.-G., Olsen, L.R., and Roderick, S.L. (2002). Structure of the *lac* operon galactoside acetyltransferase. Structure *10*, 581–588.

World Health Organisation (2015). Global Health Observatory: Meningococcal Meningitis. http://www.who.int/gho/epidemic_diseases/meningitis/en/.

Wörmann, M.E., Horien, C.L., Bennett, J.S., Jolley, K.A., Maiden, M.C.J., Tang, C.M., Aho, E.L., and Exley, R.M. (2014). Sequence, distribution and chromosomal context of class I and class II pilin genes of *Neisseria meningitidis* identified in whole genome sequences. BMC Genomics *15*, 253.

Yi, K., Rasmussen, A.W., Gudlavalleti, S.K., Stephens, D.S., and Stojiljkovic, I. (2004a). Biofilm formation by *Neisseria meningitidis*. Infect. Immun. *72*, 6132–6138.

Yi, K., Rasmussen, A.W., Gudlavalleti, S.K., Stephens, D.S., and Stojiljkovic, I. (2004b). Biofilm formation by *Neisseria meningitidis*. Infect. Immun. *72*, 6132–6138.

Zheng, J.H., and Min, J.-J. (2016). Targeted cancer therapy using engineered *Salmonella typhimurium*. Chonnam Med. J. *52*, 173–184.