University of Southampton

Faculty of Medicine

Department of Clinical and Experimental Science

'*Neisseria lactamica* in biofilm'

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ABSTRACT

UNIVERSITY OF SOUTHAMPTON
ABSTRACT
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Neisseria lactamica in biofilm
By Sara Hughes

It is widely documented that *N. meningitidis* causes bacterial meningitis, which can also lead to sepsis, pneumonia and other life threatening infections (Rouphael and Stephens, 2012). According to the Meningitis Research Foundation in 2009-2010 there were 1469 cases of meningococcal meningitis and septicaemia in the UK alone. The commensal bacteria *Neisseria lactamica* shares 60 % genetic similarity with *N. meningitidis* (Bennett *et al*. 2010). *N. lactamica* carriage is high in early years of life but declines over time, with the inverse found in *N. meningitidis* carriage. It has been proposed that *N. lactamica* confers some protection against *N. meningitidis*. Previously over 350 volunteers have been inoculated with *N. lactamica* without any complications.

Biofilms offer individual bacteria a degree of protection compared to a planktonic state and also enhances the ability to evade the hosts' immune system allowing long-term colonization (Yi and Tian, 2012). *N. meningitidis* has been shown to form colony units known as biofilms (Yi *et al*. 2004). We investigated whether *N. lactamica* is able to form a biofilm *in vitro* and establish a working model. The developed method was applied to *N. meningitidis* isolates collected from a previous carriage study (Deasy *et al.* 2015), and biofilm formation observed. Finally it was investigated what, if any, relationship *N. lactamica* has with *N. meningitidis* whilst in biofilm.

The results found that *N. lactamica* can form a stable biofilm *in vitro*. Most *N. meningitidis* carriage isolates were also found to form biofilm, although no relationship between biofilm phenotype, serotype or capsule production was found. In biofilm no correlation between biofilm phenotype or impact on growth over 7 days was observed.

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Academic Thesis: Declaration Of Authorship

I, ... [please print name]

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.

.....*Neisseria lactamica* in biofilm.....

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LIST OF ABBREVIATIONS

A
API – Analytic Profile Index
AST – Antibiotic Susceptibility Testing

B
BHI – Brain Heart Infusion
BIGSdb – Bacterial Isolate Genome Sequence Database

C
CBA – Columbia Blood Agar
CDC – Centers for Disease Control and Prevention
CEACAMs – Carcinoembryonic Antigen-related Cell Adhesion Molecules
CFU – Colony Forming Unit
CO_2 – Carbon Dioxide
CPD – Critical Point Drying
CSF – Cerebral Spinal Fluid
CV – Crystal Violet

D
DIC – Disseminated Intravascular Coagulation
DNA – Deoxyribonucleic Acid
DMSO – Dimethyl Sulfoxide

E
eDNA – extracellular Deoxyribonucleic Acid
EMA – European Medicines Agency
EPS – extracellular polymeric substance

G
g – Gravitational Force
GC- selective – Gonococcus selective agar
GSK – Glaxo Smith Kline

H
HBE cells – Human Bronchial Epithelial cells
HBSS – Hank's Balanced Salt Solution

I
ID – Identification
Ig – Immunoglobulin

J
JVCI - Joint Committee on Vaccination and Immunisations

L
LPS – Lipopolysaccharide

LIST OF ABBREVIATIONS

M
MBC – Minimum Bactericidal Concentration
MIC – Minimum inhibitory Concentration
MLST – multi locus sequence typing
MSC – Microbiological Safety Cabinet

N
NHS – National Health Service
NK cells – Natural killer cells
Nuc – Nuclease

O
OD – Optical Density
OMP – Outer Membrane Protein
OMV – Outer Membrane Vesicle

P
PCR – Polymerase Chain Reaction

S
SEM – Scannning Electron Microscope

T
TfP – Type IV pili
TM agar – Thayer-Martin Agar
tRNA – transfer ribonucleic acid
TSB – Typtic Soy Broth

U
UV – Ultra Violet

V
V/V – volume to volume
VBNC – viable but non-culturable

X
X-gal – 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

INTRODUCTION CHAPTER 1

"Why is the study of Neisseria important?"

1.1 INTRODUCTION

The genus *Neisseria* is generally limited to modern Humans, named after the discoverer Albert Neisser, who in 1879 observed 'micrococci' from 26 adults with gonorrheal urethritis (Ligon, 2005). Eleven species are known to colonize humans, but only two of these are pathogenic; *Neisseria meningitidis* which frequents the nasopharynx, and *Neisseria gonorrhoeae* which occupies the urogenital tract. Common interactions between *Neisseria* and humans tend to be silent and non-threatening, with the micro-organism occupying the mucosal recess as a commensal without the host being aware of its presence. Should *N. meningitidis* enter the bloodstream however, the subject is at risk of sepsis, meningitis and possible death if left untreated. The first meningitis outbreak was recorded in Geneva in 1805 (Greenwood, 2006), this was followed by several outbreaks across Europe and the United States. Survivors of aggressive *N. meningitidis* infections are often left disabled, with a loss of hearing or complete deafness, amputation and the effects of amputation or neurological impairments (Viner *et al.* 2012). The ability of *N. meningitidis* to maintain carriage is impressive, with some individuals enabling carriage for up to 12 months (Trotter, 2006). Quiet non-invasive carriage demonstrates that invasion into the bloodstream is a rare event, suggesting some serendipity is required with the organism preferring a commensal state. The mechanism of long term carriage is not yet fully understood. This thesis explores the theory that carriage could be promoted by biofilm formation and that long-term carriage of commensal *Neisseria* confers some competition to invasive *Neisseria meningitidis*.

CHAPTER 1 INTRODUCTION

1.2 MICROBIOLOGY

Neisseriaceae are a family of β-Proteobacteria of gram negative bacteria from 14 genera including *Neisseria* (Whitman, 2005). Typically aerobic to facultative anaerobic, the coccoid bacteria normally present in pairs. They are commonly found as part of the oral flora and are seen as commensal organisms, with the exception of the potentially invasive *N. meningitidis* and the sexually transmitted *N. gonorrhoeae*.

1.2.1 MULTI-SPECIES FLORA

Neisseria is not the only species to exclusively colonise the nasopharynx, laryngotracheal stents are often found to be colonised with multispecies biofilms containing the *Neisseria* genera, however it has yet to be determined if colonisation is due to oral or nasopharyngeal *Neisseria* (Neil and Apicella, 2009). This microbial niche is shared with other commensal bacteria such as *Streptococcus*, *Staphylococcus* and *Haemophilus* to name a few. Whilst all these bacteria have evolved specifically for colonisation on epithelial cells, they also need to compete with each other for the sparse resources available. Oral flora species appear to be dependent on many factors including age and life style habits. The interactions between *Haemophilus influenzae* and *Streptococcus pneumoniae* for example are well documented and the two species are regularly co-cultured together. Studies indicate that both species exist together in a synergistic state as a biofilm (Tikhomirova and Kidd, 2013). Additionally, it has been suggested that there may also be survival benefits for these species when found together; this may be due to the biofilm environment and the wider genetic reservoir available in a multi-species biofilm. There is limited literature regarding the impact other species have on *Neisseria* and *vice versa*; however it has been suggested that previous colonisation of *N. lactamica* may give some protection against *N. meningitidis* due to cross-reacting antibodies. Given that colonisation of the upper airway tract is almost immediate from birth, the relationship between species like *Neisseria*, *Haemophilus*, *Staphylococcus* and *Streptococcus* has yet to be fully explored and understood.

1.2.2 GENEALOGY

N. lactamica and *N. meningitidis* are both closely related, along with *N. gonorrhoeae*, suggesting that divergence is a somewhat recent event (Bennett *et al*. 2010). All three have a similar genome in size, composition and architecture. From the phylogenetic tree (Figure 1) it is clear to see how the *Neisseria* species has diverged. The close proximity between *N. lactamica* and *N. meningitidis* means they share 60% genetic similarity (Bennett *et al*. 2010). This idea of *N. meningitidis* being a recent pathogen also corroborates with the medical literature. The first outbreak of meningitis wasn't fully documented until 1805 although ancient medical texts do describe meningitis-like symptoms commonly associated with meningitis, it is not commonly documented unlike the symptoms for influenza that appear frequently throughout history across the globe.

Cross species recombination of the core genome has been identified as rare; this was demonstrated by a particular core nucleotide sequence diversity of each group. Genetic analysis has demonstrated that cross species recombination does occur in many other genes, with some evidence of hitch-hiking within the housekeeping genes. It was also shown that the *cps* region in *N. meningitidis* is exclusive; this particular region encodes capsule production. By comparing the genomes of both *N. meningitidis* and *N. gonorrhoeae* with *N. lactamica*, no single set of genes was identified as being essential for disease. The lack of this 'pathogenome' demonstrates that virulence amongst pathogenic isolates is complex (Bennett *et al*. 2010).

CHAPTER 1 INTRODUCTION

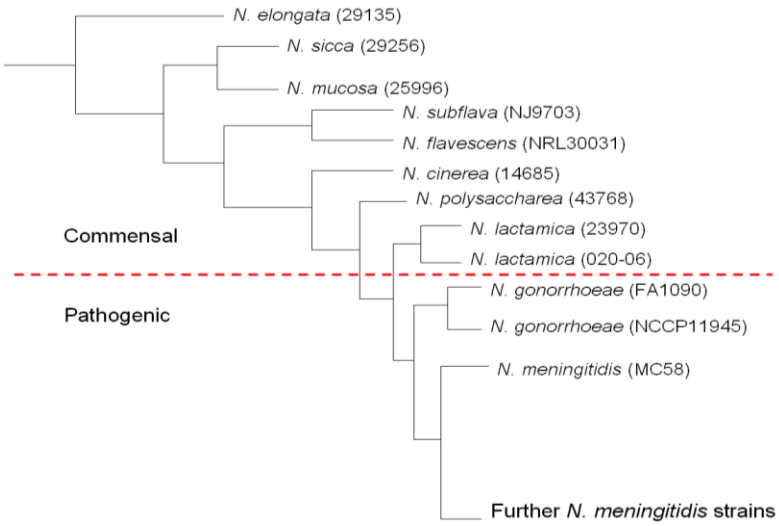


Figure 1 – A simplified schematic drawing of the *Neisseria* phylogenetic tree

The schematic diagram visually demonstrates the close relationship between the commensal *N. lactamica* and the pathogenic *N. meningitidis*, showing that divergence is relatively recent.

1.3 ADAPTATIONS FOR CARRIAGE

The *Neisseria* species is extremely well adapted to life on a mucosal surface, with the exception of *N. gonorrhoeae* the majority of species are found in the upper airway tract and nasopharynx (Figure 2). The presence of non-virulent meningococci in the oropharynx produces an antibody response within a few weeks of acquisition resulting in clearance (Yazdankhah and Caugant. 2004). The ability to transmit via aerosolised water droplets, attach to mucosal surfaces, replicate in nutrient poor environments, evade the immune system and thrive in a polymicrobial environment are all adaptations required to survive in the nasopharyngeal niche.

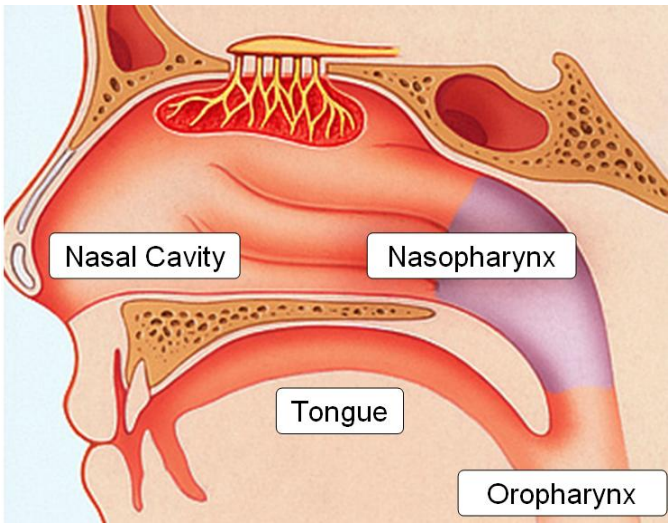


Figure 2 – Diagram showing the location of the nasopharynx within the upper respiratory system.

The nasopharynx is located at the back of the nasal cavity, above the soft palette and before the oropharynx.

1.3.1 EPIDEMIOLOGY

Young infants typically carry the non-capsulated commensal *N. lactamica*, which is commonly lost as they grow into toddlers; the exact mechanism for this is poorly

described in the literature. The occurrence of *N. meningitidis* starts to increase from this age and peaks in adolescence, thus suggesting a possible link between *N. lactamica* and *N. meningitidis* (Figure 3). Overall community carriage of *N. meningitidis* is between 10-25%, depending on population, age group and social behaviour (Caugant *et al.* 2007). Contributing factors of carriage are usually smoking and close contact with other individuals, including close living quarters (Trotter *et al*. 2006) such as in army barracks, prisons and student populations. In student populations up to 55% carriage can be observed; however this percentage is temporary and generally drops post-Freshers week (Ala'aldeen *et al*. 2011; Neal *et al*. 2000).

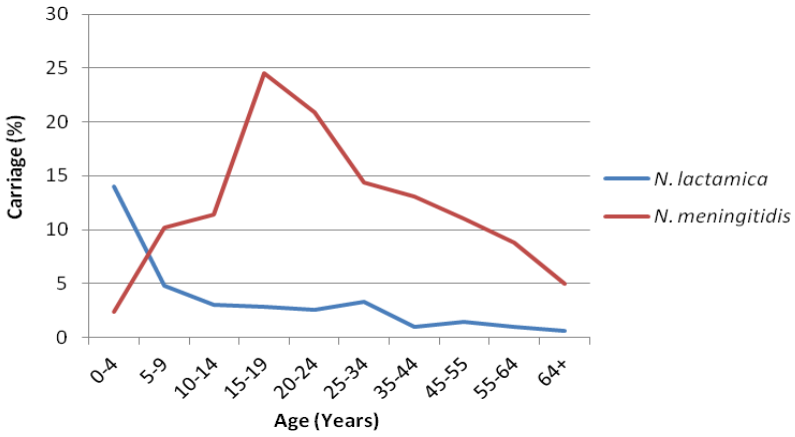


Figure 3 – Graph demonstrating the carriage rate of both *N. lactamica* and *N. meningitidis* during the 1986 *N. meningitidis* outbreak in the UK.

As carriage of *N. lactamica* decreases there is an increase in the carriage rate of *N. meningitidis*.

Source: Data was taken from Cartwright *et al*. (1987) The Stonehouse Survey – nasopharyngeal carriage of meningococci and *Neisseria lactamica*.

1.3.2 CAPSULE

One important and well-studied virulence factor which has been identified and exclusive to *N. meningitidis* in the *Neisseria* genus is the capsule (Figure 4). This

polysaccharide case confers resistance to complement-mediated killing (Kahler *et al*. 1998) and phagocytosis, as well as preventing toxic materials such as detergents from direct contact with the cell. Disease-causing *N. meningitidis* typically expresses one of six individual capsular polysaccharides; A, B, C, W, X and Y. However, it is the non-capsulated *N. meningitidis* that are commonly isolated from asymptomatic healthy humans (Arenas *et al.* 2015). It has been previously demonstrated that the capsule hinders attachment and adhesion of the bacteria to the nasopharynx mucosal epithelia (Virji *et al*. 1993). However, the polysaccharide capsule is generally credited with sustaining and aiding transport by preventing desiccation of the cell. Although each polysaccharide sub-group has a different rate of desiccation *in vitro*, especially in those bacteria expressing serogroup B and those that expressed different variants of PorA (Swain and Martin, 2007), there is little literature describing the exact nature of how *Neisseria* are adapted for aerosol transmission. Listed as highly infectious by most public health organisations across the globe, *N. meningitidis* is transmitted by a colonised individual through water droplets. This may be via general coughing or sneezing, sharing drinks or intimacy. It had been previously suggested that transfer may be possible from infected receptacles such as glassware which has not been properly cleaned and sterilised, however evidence for this is sparse (Brigham and Sandora, 2009). Clinically individuals will remain infectious so long as there are bacteria present for 24 hours post antibiotic treatment.

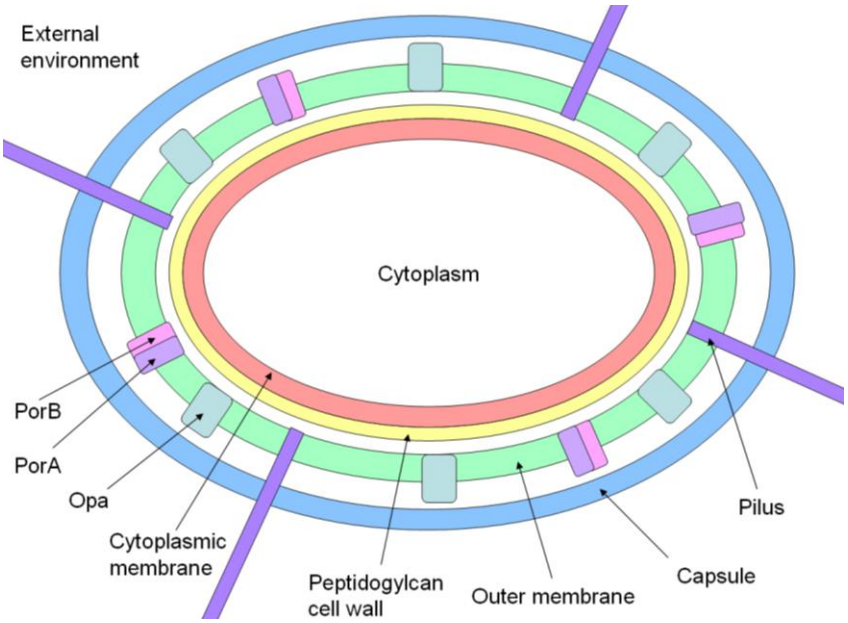


Figure 4 – Schematic diagram of *N. meningitidis*.

The Capsule provides an additional protective layer to the cell against the hosts' immune system, and against desiccation during transmission. However the role of capsule in biofilm formation is hypothesised to be a hindrance as outer membrane proteins such as NadA have been shown to be important in adhesion of the bacterium to the host membrane.

1.3.3 TYPE IV PILI

Several surface antigens have been shown to play a critical role in colonisation and cell adherence. Type IV pili (TfP), Opc, Opa and NadA are all inter-strain variable and have been shown to be important in cell adhesion and colonisation (Trivedi *et al*. 2011). TfP enable initial attachment to both non-ciliated epithelium and the mucoidal layer of the nasopharynx (Trivedi *et al*. 2011). Meningococci pili are composed of several pilin proteins, Pilin E being the most predominant, enabling adhesion to both endothelial and epithelial cells. Pilin C (PilC), at the end of the pilus is commonly employed to mediate the binding of the bacterium to the epithelial receptors of the host. PilC is encoded by two alleles; *pilC1* and *pilC2*. Adhesion to human epithelial cells only

requires PilC1; however, PilC2 has been shown *in vitro* to adhere to cancerous cervical ME180 cells, so it is no surprise that PilC2 is also expressed in *N. gonorrhoeae* (Morand *et al.* 2009). The minor subunits Pilin V and Pilin X are found at the base of the pilus, and serve as a foundation to enable micro-colony formation and aggregation (Helaine *et al*. 2005). Pilin Q forms a pore within the outer membrane in which the pilus can protrude. Pilin F, Pilin M, Pilin N, Pilin O and Pilin P all contribute to pilus synthesis, whilst Pilin G, Pilin H, Pilin I, Pilin J, Pilin K, and Pilin W have been identified with functional maturation and stability (Carbonnelle *et al*. 2005). Pilin V has been shown to enable survival in environments where sheer stress is exhibited, such as in the blood stream, conferring another virulence factor to the meningococcus (Takahashi *et al*. 2012). TfP are also phase variable and glycosylation of phase variable pili may allow colonisation on other epithelial surfaces by releasing bacteria from larger micro-colonies.

1.3.4 OUTER MEMBRANE PROTEINS

Opa and Opc are outer membrane proteins which are significant in adhesion to epithelial, endothelial and polymorphonuclear neutrophil cells. *N. meningitidis* commonly encodes four Opa proteins with the loci dispersed throughout the whole of the genome. Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are a group of immunoglobulin proteins important in cell-to-cell recognition, enabling T-cell migration, tissue shaping and organisation. CEACAMs are found on a wide range of cells, from epithelial cells to lymphocytes. The *Neisseria* species have fully exploited this group of proteins; CEACAM-specific Opa proteins are abundant on the *Neisseria* cell surface which adheres to CEACAMs present on the host cell surface. After initial attachment by pili, the pilus contracts and enables long-term attachment to epithelial cells via CEACAM-specific Opa proteins. This attachment allows the meningococci to be engulfed and pass through or in between epithelial cells (Figure 5). Like TfP, opa and opc are also subject to phase variation (De Jonge *et al*. 2002).

T lymphocytes play a huge part in mediating an immunological response to an infectious agent as well as immune memory and cell-mediated immunity. Lymphocytes

make up the majority of cells involved in adaptive immunity with the average human adult containing around 2 x 10^{12} lymphocytes (Albert *et al*. 2002). The group consists of T cells, B cells and natural killer (NK) cells (which are also involved in innate immunity). T and B cells are able to tailor their response to a pathogen to optimise clearance, whereas NK cells release cyto-toxic granules to kill infected cells. Experiments by Wiertz, *et al* (1991) with Opa demonstrated that T lymphocytes produce a strong immune reaction, more so than against any of the other virulent outer membrane proteins (OMPs). The group used 16 synthesized class 1 OMP-derived peptides and exposed them to T-cells. However, other studies have shown that when CD4+ T lymphocytes were exposed to Opa-expressing *N. meningitidis*, their activation and immunological response was prevented (Sadarangani *et al*. 2011). This effect was due to the Opa binding to CEACAM-1 present on T lymphoctes, but was not seen in other variants of CEACAM. By preventing T lymphocyte activation meningococci are able to avoid an escalated attack.

Neisseria adhesin A (NadA) is also involved in adherence of the bacteria to the host cell, however the *nadA* gene is only present in 50% of three of four hypervirulent *N. meningitidis* isolates and is completely absent in commensal *Neisseriae* and *N. gonorrhoeae* (Capecchi *et al*., 2005). It has therefore been identified as a vaccine target. NadA has been shown to be closely related to YadA found in the *Yersinia* genus, which is primarily involved in adhesion to the host epithelium and therefore an increase in virulence (Nagele *et al*. 2011). Even in the presence of the *nadA* gene the amount of NadA expressed varies greatly *in vitro* when different strains are compared. This not only makes NadA difficult to study but also implies the mechanism of *nadA* regulation is more complicated than first anticipated (Fagnocchi *et al*. 2013).

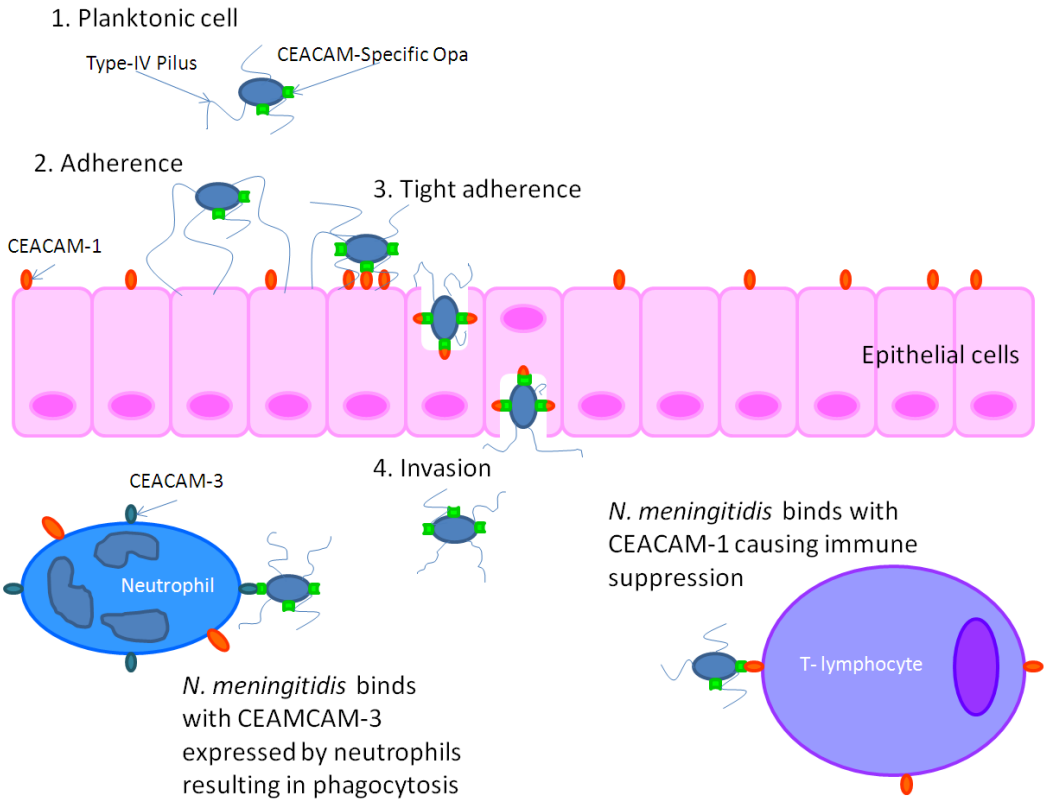


Figure 5- Diagram demonstrates the ability of the meningococcus to use CEACAM-specific proteins to avoid detection by the host immune system.

CEACAM-specific Opa proteins are able to interact with CEACAM-1 to allow *Neisseria* to adhere and pass through the epithelial layer. *N. meningitidis* is able to bind with T-lymphocytes to suppress the immune system. If *N. meningitidis* binds with CEACAM-3 expressed on the surface of neutrophils, the pathogen is phagocytosed.

1.3.5 BIOFILM

Interest in biofilm-forming bacteria has been steadily increasing over the past decade due to the recognition of the role they play in chronic infection (Bjarnsholt 2013). A biofilm is generally described as a community of organisms, encased within a self-produced extracellular matrix and associated with a surface. They can be found in the human host, the natural environment, on abiotic surfaces, and on other living matter (Karatan and Watnick. 2009). Studies in a variety of bacterial species have

demonstrated that bacteria have an increased survival rate in the biofilm phenotype when compared to the planktonic mode of growth. This protection includes fortification from UV light, reactive oxygen species, dehydration, changes to osmolarity, and antimicrobial agents (Hall-Stoodley *et al.* 2004). Biofilm formation also enhances the ability to evade the host immune system allowing long-term colonization. Biofilms pose problems in hospitals as colonisation of medical devices, especially venous and urinary access ports, lead to additional infection(s) and further inhibit medical treatment.

It is generally accepted that biofilm formation occurs in five stages;

- Stage I – initial reversible surface attachment
- Stage II – Irreversible adherence where the pilus contracts and attachment to the host occurs (in *Neisseria* this is via CEACAM-Specific Opa and the host CEACAM-1
- Stage III – Bacterial aggregation and micro-colony formation
- Stage IV – Biofilm maturation and extracellular matrix production
- Stage V – Detachment of sections of the biofilm to enable colonisation of a new area.

The length of these stages varies from species to species, and even between various isolates.

The human host can carry both *N. lactamica* and *N. meningitidis* asymptomatically for a considerable period of time in the nasopharynx (Bennett *et al*. 2010). The often-asymptomatic *N. gonorrhoeae* is also competent at evading clearance, suggesting a role for the biofilm phenotype in carriage and disease pathogenesis (Greiner *et al*. 2005). Biopsy samples have shown *N. gonorrhoeae* forms biofilms as part of the natural infection of the cervix, in continuous flow systems, in static infected cervical epithelial cells and primary human urethral cells *in vitro* (Steichen *et al*. 2008). *N. gonorrhoeae* lacks the genes required for production of an exopolysaccharide coat,

and instead membrane blebbing (the bulging of the plasma membrane of a cell) is required for biofilm formation (Steichen *et al*. 2011). It was thought the polysaccharide capsule of some meningococcal species inhibits biofilm formation as uncapsulated meningococci form biofilms using standard methods (Yi *et al*. 2004., Neil *et al*. 2009). However, biofilm formation of encapsulated meningococci was shown using an epithelial model and demonstrated how long-term carriage could be achieved (Neil *et al*. 2009). It is theorized the *Neisseria* species forms biofilms on human mucosa to survive.

Although evidence of respiratory biofilms i*n situ* from patients with chronic infections have been identified (Hall-Soodley and Stoodley, 2009), evidence of meningococcal biofilms *in situ* is extremely limited. Current identification methods often involving nasopharyngeal swabbing to detect carriage, which are not compatible with biofilm identification. A large widespread study of excised tissue (adenoidectomy samples from children, tonsillectomies, sinus repair surgery samples, etc.) would be preferential and more beneficial. There are also limitations with nasopharyngeal swabbing which has previously been shown to be selective. Sim *et al* (2000) swabbed 32 patients prior to tonsillectomy surgery. Of those swabbed 10% tested positive for *N. meningitidis* using traditional identification methods; however immunolabeling methods, targeting the outer membrane protein PorA, increased the rate of positive detection to 45% of the excised tissue. PorA is exclusive to meningococcus, demonstrating that nasopharyngeal swabbing alone is not a conclusive indicator of carriage.

Purpura fulminans occurs in 10-20% of severe meningococcal sepsis and can in extreme cases lead to thrombosis of large vessels (Harrison *et al*. 2002). The ability of the meningococcus to interact with the host tissue, especially with inflammatory cells and the endothelium lining of the blood vessels, is impressive for a bacterium suited for the nasopharyngeal environment. Skin biopsy samples taken from patients with purpuric lesions showed that *N. meningitidis* clusters and aggregates into micro-colonies around the blood vessels (Harrison *et al*. 2002). It is possible that these micro-colonies are the beginnings of biofilm formation in the tissue.

CHAPTER 1 INTRODUCTION

Evidence of *N. meningitidis* forming biofilm *in vitro* is limited in the literature, and the debate of whether encapsulated meningococci are able to form biofilm is still hotly deliberated. It is possible that *N. meningitidis* is able to down-regulate capsule production and therefore allow biofilm formation (Neil *et al*. 2009). Encapsulated *N. meningitidis* has been shown to form biofilm in vitro using an epithelial model (Lappann *et al*. 2010), but this has yet to be identified in a clinical setting. *N. meningitidis* isolates identified from diseased patients tend to be capsulated (Johswitch *et al*. 2012).

Proteomic studies comparing *N. meningitidis* in a planktonic and biofilm state demonstrated that proteins associated with oxidative stress (SodC and MntC) are highly up-regulated in biofilm (van Alen *et al*. 2010.).The protein LeuA involved in amino acid synthesis was also found to be up-regulated (van Alen *et al*. 2010.). van Alen *et al* (2010) theorised that the production of reactive oxygen species (ROS) in biofilms is a by-product of aerobic respiration due to the close proximity of the cells in a matrix, and therefore the species has adapted a coping mechanism to deal with elevated levels of ROS.

Evidence of *N. lactamica* biofilms is poorly documented in the literature but could offer an explanation of how the commensal is able to maintain carriage for long periods of time (Neil and Apicella 2009).

1.4 DISEASE

Although generally a commensal, invasive *N. meningitidis* has an incubation period of 2-10 days with invasive infection occurring within 14 days of acquisition. Should *N. meningitidis* enter the blood stream the subject is at risk of sepsis, meningitis and potentially death if left without treatment. Disease is caused when the bacteria break through the epithelial barrier within the nose or throat and access the blood stream. Although the exact mechanism of this is unknown, it is generally accepted that a range factors influence cell invasion, including the hosts' predisposition to infection, the environment, and the particular stain carried. Despite advances in modern medicine and improved medical care bacterial meningitis is still prevalent. Mortality rates have been reported as high as 34% with up to 50% of survivors suffering long-term consequences of the disease (Hoffman and Weber, 2009).

1.4.1 EPIDEMIOLOGY

Although *N. meningitidis* has a fierce reputation, it is best suited to being a commensal not an invasive organism. Indeed the relatively recent documentation of bacterial meningitis suggests that *Neisseria* have been extremely successful in occupying this commensal niche. *N. meningitidis* in Europe today causes meningococcal disease with an average annual incidence of 1:100,000 persons with a case fatality rate of 8% (Trotter and Ramsay, 2007). The average annual incidence during African epidemics is around 100-800:100,000 persons (http://www.meningvax.org/epidemics-africa.php). Although *N. meningitidis* can be isolated from asymptomatic carriers throughout the year, like many respiratory pathogens, in Europe incidence peaks during mid-winter to early spring (Rosenstein *et al.* 2001). Incidence peaks across the Sub-Saharan during the dry season between (World Health Organisation, 1998). The higher fatality rate can be contributed to the climate, overcrowded housing, social habits and customs, and regional traditional markets increasing transmission rates (http://www.who.int/mediacentre/news/notes/2007/np12/en/).

1.4.2 GENE REGULATION

Genetic analysis of *N. meningitidis* Men B (strain MC58) on epithelial 16HBE14 cell lines has shown that 7-14 % of the genome is differentially regulated over 21 days. Unsurprisingly genes known to encode adhesion proteins were shown to be among those up-regulated (Hey *et al*. 2013). Late invasive infection after long-term colonisation seems to be rare, so incidences of disease are thought to occur soon after colonisation. The interaction between bacterial pili and the host epithelium is well studied, and it has been suggested that it is these interactions which fuel the mutations and changes of the meningococcal gene pool. Bacteria that do enter the blood stream are often not afforded the chance to diversify due to the bactericidal activity of the hosts' immune system, hospital treatment or death of the host (Budroni *et al*. 2011).

1.4.3 SEPSIS

Meningococcemia as a cause of sepsis is widely documented. Like several other gram-negative bacterial infections, meningococcemia can cause disseminated intravascular coagulation (DIC) (Campbell *et al.* 1997). In normal, healthy individuals proteins found in blood that assist with clotting travel to the site of injury. In subjects with DIC, these proteins are abnormally active causing small clots (thrombi). Organ damage occurs when thrombi obstruct blood flow, leading to poor and restricted blood supply to host organs and can also cause erythrocytes to lyse leading to increased levels of free iron in the body (McKay, 1968). The classic skin rash associated with meningitis is caused by small bleeds, which have resulted from the destruction of the small blood vessels by toxins. Invasive meningococci release blebs of outer membrane vesicles into the bloodstream; these vesicles are rich in endotoxin (van Deuren *et al*. 2000). Endotoxins are often referred to as lipopolysaccharide (LPS), and form part of outer membrane of gram-negative cells so therefore pose no enzymatic activity. The immune system is highly sensitive to the polysaccharide component, so activates the complement system (Hellerud *et al*. 2008). Initial symptoms are similar to those suffering from influenza; however later symptoms include septic shock (possibly resulting in multiple organ dysfunction syndrome), seizures and hypotension (Coureuil *et al*. 2013).

1.4.4 MENINGITIS

Meningitis is the inflammation of the meninges, the protective lining surrounding the central nervous system, including the spine and the brain. Invasive *N. meningitidis* is able to cross first into the bloodstream and then to the normally sterile meninges (Coureuil *et al*. 2012). *N. meningitidis* is able to cross the blood brain barrier and access the subarachnoid space, from here it is able to enter the cerebral spinal fluid (CSF) (Vincent *et al.* 2011). High yields of bacteria can be identified within the hosts' CSF as the bacterial population increases and moves from the blood stream (Hoffman and Weber. 2009). Swelling is caused by increased numbers of bacteria and white blood cells migrating into the CSF and releasing inflammatory mediators, which in turn increases the permeability of the blood-brain barrier (Vincent *et al.* 2011). The increased accessibility of the blood-brain barrier has a detrimental effect to the host; neutrophils release inflammatory proteins, enzymes and proteases, which cause neuronal damage (Hoffman and Weber, 2009). The bacteria cannot however cross the sub-pia tissue and the neurological symptoms associated with meningitis are caused by pro-inflammatory cytokines, which are released in response to the bacterial presence (Vincent *et al*. 2011). Of all meningococcal deaths, septicaemia accounts for 25%, meningitis for 15% and 60% for a combination of the two (https://www.nice.org.uk/guidance/cg102/chapter/introduction?unlid=72909131020161251162).

Meningitis can progress rapidly, initial symptoms can be quite generic; sore throat, cough, headache, fever, nausea, vomiting, drowsiness, rapid breathing, etc (http://www.nhs.uk/Conditions/Meningitis/Pages/Symptoms.aspx). It is not normally until diagnostic tests are completed or the patient develops other 'classic' meningitis symptoms such as a stiff neck, dislike of bright lights (Hoffman and Weber, 2009) and a rash that a diagnosis is suspected. There are a handful of documented cases of *N. lactamica* causing disease, however these are extremely rare events

1.4.5 CURRENT TREATMENT

N. meningitidis is a gram negative bacterium, so is susceptible to the majority of common antibiotics. Antibiotic treatment of meningitis should be immediate (Hoffman and Weber, 2009) as to prevent further tissue damage. General intravenous antibiotics such as Cephalosporin (including Ceftriaxone) normally given until a confirmed microbiological culture is obtained; upon a confirmed diagnosis targeted antibiotics or if antibiotic resistance is suspected antibiotic chemotherapy (Hoffman and Weber, 2009). Treatment usually lasts between 5 – 14 days depending on the severity of the disease.

Patients with meningitis may also be given corticosteroids to reduce the intracranial hypertension and meningeal inflammation, although several studies have shown varied results of using such steroids on patients with meningitis (Hoffman and Weber, 2009).

1.4.6 PREVENTION

Preventing the spread of *N. meningitidis* during an outbreak is critical for those who are unable to be vaccinated. This is especially apparent in Africa, where access to vaccines may be limited. Although general healthy life choices and good hygiene are all acceptable prevention methods, there is very little else that can be done to prevent individuals carrying *N. meningitidis* without access to a vaccination programme. Several risk factors including current respiratory infection (either viral or bacterial), smoking (including passive smoking), and people of a low social-economic status, regardless of ethnic origin are more likely to develop meningococcal disease (Yazdankhah and Caugant, 2004). Previous silent carriage can help individuals, however it requires a subject to have previously cleared a non-virulent strain, and have developed cross-reacting antibodies.

Chemoprophylaxis could eliminate nasopharyngeal carriage, indeed in the 1950's sulphonamides successfully eradicated meningococcal carriage (Yazdankhah and Caugant, 2004). Sulphonamides were used up to 1960, since sulphonamide-resistant strains have been identified from both carriers who had not previously been exposed

to sulphonamides and patients with meningococcal disease (Yazdankhah and Caugant, 2004).

1.4.7 DIAGNOSTIC TESTING

Diagnostic tests for patients suspected of suffering from suspected meningitis are highly invasive. A lumbar puncture is performed in order to extract cerebral spinal fluid (CSF). The CSF can then be analysed for the presence of bacteria (Hoffman and Weber, 2009), given that several species of bacteria can cause meningitis PCR is often a quick diagnostic tool of choice alongside traditional culturing methods, which take longer but have the benefit of being cheaper.

1.5 VACCINES

Vaccinations against *N. meningitidis* have proved tricky due to the variability of the species. Polysaccharide vaccines typically contain the polysaccharide capsule of the bacterium, generally considered to be a major virulence factor. Polysaccharide vaccines elicit an immune response without T-Cells. In contrast conjugate vaccines promote a greater immune response as they usually contain an additional protein carrier to the original polysaccharide. The addition of this protein carrier produces a response in T-Cells, which results in the production of IgG antibodies, and longer lasting B Cells. (Granoff, 1997) The majority of polysaccharide vaccines are modest and fail to induce a lasting immunological memory (Arenas *et al*. 2015). There are several vaccines available on the market but these are usually limited to the serogroups B, C and Y and are not 100% effective.

1.5.1 CURRENT VACCINES

There are currently three meningococcal vaccines available in the UK; all are currently available and given as part of the National Health Service (NHS) vaccination programme. The Hib/MenC vaccine given at one year offers protection to both *Haemophilus influenza* type B and meningococcal group C, and the MenACWY vaccine currently only offered to teenagers and first-time students. The MenACWY and Hib/MenC vaccines have replaced the older MenC vaccine that was first given in 1999. Those who have already received the MenC vaccine are still eligible to have the MenACWY or Hib/MenC should they require it. The third vaccine offers some protection against MenB, developed by Novartis and manufactured by GlaxoSmithKline (GSK), Bexsero® (also known as 4CMenB vaccine) was licensed for use in 2014. (http://www.nhs.uk/Conditions/vaccinations/Pages/men-acwy-vaccine.aspx). In 2015, following advice from the Joint Committee on Vaccination and Immunisations (JCVI) the department of health announced that the vaccination programme was including Bexsero®, this vaccine is now available routinely to infants from 2 months of age.

A study conducted following the UK national Meningococcal Conjugate (MCC) vaccine programme (NHS MenC vaccine) in 1999 demonstrated that there was a major reduction of *N. meningitidis* serogroup C carriage but had little to no effect on other serogroups (Maiden *et al*. 2002). The MenC vaccine is an inactive, conjugate vaccine which has seen annual confirmed cases of meningococcal disease caused by serogroup C decline dramatically since introduction (Figure 6).

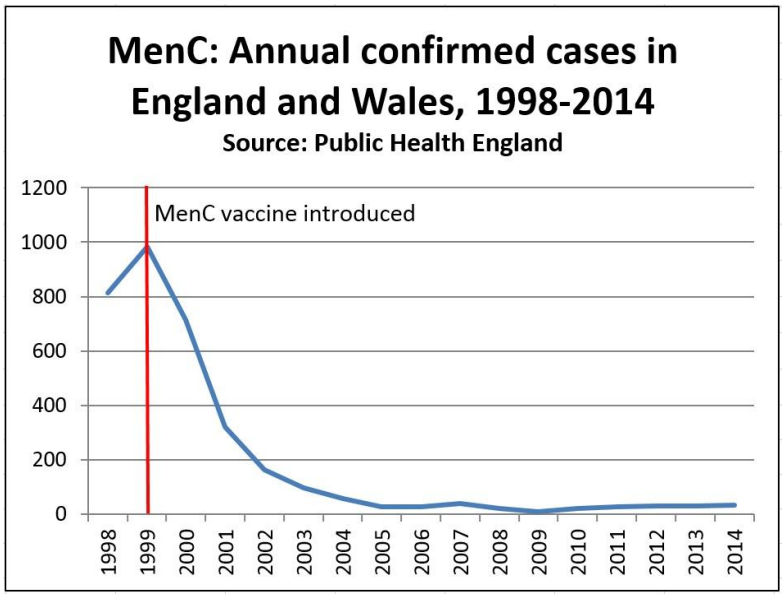


Figure 6 – The dramatic effect of immunisation against Men C.

The vaccine was first dispensed in 1999 and has resulted in a 90% decrease in Men C cases in the UK.

Source: Public Health England

Initial studies have shown that Bexsero® is cost effective. Christensen *et al* (2014) estimated that 1447 cases of meningococcal disease and 59 deaths could occur in the UK without vaccination, this number is decreased by 26.3% within the first five years of Bexsero® being introduced in the vaccination program. This number then further decreases by 48.8% in 10 years and 59.7% in 20 years, assuming that public opinion

remains favourable towards vaccines and vaccination programs. A large phase III study involving 3,630 infants demonstrated that compared to routine vaccination of MenC alone, Bexsero® vaccinated infants produced more meningococcal C bactericidal antibodies (81.7% compared to 99% respectively) implying better protection (Gossger *et al*. 2012). The JCVI however, concluded that there was not enough evidence to suggest these results are replicated in older children, teenagers and adults.

Since beginning the Bexsero® vaccination programme in the UK, Parikh *et al* (2016) demonstrated that uptake of the vaccine was good (94.8-95.5 % for the first dose at 2 months of age, and 84.8-88.6 % for the second dose at 6 months of age). A 50% reduction in the incidences of Men B cases was recorded between 1st September 2015 and 30th June 2016 in vaccine eligible infants when compared to cases in previous years. The biggest improvement was noted in the 'catch-up' group where 58 % relative reduction was observed (Parikh *et al*. 2016). The 'catch-up' group defined as those infants born between 1st May 2015 and 30th June 2015 (Meningococcal B immunisation programme Factsheet for healthcare professionals, 2017 http://www.publichealth.hscni.net/sites/default/files/Men%20B%20Factsheet%202017_1.pdf).

It was concluded that the vaccine programme aligned well with the health economic model previously used to estimate meningococcal cases (Parikh *et al* 2016).

The model predicted that Bexsero® will provide cover for 88 % of Men B strains, which will result in 94.2 % coverage against vaccine-preventable strains (Christensen *et al.* 2014).

In 2015 the US licensed Pfizer Trumenba® vaccine launched. Aimed at 10-25 year olds, the vaccine targets serogroup B (Shirley and Dhillon, 2015). The vaccine contains two variants of the meningococcal factor H-binding protein (fHBP) (Shirley and Dhillon, 2015). FHBP is a surface protein which inhibits the complement pathway, and thus promotes immune evasion (Rossi *et al.* 2016). The European Medicines Agency (EMA) confirmed the vaccine can be given to patients who have previously received the MenACWY (Product information WC500228995 pdf - http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/004051/WC500228995.pdf). The Centers for Disease

Control and Prevention (CDC) later confirmed that either Trumenba® or Bexsero® could be administered to offer protection against serogroup B meningococcal disease, but that the two vaccinations are not interchangeable and a full course must be completed.

A total of 170 11 to 18 year old volunteers were vaccinated between March 2010 and Feb 2011 with Trumenba® (Clinical Trial number: NCT00808028). Marshall *et al* (2017) found that after three doses of Trumenba®, protective human serum bactericidal activity (hSBA) remained high in 50% of the participants after four years. The group concluded that further studies would be required to assess the need for a booster and any possible effect on herd immunity.

1.5.2 Effect of Vaccines on Carriage

There have been several studies conducted in the UK investigating the effect of herd immunity, carriage rates and reduction in disease. When first introduced the effect of the meningococcus C conjugate vaccine on carriage rates was unclear but previous vaccines, such as the Hib conjugate vaccine, had successfully shown to reduce carriage in those individuals who had received the vaccine (Barbour *et al.* 1995). Vaccination with serogroup C polysaccharide produced mixed results on meningococcal carriage. Several groups reported that local immunity in the nasopharynx toward meningococci had increased (Sandarangani *et al*. 2011). However, this effect appears to have been a short-term one, as seroprevalence studies later indicated that antibody levels drop and return to pre-vaccination level as soon as 6-12 months post-vaccination. The effect of serogroup A and C polysaccharide was observed in Spanish children and adolescents. Researchers observed a decrease in group C carriage among 10-14 and 15-19 year olds; however the same effect was not seen 5-9 year olds (Fernandez *et al*. 2003). A separate investigation of serogroup A and C polysaccharide vaccination was observed in Italian army recruits. Again a similar decrease was observed but notably an increase in serogroup Y was discovered (Di Martino *et al*. 1990).

The UK Meningococcal Carriage (UKMenCar) study commenced in 1999. The study investigated the rate of meningococcal C carriage in individuals aged between 15-17

during 1999 and compared the results individuals aged 15-17 years in 2000. 14,064 students were surveyed in 1999 and compared with 16,583 students in 2000. The study sampled students from 5 sites in England, 2 in Wales and 1 in Scotland. Oropharyngeal swabs cultured using standard microbiological techniques and those meningococcal positive swabs were sent to either Public Health England in Manchester or the Meningococcus and Pneumococcus Reference Laboratory in Scotland. The study concluded that the carriage of meningococcal serogroup C was reduced by 66%. However the vaccine had no significant effect on carriage of other meningococcal serogroups.

Dellicour and Greenwood (2007) conducted a review of 29 studies that compared carriage rates of meningiccoci pre and/or post vaccination. The group analysed papers that specifically reported on meningococcal carriage and vaccination of several serogroups. They concluded that in high-risk groups, such as military recruits, carriage rates could be temporarily reduced by the introduction of a vaccination programme and thus promoting herd immunity. The same was not so clear within a civilian population however. The group concluded that polysaccharide vaccination alone has little to no impact on carriage, however they discovered that the majority of literature published was from studies conducted during an outbreak of disease. These studies often had no control group and those studies that did include a control group were flawed by poor randomisation ratios. Dellicour and Greenwood (2007) noted that the follow up periods of vaccinated individuals is relatively short and although carriage rates may be reduced in one serogroup, as was seen with pneumococcal conjugate vaccines, serogroup replacement may occur.

From these observational studies there is a clear impact on meningococcal carriage from the introduction of the serogroup C conjugate vaccine. This decrease in carriage is beneficial in enabling herd immunity for those individuals that cannot be vaccinated. However, further investigation is needed as these vaccines only target those bacteria expressing capsule or those bacteria which are able to down-regulate capsule production. A balance of vaccine efficacy against carriage and a wide coverage against targeted serogroups and those strains that compete with them is required. The

potential for a vaccine to limit the transmission of particular encapsulated group could be seen as beneficial. However this has the potential to create an unwanted side effect; allowing the strains that compete with the targeted serogroup to continue unhindered as they are neither covered by the vaccine nor have the competition previously encountered (Anderson and May, 1985). Therefore it is likely that a multi-vaccine approach will be necessary to eliminate virulent strains from the population.

1.6 IMMUNITY

Humans do have some natural immunity to *Neisseria*, as with most bacteria, viruses, fungi and parasites the innate immune system will clear most of these pathogens. Immune cells populate skin tissues and mucus membranes, which are generally considered to be the first physical barrier to microbes. Should this fail however the adaptive immune system is activated and once the pathogen has been eliminated a 'memory' immune response is created and, should the pathogen be identified again, it can be eliminated much quicker. However, typical meningococcal polysaccharide vaccines designed in the 1970's were poorly immunogenic and did not confer a long lasting immune response. Polysaccharide vaccines typically stimulate B cells but not T cells, conjugate vaccines enhance immunity by stimulating T cells (Lee *et al.* 2002).

1.6.1 INNATE IMMUNITY

Innate immunity is a non-specific immune response against a pathogen. This can include anatomical barriers like the skin, mucus in the respiratory tract and the normal gut flora. Innate immunity does not provide long-lasting immunity, but does encourage migration of immune cells to the site of infection. The complement system is part of the innate immune system. It is a system of plasma proteins that become activated by pathogens or antibodies bound to pathogen, leading to a cascade event. This cascade event helps phagocytic cells to remove pathogens and damaged cells from the host.

Neutrophils account for 40-70% of white blood cells (Raven *et al.* 2015.) in healthy humans; these short-lived cells are highly motile and easily navigate to a site of infection guided by cytokines released by endothelium cells and macrophages (Ear and McDonald 2008). Should *N. meningitidis* migrate across the epithelial barrier the Opa-expressing bacteria are likely to encounter neutrophil cells in the hosts' endothelial tissue. Contact between the neutrophil cells and Opa-expressing *N. meningitidis* enables the phagocytosis and other bactericidal affects of the hosts cells against the bacteria (Sadarangani *et al*. 2011). This is in contrast with T lymphocytes, which are de-activated and suppressed by CEACAM-specific Opa (Sadarangani *et al*. 2011). Recent *in vitro* studies have shown that neutrophils use CEACAM-3, highly similar to the T

lymphocyte CEACAM-1, effectively tricking *Neisseria* into binding and subsequent elimination. Neutrophils express several CEACAMs, although it is thought that CEACAM-3 is exclusively expressed in humans (Scmitter *et al*. 2004). Neutrophils are also activated by Opa-dependant blebs, which are released during invasive *N. meningitidis* infection (Sadarangani *et al*. 2011), however this may also serve as a diversion enabling the meningococci to thrive unhindered.

1.6.2 ADAPTIVE AND HUMORAL IMMUNITY

Humoral immunity is also known as antibody-mediated immunity. Unlike innate immunity, adaptive immunity elicits a specific response to an antigen. It is also responsible for long lasting memory immunity, which can provide protection for life against some pathogens (Janeway *et al*. 2001.).

Humoral immunity has also been shown to have limited effect on meningococcal carriage (Jones *et al.* 1998) but is important against infection. Carriage alone naturally creates an antibody response, during infection and increase in the antibody titres can be detected from Day 4 (Gasparini *et al.* 2015). Antibody studies have demonstrated that the carriage of certain strains leads to a direct attack on OMPs. This attack was directed against LPS, PorA, PorB, and Opa (Jones *et al.* 1998). Although these interactions were largely strain specific, there was some cross-reactivity with some heterologous strains. High levels of antibody were still recorded even after several months post clearance. In those patients with acute disease, the number of T-cells declines whilst the amount of B-cells increases.

The exact relationship between cytokine production and cellular immunity against meningococcal disease and carriage is yet to be fully understood. There is no perfect animal model for observing meningococcal infection (Coureuil *et al*. 2013) so observations are made *in vitro* using cultured human epithelial cells and human endothelial cells. Recently mice expressing Human Factor H and Human Transferrin were injected with one of two *N. meningitidis* isolates (serogroup C LNP24198 or LNP27704). The experiment investigated the antibiotic susceptibility testing (AST) of the two isolates. The group concluded the use of an animal model combined with

bioluminescent imaging in vivo is a rapid and sensitive way to observe infection (Belkacem *et al.* 2016).

Meningococcal incidence rates fall as age increases (Trotter *et al*. 2006), a likely outcome from several silent colonisation episodes throughout earlier stages of life. A seroprevalence study conducted in England demonstrated that antibodies toward *Neisseria* are low in childhood, with the exception of children aged 3-11 months due to maternal immunity, and rise throughout childhood, peaking in the teenage years with meningococcal incidence (Trotter *et al*. 2007). The study demonstrated that the rise of anti-meningococcal antibody expressed in children and young adults without a history of invasive meningococcal disease could be due to non-invasive meningococcal carriage. Meningococcal carriage could consist of either cognate serogroups or non-groupable strains which provide cross-protective immunity (Trotter *et al*. 2007). Immunity from *N. meningitidis* is reliant upon the presence of IgG produced by plasma B cells (Yazdankhah and Caugant, 2004). IgG has bactericidal properties and attaches to the bacterial cell surface rendering the bacteria less motile or completely immotile, labelled for complement attack and cause agglutination. IgG labelled bacteria are then engulfed by phagocytic cells such as macrophages.

Complement-mediated antibody specific bactericidal activity is the activation of complement by an antibody bound to the surface of a bacterial cell. The binding of the antibody with the cell antigen causes bacteriolysis and eventual cell death (Mountzouros and Howell, 2000). It has been previously demonstrated the important role that antibodies have in meningococci protection (Goldschneider *et al*. 1969). Among military recruits it was shown that in prospective cases of meningococcal disease, patients lacked antibodies not only to the patients' strain but also to a wide variety of meningococcal strains which suggests a general lack of immunity to all pathogenic meningococci (Goldschneider *et al*. 1969).

1.6.3 CROSS-PROTECTIVE IMMUNITY GENERATED BY N. LACTAMICA

It has been theorised that *N. lactamica* carriage could confer immunity to *N. meningitidis* colonisation and subsequent carriage (Wong *et al*. 2011). *N. lactamica* is

commonly isolated from infants and young children at a time when *N. meningitidis* carriage is particularly low (Bennett *et al*. 2005). The Seroprevalence study found that there was little difference in serum bactericidal antibody (SBA) activity or anti-outer membrane vesicle (OMV) IgG titres at the age of *N. lactamica* carriage (Trotter *et al*. 2007). This would suggest that instead of *N. lactamica* providing an immune response to *N. meningitidis*, *N. lactamica* out competes *N. meningitidis* in the nasopharyngeal niche. This is contrary to the study conducted by Gold and colleagues (Gold *et al*. 1978) where results demonstrated that children and infants develop cross-reacting antibodies to both *N. meningitidis* and *N. lactamica*, post asymptomatic carriage of *N. lactamica*. Evans *et al* (2011) challenged 41 healthy volunteers who had been identified as *Neisseria* negative with *N. lactamica*. The group found an increase in serum IgG in those volunteers challenged with *N. lactamica* compared to the un-challenged controls. They also found that volunteers who had been challenged with *N. lactamica* were able to produce IgG within four weeks of colonisation, and yet the organism could be carried for 24 weeks. This would suggest the organism is either able to evade the immune system, possibly via biofilm formation, or that *N. lactamica* is able to replicate and survive quicker than the immune system can clear it. Rapid replication seems unlikely as a proportion of the subjects were resistant to re-inoculation of *N. lactamica*, even with a 10-fold higher dose. The group did demonstrate that colonisation did result in cross-reactive opsonophagocytic antibodies against *N. meningitidis*, but not bactericidal antibodies. They also demonstrated that *N. lactamica* limited the colonisation of *N. meningitidis* (Evans *et al.* 2011), which supports previous findings of observational studies (Olse *et al.* 1991). This restriction of *N. meningitidis* by *N. lactamica* could be due to direct competition, with *N. lactamica* better suited as a commensal in the nasopharyngeal niche. Deasy *et al.* (2015) also demonstrated that the ability of *N. lactamica* to inhibit *N. meningitidis* carriage was more potent in subjects who had received the glycoconjugate meningococcal vaccination.

Although encouraging, little work of this nature has been completed since. The complex relationship between *Neisseria* and other organisms still remains to be

completely understood, and in particular the relationship between *N. lactamica* and *N. meningitidis*

1.7. AIMS AND OBJECTIVES

Chapter One has given a broad review of the field of *N. meningitidis* and the non-invasive commensal *N. lactamica*, exploring how both organisms have evolved to survive in the nasopharyngeal niche, the effects of untreated meningococcal disease, the hosts' immune response to carriage and disease, and the latest vaccine developments.

Chapter Two describes the methods and materials used throughout this thesis.

Chapter Three explores the theory that long term colonisation of the host by *N. lactamica* is promoted by biofilm formation. There is extremely little evidence in the literature to support the ability of *N. lactamica* in single culture to form biofilms, and this chapter aims to investigate whether *N. lactamica* has the potential to form a biofilm *in vitro*. If formation of *N. lactamica* biofilms is possible, then this may at least partially explain how *N. lactamica* can be carried for long period of time in infants before being cleared by the immune system or spontaneously lost. The formation, and presence of biofilm, may alternatively indicate why carriage is lost and replaced with organisms like *N. meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. The work comprising this chapter aims to create a working biofilm model that can be standardised and used in combination with other *Neisseria* species.

Chapter Four investigates the biofilm-forming properties of several *N. meningitidis* isolates collected from a previous investigational study. Those isolates that successfully demonstrated biofilm growth in 100% media were studied using the working biofilm model developed for *N. lactamica* in Chapter Three. Using the identification methods from the previous chapter, several isolates were investigated further in detail to demonstrate biofilm production. Upon identification of viable biofilm, the isolates were categorised according to biofilm phenotype for future work. The characterisation was compared with the carriage data collected during the investigational study. The

chapter aims to discover if there is a correlation between biofilm phenotype and success of carriage.

Chapter Five investigates further some of those *N. meningitidis* isolates that express viable biofilm formation. All the isolates were 'challenged' with a predetermined concentration of *N. lactamica* in the planktonic state and results were compared to observations made from isolated planktonic growth. Those isolates that exhibited a negative effect when grown in a planktonic culture with *N. lactamica* were further investigated in biofilm over 1 to 3 days. Our hypothesis is that carriage of *N. lactamica* confers some protection against co-carriage of *N. meningitidis* by an unknown and currently uncharacterised competitive relationship.

Chapter Six will discuss the results of this thesis, the limitations of the experiments and further work that is required.

CHAPTER 2 MATERIALS AND METHODS

2.1 MEDIA AND REAGENTS

2.1.1 MEDIA

2.1.1.1 LIQUID MEDIA

NB: All media was pre-warmed to 37 ^{o}C before experimental use.

2.1.1.1.1. Tryptic soya broth supplemented with 0.2 % yeast extract (TSB)

Tryptic Soya broth powder was purchased from Oxoid. TSB was prepared by dissolving 30 g of powder into 1 litre of distilled water and sterilised by autoclaving at 126 ^{o}C for 20 minutes 30 PSI.

2.1.1.1.2. Brain Heart Infusion broth (BHI)

Brain Heart Infusion broth powder was purchased from Oxoid and prepared by dissolving 37 g into 1 litre(s) of distilled water. BHI was sterilised by autoclaving at 126 ^{o}C for 20 minutes.

2.1.1.1.3. Reduced concentration media.

All media were prepared fresh weekly, as described in Appendix 1. Where 'reduced concentration' medium is used in experiments, the appropriate medium was diluted to the stated percentage (v/v) with sterile water: i.e. 25% TSB media was prepared by mixing 37.5 mL sterile water and 12.5 mL TSB media.

2.1.1.2 SOLID MEDIA

2.1.1.2.1 Columbia Blood Agar (CBA)

Columbia agar plates (hereafter, CBA) supplemented with 5% v/v horse blood were purchased from Thermo Scientific.

2.1.1.2.2. Thayer-Martin selective agar (TM agar)

Thayer-Martin selective agar plates (BBL modified Thayer-Martin MTM II agar) were purchased from Becton Dickinson UK.

2.1.2 REAGENTS

2.1.2.1 X-GAL

Bacterial colonies were stained with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside. Sigma) solution. A 20 µg/mL solution was prepared using dimethylsulfoxide (DMSO) as per manufacturer instructions. X-gal was used as *N. lactamica* contains a β-galactosidase, which in the presence of X-gal turns the colonies from gray to blue. *N. meningitidis* does not contain a β-galactosidase enzyme and so is unable to break down the x-gal and remains light gray in colour. 20 µL of solution was dropped directly on to colonies and left to air dry at room temperature. Fresh solution was made monthly and covered with tin foil to prevent UV damage.

2.1.2.2 KANAMYCIN

The antibiotic Kanamycin, an aminoglycoside which inhibits protein synthesis, was used for all antibiotic experimentation. An initial working stock of 500 µg/mL was serially diluted 10-fold to a minimum of 5 ng/mL in 100% TSB.

2.1.2.3 CRYSTAL VIOLET

Crystal violet solution (Pro-Lab Diagnostics, PL7002) was made to various concentrations (ranging from 10 % to 0.1 %) using deionised water. The stain was applied for 10 minutes, twice washed with sterile water and eluted using 100% ethanol with gentle agitation.

2.1.2.4 GRAM STAINING

The gram stain comprised of 3 different stains and 100% ethanol. Pure crystal violet (Pro-Lab Diagnostics, PL7002), Grams Iodine solution (Sigma-Aldrich, HT902-8FOZ) and Carbol Fushsin (Sigma-Aldrich, C4165).

Bacteria were smeared onto uncoated microbiological slides and allowed to air dry in a Class 2 microbiological safety cabinet. The slide was then heat fixed using an open flame. Crystal violet was applied to the slide, left to stain for 2 minutes and removed by blotting at the end of the slide. Iodine was applied to the slide, left to stain for 2 minutes and removed by blotting the end of the slide. 100 % ethanol was used to wash the slide until the precipitate ran clear. The counter stain Carbol Fuchsin was used to flood each slide, allowed to stand for 2 minutes and then removed using sterile water. The slide(s) were left to air dry at atmospheric temperature prior to being viewed using a Leica DM 1000 LED microscope and a HI PLAN 100x oil immersion lens.

2.1.2.5 INDIA INK CAPSULE STAINING

India ink (VWR, J61007.AP) Bacterial colonies were grown overnight on CBA plates, and a single colony was re-suspended into 10 µL of India ink and spotted onto an uncoated microscope slide. A bacteria smear was created using a fresh slide and left to air dry. The slide was not heat fixed to preserve the capsule and allowed to air dry. The slide was flooded with 50 % v/v crystal violet, left to stain for 60 seconds and washed off with sterile water. The slides were left to completely dry at atmospheric temperature prior to being viewed using a Leica DM 1000 LED microscope and a HI PLAN 100x oil immersion lens.

2.2 BACTERIAL ISOLATES

NB: All bacteria were maintained as frozen glycerol stocks at -80 ^{o}C and grown on Oxoid Columbia Blood Agar (CBA) media (Section 2.1.1.2.1).

2.2.1 NEISSERIA LACTAMICA

N. lactamica strain Y92-1009 (sequence type 3493, clonal complex [CC] 613) was originally isolated from a carriage study conducted in Northern Ireland in 1992. The isolate has previous been used in several observational studies (pubmlst ID: 4945).

2.2.2 NEISSERIA MENINGITIDIS

2.2.2.1 LABORATORY STRAINS

N. meningitidis strain MC58 (pubmlst ID: 240) was first isolated in 1983 from a meningococcal case in the Gloucester region (McGuinness *et al*. 1991).

2.2.2.2 NASOPHARYNGEAL CARRIAGE ISOLATES

Twenty seven *N. meningitidis* isolates were obtained from healthy asymptomatic carriers via nasopharyngeal swabbing during the clinical trial NCT02249598 (Deasy *et al*. 2015). The isolates were identified by overnight growth on GC-selective media (E&O Laboratories), Colonies were then exposed to a solution of X-gal in PBS to discriminate colonies of *N. lactamica*, which turned blue. Colonies without B-galactosidase enzyme activity, which remained white after exposure to X-gal, underwent analytical profile index (API) strip testing to verify their identity as *N. meningitidis* (Deasy *et al.* 2015). Each isolate was given a unique identifier.

2.3 BACTERIAL CULTURE TECHNIQUES

2.3.1 SOLID PHASE CULTURE

2.3.1.1 BACTERIAL RECOVERY FROM FROZEN STOCKS

Aliquots of frozen stock were transferred aseptically to CBA plates in a Class II microbiological safety cabinet (MSC) and repeatedly streaked across the plate. Fresh microbiological loops were used with each streak to reduce the number of viable bacteria being displaced. Streaked plates were then incubated for 16 hours at 37 ^{o}C, 5 % (v/v) CO_2 to produce single colonies.

The assumption is that a single viable bacterium gave rise to each individual colony.

2.3.1.2 SCREENING MASTER STOCKS

A loop-full of frozen master stock was streaked onto CBA plates. Plates were incubated for 16 hours at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2. Those plates which visually were identified as containing a contaminant were re-streaked on Thayer-Martin selective agar plates (section 2.1.1.2.2) and incubated for 16 hours at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2. 1 colony per mL was picked and fresh stocks made using methodology described in section 2.3.2.2

2.3.1.3 ESTIMATING BACTERIAL VIABILITY

An aliquot of bacterial culture was serially diluted tenfold across an appropriate range (typically from 10 – 10^{-8}). Three 10 µL aliquots of each dilution were spotted onto CBA plates (section 2.1.1.2.1) in order to establish the number of bacteria (Miles *et al.* 1938). Bacterial spots were allowed to dry in a Class II MSC. Plates were incubated at 37 ^{o}C, 5 % v/v CO_2 overnight to produce colonies. Where the number of colonies in each spot was between 10-50, the average number of colonies across all three spots was multiplied by 100 (to adjust for the volume of the aliquot plated) and then by the dilution factor. The resulting number is an estimation of the number of viable bacteria (colony forming units or CFU) per millilitre of the original culture (CFU/mL).

2.3.2 LIQUID CULTURE

2.3.2.1 MEASURING OPTICAL DENSITY (OD)

Optical density measurements were taken at a wavelength of 600 nm using a Jenway 6300 spectrophotometer in transparent 1.6 mL cuvettes. Prior to measurement, the spectrophotometer was blanked with a 1 mL aliquot of sterile medium of the appropriate type. Because the linear relationship between OD and cell number is only maintained between zero $\leq OD_{600nm} \leq 0.8$, wherever culture OD_{600nm} exceeded 0.8, turbidity was re-measured using a dilution of the culture. The resultant OD_{600nm} was multiplied by the dilution factor to provide a more accurate estimate of the culture turbidity.

2.3.2.2 CREATING LIQUID CULTURES FROM CBA PLATES

Oxoid CBA plates were streaked with viable bacteria from frozen master stocks (section 2.3.1.2) and were incubated overnight at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2 to produce single colonies. Individual colonies were re-suspended (1 colony/mL) into the appropriate volume of TSB or BHI (Section 2.1.1.1.1 or Section 2.1.1.1.2 respectively). Broth cultures were incubated at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2 with agitation (320 rpm).

2.3.2.3 PREPARATION OF FROZEN STOCKS

Cultures growing in liquid media (section 2.3.2.2) were used to generate stocks for both long term storage, and for repeated, regular sub-culture. To protect bacteria against damage from freezing, culture of the appropriate stage of growth was mixed 1:1 with an equal volume of a 60 % v/v glycerol (60 % glycerol, 40 % PBS mixture) to give a final concentration of 30 % glycerol. Stocks were stored in 2 mL cryovials and stored at -80 ^{o}C until required. Working stocks were frozen/thawed a maximum of five times before being discarded.

2.3.2.4 PLANKTONIC REGRESSION ANALYSIS

NB: Regression analysis compares optical density of a culture and colony forming units (CFU). It provides an estimate of how many viable bacteria there are in a given unit volume, and allows for adjustment of the number of viable bacteria to suit the needs of the protocol.

Several regression experiments were performed for each isolate to create an average value. Table 1 lists the corresponding average concentration of colony forming units per mL (as described in Section 2.3.1.3).

Bacterial cultures were grown as specified in section 2.3.2.2 with optical density readings taken every 30-60 minutes depending on the rate of growth by each isolate (section 2.3.2.1). From each OD measurement the viability was determined by calculating the CFU/mL as described in section 2.3.1.3.

Bacterial isolate	OD value λ600nm	Concentration (cells/mL)
N. lactamica Y92-1009	0.23	1.0×10^8
N. lactamica Y92-1009 *aphA3* (Kan^R)	0.29	9.4×10^7
N. meningitidis MC58	0.13	9.3×10^7
N. meningitidis R022	0.14	3.3×10^6
N. meningitidis R145	0.30	1.7×10^8
N. meningitidis R191	0.41	2.4×10^7
N. meningitidis R210	0.24	1.3×10^7
N. meningitidis R221	0.32	9.6×10^7
N. meningitidis R222	0.20	1.6×10^8
N. meningitidis R242	0.15	6.0×10^7
N. meningitidis R279	0.23	2.8×10^7

Table 1. Average optical densities of *Neisseria* suspensions and corresponding CFU measurements. Experiments repeated in duplicate on six separate occasions, n=6.

2.3.3 BIOFILM GROWTH AND MAINTENANCE

All biofilms were grown using the static microtitre method (Merritt *et al.* 2005) in enclosed uncoated plastic tissue culture plates. Biofilms were grown on glass coverslips for imaging as described in section 2.3.3.3.4. All work was performed in a sterile class 2 microbiological safety cabinet.

2.3.3.1 STARTING CULTURE FOR BIOFILM GROWTH

Colonies were grown onto CBA plates as described in section 2.3.1.1, TSB media was inoculated as previously described in section 2.3.2.2.

2.3.3.2. INITIAL BIOFILM GROWTH

From the starting culture described in section 2.3.2.2, bacteria were grown to OD_{600nm} = 0.7 and the culture was centrifuged at 3500 x *g* for 4 minutes to pellet the cells. Spent media was aspirated, replaced with fresh media (section 2.1.1.1) and the pellet re-suspended. To seed biofilm, equal volumes of fresh media and bacterial suspension were added to each well of the appropriate cell culture plate (6-well plates: Fisher Scientific, 12-well plates and 24-well plates: VWR Jencons). Plates were incubated for various time periods as required for each experiment at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2 and maintained as described in section 2.3.3.4.

2.3.3.3 METHOD DEVELOPMENT FOR BIOFILM GROWTH

2.3.3.3.1 Method 'A'

Log phase cells (OD_{600nm} = 0.2) were added in equal concentration to fresh sterile TSB media in a 6-well tissue plate and incubated for 24 hours at 37 ^{o}C and 5 % (v/v) CO_2. All spent media was removed and replaced with fresh sterile reduced media of either 25 %-TSB media or 50 %-TSB media (section 2.1.1.1.3). Plates were then incubated for a further 24, 48 or 72 hours as required and maintained as described in section 2.3.3.4. The plates were stained with CV as described in section 2.1.2.3 for analysis.

2.3.3.3.2 Method 'B'

Log phase cells (0.2 OD_{600nm}) were re-suspended in fresh sterile media of either 25 %-TSB media or 50 %-TSB media (section 2.1.1.1.3). To each well an equal concentration of the re-suspended log phase cells and the fresh sterile reduced-media was added. Cells were incubated at 37 ^{o}C and 5 % (v/v) CO_2 and maintained for up to four days as described in section 2.3.3.4. The plates were stained with CV as described in section 2.1.2.3 for analysis.

2.3.3.3.3 Adapted reduced media biofilm growth

Reduced 25 %-TSB media and reduced 50 %-TSB media (section 2.1.1.1.3) were used to grow biofilm and investigated over three days.

TSB media was inoculated as previously described in section 2.3.2.2. Bacteria were grown to log phase as measured via optical density at 600 nm (OD_{600nm}) as described in section 2.3.2.1. The culture was then centrifuged at 3500 *x g* for 4 minutes to pellet the cells. All spent media was removed and replaced with full strength TSB (hereafter 100 %-TSB media), 25 %-TSB media or 50 %-TSB media (section 2.1.1.1.3), and the pellet re-suspended. Equal volume of suspension was added in equal measure to fresh media (of the required concentration) in a 1:1 ratio in a well of a cell tissue culture plate. Biofilms were incubated at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2 and maintained as described in section 2.3.3.4.

2.3.3.3.4 Biofilm growth on glass cover slips

Biofilms were grown as described in section 2.3.3.3.3, however prior to adding the bacterial suspension to a tissue culture plate a single cover slip was added to each well. Cover slips were prepared by slightly bending a single edge under a blue flame. This allowed the cover slip to be removed with forceps from each well without significantly damaging the biofilm. Cover slips were stored in ethanol and allowed to air dry before added to each tissue plate.

2.3.3.3.5 Biofilm grow in CELLview™ culture dish

CELLview™ culture dishes are specifically designed for microscopy work, comprising of polystyrene with an integrated glass bottom which reduces the amount of refractive light. A starting culture (section 2.3.2.2) of log-phase cells (*N. lactamica* 0.2 OD_{600nm}, N. meningitidis R083 0.3 OD_{600nm}) was centrifuged at 3500 *x g* for 4 minutes to pellet the cells. All spent media was removed and replaced with 50 %-TSB media (section 2.1.1.1.3). Cells were re-suspended by gentle vortexing. To the CELLview™ plate 2 mL of suspension was added to 2 mL of 50 %-TSB media. The plates were incubated at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2 and maintained as described in section 2.3.3.4.

2.3.3.4 BIOFILM MAINTENANCE

To maintain successful biofilm growth over a long period of time every 24 hours spent media was removed and replaced with fresh media of the same concentration. Media was removed and added from the same location each time.

2.3.4 CALCULATING SUSCEPTIBILITY

2.3.4.1 MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that will inhibit visible growth of a bacterial suspension after a pre-determined incubation period.

The MIC of appropriate compounds was measured by re-suspension of bacteria (1 colony per mL) into TSB into a 96-well tissue plate. Into each well a known concentration of antimicrobial agent was introduced (section 2.1.2.2), and the plate was incubated for 16-20 hours at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2. Culture OD at 550 nm was measured using the Biochrom EZ read 400 plate reader. OD readings were taken at intervals over an eight hour time period and again at 24 hours. OD readings were compared to wells that contained no antimicrobial agent and wells containing only TSB (section 2.1.1.1.1).

MIC was determined as the lowest concentration of antimicrobial agent with OD reading similar to those wells that contained only TSB.

2.3.4.2 MINIMUM BACTERICIDAL CONCENTRATION

The minimum bactericidal concentration (MBC) is defined as the lowest concentration of an antimicrobial agent that will kill the bacteria. The MIC and MBC values of the same antimicrobial agent may differ.

An MBC value was measured by re-suspending planktonic bacteria grown to an OD_{600nm} of 0.2 into TSB media in a 96-well plate. Each well contained a known concentration of antimicrobial agent (section 2.1.2.2), and the plate was incubated for 16-20 hours at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2. Ten microliter aliquots of each overnight suspension were plated on CBA plates. The plates were then incubated for a further 16-20 hours at 37 ^{o}C in an atmosphere of 5 % (v/v) CO_2 and the numbers of viable colonies enumerated (section 2.3.1.3).

2.3.4.2.1 MBC of biofilm

MBC analyses of biofilms were adapted from the planktonic methodology. Individual biofilms were twice washed with sterile TSB prior to all media being completely aspirated. A known concentration of antimicrobial agent was added as described in section 2.3.4.2. Upon incubation biofilms were twice washed with sterile water and then re-suspended into 1 mL of sterile water. 10 µL of each bacterial culture was drop plated onto CBA plates and incubated for 18 hours at 37 ^{o}C in an atmosphere 5 % v/v CO_2.

2.4 VIABILITY TO ANTIBIOTIC

2.4.1 PLANKTONIC ASSAY

One colony/mL was re-suspended into fresh sterile TSB (section 2.1.1.1.1). 180 µL of each antibiotic dilution was added to a sterile 96-well plate. 20 µL aliquots of bacterial suspension were added to each concentration and incubated at 37 ^{o}C in an atmosphere of 5 % v/v CO_2. OD600nm was measured at 18 hours and 10 µL of culture from each well was drop plated onto CBA plates and incubated for 20 hours to determine MIC (section 2.3.4.1).

2.4.2 BIOFILM ASSAY

Log phase bacteria (0.2 OD_{600nm}) were re-suspended into fresh 50 %-TSB media. 1 mL of the bacterial suspension was added to each well which contained 1 mL of fresh 50 %-TSB media and left to incubate overnight at 37 ^{o}C and 5 % v/v CO_2 to create a 1-Day biofilm.

A solution of kanamycin (50 µg/mL) was serially diluted in TSB 10-fold to give a final concentration of 50 ng/mL. Biofilms were treated directly with the antibiotic for 2 hours. Biofilms were then washed twice with sterile water. The biomass present in each biofilm was measured by CV analysis (section 2.1.2.3) and an MBC (Section 2.3.4.2.1) was calculated.

The MBC was determined to be the lowest concentration where viable growth was prevented.

2.5 MOLECULAR BIOLOGY TECHNIQUES

2.5.1 POLYMERASE CHAIN REACTION (PCR)

PCR products were electrophoresed on a 0.8 % Agarose gel (BioReagent, Sigma).

Full primer sequences used to amplify genes *ctrA*, *porA, nlaIII* and *lacZ* are located in Table 2.

2.5.1.1 CYCLE FOR GENES CTRA, PORA AND LACZ

The cycles for the genes *ctrA*, *porA* and *lacZ* were as follows;

10 minutes at 95 ^{o}C, 35 cycles of 20 seconds at 98 ^{o}C denaturing, 15 seconds at 53 ^{o}C annealing and 15 seconds at 72 ^{o}C extension. Upon completion of the cycles, the PCR product was heated to 72 ^{o}C for 10 minutes for final extension and held at 10 ^{o}C until required.

2.5.1.2 CYCLE FOR GENE NLAIII

The cycle for the gene *nlaIII* was as follows;

10 minutes at 95 ^{o}C, 30 cycles of 20 seconds at 98 ^{o}C denaturing, 15 seconds at 65 ^{o}C annealing and 90 seconds at 72 ^{o}C extension. Upon completion of the cycles, PCR product was heated to 72 ^{o}C for 10 minutes for final extension and held at 10 ^{o}C until required.

Gene	Forward	Reverse
ctrA	GTCGCGGTGATGTGGTTA	AATCTCTGCCTCACTGCCAT
porA	CTCATAGCCGCCCGTCA	GCGGTTTTGCCGGGAACTAT
lacZ	TTCCCAAAGAACGGGAAGGA	GCATTTTGCCATTGGGAACG
nlaIII	CGGGTGATTAGCTCAGTTGG	GGTTTTCATTGTGCCGATAACGG

Table 2 – Primers used for PCR

2.6 MICROSCOPY

2.6.1 LIGHT MICROSCOPY

All light microscopy was performed on a Leica DM 1000 LED microscope and a HI PLAN 100x oil immersion lens. All microscope slides used were uncoated.

2.6.2 CONFOCAL MICROSCOPY

Confocal microscopy is used for high optical magnification imaging, which enables the reconstruction of 3D images via fluorescence. The technique allows the user to view thin optical sections without the need for physical dissection. Unlike conventional microscopy which utilises a condenser lens to illuminate a wide area, resulting in an out-of-focus blur on surrounding areas, a confocal microscope is able to concentrate the light source on single particular area. This concentration not only enables a higher resolution but also prevents flooding the sample with unnecessary light and quenching the sample. By varying the distance between each image a comprehensive 3D model of the structure imaged can be built. Contrasting fluorescent stains or probes are traditionally used to help build such models.

2.6.2.1 PREPARATION FOR IMAGING

Established biofilms were stained for 15 minutes with the BacLight™ Kit (Thermo Fisher, L7012) using 9 µL of Syto-9 and 9 µL of propidium iodide re-suspended into 3 mL of sterile HBSS. The biofilm was then washed with sterile HBSS before a layer of 60 % glycerol was added. Biofilms were then kept in the dark and imaged within the hour.

2.6.2.2 IMAGING

Tissue culture plates were viewed using a Lecia TSC SP5.

A maximum wavelength of 483 nm was used for excitation and 503 nm for emission of Syto-9 and a maximum wavelength of 493 nm was used for excitation and 636 nm for emission of Propidium iodide.
Biofilms were imaged in 2 µm sections from the bottom of the well to the top of the biofilm structure.

Images were processed using the Leica software program LAS-AF software v2.1.2 (provided with the TSC SP5 microscope). Using the software the individual 2 µm images were complied together and stacked to produce a 3D image for both *N. lactamica* and *N. meningitidis* R083.

2.6.3 SCANNING ELECTRON MICROSCOPY (SEM)

Scanning electron microscopy, unlike traditional light microscopes, uses electrons to visualise a sample. Light microscopy is restricted by the wavelength of light whereas electrons have a shorter wavelength which enables a higher resolution. A concentrated beam of electrons is fired at the sample, which repel the samples' electrons and cause them scatter, known as secondary electrons. Secondary electron detectors in the microscope attract the scattered electrons and allow an image to be built of the sample. Samples for SEM have to be appropriately prepared as imaging is completed under vacuum.

2.6.3.1 PREPARATION FOR IMAGING

Day-1 and Day-2 biofilms were grown on glass cover slips (section 2.3.3.3.4) and maintained as described in section 2.3.3.4.Biofilms were fixed for 1 hour with an Alcine blue fixative (3 % glutaraldehyde, 0.1 M sodium cacodylate and 0.15 % Alcine blue). The Alcine blue fixative was replaced with 0.1 M sodium cacodylate and incubated at room temperature for 1 hour. Sodium cacodylate fixative was replaced with a 0.1 M osmium tetroxide solution and incubated for 1 hour. Osmium tetroxide solution was replaced with 0.1 M sodium cacodylate and again incubated for 1 hour. The biofilm

coverslips were then treated with a series of ethanol washes. Ten minute washes were performed with increasing concentrations of ethanol (starting at 30 % (v/v) ethanol). A final, twenty minute treatment in 100 % ethanol was followed by incubation at room temperature to allow the samples to dry. Coverslips were critical point dried (CPD) and mounted onto stubs, sputter-coated in silver and dried.

2.6.3.2 IMAGING

Random sections of biofilm were selected for viewing and imaging. All sections were viewed at 100x magnification, 800x magnification and 4000x magnification.

Images were saved as *.Tiff* files and processed and adjusted using Microsoft PowerPoint 2007.

"Can Neisseria lactamica form a biofilm in vitro?"

3.1 INTRODUCTION

The commensal *Neisseria lactamica* is a non-pathogenic commensal of the human nasopharynx, carried in the first three years of life (Wong *et al.* 2011). However, the mechanisms behind the ability *N. lactamica* to maintain carriage without being cleared by the hosts' immune system is poorly understood. If *N. lactamica* could form a biofilm in the human nasopharynx it could help prevent loss by clearance. It has also been demonstrated that colonisation of *N. lactamica* provides a degree of protection from colonisation by *N. meningitidis* (Deasy *et al*. 2015) and biofilm formation by *N. lactamica* could potentially prevent or limit carriage of *N. meningitidis*. In planktonic form both *N. lactamica* and *N. meningitidis* show no significant dissimilarities in invasion and association with epithelial cells at 3 to 5 hours (Wong *et al*. 2011). The further study of *N. lactamica* biofilms could provide more information about possible targets, treatments, long-term acquisition and dispersal of *N. meningitidis*.

The documented evidence of biofilms within the human host varies from species to species. Meningococci have been found to form micro-colonies, which resemble biofilm formation, in the nasopharynx (Arenas *et al*. 2015) but no such structure has been described for *N. lactamica*. Other respiratory pathogens such as *Pseudomonas aeruginosa* and *Haemophilus influenzae* have been shown to form biofilms on cell lines *in vitro* (Moreau-Marquis *et al*. 2010; West-Barnette *et al.* 2006) and *Streptococcus pneumoniae* has been shown to form biofilm in the murine nasopharynx (Marks *et al.* 2012). The ability to form a biofilm *in vivo* would have many advantages such as immune invasion, protection from other respiratory flora, and as a result the ability to maintain carriage for extended periods of time.

Demonstrating that *N. lactamica* can form biofilm could potentially enhance our understanding of the nasal microflora, the types of polymicrobial interactions that occur in the nasopharynx and raise the question of whether an established *N.*

lactamica biofilm could prevent the adhesion and colonisation of *N. meningitidis* to the nasal epithelial or prevent the colonisation of other respiratory pathogens within the nasopharynx. Up to 50% of the population could be carrying the pathogenic *Staphylococcus aureus* asymptomatically in their nose (Warnke *et al.* 2014) and like many other species it is theorised that biofilm formation increases the survival of *S. aureus* (Archer *et al*. 2011). Alternatively the production of biofilm may be beneficial towards other species it has been documented that *Haemophilus influenzae* and *Streptococcus pneumoniae* are able to form a multi-species biofilm, conferring protection to both species (Tikhomirova and Kidd, 2013). *S. aureus* has also been shown to have a positive relationship with the pathogenic fungus *Candida albicans* in biofilm (Archer *et al.* 2011). It could be possible that *N. lactamica* is able to form a biofilm in conjunction with other commensal species.

3.2 AIMS FOR CHAPTER 3

The aims of this chapter are as follows;

- To investigate if *N. lactamica* is able to form a biofilm *in vitro*. *N. lactamica* may simply exist in a single-cell planktonic state, or may only be able to form a biofilm under extremely specific conditions. Investigate if *N. lactamica* is only able to aggregate as seen in non-capsulated *N. meningitidis* or if *N. lactamica* expresses multiple biofilm phenotypes dependent upon environmental conditions.
- Demonstrate that *N. lactamica* is capable of forming biofilm *in vitro*, using methods established to characterize the biofilms of other bacterial biofilms. Investigate the longevity of any resultant *N. lactamica* biofilm and the viability of its constituent bacterial components. Establish that any biofilm production by *N. lactamica* functions to protect the cells for long periods of time, from environmental pressures such as antibiotics. Investigated if *N. lactamica* can be sustained in this biofilm state without a detrimental effect.
- Develop a standardised working method for biofilm growth that is able to produce replicable results. A model would be important for any future work involving potential treatments, genetic analysis or multi-species work.

3.3 BIOFILM MODEL DEVELOPMENT

The idea that *N. lactamica* is able to form a biofilm is a novel idea. Methodologies of how to create environments suitable for biofilm formation exist mainly for other species and there are no standard methodologies. Merritt *et al* (2005) suggested two methods for growing and analysing biofilms using a 'static' microtiter or a 'flow' system. As a resident of the human nasopharynx, a niche largely devoid of sheer stresses caused by high flow rates, it was decided that the static method of biofilm culture would be adapted for this project. We hypothesized that the static method would more accurately reflect the environment to which *N. lactamica* is adapted, as in a flow system fresh media is continuously supplied to the biofilm and spent media removed; models which are generally more applicable to water systems or medical catheters. The limited exterior epithelial movement in the nasopharynx means that cells are not subjected to a continuous supply of nutritional media, nor have the spent media continually removed. Bacteria need to be able to survive this difficult, nutritionally stressed environment, of which these conditions can be more easily reproduced in a static biofilm culture model.

One of the most intensively studied biofilm forming bacteria, *Pseudomonas aeruginosa* is extremely well documented and has been shown to establish biofilm as a response to limited nutrient availability. *P. aeruginosa* is an opportunistic pathogen and commonly colonises the respiratory tract and is particularly problematic for cystic fibrosis suffers. It was discovered that *P. aeruginosa* varied the mushroom-shaped structures within the biofilm according to the varied amounts of a carbon source available (Klausen *et al*. 2004). As food does not routinely pass through the nasopharynx, the idea of a nutritional stress was applied to the *N. lactamica* model in this study. We investigated the biofilm-forming response of *N. lactamica* to limited nutrient availability, when either no fresh medium was added to wells or when a diluted medium is supplied at regular intervals.

3.3.1 INITIAL BIOFILM FORMATION

There is little literature addressing *N. lactamica* biofilms. The first experiment aimed to investigate whether *N. lactamica* is capable of forming a biofilm or a structure that resembled a biofilm. To determine if *N. lactamica* attachment to a plastic surface was possible 10^8 cells/mL of *N. lactamica* suspended in TSB media were incubated in 6-well tissue culture plates for 20 hours at 37 ^{o}C supplied with 5 % (v/v) CO_2. Plates were then stained with 10 % CV (Figure 7).

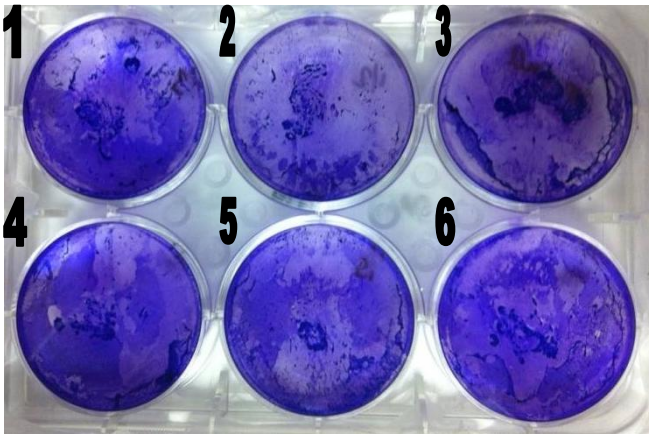


Figure 7. Day-1 *N. lactamica* biofilms stained with CV.
Biofilms were seeded with 10^8 cells/mL, bacterial inoculums composed of log phase cells. CV identified some biomass had established post 20 hours of inoculation. Removing the spent media did not remove the mass suggesting that cells had attached to the plate, n=1.

The assay demonstrated that *N. lactamica* was able to adhere to an untreated plastic surface. The dark purple areas are indicative of a larger amount of surface-bound biomass when compared to the more lightly stained areas, which in part is due to non-specific staining of the plastic by the CV.

The slight 'halo' effect around wells 2 and 3, and partiality around well 5, was identified as being due to the biofilm becoming exposed to the air. This caused detachment from the plate. Further investigation found that leaving the biofilm exposed to air caused considerable damage and promoted detachment from the plate surface. This damage could be avoided however by leaving a small amount of liquid in the well.

3.3.2 CONFIRMATION OF N. LACTAMICA BIOFILM PHENOTYPE AGAINST CONTAMINATION PHENOTYPE

Each species of biofilm-forming bacteria has different phenotypic biofilm characteristics. Visually identifying *N. lactamica* biofilms characteristics initially proved difficult with several experiments yielding two different biofilm phenotypes.
In initial experiments the biofilm produced a single surface-liquid interface; however repeated experiments exhibited an additional liquid-air biofilm (Figure 8).

Samples of each biofilm layer were streaked on CBA plates to confirm that the two layers are *N. lactamica*. The surface-liquid interface produced small circular opaque grey colonies consistent with *N. lactamica*, however the liquid-air interface produced colonies of the same size and shape as would be expected with *N. lactamica* but pure white in colour.

Colour variation of colonies does not conclusively prove there is contamination, especially with such similar colony morphology. The respiratory bacterium *S. pneumoniae* is able to produce several visually different colonies, due to the variability of the bacterial capsule when forming a biofilm (Allegrucci *et al*. 2006). This phenomenon may also apply to *N. lactamica*.

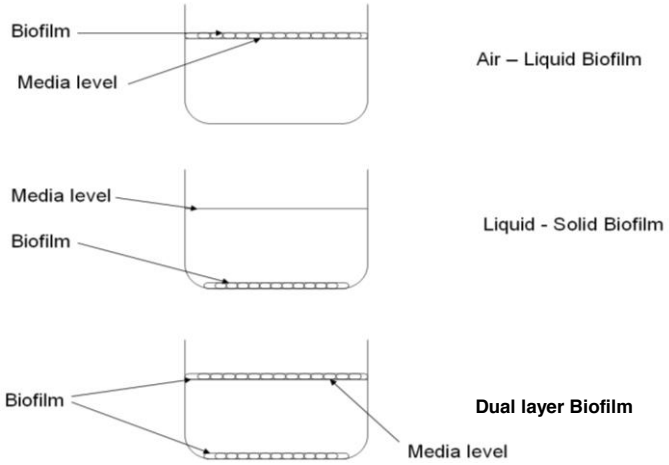


Figure 8 – Schematic drawing of air to liquid, liquid to solid and dual layer biofilm phenotype.

Air-liquid biofilms sit on top of the media level and are exposed to both the air and media; liquid-solid biofilms in contrast are positioned at the bottom of the well below the media and in contact with the polystyrene surface. Both phenotypes display confluent layers of biofilm, spanning the entire well area. A dual layer biofilm is one that contains both of these biofilms. The distance between the two different layers was between 0.8 cm and 0.9 cm (variation occurred due to volume of liquid that had evaporated).

3.3.2.1 GRAM STAINING

N. lactamica is a gram-negative diplococcus bacterium and gram staining was used as a rapid identification tool to firstly identify if the two colonies were both gram negative.

Samples were taken from both grey and white colonies and were mounted onto coated microscope slides and gram stained. The slides were viewed under a light microscope using an oil immersion lens.

Both slides showed the presence of diplococcus bacteria. The white colonies were clearly identifiable as gram-positive being purple in colour, whereas the grey colonies were gram-negative appearing red/pink in colour. To positively identify if the white colonies are a contaminant or a *N. lactamica* variant, samples from both colonies was taken for PCR analysis.

3.3.2.2 POLYMERASE CHAIN REACTION

The primers for genes ctrA, porA, lacZ are specific to the *Neisseria* species and *nlaIII* is specific to *N. lactamica*. Samples of both white and grey colonies were suspended into sterile deionised water and boiled to release the genomic DNA. The DNA sample was added to each primer and set to a specific PCR program.

DNA samples were run on a 1 % agarose gel along with a sample of known *N. lactamica* DNA and imaged (Figure 9).

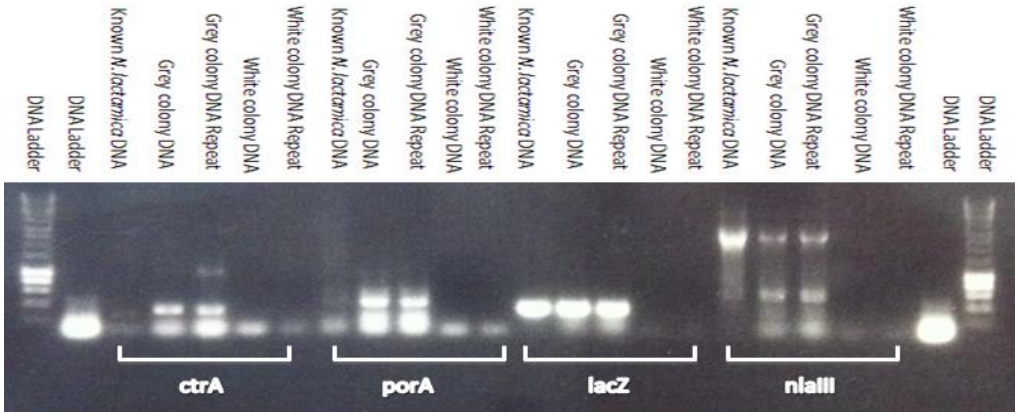


Figure 9 - Agarose gel containing PCR products.

DNA samples from both grey and white colonies were sampled in duplicate. They were then compared to a sample of known *N. lactamica* DNA. All DNA samples produced clear banding patterns with all four primers demonstrating the contaminant is not *N. lactamica*. Samples were taken from three different tissue plate wells on three separate occasions, all producing similar banding patterns.

The PCR results showed that the grey colonies were from the *Neisseria* species; the banding pattern for the grey colonies is similar to that of the known *N. lactamica* DNA sample. The gel also demonstrates that the grey colonies can be positively identified as *N. lactamica* due to the banding pattern matching the band pattern from the known sample of *N. lactamica* DNA, including the *N. lactamica* specific primer for the gene *nlaIII*.

The white colonies show no such matching banding pattern with any of the primers indicating they are not of the *Neisseria* species. It can therefore be concluded that the top liquid-air biofilm was a contaminant.

These results enable the positive visual identification of a *N. lactamica* biofilm, which produces a biofilm containing a single confluent layer that is opaque in colour. Further biofilms were tested using this methodology to confirm that visual identification could be achieved accurately and compared with biofilms suspected of containing a contaminant. The results from the repeats demonstrate that *N. lactamica* biofilms are consistent in structure and colour under these experimental conditions, and colonies are visually identified as small, round, smooth with a slight gloss and light grey in colour when grown on CBA plates with no haemolysis.

3.3.3 COMPLETE STARVATION

In vivo the ability of planktonic cells daily accessing a high volume of nutrients is unlikely. The nasopharynx is known to be a hostile environment, with limited nutrient access (Marks, *et al.* 2012a). When swallowing, chewed food products and liquids pass from the mouth down through the oropharynx, once in the oropharynx involuntary reflex actions prevent food from entering the nasopharynx and guides the food to the esophagus (Goyal and Mashimo, 2006). Further experiments investigated whether cells survived in biofilm when totally deprived from fresh media.

Biofilm formation was assessed by CV analysis over a 7-day period to determine the effect of depleted nutrient starvation on adhered biofilm mass. A starting culture of 10^8 cells/mL was used for seeding.

Visual observations noted that the *N. lactamica* biomass appeared to be established quickly - prior to the 24 hour time point (Figure 10). However, there was a significant decrease in the biomass measured at Day-2 (48 hours) (****P= 0.0001, non-parametric, unpaired t-test). No further increase in biomass was observed for the remainder of the experiment.

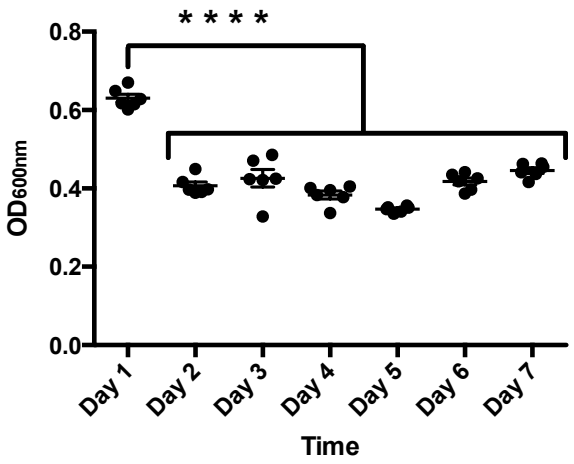


Figure 10 – Effect of nutrient starvation on results of *N. lactamica* biofilm biomass over 7 days.

The graph demonstrates that biomass can be maintained over 7 days though no fresh media was supplied. Without fresh media the biomass but does not increase in biomass suggesting that the core biomass is established early. The decrease in biomass from Day 2 to 7 is statistically significant when compared to Day 1 (****P<0.0001), however there is no significance in the difference from Day 2 to Day 7 when the time points are compared with each other. Results taken from single 6-well tissue culture plates over 7 days, n=1.

Visual inspection of the biofilms appeared thin and loosely attached to the plastic substratum post Day-1. At Day-7 the total number of viable cells was reduced to 10^5 cells/mL, which is a substantial 3-log decrease of viable cells. Spectrophotometry measurements demonstrated that the core biomass of the biofilm was established prior to the 24 hour time point and that some of this mass could be retained without nutrient replenishment. The ability to maintain all the initial biomass was poor, however the biomass was not completely dispersed despite a drop in viable cells over the 7-Day period.

These results demonstrate that some survival of *N. lactamica* in biofilm over the course of seven days without replenishment of media is possible.

3.3.4 VARIED MEDIA CONCENTRATION

The literature does not specify a standard methodology for establishing a *N. lactamica* biofilm in isolation. However examples exist for other nasopharyngeal microbial flora. Two nutritional-stress methods were adapted from previously used microtiter plate assays (Merritt *et al*. 2005); the methods examined the best conditions for biofilm growth.

3.3.4.1 CV ANALYSIS

'Method A' investigated the effect of gradual media reduction, mimicking a nutritionally variable environment.

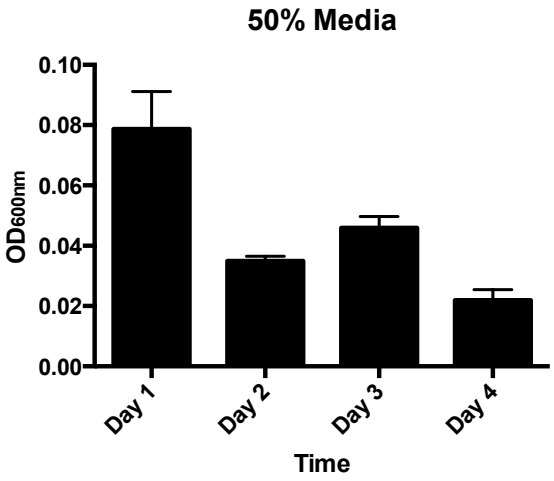
'Method B' investigated the effect of immediately nutritionally stressing the cells. This immediate stress mimics an environment in which a limited amount of nutrition would be available continually.

CV analysis was used to identify any biofilm present. *N. lactamica* was supplied with three different strengths of TSB; 100 %-TSB media, 50 %-TSB media (made with 50 % sterile water) and 25 %-TSB media (made with 75 % sterile water). The biofilms were maintained up to four days, biomass readings were taken every 24 hours in triplicate.

The biofilms were supplied with fresh reduced media every 24 hours until the required time point.

At 24 hours all concentrations phenotypically expressed a single confluent layer on the bottom of the plate and were physically robustly bound to the substratum. Upon being washed, minimal damage occurred to the confluent layer. In 100 %-TSB media the layer became thicker and visually more apparent as time increased post Day-2. At Day-2 all three media concentrations showed some removal of the biofilm layer upon washing. The layers detached from the plate with ease once stained and during the second round of washing. It was theorised that this may be due to the natural biofilm cycle or may be a cellular reaction to the environment within the well.

11a



11b

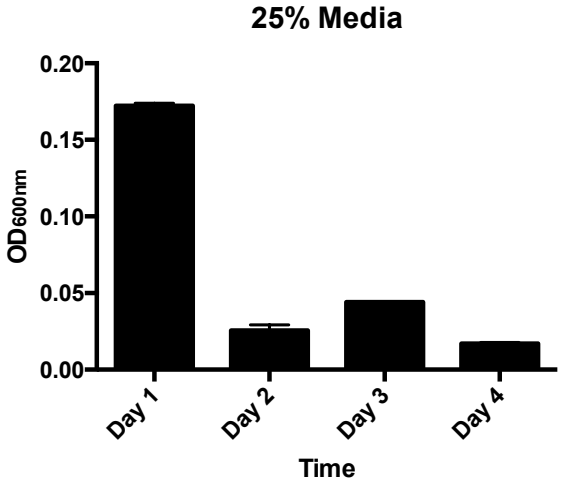


Figure 11a and 11b – Method A –50 %-TSB media and 25 %-TSB media.

Biomass fell sharply on Day-2 (Graph 11a - 0.079 to 0.029) and did not appear to recover back to Day-1 biomass. 25 %-TSB media (Graph 11b) initially demonstrates better bacterial adherence at Day 1 starts well in comparison to cells supplied with 50 %-TSB media however by Day-2 the biofilm is visually less stable, extremely poor and easily detaches from the plate during washing. Repeats conducted in separate experiments in 6-well tissue culture plates, n=3.

The biomass for 100 %-TSB media did increase somewhat by Day-3, from 0.293 at Day-1 to 0.454 at Day-3. This again indicates that biofilm establishment occurs rapidly prior to 24 hours, at Day-2 there is a slight decrease in OD, however biomass recovers by Day-3. Unsurprisingly, cells exposed to 100 %-TSB media gave the highest biomass readings.

This methodology was compared to stressing the cells immediately; observations were again repeated over four days.

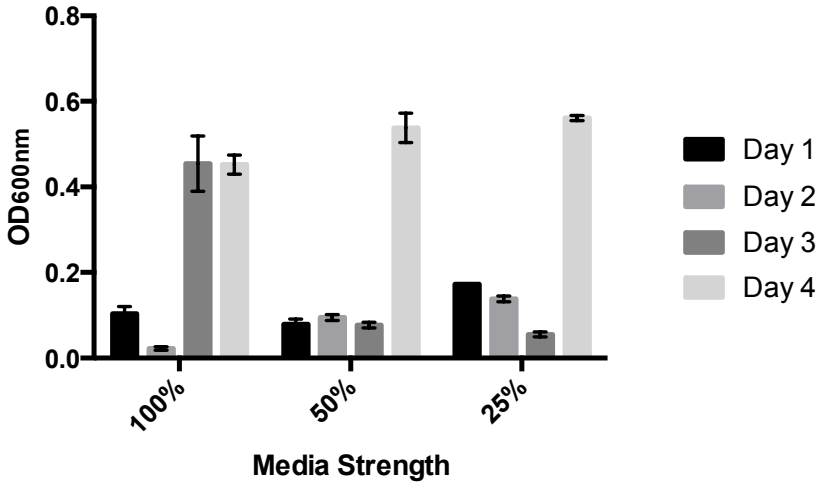


Figure 12 - Biomass using the immediate nutritional stress method.

As could be expected, cells grown in 100 %-TSB media have an extremely high biomass over time, however immediately stressing cells with 25 %-TSB media has a major effect over the four day experiment when compared to gradual stressing, overall biomass during Method A = 0.021 and biomass for Method B = 0.363. Repeats conducted in separate experiments in 6-well tissue culture plates, n=3.

Investigation into the 50 %-TSB media showed that by comparing the immediate stress method to the gradual stress method the difference in biomass between the two methodologies is statistically significant (P=0.006, unpaired t-test) over the four days.

When investigating the 25 %-TSB media, the difference between the two methods was only statistically significant on Day 2 and Day 4 (P=<0.001, unpaired t-test).

The method of immediately stressing the cells was adapted and modified for the remainder of the experiments during the project (Chapter 2 Section 2.3.3.3).

The methodology for 'Method B' was extended to cover a period of 6 days to investigate if like many other biofilm-forming bacteria, *N. lactamica* had a visible biofilm cycle. The biofilm life cycle previously described in Chapter 1 (Section 1.3.5) contains five stages.

The extension of the experiment demonstrated that although there was physically biofilm throughout all six days (Figure 13) it did not conclusively prove that all the cells were viable and alive. The increase in mass may be from an increase in sunken dead cells within the biofilm matrix. CV was again used to detect any increases, decreases or oscillations in biofilm mass. Figure 13 alludes to the biofilm cycle, although the exact biofilm stages cannot be attributed to individual days a pattern can be seen with biomass increasing and decreasing as cells adhere, multiply and disperse throughout the experiments.

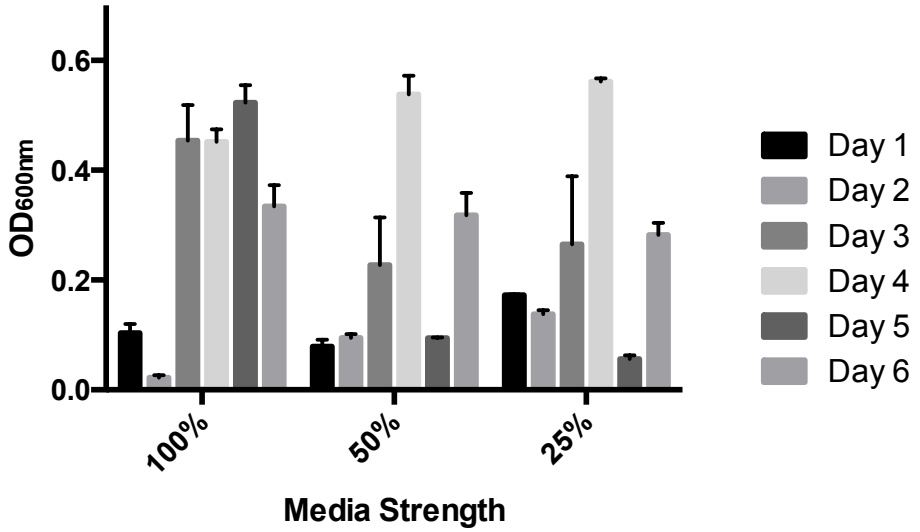


Figure 13 - Observation of Biofilm cycle stages I to V.

The oscillation of the graph alludes to the natural *N. lactamica* biofilm cycle. Groups of cells, possibly at stage IV and V within the biofilm cycle break away to allow colonisation of new areas, which results in a decrease in OD (Day 4 and Day 5 for biofilm in 50 %-TSB media and 25 %-TSB media respectively). In the experiments, the removal of some biomass is inevitable during media aspiration; however this is minimal and cannot easily explain the large decreases observed (50 %-TSB and 25 %-TSB media at Day-5). The graph demonstrates that after a decrease in biomass there is an increase, suggesting that the biofilm continues to increase. Results taken from repeated experiments, n=3.

3.3.4.2 CFU ANALYSIS

CFU data across six days was collected to determine the viability of biofilm.

Cells supplied with 100 %-TSB media demonstrated a high biomass, although the number of viable cells slowly decreases over 5 days (Figure 14).

Cells provided with 50 %-TSB media show a clear oscillation of viable cells; this is the type of pattern expected. Cell numbers peak and fall, however biomass continues to increase.

Cells supplied with 25 %-TSB media also show an oscillation of cell numbers; however this oscillation repeats less frequently than cells supplied with 50 %-TSB media, numbers peak twice in six days.

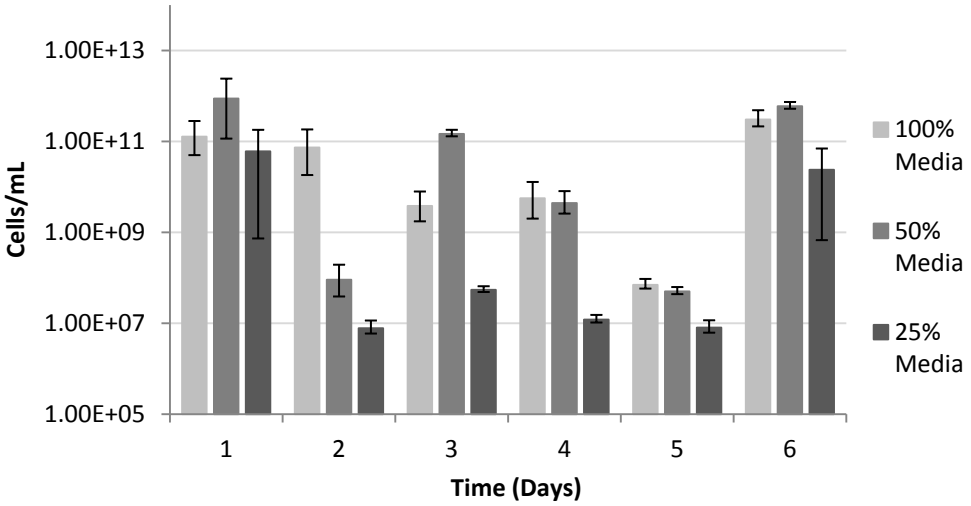


Figure 14 - Full sets of CFU counts over six days with all three different media concentrations using the immediate stress methodology.

A clear oscillation pattern is observed across all media concentrations. Biofilm grown in 100 %-TSB media at Day 5 has a low CFU count, yet a high biomass when compared to the CV data. CFU counts for biofilm supplied with 50 %-TSB media demonstrates a good growth pattern with CFU numbers remaining high from the initial 10^8 cells/mL seeding culture. Results taken from repeated experiments, n=6.

3.3.4.3 PHYSICAL OBSERVATIONS

Physical properties of the biofilm in both Method A and B experiments included a possible two-layer liquid-solid interface structure. A visual difference between where biofilm had been removed during feeding and a blank media-only well was observed. It was theorised that there is a dual layer within the biofilm consisting of an adhered layer of cells below a layer of matrix encased cells. During feeding the upper matrix

encase cells are easily removed by sheer force but are quickly replenished by the cells that are adhered to the plate. Figure 15 demonstrates the effects of removing spent media.

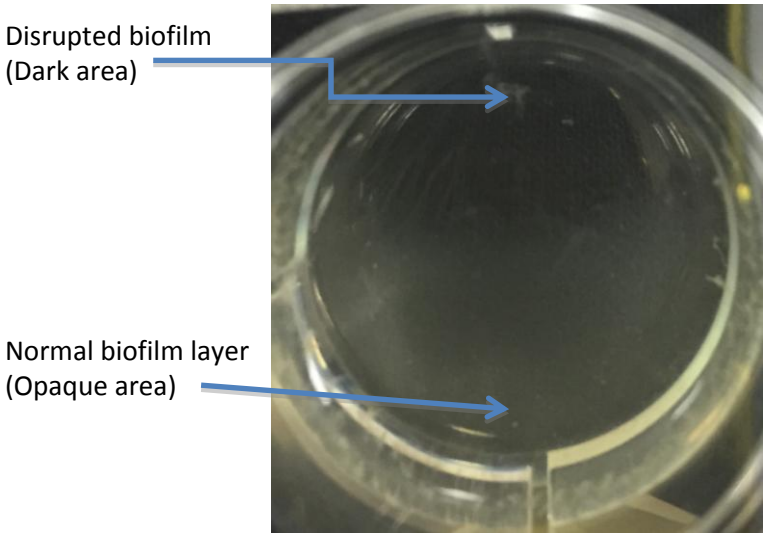


Figure 15 – Photo displaying the effects of media removal on a 1 Day *N. lactamica* biofilm.

The bottom part of the well shows the remaining biofilm after spent media has been removed; the presence of biofilm makes the well visually appear slightly yellow and cloudy. The top darker part of the well shows where the biofilm has been disrupted and a layer has been removed. After 24 hours this layer is replaced and there is little sign that any damage had previously occurred.

3.4 BIOFILM STRUCTURE

Scanning electron microscopy (SEM) was used to visually demonstrate what the bacterial structures appear like when stressed with the three media concentrations (25 %-TSB media, 50 %-TSB media and 100 %-TSB media). Although less damaging microscopy methods exist, SEM was chosen to enable an accurate visualisation of the bacterial cells *in situ* within the matrix. Highly sensitive, SEM imaging allows users to use a combination of high power microscopy and resolution to view extremely detailed structures. *N. lactamica* biofilms were grown in the required media concentration over one and two days. The biofilms were grown over glass coverslips before fixing for SEM imaging.

3.4.1 1-DAY BIOFILM

All coverslips were imaged at 100x magnification, 800x magnification and 4000x magnification (Figure 16 and Figure 17). Several images were taken at each magnification from different areas of the biofilm (four from the edges and four in the middle sections). At all media concentrations the *N. lactamica* cells adhered to the glass surface securely and biofilm covered the whole coverslip.

Figure 16 at 100x magnification 100 %-TSB media shows that there is some clumping of cells to form micro-colonies. These micro-colonies were not visible with the naked eye in the well prior to magnification. The cells appear to be adhered to one other and the glass surface, although the exact mechanism is not clear to see. Several images at 4000x magnification were taken, with all showing a similar structure; clusters of cells adhering together in a seemingly random structure. The CFU counts previously demonstrated that the amount of viable cells is high during this period at 1.58×10^{11} cells/ml (Figure 14). The 100x magnification pictures of all the media concentrations (Figure 16) do not show any fine structures present. Only biofilms grown in 100 %-TSB media showed aggregates of large groups of cells.

Initial observations at 100x magnification the 50 %-TSB media and 25 %-TSB media biofilms appeared to show that the sample processing steps required for SEM analysis had partially damaged the biofilm as visible cracks had appeared in the confluent layer. Further magnifications showed this cracking was consistent across all the samples at all concentrations.

Upon magnification at 800x and 400x it is possible to view connecting structures across the cracks. The presence of these small 'bridging' links demonstrates the impressive stability of the biofilm structure to withstand the destructive SEM processing steps.

The impact of reducing the media to 50 % has a marked effect on matrix production. *N. lactamica* grown at 25 %-TSB media exhibited a similar structure to bacteria grown at 50 %-TSB media (Figure 16), although those grown in 25 %-TSB media visually appear to have a denser structure with cells tightly aligned together when compared to those grown in 50 %-TSB media. The structures observed at both 50 %-TSB media and 25 %-TSB media are very different to the aggregating cells seen at in 100 %-TSB media.

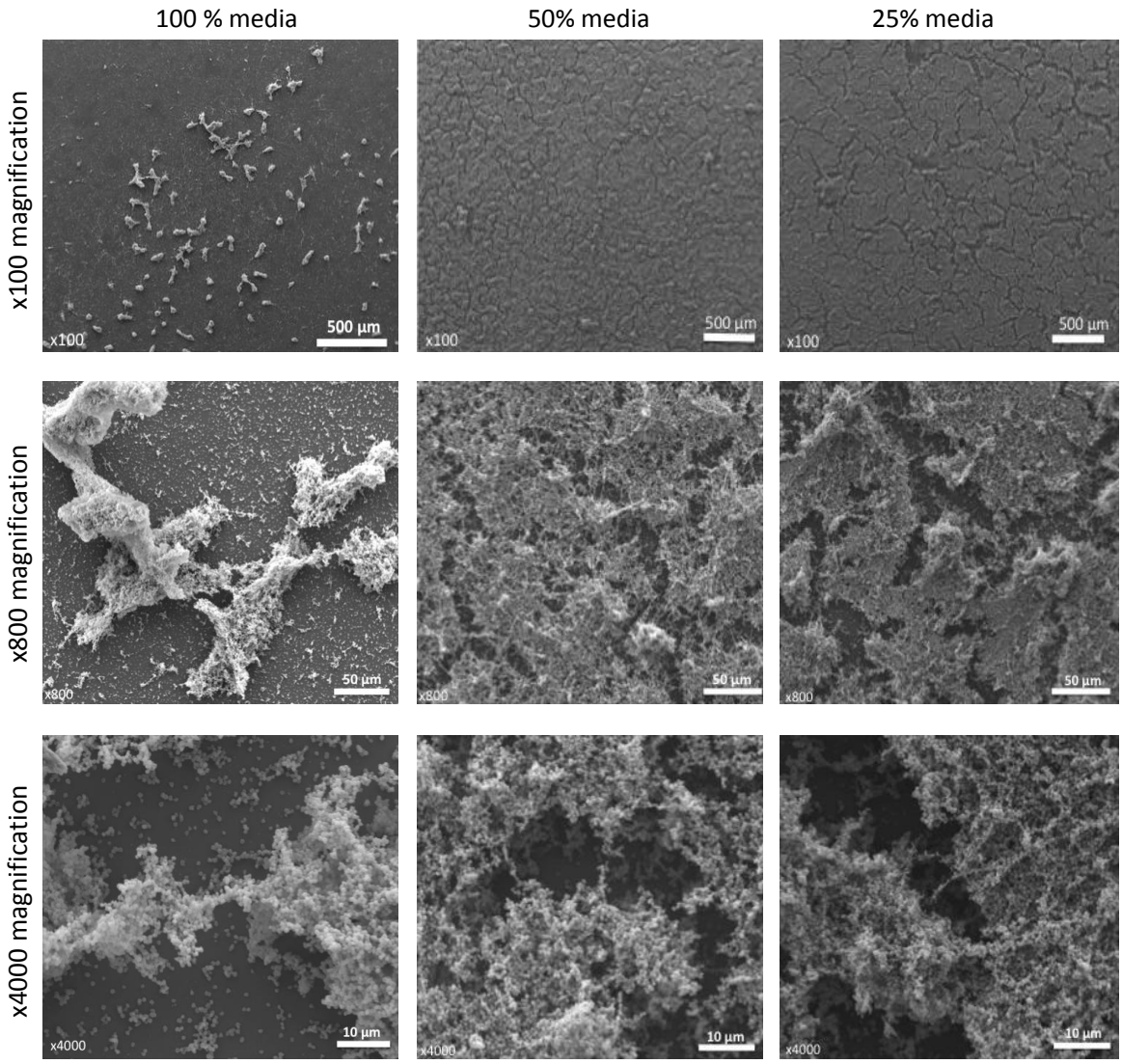


Figure 16 .Day-1 *N. lactamica* biofilm grown in 25 %-TSB media, 50 %-TSB media and 100 %-TSB media.

Samples were viewed at 100x, 800x and 4000x magnification. *N. lactamica* grown in reduced strength media (25 %-TSB media and 50 %-TSB media) appeared to be denser when compared to biofilm grown in 100 %-TSB media, n=1. Images were taken from several different section of the biofilm, including the centre and edges of the glass coverslip.

3.4.2 2-DAY BIOFILM

At Day-2 a biofilm matrix structure is now clearly visible under 800x magnification (Figure 17). Overall across all the media concentrations there visually appears to be more structure and mass when compared to the images from Day-1. At Day-2 individual cells can clearly be seen linking in lines across all media strengths at both x800 and x4000 magnification. Physical observations noted that during the washing and removing of spent media, Day-2 100 % biofilms have a thick viscous matrix layer which easily detached from the well and glass coverslip in sheet-like layers previously described in Section 3.3.3. Although some of this layer was removed Figure 17 demonstrates that there is still some structure remaining attached to the glass coverslip.

Again as seen previously in the Day-1 biofilm images the biofilm appears to have cracked. Small holes or gaps in the biofilm matrix with diminutive fine structures linking across were revealed at high magnification (4000x). This phenomenon can be seen in all media concentrations.

The CFU counts demonstrate that Day-2 cells are surviving despite an initial decrease when compared to Day-1 (Figure 14 – 100 %-TSB media = 7.35 x 10^{10} cells/ml, 50 %-TSB media = 9.05 x 10^{7} cells/ml and 25 %-TSB media = 7.78 x 10^{6} cells/ml), but that the biofilm or matrix component is easily damaged and separated from the well during the removal of spent media. At Day-2 the matrix structure is clearly defined and built with several layers of entwined cells. The images taken at both 400x and 800x magnification (Figure 17) show how *N. lactamica* is capable of producing a matrix in which individual cells can reside.

SEM is unable to distinguish between alive and dead cells so it remains unknown whether the cells at the bottom layer and within the matrix are viable. The phenotypes of the structures at Day-2 are generally less prone to damage than those compared to the structures at Day-1 when washing and feeding.

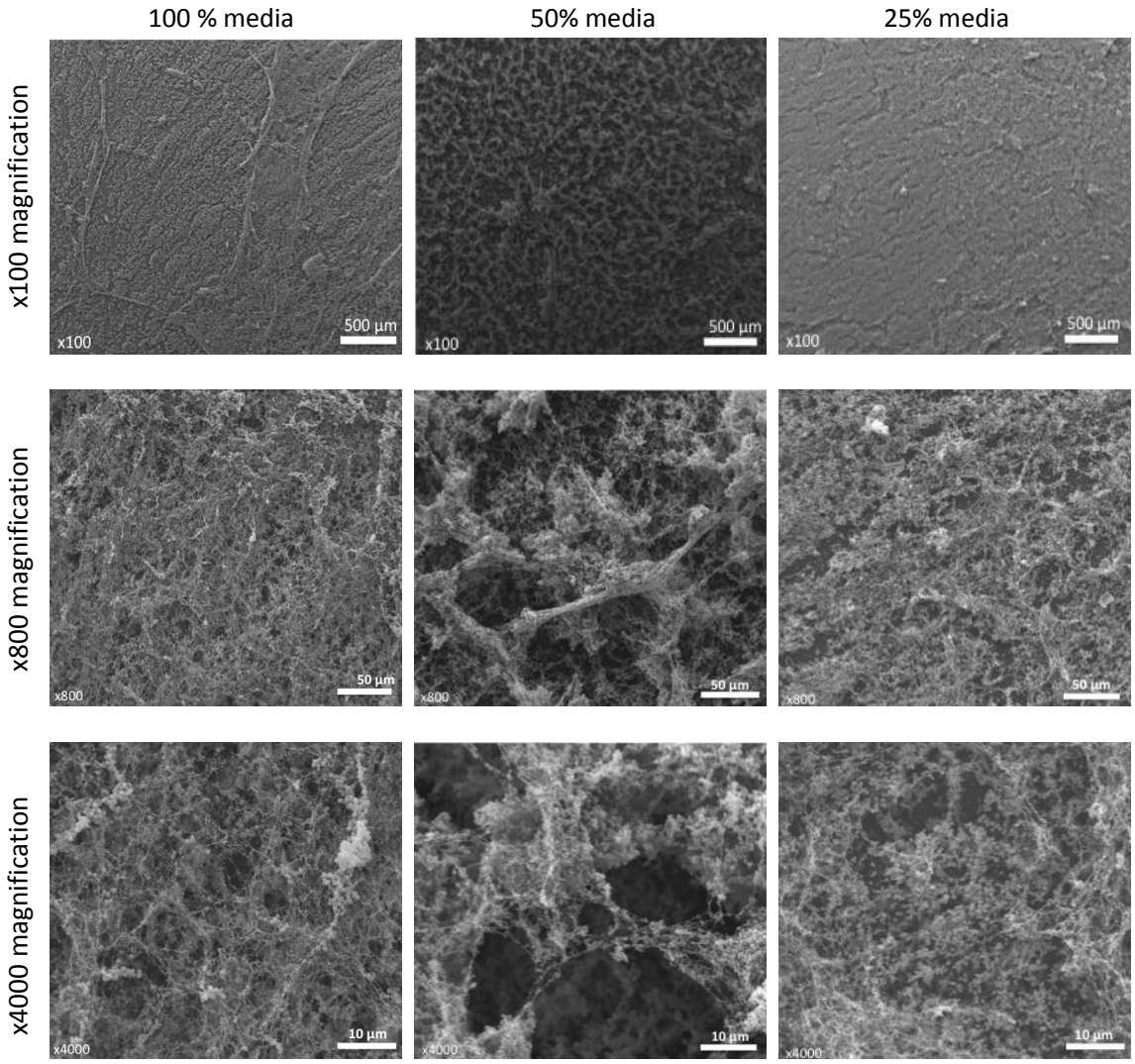


Figure 17. Day-2 *N. lactamica* biofilm grown in 25 %-TSB media, 50 %-TSB media and 100 %-TSB media.

Day-2 growth viewed at 100x, 800x and 4000x magnification. The matrix structure at Day-2 is now more defined in comparison to Day-1, removing spent media and adding fresh media to each well has not destroyed or removed the biofilm from the coverslip. Layer of cells entwined within a matrix can be seen in all media concentrations, although SEM is unable to distinguish between alive and dead cells, n=1.

3.5 ANTIBIOTIC RESISTANCE

A predominant feature described in the literature of a biofilm is its ability to increase resistance to antibiotics (Hall-Stoodley *et al*. 2004). To further demonstrate that *N. lactamica* is able to successfully form a biofilm in order to enable carriage and prevent clearance, it was investigated if *N. lactamica* cells in a planktonic state were more susceptible to antibiotic treatment compared to those in a biofilm state.

3.5.1 PLANKTONIC CULTURE

Using the methodology described in Chapter 2 section 2.4.1 the first assay investigated the effect of kanamycin on *N. lactamica* in a planktonic culture. Two strains of *N. lactamica* were used within this assay. One isolate was *N. lactamica* Y92-1009 previously used throughout this thesis; the second *N. lactamica* strain was Y92-1009 that contained a kanamycin resistance plasmid. Both MIC and MBC was identified in both isolates.

The MIC concentration was identified as 5 ng/mL for *N. lactamica* Y92-1009 (without the kanamycin plasmid); the MIC for the *N. lactamica* strain with the kanamycin plasmid was over 0.5 µg/mL (Table 3).

Spot plating each concentration of *N. lactamica* Y92-1009 showed that the MBC was also 5 ng/mL. The *N. lactamica* containing the kanamycin resistant plasmid was able to grow at 0.5 µg/mL but not at higher concentrations. The experiment demonstrated that the kanamycin resistance plasmid conferred 100-fold resistance when compared to the strain without the plasmid.

	N. lactamica Y92-1009	***N. lactamica*** **KanR**
500 µg/mL	*No growth*	*No growth*
50 µg/mL	*No growth*	*No growth*
5 µg/mL	*No growth*	*No growth*
0.5 µg/mL	*No growth*	*Growth identified*
50 ng/mL	*No growth*	*Growth identified*
5 ng/mL	*Growth identified*	*Growth identified*
Control	*Growth identified*	*Growth identified*

Table 3– The effect of kanamycin on planktonic *N. lactamica* culture.

The *N. lactamica* strain with the kanamycin plasmid confers a 100-fold increase in resistance to Kanamycin when compared to the wild type *N. lactamica* strain without the plasmid. This demonstrates that *N. lactamica* is susceptible to the antibiotic. Results taken from repeated experiments, n=3.

3.5.2 BIOFILM

The experiment was modified and repeated in biofilm as described in Chapter 2 Section 2.4.2, using only the *N. lactamica* strain Y92-1009 without the kanamycin plasmid. 1-Day *N. lactamica* biofilms were treated with each antibiotic dilution for 2 hours in triplicate. The biofilms were then twice washed with sterile water to remove any remaining antibiotic. CV was used to demonstrate that the antibiotic does not disperse the biofilm (Figure 18).

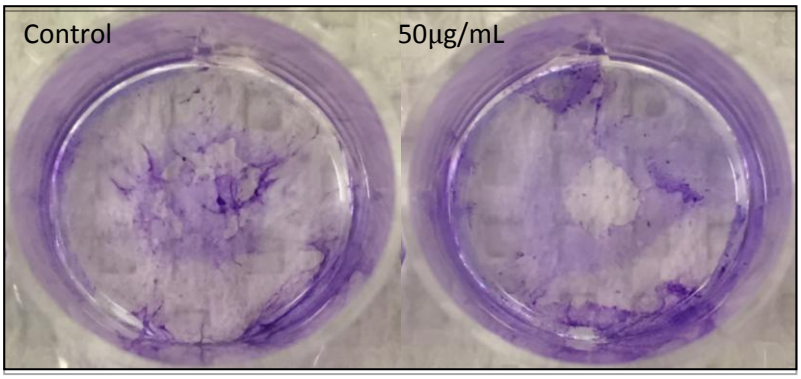


Figure 18– The biofilm remained intact after an antibiotic treatment.

During the CV washing process some damage to the biofilm did occur but the antibiotic did not physically disperse the biofilm. This was observed across all experiments and suggests that antibiotic treatment alone will not disperse biofilm.

The use of kanamycin on the biofilm did not appear to have any effect on the structure of the biofilm. The CV stain demonstrated that the biofilm was unaffected and remained intact after the treatment.

The spot plating the biofilm showed that *N. lactamica* cells were able to survive an antibiotic concentration of 50 µg/mL. When this is compared to the MBC of the planktonic assay of *N. lactamica* without the plasmid (5 ng/mL), there is a 10,000-fold increase in viability (Table 4).

	N. lactamica Y92-1009 in Planktonic		***N. lactamica* Y92-1009 in biofilm**
	MIC	MBC	MBC
500 µg/mL	*No growth*	*No growth*	*No growth*
50 µg/mL	*No growth*	*No growth*	*Growth identified*
5 µg/mL	*No growth*	*No growth*	*Growth identified*
0.5 µg/mL	*No growth*	*No growth*	*Growth identified*
50 ng/mL	*No growth*	*No growth*	*Growth identified*
5 ng/mL	*Growth identified*	*Growth identified*	*Growth identified*
Control	*Growth identified*	*Colonies observed*	*Growth identified*

Table 4 – A comparison of the effect of Kanamycin on *N. lactamica* in both planktonic and biofilm. When compared to Planktonic culture, *N. lactamica* biofilm are 10,000 times more resistant to kanamycin. Results suggest that not only is *N. lactamica* producing biofilm, but also that the biofilm confers some protection to individual cells. Results taken from repeated experiments, n=3.

3.6 CONCLUSIONS AND DISCUSSION

Overall there is good evidence within this chapter to suggest that under nutritional stress *N. lactamica* is able form a biofilm *in vitro*.

Individually no single experiment can conclusively demonstrate that biofilm formation is transpiring however in combination, using methodologies previously employed in the literature, there is strong evidence that *N. lactamica* is able to survive and thrive in a biofilm state. This perhaps should not be surprising, as many other respiratory flora species have recently been identified as able to form biofilm (Hall-Stoodley *et al*. 2004; Hanke *et al*. 2004; Marks *et al*. 2012; O'Toole *et al*. 2000) . Overall the hypothesis of nutritionally stressing *N. lactamica* appears to fit with observations of other biofilm-forming bacteria (Poole, 2012).

3.6.1 CV ANALYSIS

CV analysis was used as a rapid tool for identifying biomass and the methodology was adapted to prevent as much damage to the biofilm structure as possible. A major issue with CV staining is the damage caused to the biofilm during the washing steps. Repeated washing steps damages the biofilm structure which can then easily become detached from the plate. To minimise the amount of potential damage, removing and added liquid from the well was done in the same location each time and a small amount of liquid was maintained in the well at all times to prevent the structure drying out and disintegrating. Poor adhesion resulting from the removal of liquid suggests that *N. lactamica* biofilms are adapted to survive in a moist or humid environment.

The initial biofilm formation experiment using crystal violet (Section 3.3.1) showed that *N. lactamica* was able to adhere and maintain that adherence to a polystyrene plate (Figure 7). Adherence is a critical survival factor for respiratory commensals. Initial attachment enables the bacteria to anchor onto the epithelial layer; from this stage biofilm formation is possible (Otto, 2014). This initial experiment was encouraging,

demonstrating that cells readily adhere to the plastic surface in large multicellular structures. Further investigation focused on optimising the media and nutrient conditions needed for biofilm growth.

CV is a stain which can be used with many different types of biofilm formation (O'Toole, 2011). The 7-Day CV experiment (Section 3.3.3, Figure 10) demonstrated that biomass is established quickly (prior to 24 hours). It also demonstrated that biomass can be sustained over 7 days without nutrient replenishment, although this is not indicative of a thriving biofilm. There was also a phenotypic change in the biofilm post 24 hours, the biofilm became thicker and had a thick viscous texture when removing spent media. This is commonly seen in *Pseudomonas aeruginosa* isolates which express high levels of polysaccharide (Evans and Linker, 1973). Despite no nutrient replenishment *N. Lactamica* was able to survive in a biofilm state for seven days, although a 3-log fold drop in CFUs was observed survival was possible. This experiment also established that prolonged starvation does not disperse biofilm. Although cells could survive there was no indication that the normal biofilm cycle is present (as described in Section 1.3.5) suggesting conditions are not favourable for *N. lactamica* to thrive. Overall Section 3.3.3 demonstrated it is possible to maintain a population of *N. lactamica* cells without media replenishment, which is indicative of a biofilm.

Section 3.3.4.1 Figure 13 demonstrated that *N. lactamica* is able to produce a characteristic biofilm cycle. We hypothesize that the biofilm is able to regulate its own population size. Upon observing an increase in biomass in Figure 12 the experiment was continued for six days to identify the natural biofilm cycle described in the literature. Figure 13 demonstrates this oscillation in biomass, indicating that parts of the biofilm are able to disperse, which would enable the cells to form new biofilm colonies (Garrett *et al.* 2008). Although a small proportion of biomass loss was due to the removal of spent media, the amount of biofilm lost varied on a daily basis. This may give *N. lactamica* an advantage in the nasopharyngeal niche, as sections of biofilm containing viable cells may be able to colonise on to another surface away from the parent biofilm (Garrett *et al.* 2008). Alternatively another theory for this dispersal

could be due to a large section of cells dying. Dead areas of the biofilm could be dispersing to allow a flow of nutrients to enter and sustain the living section(s). Alternatively using quorum sensing bacteria in the biofilm could be producing enzymes which actively break down the biofilm (Garrett *et al.* 2008). Overall the CV data in Section 3.3.4.1 demonstrates that there is a maintained biomass across a prolonged period of time (Figure 13) and that this mass varies across seven days, which is suggestive of a thriving biofilm.

Although no mechanical sheer-force tests were performed, physical observations of both types of biofilm indicated that those biofilms that were stressed gradually did not attach to the plate as well as those that had been immediately stressed. This may be due to the amount of cells initially attaching to the plate to form biofilm; with fewer cells attaching to the plate for biofilm formation and producing matrix the structure would naturally be weaker. Although there is no known surface upon which bacteria cannot attach, the strength of that attachment is determined by many factors such as hydrophobicity, pili attachement and chemical composition of the surface (Paulson et al. 2009)

3.6.2 CFU ANALYSIS

The colony forming unit counts (Section 3.3.4.2 Figure 14) demonstrate that the biomass observed from Section 3.3.4.1 is viable and cells are able to survive and thrive in the environment even when under nutritional stress. The CFU numbers show that the biomass is not predominantly comprised of dead or dying cells, but a living mass of cells that are able to replicate and survive over a long period of time. The CFU counts also show that although numbers initially decrease after Day-1 the biomass continues to increase, possibly until the biofilm becomes unsustainable, and then decreases. CFU numbers only decrease by a single log-fold when compared to inoculation (each biofilm was seeded with 10^8 cells/mL) across a six-day period. This demonstrates that even when only supplied with 25 %-TSB media cell numbers can be maintained over a prolonged period in a biofilm state.

There is an observed difference between nutritional stress and gradual nutritional stress, although statistical analysis was inconclusive. By entering a low nutrient environment the bacteria quickly form a biofilm (within 24 hours), and are able to maintain the structure. This was in stark contrast with gradual stressing; it appears that the cells do not form a confluent biofilm structure that is comparable to the immediate stressing methodology. Although a small amount of biomass is clearly evident, it appears the majority of cells remain in a planktonic state. The effect of stressing immediately had a marked increase on the biomass of the biofilm (Figure 10), either from cell volume increase or EPS production as a reaction to stress (Garrett *et al.* 2008). Immediate nutritional stress produced a higher value of biomass at each time-point. This continued over time with an increase in the amount of biomass produced.

Experiments investigating CFU counts supplied with 100 %-TSB media produced an unexpected result. As the bacterial cells are not nutritionally stressed and do not have the extreme competition for nutrients unlike cells supplied with the other reduced concentrations, it was theorised that CFU counts would be continually high. The results from Figure 14 demonstrate that this was not necessarily the case and the results followed a similar pattern to 50 %-TSB media and 25 %-TSB media.

CFU counts supplied with 50 %-TSB media demonstrated an oscillation pattern similar to 100 %-TSB media. At Day-2 counts were at the lowest which could suggest that nutritionally stressed cells may be dying, becoming quiescent and/or are no longer viable using standard culturing methods, a phenomenon which is documented in the literature as a potential survival strategy by biofilm cells (Gilbert *et al.* 2002). These numbers increase post Day-2 and the oscillation continues demonstrating that the biofilm is maintainable.

Figure 14 demonstrates that cells are able to survive in 25 %-TSB media, and despite this nutrient-depleted environment biofilm formation is observed. 25 %-TSB media is not an ideal model to work with due to the length of time needed for cell numbers to increase post inoculation into a nutrient-reduced environment.

The overall drop in CFUs at Day-2 when compared to Day-1 across all media concentrations indicates that some cells have died and the biofilm may have absorbed the dead cell components. Some cell death may be beneficial in order to produce matrix and 'protect' the remaining cells. Alternatively the drop may be attributed to some cells becoming dormant and are no longer viable until more favourable conditions return. Termed 'quiescent cells' these cells are seen in biofilm populations where nutrients are particularly scarce (Gilbert *et al.* 2002).

3.6.3 CONTAMINATION IDENTIFICATION

One major disadvantage to static biofilms is that spent media is removed and fresh media supplied; this is done manually at each time point. At each time point the well is exposed to the environment and contamination can occur. Contamination rates are higher if the microbiological safety cabinets (MSCs) used to keep work sterile are multi-user with different species. Sharing MSC's in some facilities is unavoidable so extra caution must be applied. Visually spotting contaminated biofilms early can save valuable time and consumables. Another method of identifying the contaminant would be to send a sample for full PCR identification; unknown bacterial infections are routinely diagnosed using such techniques in a clinical setting. In this thesis it was decided that identifying the contaminant was not a justifiable cost as it bore no clinical significance however a samples was frozen. After deep cleaning the MSC, autoclaving equipment and using only 'biofilm-specific' consumables the contamination was only observed on five other occasions throughout this thesis. It is therefore highly likely that the contaminant is a bacterial species commonly found on the skin, *Staphlococcus epidermidis* for example is a known biofilm forming species (Archer *et al.* 2011). Each time the contaminant was discovered it was quickly eradicated by deep cleaning all equipment and using fresh consumables. Samples from three separate wells, from three separate experiments were subjected to PCR; all three produced a similar banding pattern.

3.6.3.1 GRAM STAINING

The gram staining methodology proved to be inconclusive. The presence of both gram negative and gram-positive bacteria in the liquid-surface biofilm layer could be attributed to *N. lactamica* expressing a different phenotype in biofilm. This hypothesis is unlikely however as *N. lactamica* does not have a peptidoglycan layer unlike other bacteria known to vary in biofilm.

3.6.3.2 PCR

Results from the PCR experiments enabled a positive identification of a pure *N. lactamica* biofilm. Several samples from the confluent biofilm layers were subjected to PCR testing alongside biofilm layers of questionable origin. Had the confluent layer been made by another species the banding pattern on the Agarose gel would not match the banding pattern of the known DNA sample of *N. lactamica*. The same applies if the structure had been made by another species yet contained *N. lactamica* cells as two distinct banding patterns would appear. From this set of experiments contamination could easily and quickly be identified. It confirmed that a *N. lactamica* biofilm is a smooth and confluent structure when viewed in a tissue-plate.

3.6.4 SEM IMAGING

The images of 100 %-TSB media demonstrate that *N. lactamica* is able to produce a matrix structure, without nutritional pressures, in which further cells are able to reside. This matrix can confer some protection to the cells but it is highly unlikely that cells *in vivo* will be exposed to such a media rich environment as the nasopharynx is known to be a hostile environment (Marks *et al*. 2012a). Biofilm grown in 100 %-TSB media easily detached from the substratum in sheet-like layers. These sheet-like layers encasing the cells could be an example of the basal layer of cells creating an extra-cellular matrix cover, upon which further cells attach and further create more extra-

cellular matrix. This dual sheet-like layer was exclusive to the 100 % media suggesting that due to the abundance of media the cells were able to form a biofilm but it was perhaps established slightly later than those exposed to the reduced media and therefore initial attachment between cells is weaker.

From the images shown in Figure 16 it was demonstrated that cells exposed to 100 % media initially adhere to each other, prior to matrix production and visualisation. From the magnification images it is unclear if the cells attach to the surface first then to one another, or if cells adhere to one another when in a planktonic state fall to the bottom of the well and then adhere to the surface. This clumping could be a process known as bacterial coaggregation, and has been shown to occur in dental biofilm. It is theorised that bacterial coaggregation allows the development of oral multi-species biofilm (Rickard *et al.* 2003). The initial 100x magnification (Figure 16) does not show any fine structures present, only large groups of cells. This is especially evident when compared to the images at Day-2 (Figure 17). Several images were taken from numerous different parts of the biofilm with all images demonstrating similar structural conformation. This may be because there is no limited nutritional strain on the cells compared to the other media concentrations, and therefore there is no little requirement for a robust matrix structure.

The SEM processing is extremely damaging to delicate structures, during processing the spent media is removed; the biofilm is washed to remove planktonic cells, and finally prepared for analysis via several ethanol submersion steps. The processing visually appeared to destroy a large proportion of the biofilm material and through each processing stage parts of the biofilm structure and cells would have been lost regardless. However, despite this intensive processing some biofilm structure did survive.

Although there does not appear to be any order to the structure, this may be due to the amount of disruption the biofilm underwent during the processing stages. Visually during processing there appear to be very little structure remaining.

Figure 16 and Figure 17 both display evidence of the biofilm cracking. Dehydration steps involved in SEM processing could cause the cracks seen in the samples alternatively it is possible that these are natural structures. We theorised that the cracks are in fact water and nutrient channels that enable the biofilm to support growth and maintain cell numbers by allowing fresh water and media to circulate via capillary action. These nutrient channels have been previously seen in other biofilms (Yi *et al.* 2004) and are important in biofilm maintenance. The Day-2 images (Figure 17) show that the 'cracks' appear to be heavily surrounded with matrix in all media concentrations.

When the limited media images are compared to the 100 %-TSB media biofilm on Day-1, the cells supplied with 50 %-TSB media appear to have thicker matrix, with cells along the surface of the glass slip with a mixture of matrix and cells on top. Cells that were supplied with 50 %-TSB media had a denser matrix structure when compared to cells supplied with 100 %-TSB media during washing. A reduced media concentration would also more accurately reflect the challenging environment cells have to survive in the nasopharynx. The denser matrix produced by the 50 %-TSB media and the 25 %-TSB media may be due to a higher volume of EPS being produced. The production of EPS under high nutrient stress is also seen in *Bacillus subtilis* (Zhang *et al*, 2014). Zhang *et al* (2014) identified that when *B. subtilis* is nutritionally stressed more matrix is produced. A similar phenomenon can be seen here in *N. lactamica*.

Physical observations noted very little phenotypic difference between cells supplied with 50 %-TSB media and 25 %-TSB media; with the exception that biofilm supplied with 25 %-TSB media were more susceptible to damage at the earlier stages of growth (up to Day-2) from removing spent media and adding fresh than those grown at 50 %-TSB media. Large vibrations from other lab users and lab equipment could damage the initial attachment stages of a biofilm and therefore delay visible biofilm formation. Biofilms supplied with 25 %-TSB media are more fragile when compared to other media concentrations however the surface area of a cell culture well is markedly larger than the recesses and crevices found in the hosts' nasopharynx. Demonstrating that *N.*

lactamica can successfully survive in a depleted nutrient environment that could potentially correspond to the environment found within the nasopharynx. By occupying a small recess within the nasopharynx *N. lactamica* could thrive in small pockets in a biofilm state, prolonging carriage and avoiding clearance in a similar way in which many other respiratory and oral bacterial species can be identified from the pockets, crypts and surfaces on adenoids (Nistico *et al*. 2011).

There were no phenotypic differences noted between biofilms grown on a glass surface compared to those grown on plastic, both biofilms behaved the same way during adding fresh media and removing spent media. There was neither a visual difference to the amount of biomass in each well nor a difference in the ability for the biofilm to adhere to the glass surface. This supports the theory that bacterial biofilms are able to adhere to most surfaces provided there is an appropriate foundation upon which cells can adhere (Garrett *et al.* 2008)

Overall the SEM images demonstrate that under nutritional stress (50 %-TSB media and 25 %-TSB media) the cells produce a matrix prior to sampling at 24 hours. An essential component of a biofilm, the matrix provides structural support for the cells and well as protection from the environment. Although the SEM images do not detail what the matrix is comprised of, the images do show that cells are able construct the matrix in a way that allows for some nutrients and water to pass through, maintaining the cell viability and enabling growth. The matrix is an extremely strong structure, surviving the SEM processing stages that involve several dehydration steps. The presence of the matrix in the SEM images demonstrates that the structure is able to withstand some stress from sheer forces. Although inevitably some of the structure was lost, enough remained to view. The images also appear show that the more *N. lactamica* is subjected to nutritional stress the more matrix/EPS is produced. Although the SEM is unable to quantify the amount of matrix produced, the images show that the cells are situated closer together and more tightly packed at 25 %-TSB media (x800 magnification in Figure 16 and Figure 17) than at 50 %-TSB media (x800 magnification in Figure 16 and Figure 17).

3.6.5 ANTIBIOTIC RESISTANCE

The antibiotic Kanamycin is used for treating severe bacterial infections and usually given as an intramuscular injection. Kanamycin is an aminoglycoside antibiotic that was originally identified from *Streptomyces kanamyceticus*. The antibiotic is effective against bacterial growth due to binding to the bacterial 30S ribosomal subunit. This binding causes the ribosome to mis-read the tRNA and leaves the bacteria unable to synthesize proteins. Once protein synthesis has been disrupted the bacterial cell is unable to produce these vital proteins required for sustained life (https://www.drugs.com/pro/kanamycin.html).

The experiment demonstrated that the biofilm offered protection to the cells and was not dispersed by the antibiotic. This effect is also seen in other biofilm-forming species such as *Escherichia coli* (Bernier *et al.* 2013) Some disruption to the biofilm occurs normally when adding and removing liquid from the well. Figure 18 demonstrates that there was minimal disruption of the biofilm during the experiment with a substantial amount of biomass remaining. The biofilm offered the cells protection from the antibiotic and was not dispersed ensuring cells remained viable.

To enable an accurate MIC, the planktonic cultures were incubated with Kanamycin for 18 hours. This is in contrast with the biofilm cultures that only received a 2-hour treatment. A shorter contact time between antibiotic and biofilm is more medically relatable. Patients with infection who medicate at home are highly likely to take the antibiotic prescribed in tablet form. This would mean that the amount of antibiotic circulating in the patients body is not only relative to body mass but also would greatly reduce the contact time due to enzymatic activity.

3.6.6 BIOFILM FORMING SPECIES

The combination of all these experiments demonstrates there is extremely strong evidence that *N. lactamica* is a biofilm-forming species *in vitro*. Although there are limitations for any experiment the volume of evidence accrued indicates that *N.*

lactamica does produce a biofilm when under nutritional stress and is able to maintain that biofilm over a prolonged period of time successfully.

CHAPTER 3 *NEISSERIA LACTAMICA* BIOFILM

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Can all isolates of Neisseria meningitidis form a biofilm?

4.1 INTRODUCTION

Neisseria meningitidis was first isolated from a diseased patient in 1887 by Anton Weichselbaum, and was identified as the causative agent of meningitis (Cartwright, 2001). Modern medicine is able to demonstrate that *N. meningitidis* rarely causes disease, instead preferring a commensal state (Yazdankhah and Caugant, 2004). The prevalence of meningococcal carriage is seen across all ages, although carriage is generally more common in childhood peaking in late adolescence, adults are not excluded. Although it is less common in adulthood, it is estimated that 7.8 % of 50 year olds in Europe are asymptomatic carriers; this is compared to 4.5 % and 23.7 % for children and teenagers respectively (Christensen *et al.* 2010). This persistent carriage rate demonstrates that *N. meningitidis* is well adapted for the nasopharyngeal niche, however the length of time that asymptomatic carriage is maintained varies; carriage can be for a few weeks to several months (Yazdankhah and Caugant, 2004). The presence of meningococci in the oropharynx produces an antibody response after a few weeks that will clear the organism. This implies *N. meningitidis* avoids clearance by the immunity-associate cells, however the exact mechanism is yet to be fully concluded.

Long-term carriage may be enhanced by a biofilm state. It has been previously suggested that biofilm formation is hindered by the production of capsule, a known virulence factor in *N. meningitidis*. The majority of *N. meningitidis* isolates found in healthy asymptomatic individuals do not express capsule. However, virulent encapsulated *N. meningitidis* have been shown to form biofilms on abiotic surfaces (Lappann and Vogel, 2010), although poorly. The majority of virulent isolates appear to be inhibited by capsule production, which is important in providing protection from the hosts' immune system during disease (Cress *et al*. 2014). Capsule production alone however does not mean that an isolate of *N. meningitidis* is virulent. It has been shown that down regulation of capsule production in some meningococcal strains is

possible (Swartley *et al.* 1997), enabling the ability for biofilm formation and long-term carriage.

4.2 AIMS FOR CHAPTER 4

The aims for this chapter are as follows;

- Demonstrate that *N. meningitidis* isolates previously isolated from asymptomatic carriers are sustainable in TSB supplemented with 0.2 % yeast extract. Expand on previous work (conducted by another member of the team), which identified TSB as a suitable medium for *N. lactamica* and a small selection of *N. meningitidis* isolates. Identify if TSB is a suitable medium for the remaining isolates that have been sequestered from asymptomatic carriers.

- Identify whether any isolates sustained by TSB are able to form a biofilm or a structure(s) that phenotypically expresses biofilm characteristics when grown in full strength TSB. Identify and classify, using the model previously developed in Chapter 2 any of the *N. meningitidis* isolates which form a biofilm.

- Identify any common characteristics between biofilm phenotype and the origin of the isolates. Small amounts of information are available about the strains; characterise any common factors between isolates that are good biofilm-formers and the length of carriage within the observational clinical study.

4.3 *NEISSERIA MENINGITIDIS* BIOFILM SCREENING

An investigative study in 2011 collected over 50 *Neisseria meningitidis* isolates by nasopharyngeal swabbing healthy individuals (Deasy *et al*. 2015; Clinical trial study ID number: NCT02249598). These healthy individuals were screened for *Neisseria* carriage using gonococcus-selective media. The participants were randomized to receive either a controlled inoculation of *N. lactamica* (10^4 colony-forming units) or to receive a control solution of phosphate-buffered saline solution. The volunteers were observed for a total of 28 weeks post screening with an additional inoculation of *N. lactamica* at week 26. Any *N. meningitidis* isolates identified during screening were cultured and given a randomisation letter and number (letter R, number 1-300). Nasopharyngeal swabbing was conducted throughout the visits during this period (at weeks 2, 4, 8, 16, 26 and 28). Throughout the duration of the study some of the *N. meningitidis* isolates were lost, replaced with another *N. meningitidis* strain, maintained or 're-acquired' after appearing to have been lost. The *N. meningitidis* isolates that appeared to displace a previous isolate were also collected, cultured and given a randomised letter and number combination (Letters S-Z, number 1-300). For this thesis only those *N. meningitidis* isolates identified at screening from asymptomatic students (R coded isolates) were investigated. The study was known locally as the 'Lac2 study' and shall be referred to as such throughout the remainder of this chapter and thesis.

The research group demonstrated from the Lac2 study that *N. meningitidis* carriage in the control group slowly increased during the study peaking at 33.6 %, compared to *N. meningitidis* carriage rates of 14.7 % in those volunteers who had been inoculated with *N. lactamica*.

N. lactamica colonisation further inhibits the carriage of *N. meningitidis*, especially when in combination with the glycoconjugate meningococcal vaccine. They suggested that *N. lactamica* carriage conferred some natural immunity to the host, and *N.*

lactamica or some of its components could be used to suppress further meningococcal outbreaks in the future (Deasy, *et al*. 2015).

We theorised that the *N. meningitidis* isolates identified during the screening process may be biofilm-forming isolates. It was hypothesised that these collected isolates may exhibit a positive correlation between length of carriage and successful biofilm formation. To prevent any bias interpretations, all information collected about the isolates was withheld until the end of the screening process. Only the screening identification numbers were disclosed. All phenotypes and observations were documented.

4.3.1 N. MENINGITIDIS GROWTH IN TSB

Master stocks were fully screened for any contaminates. Master stocks had previously been obtained from the nasopharyngeal swabs of the healthy volunteers.

From the *N. meningitidis* stocks 1 colony/mL was added to fresh TSB to ascertain growth. The inoculum was grown with agitation at 37 ^{o}C and 5 % (v/v) CO_2, OD_{600nm} readings were taken hourly to ensure an increase in biomass and therefore growth. Those isolates that failed to grow in 100 %-TSB media were examined for growth in bovine BHI media; this ensured the original stock was still viable. Isolates that grew in either TSB or BHI were checked for any contamination.

Of the 27 isolates examined all except two demonstrated successful growth in TSB (Table 5). The two strains R034 and R146 failed to survive in TSB but successfully grew in BHI media; this indicated that the problem was not with the master stock but the media source available. The isolates that demonstrated positive growth in TSB were further screened for biofilm formation.

4.3.2 SCREENING ISOLATES FOR BIOFILM GROWTH

Using the *N. lactamica* biofilm model developed during Chapter 3, the 27 *N. meningitidis* isolates and an additional, fully sequenced serogroup B isolate, *N. meningitidis* MC58 were individually screened for recognisable biofilm growth.

To encourage biofilm formation each isolate was grown in full strength and 50 % reduced TSB supplemented with 0.2 % yeast extract media in static non-coated plastic tissue culture plates. Biofilms were screened for growth over a four day period. 1 mL of spent media was removed and 1 mL of fresh media was applied every 24 hours. The experiment was conducted in duplicate with both 50 %-TSB media and full strength 100 %-TSB. At each time point, one biofilm from each isolate was twice washed with sterile water and stained with 10 % CV to rapidly identify any biofilm growth and visually assess any biofilm attachment to the well. The second biofilm of each isolate was twice washed with sterile water, removing the majority of planktonic cells and re-suspended in 1 mL of sterile water. Plate scrapers were employed to remove any remaining biofilm from the plastic well surface. A single loop of inoculate was streaked onto CBA plates and incubated at 37 ^{o}C and 5 % (v/v) CO_2 for 16 hours to determine if cells were viable.

The phenotype of each isolate was recorded over the four day time period (Table 5). MC58 for was selected for comparison as it has been widely studied in the literature and genetic information is available. It has also been previously demonstrated that despite MC58 expressing capsule the isolate is able to successfully form a biofilm on HBE cells grown *in vitro* on a flow cell model (Neil *et al*. 2009).

Isolate number	Biofilm Growth (in 50 % media)	Phenotype visual observations
R010	Yes	Similar properties to *N. lactamica* (Very thin)
R022	Yes	Aggregates/'clumps' within a extremely thin matrix
R034	No	Cannot survive at 50 % struggles to survive at 100 % past 24hrs. Grows well in BHI.
R046	Yes	Similar properties to *N. lactamica*
R049	Yes	Similar properties to *N. lactamica.*
R054	Yes	Similar properties to *N. lactamica*
R059	Yes	Presents an air-liquid and liquid-surface interface
R079	Yes	Aggregates or 'Clumps' to the plate
R083	Yes	Only aggregates, but really adheres to the plate
R104	No	Aggregates into large clumps within the media, no adhesion.
R119	Yes	Similar properties to *N. lactamica*
R128	Yes	Presents an air-liquid and liquid-surface interface
R129	Yes	Presents an air-liquid and liquid-surface interface
R136	Yes	Similar properties to *N. lactamica*
R145	Yes	Similar properties to *N. lactamica*
R146	No	Unable to thrive in 50 %, struggles in 100 %. Grows in BHI with no problems.
R191	Yes	Presents an air-liquid and liquid-surface interface
R196	Yes	Poor adhesion but produces a single confluent layer, Similar properties to *N. lactamica*
R200	Yes	Presents an air-liquid and liquid-surface interface, best after day 3
R210	Yes	Forms micro-colonies in a thin biofilm
R221	Yes	Single confluent layer, thin air-liquid interface produced think sheet-like layers which broke when disturbed
R222	Yes	Presents an air-liquid and liquid-surface interface
R234	Yes	Very viscous dual layer, produces micro-colonies
R242	Yes	Dual layer with micro-colonies
R262	Yes	Similar properties to *N. lactamica* but a weaker structural biofilm.
R263	Yes	Similar properties to *N. lactamica*
R279	Yes	Similar properties to *N. lactamica* but appears weaker and sometimes doesn't thrive.
MC58	Yes	Similar properties to *N. lactamica* but struggles to survive in 50 % media (very low yield)

Table 5 - Phenotype observations from Day 4 biofilm screening.

Isolates were supplied with fresh 50 %-reduced media daily for 4 days. Visual appearances were carefully noted and samples from each strain taken at day 4 to determine if survival at 50 % was possible.

Of the 28 isolates screened, 25 isolates demonstrated some recognisable biofilm growth and three did not visually form a biofilm or were able to survive under nutritional stress conditions. The three isolates which did not survive in TSB (R034, R104 and R146) all demonstrated successful planktonic growth in BHI. It was expected that R034 and R146 would be unable to form characteristic biofilms as both had failed to survive in TSB during the planktonic screening experiment (Section 4.3.1.). R104 demonstrated some survival but showed little to no attachment to the plastic tissue plate surface over four days. These same isolates were again grown in full strength BHI for comparison. All three isolates were able to successfully grow in BHI but still produced poor or no biofilm.

The remaining 25 isolates (with the single exception of isolate R221) could be categorised based on the observed phenotype into one of three categories;

- Confluent biofilm forming isolates – those isolates which formed a biofilm similar to *N. lactamica* i.e. a uniform single liquid-surface layer (Figure 19A). Although the ability to maintain attachment varied from isolate to isolate.

- Aggregating biofilm forming isolates – those Isolates that aggregated, formed micro-colonies or clumped yet still maintained an attachment to the surface of the well (Figure 19B). These isolates typically adhered to the plate more successfully than those biofilms that formed a single confluent layer. These biofilms however also developed slower (post 16 hours) when compared to the confluent layer isolates.

- Dual biofilm layer isolates – those isolates that had a confluent liquid-air layer, and as time progressed, an additional liquid-surface layer. Typically the liquid-air layer contained small micro-colonies (Figure 19C). These liquid-air biofilms appeared to be extremely structurally sound, with little to no damage during feeding and removing spent media.

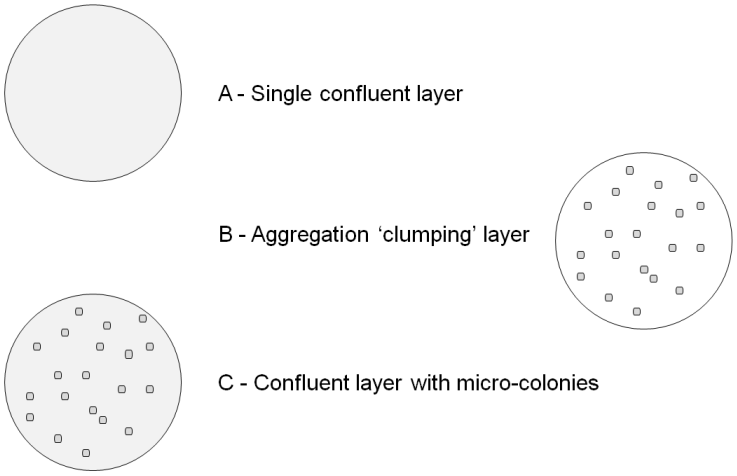

Figure 19 A-C – A schematic diagram showing an aerial viewpoint of the three phenotypes of biofilm formation observed in the *N. meningitidis* isolates.

With the exception of R221, the 24-screened isolates could be categorised into one of three biofilm 'types'. Occasionally isolates that exhibited phenotype C revealed a dual biofilm layer (both liquid-air and liquid-solid) all isolates in this category displayed a liquid-air biofilm at Day 1.

Isolates that formed a dual layer of solid-liquid and liquid-air biofilms were extremely resistant to external disturbance. The bottom liquid-surface layer was weaker and prone to more damage during liquid aspiration and media replacement than its liquid-air counterpart. The liquid-air biofilm layer in contrast however was slow to appear when compared to liquid-surface biofilm.

The isolate R221 did not fit into any of the three categories. Instead R221 displayed a confluent liquid-air layer, a phenotype similar to *N. lactamica.* However if the biofilm was disturbed the liquid-air layer biofilm would split into small sheet-like layers and sink to the bottom of the well (Figure 20). A replacement air-liquid biofilm would form over a 16-20 hour period.

Determining whether the disturbed layers were able to survive in biofilm submerged at the bottom of the well was difficult to assess. The process of removing the replenished liquid-air layer would result in further disruption and sections of the biofilm would sink to the bottom, with several attempts proving to be inconclusive. By growing the biofilm in a smaller well on a 24-well plate the surface area was reduced enabling the removal of all the liquid-air biofilm in a single step without any sections breaking away. The CFU counts showed that sunken biofilm layer was able to survive, counts were an average 10^6 cells/mL on a Day 3 biofilm (biofilm inoculation was 10^8 cells/mL).

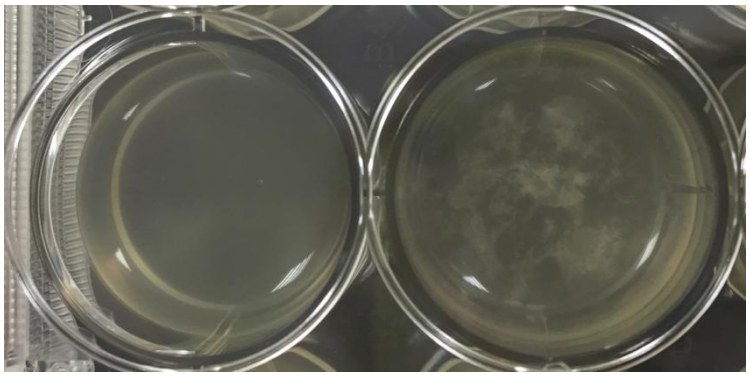

Figure 20 - Photograph of *N. lactamica* (left) and isolate R221 (Right) in biofilm.

The phenotype for R221 was unique amongst the 24 isolates forming small sheet-like layers that could be easily disturbed, but replaced quickly. The disturbed layers would sink to the bottom of the well. The image displays the thin undisturbed top layer with older previously disturbed layers at the bottom of the well (Right) next to a *N. lactamica* biofilm (Left).

4.4.3 THE BIOFILM PHENOTYPE AND CARRIAGE

Upon phenotypic biofilm characterisation of each isolate had been established, the results of carriage from the Lac2 study were revealed. Interestingly all of the isolates screened could not be identified from any of the volunteers by

nasopharyngeal swabbing at week 28 of the study. The majority of isolates (51.85 %) were however still present at their week 26 visit.

Of the 27 working isolates identified at screening;

- 7.41% of the isolates were lost after screening and prior to week 2.
- 3.70% of the isolates were last post week 2 but prior to week 3
- 7.41% of the isolates were lost between week 4 and week 8
- 7.41% of the isolates were lost between week 8 and week 16
- 18.52% of the isolates were lost between week 16 and week 26
- 51.85% of the isolates were lost between week 26 and week 28.
- No *N. meningitidis* isolates were identified/remained at week 28.

As the majority of isolates could be carried for longer than 16 weeks it was theorised that biofilm formation had increased the success of asymptomatic carriage. All the isolates that were present post week 16 successfully formed biofilms *in vitro* using the developed *N. lactamica* model.

Efforts to discover a correlation between biofilm phenotype and the length of carriage proved to be elusive and insignificant. A comparison of those isolates that were lost between week 26 and week 28 also demonstrated no correlation between biofilm phenotypes and length of carriage. When a comparison between all the biofilm phenotypes produced by the isolates and carriage length on a week-by-week basis, there was no correlation and results were again shown to be insignificant.

4.4 CAPSULE EXPRESSION

The expression of capsule has been identified as one of many virulence factors in *N. meningitidis* disease. It has been previously theorised that the production of capsule inhibits the formation biofilm (Yi, *et al* 2004). It was hypothesized that those isolates which expressed a 'clumping' phenotype when nutritionally stressed, were in fact expressing capsule and aggregating. Those isolates which do not express or contain the genetic machinery to produce a capsule therefore would produce a confluent biofilm phenotype. To determine if this hypothesis was correct traditional microbiological staining methods were first used to visualise the capsule. Some basic genetic information about the isolates was collected during the Lac2 study, using this information in collaboration with a Bacterial Isolate Genome Sequence Database (BIGSdb) software programme; it is possible to screen the isolates for known capsule production genes.

4.4.1 INDIA INK CAPSULE STAINING

There are several staining methods which can be used to identify capsule; all the methodologies are based on the principle of creating a cell suspension on a dark background and staining the cells in order to view the unstained capsule (Breakwell, *et al.* 2009). For this thesis India ink and crystal violet was used.

India ink provides a dark opaque black background which leaves the cells fully intact whilst CV stains the lipopolysaccharide (LPS) layer giving the cell a purple colour. Cells that express capsule appear to have a white halo surrounding them when viewed at 100x magnification. Those that do not express capsule simply appear as purple bacteria within a black background (Figure 21).

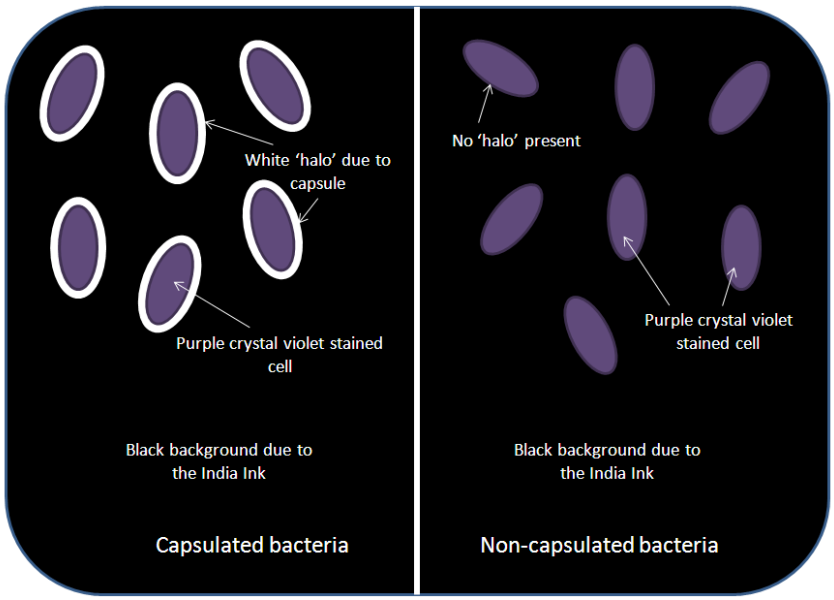

Figure 21. Schematic diagram of *N. meningitidis* when stained with CV and India Ink.

This crude methodology is able to identified both capsulated and capsule-deficient bacteria when viewed under a light microscope. The encapsulated bacteria appear to have a white halo surrounding them, in comparison to those capsule-deficient bacteria which remain purple against a dark black background.

Those isolates, which were identified as expressing a clumping biofilm phenotype, were R022, R079 and R104. MC58 was screened as a control as it is a known to be an encapsulated strain. When viewed under the microscope all these isolates had a halo surrounding them and therefore appeared to express capsule.

To ensure that it was not coincidental that all isolates presented with capsule, those isolates that expressed a confluent layer phenotype similar to *N. lactamica* were also screened for capsule expression. *N. lactamica* was used as a control as it is capsule-deficient.

A total of 11 isolates were examined which had all previously exhibited a single confluent biofilm similar in phenotype to *N. lactamica*. Of these 11 only seven

could be confidently identified as capsule negative. The remaining four could not be positively identified as either capsule positive or negative. Repeated slides demonstrated a mixture of capsulated and non-capsulated cells, indicating these isolates may be able to regulate capsule production.

Three of these isolates produced a confluent biofilm phenotype that exhibited poor adhesion to the plastic tissue well.

4.4.2 POTENTIAL GENES FOR CAPSULE EXPRESSION

The *N. meningitidis* isolates collected at the screening process during the Lac2 study underwent whole genome sequencing. From the data it is possible to look for known genes which enable capsule production, although further proteomic analysis would be required to investigate whether genetic expression corresponds to downstream protein expression during planktonic and biofilm formation.

There are several genes required for capsule synthesis and expression, with each serogroup coding its own loci. Harrison *et al.* (2013) described 14 serogroups, nomenclature of capsule genes and proposed gene orientation. The results generated from the paper were used to assist identification of the Lac2 isolates.

By using PubMLST Neisseria (https://pubmlst.org/neisseria/) it was possible to input the genetic information of each strain and isolate genes of interest. This gene identification tool was used in conjunction with the Bacterial Isolate Genome Secquence Database (BIGSdb) software. Isolates were screened for not only capsule synthesis genes but also for capsule transport genes. BIGSdb generates a spreadsheet within the PubMLST site which lists all of the known genes along the Y-axis and lists all the isolates screened along the X-axis. Genes which are positively identified are highlighted with the number of alleles associated with that particular gene. Those which do not match are simply marked with a black cell and white 'x'.

12 *N. meningitidis* serogroups (A, B, C, W, Y, E, H, Z, I K, L, X) with capsule production capabilities were identified as having 6 regions within the *cps* locus (Harrison, *et al*. 2013). Each region codes a specific aspect of capsule production and expression. The three main regions concentrated on were Region A – capsule synthesis, Region C – capsule transport and Region B - capsule translocation.

Using the data generated by PubMLST *Neisseria* it was possible to screen through each region of the *cps* locus and identify positive gene matches. For example isolates which were determined as serogroup E were identified as positive matches with Region A *cseA* to *cseG* genes but negative matches for genes *cszA* to *cszD*. This screening method was used to identify, if possible all the serogroups of all the isolates.

The results, shown in Table 6, lists all the Lac2 isolates previously used in this thesis, each respective biofilm phenotype of that isolate and the serogroup identified with that isolate if applicable. Some isolates could not be conclusively grouped as no positive matches were identified or only the conservative *cssA* to *cssF* genes matched. The *cssA* to *cssF* genes are found in serogroups B, C, W and Y and without positive matches to other known serogroup genes it is difficult to differentiate between them. Small selections of isolates were shown to lack capsule synthesis genes and therefore capsule transport and capsule translocation genes, these were therefore identified as capsule negative isolates.

Table 6 demonstrates that there is no distinct correlation between biofilm phenotype and the presence of capsule synthesis genes. It is not possible to determine the correlation between capsule and biofilm phenotype without examining whether gene expression also correlates with polysaccharide production. Those isolates which did not match any capsule production genes, and are therefore assumed to be capsule deficient, equally do not share a biofilm phenotype.

Of the 27 *N. meningitidis* isolates used 14 are non-invasive capsular serogroups, 12 are identified as potentially invasive capsular serogroups and a single isolate could

not be positively identified. Although 12 isolates were identified as potentially virulent, the presence of capsule alone does not infer that disease will occur.

Isolate	Biofilm type	Capsule present – genetic analysis	Serotype (if known)
R010	Confluent	Capsule present	B/C/W or Y
R022	Aggregates	Capsule present	E
R046	Confluent	Capsule present	B/C/W or Y
R049	Confluent	Capsule present	Z
R054	Confluent	Capsule present	B/C/W or Y
R059	Dual	Capsule present	E
R079	Aggregates	Capsule synthesis	Not known
R083	Aggregates	No capsule present	Not applicable
R104	Aggregates	Capsule present	B/C/W or Y
R119	Confluent	No capsule	Not applicable
R128	Dual	Capsule synthesis	Not known
R129	Dual	Capsule present	B/C/W or Y
R136	Confluent	No capsule	Not applicable
R145	Confluent	Capsule present	B/C/W or Y
R191	Dual	No capsule present	Not applicable
R196	Confluent	Capsule present	B/C/W or Y
R200	Dual	Capsule present	B/C/W or Y
R210	Aggregates	Capsule present	X
R221	Sheet	Capsule present	E
R222	Dual	Capsule present	E
R234	Dual	Capsule present	B/C/W or Y
R242	Dual	Capsule present	X
R262	Confluent	Capsule present	B/C/W or Y
R263	Confluent	No capsule present	Not applicable
R279	Confluent	Capsule present	E
MC58	Confluent	Capsule present	B
N. lactamica	Confluent	No capsule present	Not applicable
R034	No biofilm	Capsule present	B/C/W or Y

Table 6. Capsule expression and biofilm phenotype across all isolates as determined by PubMLST *Neisseria*.

Some isolates could not be conclusively grouped due to a lack of positive matches with the MLST spreadsheet. Further analysis of these isolates would be required for a conclusive identification.

4.5 ANTIBIOTIC RESISTANCE

In the previous chapter (Chapter 3.5) it was demonstrated that *N. lactamica* biofilms were 20-fold more resistant to the antibiotic kanamycin when compared to a *N. lactamica* planktonic culture. The same experiment was applied to three isolates expressing each biofilm phenotype.

Using five isolates the experiment was repeated in planktonic culture. The five isolates were selected due to their biofilm –forming ability. Each isolate expressed a different biofilm phenotype. The isolates used were;

- *N. lactamica* Y92-1009 – as used previously throughout this thesis. Results demonstrated that N. lactamica was only able to thrive in concentrations below 50 µg/ml.
- MC58 – a slow biofilm – former, capsule positive that may confer more antibiotic resistance when compared to other isolates.
- R022 – produces a confluent biofilm containing small micro-colonies within the thin matrix structure.
- R083 – aggregates and produces small micro-colonies that adhere to the well surface.
- R145 – produces a confluent layer and exhibits a phenotype similar to a *N. lactamica*.
- R221 – Produces a dual layer biofilm, the air-liquid interface with sheet-like layers which dispersed and sink to the bottom of the well when disturbed.

The individual colonies were picked and re-suspended into 100 % TSB supplemented with 0.2 % yeast. A starting antibiotic concentration of 100 µg/mL was diluted to 1 µg/mL. 20 µL of re-suspended culture was added to each antibiotic dilution in a 96-well plate in duplicate and incubated for 18 hours at 37 ^{o}C and 5 % (v/v) CO_2. After

incubation an OD reading was taken and each well that demonstrated no growth was spot plated for growth. The MIC concentration was identified as 25 μg/mL for *N. lactamica*, 50 μg/mL for MC58, 25 μg/mL for R022, 25 μg/mL for R083, 50 μg/mL for R145 and 25 μg/mL for R221.

Spot plating each concentration of both *N. lactamica* and the other isolates showed that the MBC was the same as the MIC.

The experiment was modified and repeated in biofilm using the same *N. lactamica* strain and *N. meningitidis* isolates. 1-Day (24 hour) biofilms were treated with each antibiotic dilution (1 mg/ml to 1 μg/mL) for 2 hours in duplicate. The biofilms were then twice washed with sterile water to remove any remaining antibiotic. Treated biofilms were re-suspended in 1 mL of sterile water, 20 μL of each treated biofilm was spot plated onto CBA plates. Plates were then incubated overnight at 37 ^{o}C and 5 % CO_2 (v/v).

The experiment showed that for *N. lactamica*, MC58 and R022 the antibiotic was able to penetrate the biofilm and was effective at concentrations of 100 μg/mL and higher, indicating that in a biofilm state there is an increase in antibiotic resistance when compared to a planktonic state (*N. lactamica* and R022 = 25 μg/mL in planktonic to 100 μg/mL in biofilm, and MC58 = 50 μg/mL in planktonic to 100 μg/mL in biofilm). Isolates R083, R145 and R221 were able to survive better, showing growth at 1 mg/ml (Table 7).

There was no correlation between ability to survive higher concentrations of antibiotic compared to biofilm phenotype. All the isolates tested expressed a biofilm and all the isolates demonstrated an increased ability to survive in the presence of an antibiotic this demonstrates that biofilm formation not only enables a higher survival rate than planktonic cells alone.

	Planktonic - MBC					
	N. lactamica	MC58	R022	R083	R145	R221
1000 µg/mL	*No growth*	*No growth*	*No growth*	*No growth*	*No growth*	*No growth*
100 µg/mL	*No growth*	*No growth*	*No growth*	*No growth*	*No growth*	*No growth*
50 µg/mL	*No growth*	*Growth*	*No growth*	*No growth*	*Growth*	*No growth*
25 µg/mL	*growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
10 µg/mL	*growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
1 µg/mL	*growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
Control	*growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
	Biofilm - MBC					
1000 µg/mL	*No growth*	*No growth*	*No growth*	*Growth*	*Growth*	*Growth*
100 µg/mL	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
50 µg/mL	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
25 µg/mL	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*
Control	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*	*Growth*

Table 7. Planktonic verses Biofilm growth when under antibiotic stress.

When in a biofilm state *N. lactamica* and all the *N. meningitidis* isolates examined exhibited an increase in antibiotic resistance when compared to a planktonic state. This ability to increase antibiotic resistance is a classic phenotype of biofilm formation. Further demonstrating that *N. lactamica* and *N. meningitidis* is able to form a biofilm when under nutritional stress *in vitro*. Experiments were conducted in 6-well tissue culture plates and repeated in triplicate, n=3.

4.6 CONFOCAL MICROSCOPY

SEM imaging used in the previous chapter (Chapter 3.4) demonstrated the *N. lactamica* is able to form a large structure that was able to adhere to the substratum and survive the SEM processing. Confocal processing involves less washing steps and therefore is a good platform to image the cells within the structure of a biofilm. Confocal imaging is frequently used in the literature when studying biofilms (Yi *et al.* 2004; Maeyama *et al.* 2004; Steichen *et al.* 2008; Neil and Apicella, 2009; Neil *et al.* 2009; Lappann *et al*. 2010).

In this section only *N. lactamica* and *N. meningitidis* isolate R083 were observed using confocal microscopy. *N. lactamica* produces a confluent biofilm however, R083 aggregates and was shown to be capsule deficient.

SEM imaging had previously demonstrated that *N. lactamica* has the ability to produce a biofilm structure, but SEM is unable to distinguish between cells that are dead and those that are viable. Using a live/dead stain, the biofilm structure was visualised with the additional information of which areas or layers remain viable.

Day-1 biofilms were stained with two fluorescent stains. Syto®-9, a green fluorescent stain with a high affinity for DNA and Propidium iodide, a red fluorescent fluorophore and nuclear counter-stain; this dye is generally excluded from viable cells with intact membranes. The two stains when combined allow the visualisation of alive (green) and dead (red) cells within a structure.

Three randomly selected areas were imaged from each biofilm. Image slices were taken every 2 µm from the bottom of the well to the top structure. Any remaining planktonic, other free-floating cells or areas of disturbed and unattached biofilm are easily identified when imaged as they slightly move creating a blurry image in contrast to fixed structures.

4.6.1 SINGLE LASER CHANNEL VIEW

An advantage of using confocal microscopy is the ability to view different areas of the biofilm using different channels. In this section a single channel was used to image the live green cells and another one to view the red dead cells. The ability to switch between the two channels enabled an accurate comparison of the distribution between live and dead.

The biofilm of *N. lactamica* ranged from 50 – 67 µm thick. During the processing minimal visible damage occurred to the biofilm.

The 3D confocal image (Figure 22A-B) shows that there are various areas of both red and green cells. When the image is viewed using a single laser channel Figure 22B shows that the bottom basal layer of the biofilm is primarily comprised of red dead cells however it also demonstrates that within this layer a few green fluorescing cells are spread throughout. The majority of the green cells appear to be clustered in larger peaks upon the basal predominantly red cell layer. These peaks are firmly attached and built upon the basal red layer (Figure 22A).

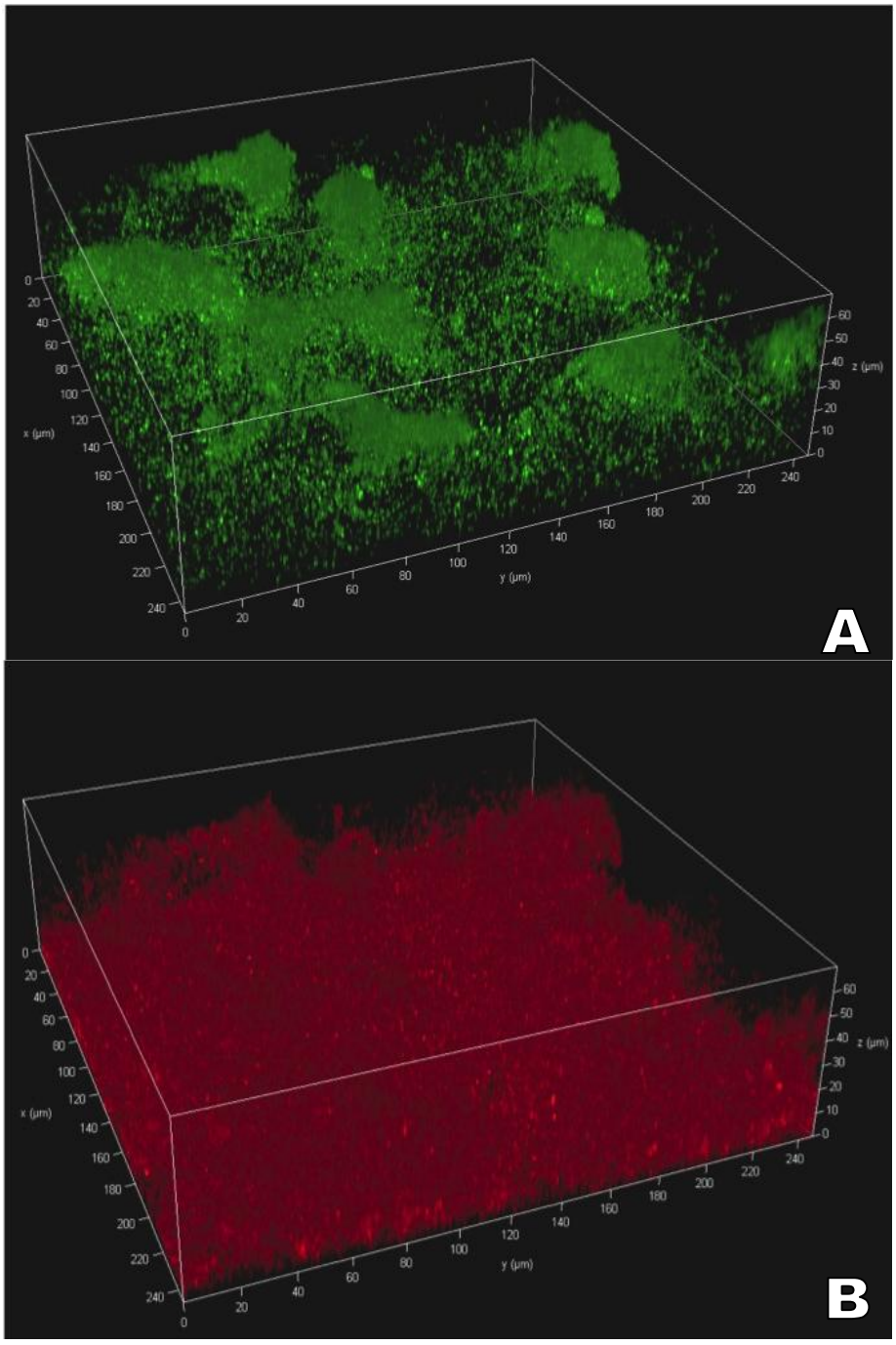

Figure 22A-B Confocal 3D image of a Day-1 *N. lactamica* biofilm. The same image was viewed using the Red and Green channels to view the live (green) cells and the dead (red) cells separately. The image demonstrates that the majority of live cells are positioned in peaks upon a predominately dead basal layer.

The biofilm thickness for *N. meningitidis* R083 measured from 51 µm to 54 µm at the highest attached peak, measuring slightly smaller compared to the *N. lactamica* biofilm. This observation also corroborates with biofilm thickness previously described by Yi *et al.* (2004) when the group investigated the biofilm forming properties of 39 *N. meningitidis* isolates.

The generic organisation of the cells also appears to be quite concentrated in the micro colony clusters (Figure 23A-B)

N. meningitidis R083 differed in biofilm phenotype from *N. lactamica* by producing an aggregating phenotype and forming micro colonies, which maintained adherence to the plastic well during the washing stages of confocal processing. Figure 23A-B demonstrates that there is a good distribution of green and red fluorescent cells distributed throughout the entire biofilm.

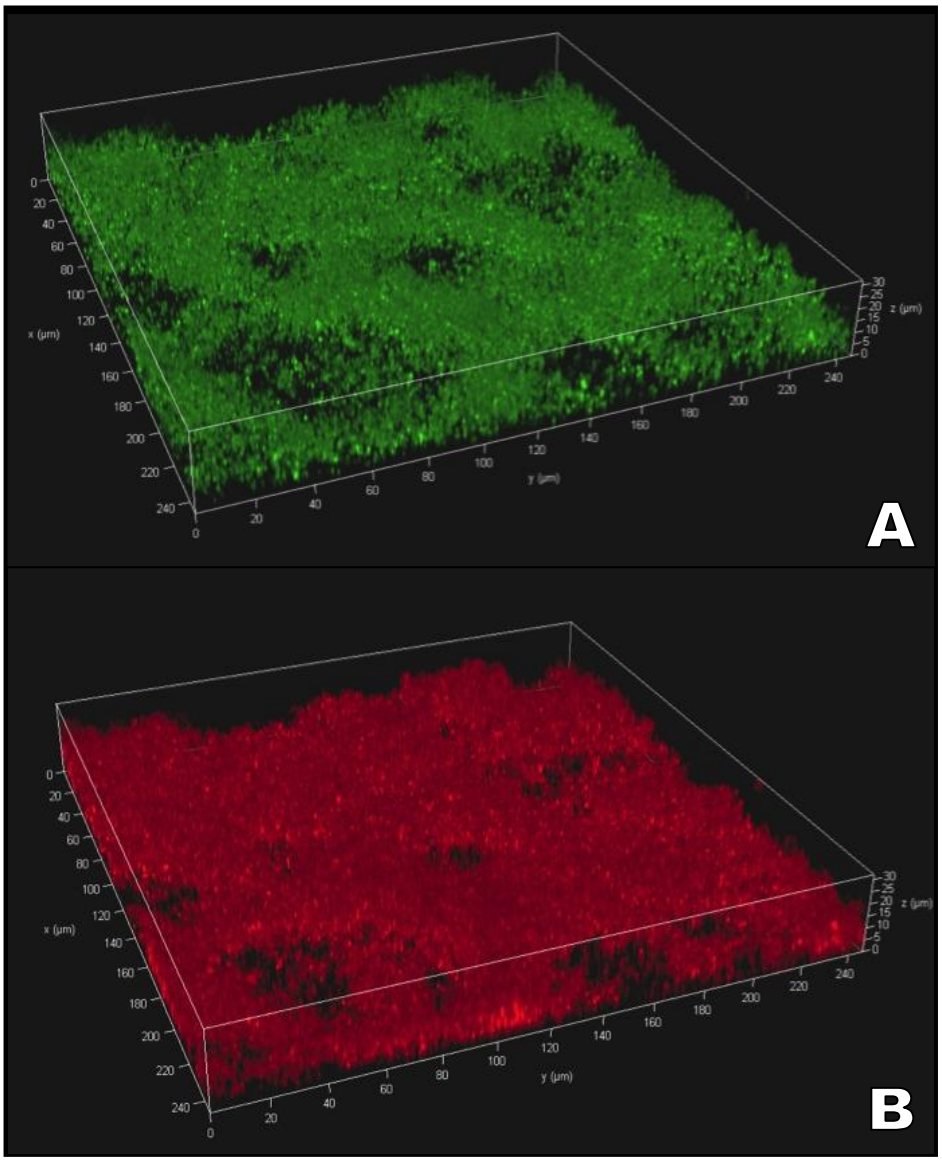

Figure 23A-B Confocal 3D image of a Day-1 *N. meningitidis* R083 biofilm. The same image was viewed using the Red and Green channels to view the live (green) cells and the dead (red) cells separately. The image shows an even distribution of both green and red cells.

4.6.2 DIRECT COMPARISON OF BIOFILM STRUCTURE

Viewing both the red and green channels together produced a combined image of the biofilms. The combination images allow a direct comparison between the biofilm structure of *N. lactamica* and the structure of R083 (Figure 24A-B).

N. lactamica produced a biofilm with two distinct areas which gives the appearance of a layer of dead cells upon which large clusters of viable green cells reside. CFU counts (Figure 14 - Chapter 3) demonstrate that the biofilm remains viable for a prolonged period of time successfully. It was also demonstrated in Chapter 3 (Figure 10) that depriving a *N. lactamica* biofilm of fresh media for 7 days did not result in complete cell death, only reduced the number of viable cells.

The biofilm structure of R083 is distinctly different from that on *N. lactamica*. R083 appears to form micro-colonies that merge into one another. Unlike the green peaks seen in the *N. lactamica* biofilm, the distribution of live and dead cells appears even with no clusters of either alive or dead concentrated to a particular area.

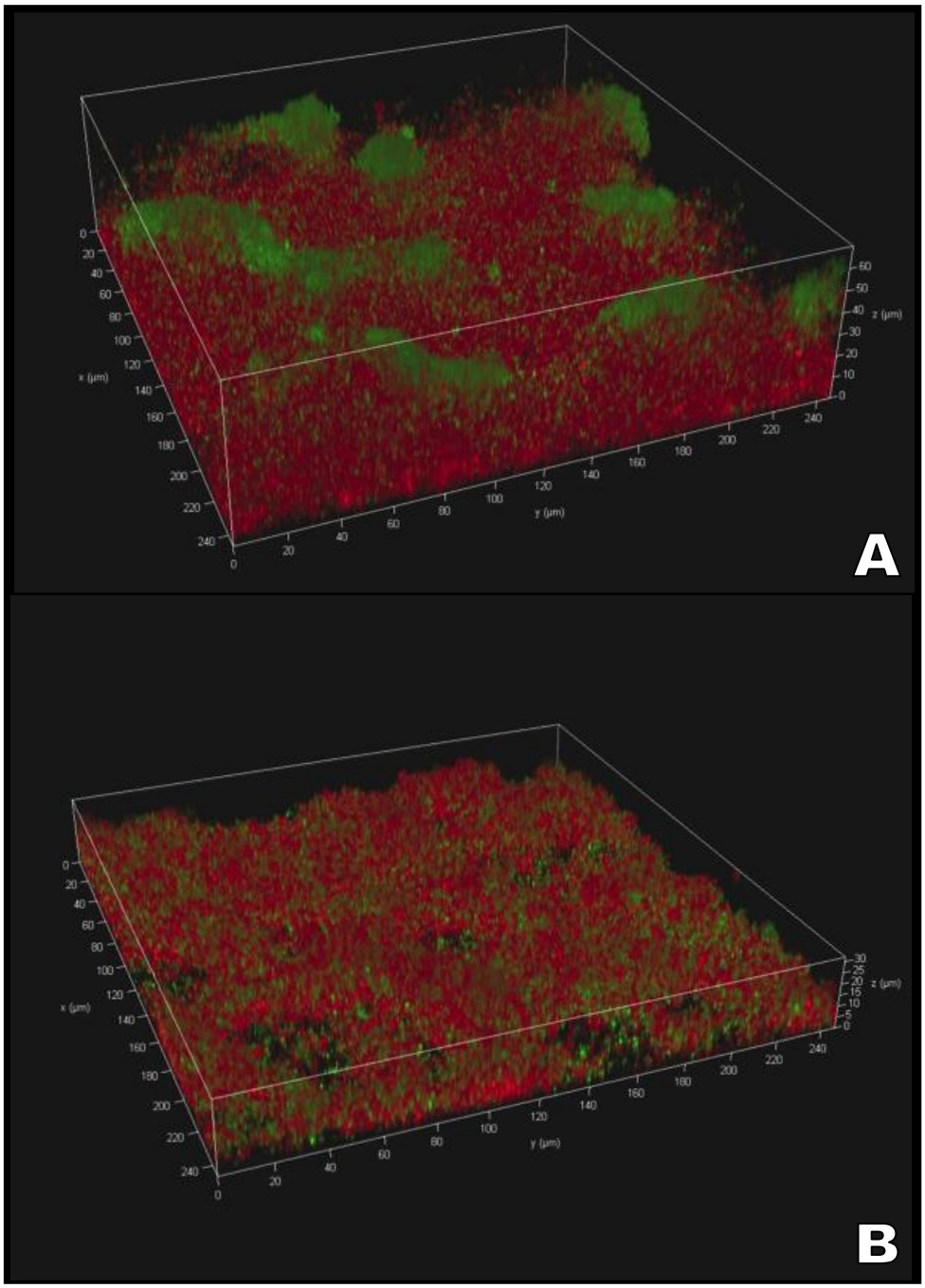

Figure 24A-B Comparison confocal 3D image of both *N. Lactamica* and R084 biofilms. The two images combine both the Red and Green channels to give an overall view of biofilm structure. The two biofilms express very different structures; with *N. lactamica* live cells predominantly situated at the top of the biofilm, whereas R083 has an even distribution of live and dead cells together.

4.7 CONCLUSIONS AND DISCUSSION

The ability of *N. meningitidis* to form biofilm is well documented in the literature. Yi *et al.* (2004) investigated the biofilm-forming properties of 39 isolates, 16 of which were invasive. The group discovered that only 12 % of the invasive isolates were able to form a recognisable biofilm, compared to 30 % of the carriage strains indicating that biofilm production may aid carriage but is not essential. The group also concluded that the virulent-associated capsule found in invasive *N. meningitidis* hinders carriage.

Using the *N. lactamica* biofilm model, 25 isolates of *N. meningitidis* from the Lac2 study were successfully assayed for the ability to form biofilm. The capability to form biofilm formation demonstrates the flexibility of *N. meningitidis* to adapt to external stresses, in the case of this thesis; a nutritional stress. An in-depth proteomic comparative study of those isolates that are able to successfully form biofilm to those that did not, may indicate which isolates are quickly able to down regulate capsule production and therefore aiding in biofilm formation.

4.7.1 BIOFILM PHENOTYPE

Three main biofilm phenotypes (excluding the phenotype expressed by the isolate R221) were identified among the Lac2 isolates. Those isolates which exhibited a dual layer biofilm were particularly resistant to damage. The top liquid-air biofilm layer was able to flex and move with the media, especially when removing spent media and adding fresh. This meant very little disturbance occurred during these processes.

No correlation between the length of carriage and biofilm phenotype could be established. Exactly which alleles determine each biofilm phenotype in these *N. meningitidis* isolates is currently unknown, and the genes involved in each phenotype is likely to be varied. Harrison *et al* (2013) discovered that a number of

genes are involved in capsule production; transport and translocation are shared among other biofilm-forming species. Serogroups W and Y were found to contain *galU*, which is shared with *Streptococcus pneumoniae* and is responsible for capsule formation. It was also found that serogroup E *N. meningitidis* isolates contain the gene *cseF* which shares 66 % genetic similarity with 3-deoxy-D-manno-octulosonate cytidylyltransferase found in *Escherichia coli* which assists in lipopolysaccharide biosynthesis. The group determined that in serogroups H, I, K, and Z there was evidence for horizontal gene transfer of the *cps* genes found in region A which suggested acquisition from other bacterial species. It has been previously demonstrated that the biofilm formation and horizontal gene transfer are connected and that the gene transfer rates are high in biofilm communities when compared to planktonic cultures (Madsen *et al*. 2012).

Many other biofilm-forming bacteria are known to produce different biofilm phenotypes naturally (Handke *et al*. 2004) however many other these have since been found to contain specific mutations. The isolates used in this work are known to be carriage isolates among a specific population. Further genetic analysis would need to be performed to discover if any of the isolates contained similar genetic mutations.

Steichen *et al.* (2008) demonstrated that pathogenic *Neisseria* isolates that contain a mutation in the *msbB* gene have a reduced ability to form biofilms. The mutation reduces the ability of the isolate to bleb its outer membrane hindering matrix formation. It was hypothesised that the three isolates (R034, R104 and R146) have this or a similar mutation due to the lack or poor biofilm produced. As isolate R034 had failed to survive using the developed *N. lactamica* model it was not possible to characterise the biofilm phenotype. Although this does not mean that R034 was completely unable to form biofilm; conditions required for biofilm formation can vary from species to species and strain to strain. It is also possible that R034 and the other two isolates (R104 and R146) that failed to produce viable biofilm using the *N. lactamica* model are naturally biofilm-deficient isolates. Instead they

increase carriage survival by cohabiting with another respiratory species that does form a biofilm. This is seen with other respiratory organisms such as *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* (Varposhti *et al*. 2014) where it was demonstrated that *P. aeruginosa* biofilm production increased 102-fold when in the presence of *S. maltophilia*. *Haemophilus influenzae* and *P. aeruginosa* have also been shown to have a synergistic relationship with one another (Weimer *et al*. 2010).

The isolate R221, which produced a different biofilm phenotype was not identified during swabbing after week 3, appearing to have been replaced by *N. lactamica*, however the isolate was later re-identified at week 16 in conjunction with *N. lactamica* before being lost again/unidentifiable. Although no conclusions can be made about a single isolate, the apparent disappearance of R221 demonstrates the possible disadvantages of nasal swabbing. The isolate may have been present throughout the study but remained undetected. As the *in vitro* biofilm screening experiment demonstrated R221 has the ability to break up its biofilm structure when disturbed, this biofilm phenotype may allow the isolate to colonise multiple minute sites within the nasopharynx and maintain carriage even once cleared from a single site. The sheet-like appearance of R221 is unusual amongst the *N. meningitidis* isolates screened. Commonly the appearance of a sheet-like biofilm is found in *Pseudomonas fluorescens* (Baum *et al.* 2009), which is regularly found in the soil around plant roots and in water. Although the air-liquid interface could easily be disturbed once the layers had sunk to the bottom of the well, they attached well to the surface.

It was also hypothesised that those isolates which aggregated and did not form a single confluent layer, expressed capsule as has previously been described in the literature. Alternatively, as these biofilms were grown on abiotic surfaces the observations may corroborate what Lappann *et al* (2010) observed when investigating the role of eDNA. The group identified that eDNA is an essential component to the structure of *N. meningitidis* biofilms. Adding DNAse I to a starting biofilm culture suppressed biofilm

formation, however DNAse I had little visible effect on a 12-hour biofilm. It is possible that these clumping isolates are eDNA-independent strains, which tend to grow and form biofilm slower than their eDNA-dependant counterparts.

The majority of isolates (51.85 %) were however still present at their week 26 visit. This demonstrates that meningococcal carriage can persist for a substantial period of time in a single healthy individual, without being cleared by the immune system or spontaneously lost. Although inconclusive, the lack of correlation between phenotype and length of carriage may simply suggest that the ability to form any biofilm, regardless of the phenotype, may have a positive impact on carriage length and the ability to evade the hosts' immune system. Even weak biofilm production could give individual isolates an advantage over non-biofilm forming bacteria.

Although nasopharyngeal swabbing is not 100 % conclusive the likelihood that the swabbing had missed all the isolates is extremely low. Neither did the lack of identifiable isolates conclusively demonstrate that carriage had been displaced by *N. lactamica* as only two subjects had viable *N. lactamica* on their final nasal swabs. Given that it is unclear how long each volunteer had previously carried the strain prior to screening, the time of year that swabbing occurred and general health of each volunteer it is difficult to draw a definitive conclusion on the lack of *N. meningitidis* at week 28. There is also no information available about the volunteers themselves. The study specifically only recruited non-smoker, healthy individuals aged between 18-25 years. Given that the majority of these volunteers would have been students this is a very select population, albeit a medically relevant one. Establishing the origin of these isolates may also enable a better understanding of carriage. As few as 7 % of the student population choose a university in their home town (https://www.timeshighereducation.co.uk/news/a-level-students-still-look-to-move-away-for-university/2010056.article) which gives rise to the possibility that many of the isolates may have originated from different areas across the UK and possibly further afield. The ability for the majority of

isolates to maintain carriage for 26 weeks would increase the chance of that particular isolate spreading from one location to another.

4.7.2 PRESENCE OF CAPSULE IN A BIOFILM

MC58 surprisingly formed a biofilm; as a capsulated strain it had been theorised that this would not be possible as it is widely documented that capsule inhibits biofilm formation (Yi *et al.* 2004). However, Hey *et al.* (2013) had previously observed a biofilm-like structure when MC58 was added to established 16HBE14 cell lines. At 48 hours the group observed 'elongated filopodium-like protrusions' which they termed 'Filopodia'. The Filopodia entangled the MC58 cells into a web-like structure, giving the bacteria a structural support in which to thrive. The group documented that the Filopodia increased over 21 days, along with CFU counts. Although the group never used the word biofilm as a definition for these structures, the observations do fit with what is generally described as a biofilm. One possible reason for the observation of these Filopodia-structures is that MC58 is able to regulate its capsule production. In a planktonic state capsule is up regulated to increase survival within the host, however to form a biofilm and/or maintain carriage the isolate down-regulates capsule production. This regulation of capsule is seen in other respiratory bacteria such as *Streptococcus pneumoniae* (Hall-Stoodley *et al.* 2008). Another reason for MC58 biofilm formation is it is a piliated strain; piliation of *N. meningitidis* has also been shown to enhance biofilm formation (Neil *et al.* 2009). Neil *et al.* (2009) demonstrated that both capsulated and uncapsulated MC58 could produce biomass when grown on HBE cells, however capsule production has previously been shown to be a hindrance when grown on glass (Lappann *et al.* 2006). This would suggest that MC58 requires an interaction to form a biofilm, which is unlikely to have occurred using plastic tissue culture plates. We therefore hypothesise that in this thesis MC58 was able to down regulate capsule production and form a biofilm.

R079 had the largest and most visible 'halo' of all the five isolates that expressed a clumping phenotype. Although this capsule identification method is not 100 % conclusive it has previously been used in other encapsulated bacteria such as *Klebsiella pneumoniae* successfully. Isolates which are able to regulate capsule production would have an increased survival against phagocytosis by the hosts' immune system whilst expressing capsule and by down regulating capsule be able to colonise and invade the nasopharyngeal barrier.

There was no correlation between capsule expression and biofilm phenotype or serogroup. This is not entirely surprising as serogroup typing only identifies a single antigen present on the surface of the bacterium, whereas the biofilm phenotype could be influenced by many factors such as matrix production, DNAses present, competition for resources, etc. Those bacteria which were determined to be capsule deficient did not produce a common biofilm phenotype either. This could indicate that those isolates which produced a clumping phenotype aggregate in planktonic culture prior to attachment to the surface. This phenomenon can be seen in *P. aeruginosa* where aggregates of bacteria have been found in non-healing wounds and in the respiratory system of cystic fibrosis patients (Alhede *et al*. 2011).

4.7.3 ANTIBIOTIC RESISTANCE

As previously established with *N. lactamica* in Chapter 3 (3.5 Antibiotic Resistance) the selected *N. meningitidis* isolates MC58, R022, R083, R145 and R221 all demonstrated an increase in antibiotic resistance when in biofilm, when compared to planktonic culture. The isolates R083, R145 and R221 all showed antibiotic resistance to kanamycin up to 1 mg/ml, demonstrating at least a 20-fold increase when compared to a planktonic culture. The biofilms examined all produced different biofilm phenotypes demonstrating again that biofilm formation alone increases bacterial survival in the presence of an antibiotic. The increase in survival also demonstrates that regardless of the phenotype, any biofilm

production is advantageous. As these isolates were taken from asymptomatic carriers and it is unlikely that these isolates would cause disease as invasive disease usually presents within 14 days of acquisition and none of the cohort reported any meningitis symptoms over the study. Further genetic analysis would be needed to identify if each of the isolates are related to other known asymptomatic isolates. All the isolates examined (with the exception of MC58 and *N. lactamica*) came from volunteers living in a single area for the duration of the study; however each of those swabbed may have originated from another entirely different area, as is common with a student population. It is unknown if these isolates brought to the area or are locally identified isolates. Although this question may be difficult to answer, another large participant study in several locations would have to be conducted.

4.7.4 BIOFILM STRUCTURE

The confocal images demonstrate further evidence that *N. lactamica* and *N. meningitidis* isolate R083 are both able to form biofilm *in vitro*. Individual *Neisseria* bacterial cells range from between 0.6-1.0 µm, other respiratory bacteria similar in size, like the pneumococcus, have been shown to have biofilms 25-30 µm thick (Domenech *et al*. 2012). A similar thickness of biofilm was seen in both *N. lactamica* and R083.

The confocal images also show that the fluorescent green peaks seen in *N. lactamica* are similar to those found in other respiratory commensal biofilm-forming species such as some isolates of *Pseudomonas aeruginosa* (Klausen *et al.* 2004). Although it appears there is a high volume of dead cells, the literature suggests that this is a normal occurrence and has been observed with other respiratory biofilm-forming commensals (Bjarnsholt *et al*. 2013).

The *N. lactamica* biofilm morphology could allow the top green-alive peaks to be naturally cleaved and dispersed during the biofilm cycle. This would allow a significant

number of viable cells to colonise a new area whilst the remaining cells re-populate the biofilm.

The *N. lactamica* biofilm (Figure 22A-B) has a higher number of red-dead cells along the bottom basal layer than R083 (Figure 23A-B and Figure 24A-B). This high number of dead cells may serve to protect any of the viable cells from environmental pressures. Alternatively it is possible that the high volumes of fluorescent red cells in the basal layer are not dead, only cells with a permeated membrane will take up the PI stain. During biofilm production in some respiratory bacterial species it has been shown that extracellular DNA (eDNA) is an essential component. The eDNA is used as a bacterial adhesion material and has been demonstrated to be produced by active excretion in *P. aeruginosa* (Okshevsky and Meyer, 2015). By actively secreting eDNA the membrane temporarily becomes permeable, which would allow the uptake of the stain. Another more frequently observed phenomenon is controlled cell lysis (Okshevsky and Meyer, 2015), with individual areas of cells lysing and releasing eDNA. The eDNA from the lysed cells enables biofilm formation although it is not yet fully understood the exact mechanisms and how the eDNA interacts with the cells and other matrix apparatuses. Lappann *et al.* (2010) demonstrated that eDNA is an essential component of *N. meningitidis* biofilms and *N. gonorrhoeae* has been shown to clump in the presence of eDNA (Jakubovics, *et al*. 2013), therefore it is possible that *N. lactamica* uses eDNA for the same purpose.

The distribution of live/dead cells in the R083 biofilm appears to be more random than the peaks seen in the *N. lactamica* biofilm. This may be due to the matrix production of both species. *N. lactamica* produces a confluent biofilm and the previous SEM images have demonstrated that cells sit within the matrix. *N. meningitidis* R083 aggregates and does not form a confluent biofilm, which may indicate that that matrix production is lower and/or slower than that produced by *N. lactamica*. Instead the R083 cells adhere to each other and the glass surface. A similar phenomenon is commonly found in the natural environment. It has been demonstrated with motile *Escherichia coli* that bacteria can adhere to the surface and then to one another

creating filamentous biofilms commonly known as streamers (Yazdi and Ardekani, 2012), which can survive and replicate in flow systems successfully. To confirm this hypothesis further COMSTAT 2 (or similar) analysis is required.

Some of these isolates investigated in this chapter exhibited biofilm, it would be interesting to undertake further imaging to determine if there any structural similarities between biofilm phenotype and structural arrangement. For example do the *N. meningitidis* isolates identified as capsulate produce a similar matrix structure to either *N. lactamica* or R083.

CHAPTER 4 *NEISSERIA MENINGITIDIS*

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"Can *N. lactamica* and *N. meningitidis* co-exist in a biofilm together?"

5.1 INTRODUCTION

Evidence of bacteria-forming biofilms in the literature is plentiful. Various studies using *N. meningitidis* isolates from healthy patients have been shown to form biofilm in both static and flow models. Yi *et al*. (2004) identified that biofilm formation was higher amongst isolates that had been identified in carriage than those that were known to cause invasive disease. The group theorised that this was due to the expression of a capsule; the group even demonstrated that a capsule deficient isolate was able to form biofilm when the parent encapsulated isolate was not. This would suggest that all *N. meningitidis* isolates have the machinery to form a biofilm but capsule production is not adapted for initial attachment and further biofilm formation. If isolates are able to regulate capsule production and it can be demonstrated that biofilm formation enables carriage, then investigation into biofilm dispersal to prevent disease could be a novel treatment strategy.

Natural biofilm dispersal has been demonstrated in other *Neisseria* species, *Neisseria subflava* was shown to disperse after 9-10 hours in a static model (Kaplan and Fine, 2002), and *N. gonorrhoeae* was shown to contain a thermonuclease (Nuc) which disperses biofilm (Steichen *et al*. 2011). This natural dispersal enables the bacteria to colonise new areas away from the main biofilm.

Having previously demonstrated in Chapter Two that *N. lactamica* is able to form biofilm, and that the majority of the isolates in Chapter Three are also able to form biofilm, it was hypothesised that *N. lactamica* provides protection to the host via competition with *N. meningitidis*. This theory was investigated using the Lac2 study isolates. If *N. lactamica* is able to out compete *N. meningitidis* for resources, carriage for *N. meningitidis* could be lowered.

5.2 AIMS FOR CHAPTER FIVE

The aims for this chapter are as follows;

- Determine if *N. meningitidis* and *N. lactamica* are able to co-exist together in a planktonic state over 24 hours, using the characteristics of the growth profiles.

- Investigate the effect of *N. lactamica* on *N. meningitidis* biofilm formation. Through comparative characterisation of the phenotype and assessment of viability.

- Further investigate isolates that displayed interesting phenotypes in the previous Chapter 4, further investigated and demonstrate any possible connection between those isolates and identify any major differences when compared to other isolates.

5.3 ONE TO ONE CHALLENGE

This series of experiments investigated the hypothesis that *N. lactamica* is able to offer some protection to the host from *N. meningitidis* colonisation by displacement, growth limitation, or by microbial competition. 'Challenging' the two species against each other, under high nutritional stress, may provide insight as to why the two species are rarely identified together in a single host. *N. lactamica* is identified in early childhood (0 to 3 years) and then replaced by carriage of *N. meningitidis*. If it could be demonstrated that the two are completely incompatible to inhabit the same niche it would suggest that carriage of *N. meningitidis* could be prevented by the presence of *N. lactamica*. Deasy *et al.* (2015) demonstrated that co-colonisation of both *N. lactamica* and *N. meningitidis* was a rare occurrence. Only 27 of the 469 volunteers swabbed during the study exhibited a positive yield for both species over a 28 week period. This would suggest that the two species do not have a positive relationship, although swabbing methodologies are not 100 % accurate in identifying nasopharyngeal flora.

5.3.1 PLANKTONIC CO-HABITATION OVER 24 HOURS

Firstly to discover if a positive, negative or neutral relationship pre-exists between *N. lactamica* and *N. meningitidis*, could co-exist under planktonic conditions. Inoculated broths containing 2 colonies per mL were added to a 96-well plate. Growth of both *N. lactamica* and *N. meningitidis* were observed together and in isolation in parallel over a 24 hour period at 37 ^{o}C and 5 % (v/v) CO_2 without agitation. Readings were taken hourly for the first eight hours and with a final reading was taken at 24 hours. Growth was measured by absorbance ($OD_{600\ nm}$). Six of the *N. meningitidis* isolates from previous screening (Chapter 4.3.2) each isolate had previously demonstrated biofilm growth in 100 % and 50 %-TSB was randomly selected.

Figure 25 demonstrates that in isolation the all bacterial isolates demonstrate an expected growth pattern, increasing in biomass over time.

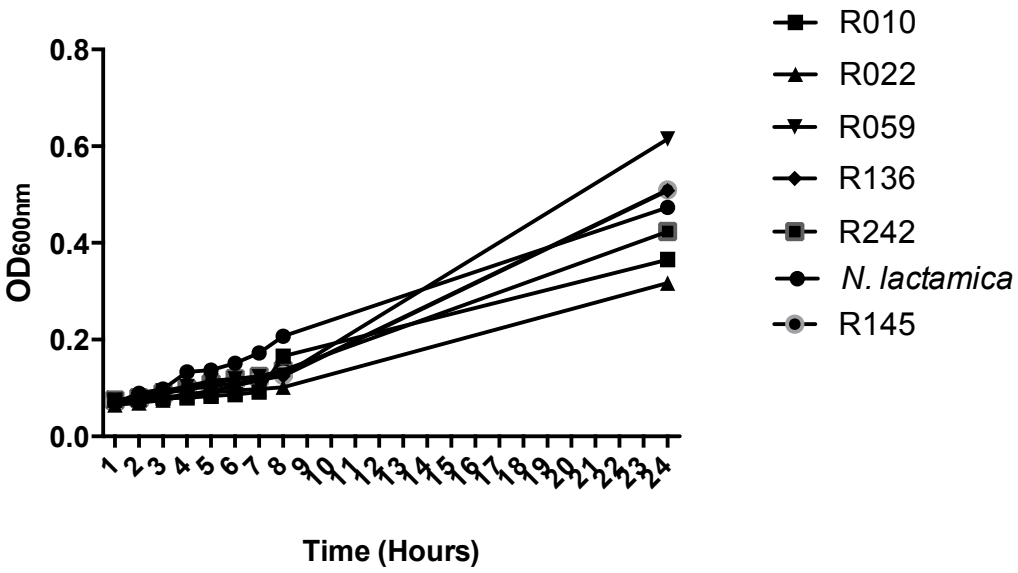

Figure 25. Planktonic Growth analysis in isolation.

The results demonstrate *N. lactamica* and six *N. meningitidis* isolates growth in 100 %-TSB over 24 hours. Readings were taken every 8 hours with a final reading at 24 hours. All the isolates demonstrated an increase in biomass over 24 hours indicating growth. Readings were taken on two separate occasions and were performed in 96-well plates with each isolate grown in 10 wells per experiment.

All the isolates demonstrated an expected increase in optical density when grown in isolation. Although isolate R022 increased slower and had an overall lower optical density measurement at 24 hours when compared to the other isolates, there is still a clear increase in biomass indicating growth. All the isolates recorded an increase in optical density over a 24-hour period demonstrating they are all able to replicate in 100 %-TSB successfully.

The second part of this assay in parallel with the previous assay investigated if *N. lactamica* had any effect on the *N. meningitidis* isolates in a planktonic culture. Figure

25 demonstrates that *N. lactamica* is able to increase mass quickly, at 8 hours *N. lactamica* has an OD reading double that of isolate R022 (0.207 and 0.102 respectively); this ability to quickly replicate may give *N. lactamica* an advantage over *N. meningitidis* colonisation. From the first part of the assay it was hypothesised that there would be an overall increase of biomass as *N. lactamica* had demonstrated that it was able to grow quickly when compared to the *N. meningitidis* isolates, and therefore the readings overall would be higher due to this increase in growth.

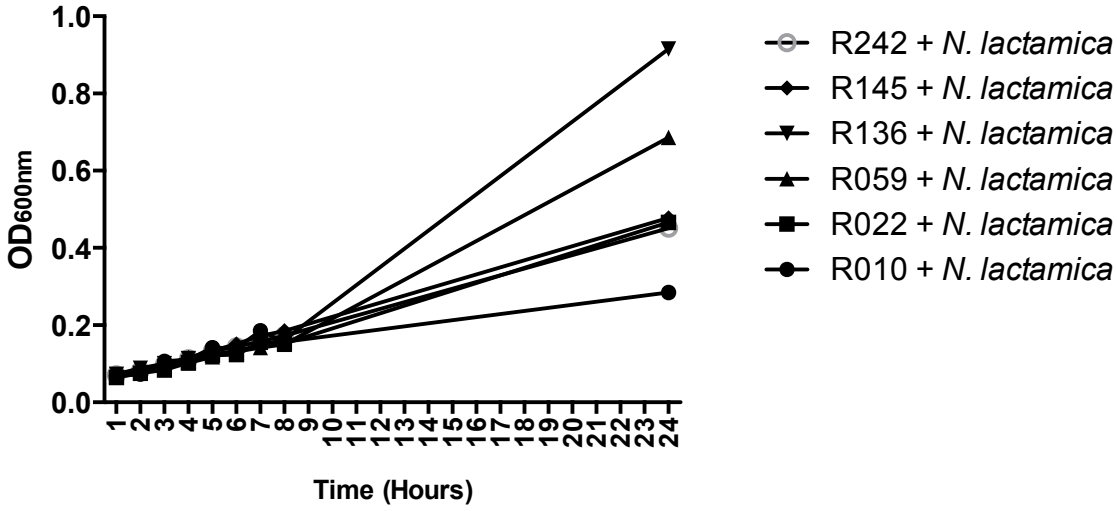

Figure 26. Planktonic growth analysis of both *N. meningitidis* isolates with *N. lactamica* in co-culture.

The same *N. meningitidis* isolates as in the previous experiment, on in a separate experiment, were grown in co-culture with *N. lactamica* over a 24-hour period. All the isolates again demonstrated an increase in biomass over 24 hours indicating growth. Readings were taken on two separate occasions, were performed in 96-well plates with each isolate grown in 10 wells per experiment and performed on a separate occasion to Figure 25 to minimise cross contamination.

When the *N. meningitidis* isolates are grown in the presence of *N. lactamica* there does not appear to be any immediate negative effects. The two species do not kill each other outright as the optical density readings continue to increase over time. This suggests that growth is possible in combination and maintained; however this does not mean that one species is not killing or inhibiting the other whilst the other continues growth. The initial results show that in combination there is a sustained increase in growth. Overall there is in an increase in optical density for five isolates when compared with the previous assay.

Further investigation would be required to demonstrate if this increase in growth is for both *N. meningitidis* and *N. lactamica* or simply one species. No firm conclusions can be made from this experiment as no CFU counts were performed. Future experiments included CFU counts.

The only isolate that did not significantly increase in optical density, was isolate *N. meningitidis* R010. In isolation R010 reached an optical density of 0.366, in combination with *N. lactamica* R010 reached an optical density of 0.285. Although this difference is not statistically significant (P= 0.1296 unpaired, non-parametric t-test) this was the only isolate not to have an increase in optical density when grown in the presence of *N. lactamica* than compared to in isolation. Isolate R136 in particular showed an increase in optical density with *N. lactamica* when compared to growth in isolation.

Growth was higher on average for all of the isolates, likely due to rapid *N. lactamica* growth rate observed in the previous experiment. The growth curves of all isolates follow a similar pattern as *N. lactamica* in isolation. This assay indicates that overall growth, or at least an increase in biomass, is achieved when *N. meningitidis* and *N. lactamica* are grown in the presence of each other. Having previously identified these isolates as able to form a biofilm (Chapter 4.4.2), further investigation continued to assess the effect of both species in combination under biofilm forming conditions.

5.3.2 REGRESSION ANALYSIS

Accurately challenging *N. meningitidis* and *N. lactamica* together in biofilm requires an identical number of cells/mL in the initial biofilm set up. For this inoculation number to be achieved several regression experiments were performed to achieve a growth curve that contained both cells/mL and optical density data. Using the data from each graph, each isolate could be grown to a specific optical density that would then correlate with a specific concentration of cells/mL.

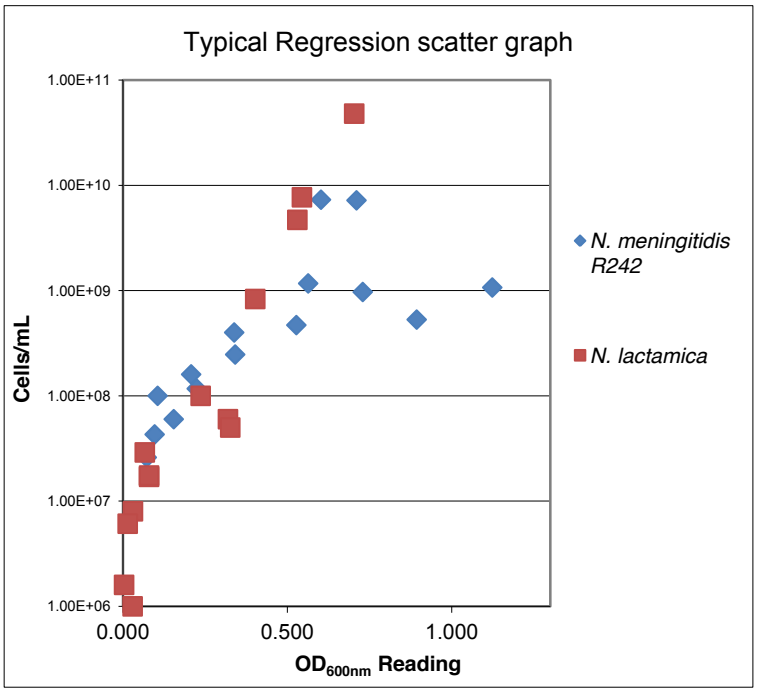

Figure 27 An example of a combined regression scatter graph

The graph demonstrates two regression data sets. The data produces a growth curve which can be measured by OD to give a determined bacterial concentration. Culture readings were taken over an 8-10 hour period (depending on the isolate) and repeated on separate occasions in triplicate n=3. The same master stocks were used on each occasion. Readings over 1.2 OD were not taken to ensure accuracy of the CFU counts.

Figure 27 shows a clear trend is visible with growth increasing over time. This was repeated multiple times for all of the isolates of interest. To create a starting culture for biofilm all the *N. meningitidis* isolates plus *N. lactamica* were grown until an optical density which indicated 10^8 cells/mL had been achieved for biofilm seeding. As these experiments aimed to investigate the relationship between *N. lactamica* and *N. meningitidis* the biofilm set up methodology was slightly altered.

5.3.3 BIOFILM EXPERIMENTS

From the planktonic data, *N. lactamica* and *N. meningitidis* do not kill each other outright, and growth is still maintained over a 24-hour period. In the presence of one another some of the isolates exhibited an increase in growth when compared to growth in isolation. We theorised that long term *Neisseria* carriage is possible because the bacteria exist in a biofilm state. The planktonic experiment was therefore repeated in biofilm.

Initially four isolates were picked to investigate further; each isolate expressed a different biofilm phenotype.

- MC58 – Capsulated Men B strain
- 022 – aggregates/clumps within a thin matrix on the liquid – surface layer
- 145 – similar properties to *N. lactamica*, however produces a thinner matrix
- 242 – forms micro-colonies in the air-liquid layer, good biofilm growth on liquid – surface layer

All the *N. meningitidis* isolates and *N. lactamica* were grown to give an initial starting concentration of 10^8 cells/mL prior to inoculation. 1:1 concentrations of equal cells/mL of *N. lactamica* and *N. meningitidis* were added to 50 %-TSB media in equal volumes. In wells containing only *N. lactamica* or *N. meningitidis* 2 mL of inoculum was

added per 1 mL of fresh 50 %-TSB media to ensure equal cell numbers across all wells. Experiments were conducted using a 12- well plate system, in triplicate. Both *N. lactamica* and the *N. meningitidis* isolate were added to each well at the same time point.

The 12-well plates were incubated at 37 ^{o}C and 5 % (v/v) CO_2 for up to three days. Every 24 hours 1 mL of spent media was removed and replaced with a 1 mL of fresh 50 %-TSB media. CFU counts performed on each day. To distinguish between *N. lactamica* colonies and *N. meningitidis* colonies, X-gal was used.

N. lactamica contains a β-galactosidase, which in the presence of X-gal turns the colonies blue. *N. meningitidis* does not contain a β-galactosidase enzyme and so is unable to break down the X-gal and remains white/gray in colour (Figure 28). After 16 hours incubation 20 µL of X-gal was drop plated onto each dilution concentration with visible colonies. The X-gal was left to dry. Using X-gal the colonies could then easily be distinguished between white and blue and counted accordingly.

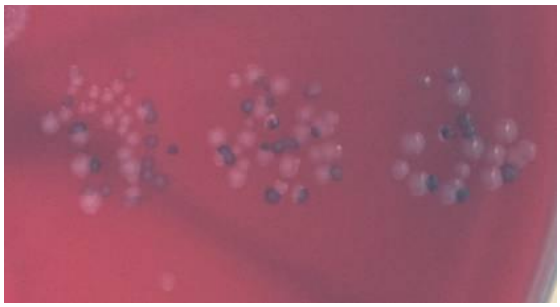

Figure 28. CBA plate containing both *N. lactamica* colonies (blue) and *N. meningitidis* colonies (white).

In the presence of X-gal the *N. lactamica* colonies turned from grey to blue making them easily and clearly identifiable as both species have a similar morphology. The *N. meningitidis* remains white in colour in the presence of X-gal as it does not contain a β-galactosidase enzyme. The *N. lactamica* colonies remained blue for up to two days, if refrigerated, before fading in colour.

5.3.3.1 N. MENINGITIDIS MC58

Prior to the challenge experiment, screening had demonstrated that MC58 did not thrive particularly well in 50 %-TSB media (Table 5). Although a fragile biofilm structure formed, it was not until after Day 2 that a recognisable biofilm structure could be physically observed. This observation is reflected in the large oscillation of CFU counts, with viable cells remaining high (4.07 x 10^7 cells/mL) after 24 hours post- inoculation but slowly dropping to 10^6 cells/mL on Day 2. Post Day 2 CFU counts do start to increase and slowly recover, however they still remain comparatively low when compared to *N. lactamica* (2.42 x 10^6 cells/mL and 4.33 x 10^7 cells/mL respectively).

Growing *N. meningitidis* MC58 and *N. lactamica* together demonstrated that MC58 thrives better in co-culture (Figure 29) with *N. lactamica* then compared to the viable counts in isolation. This positive increase is statistically relevant by Day-3 (** P= 0.0043, non-parametric Mann-Whitney t-test).

This increase in viable MC58 CFU's did not appear to have a negative effect on *N. lactamica* growth over the three-day period. There was no significant difference between *N. lactamica* in co-culture when compared to CRF counts in isolation (Day 1 – P=0.625, Day 2 – P= 0.269 and Day 3 – P= 0.131 multiple t-test using the Holm-Sidak method).

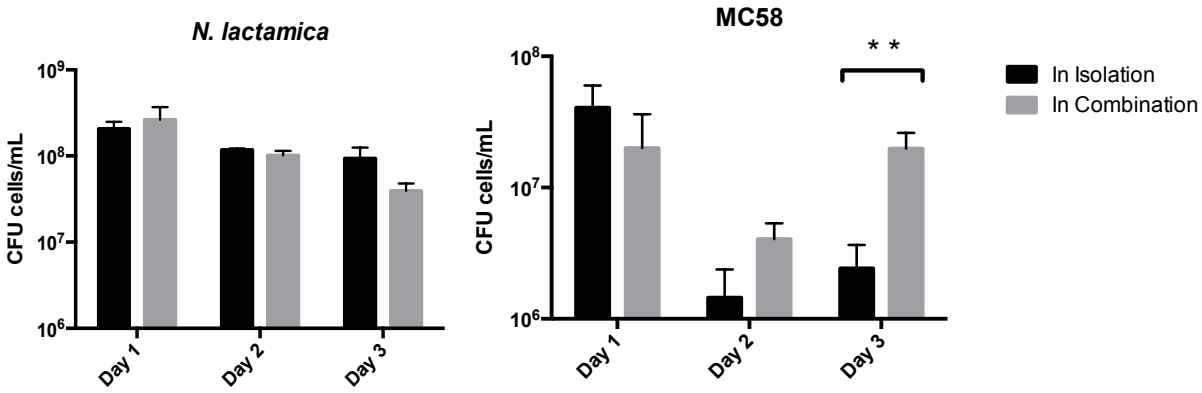

Figure 29 1:1 Challenge experiment for MC58 with *N. lactamica* over 3 days.

Co-colonisation has no effect on the number of N. lactamica CFUs in a biofilm state, but the number of MC58 CFU's does increase at Day-3. No other significant results were observed at Day 1 or Day 2. Experiments were performed in 6-well tissue culture plates and repeated in triplicate. The same master stocks were used in all experiments, n=6.

5.3.3.2 N. MENINGITIDIS R022

Isolate R022 was one of 14 isolates still identified at week 26. This suggests that long term carriage of this isolate is possible. R022 had sustained carriage throughout several previous visits and the isolate was not identified at any time point in combination with *N. lactamica*. This could be for a number of reasons, the two may be unable to co-exist together or that in that particular volunteer *N. lactamica* was unable to colonise due to the host clearing the commensal effectively. It is also quite possible that both species had colonised the nasopharynx but swabbing methods had only identified one.

R022 demonstrated a slightly slower increase in growth during the planktonic experiment and after 24 hours had the lowest optical density reading of all the isolates (OD at 24 hours = 0.317) and in comparison to *N. lactamica* (OD at 24 hours = 0.474). In combination with *N. lactamica* during the planktonic experiment there was continued growth (OD at 24 hours = 0.466).

In biofilm R022 is able to survive well; initial screening (Section 4.3.2) demonstrated increase in biomass was seen over the 4-day observation period. R022 forms a thin matrix, which contains clumps of micro colonies that aggregate together. Genetic analysis of the isolate revealed that the isolate has the machinery to express capsule (Section 4.4.2) which was further confirmed during the India ink screening where the isolate was identified as capsule positive (Section 4.4.1).

In this experiment R022 in isolation alludes to a natural biofilm cycle, increasing and decreasing in CFU counts (Figure 30). When compared to the CFU counts in isolation, R022 thrives better in a co-culture with *N. lactamica*. Day 1 * P = 0.009. This is especially evident at the Day-2 time point when R022 averages at 1.13 x 10^6 cells/mL in isolation compared to 6.21 x 10^7 cells/mL when in combination with *N. lactamica*. This is a significant result *P= 0.005 (Multiple t-test Holm-Sidak method).

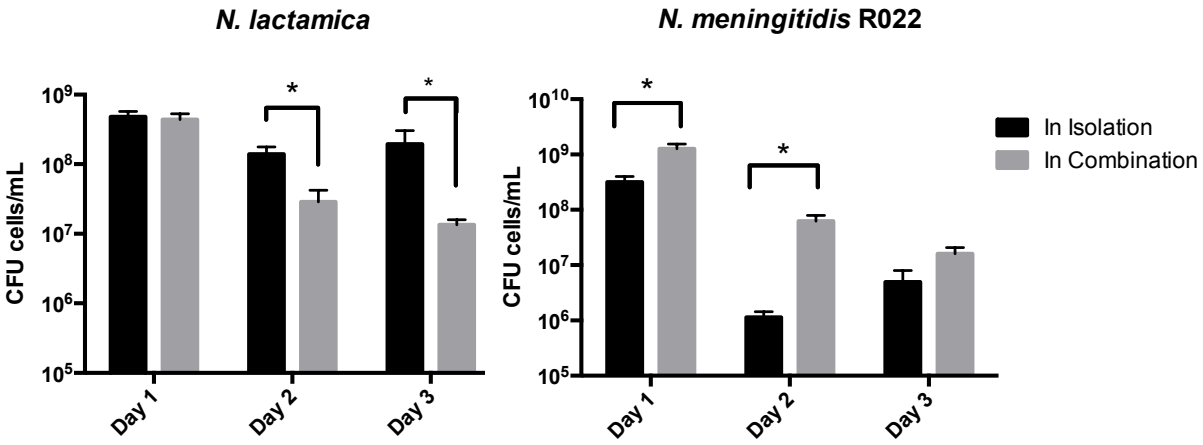

Figure 30 1:1 Challenge experiment of R022 and *N. lactamica*.

In co-culture R022 CFUs increases at Day-1 and Day-2, however the number of viable *N. lactamica* decreases at Day-2 and Day-3 indicating that R022 has a negative impact on *N. lactamica*. Experiments were performed in 6-well tissue culture plates and repeated in triplicate. The same master stocks were used in all experiments n=6.

Unlike the relationship between MC58 and *N. lactamica*, R022 does appear to have a negative effect on *N. lactamica* CFU counts. When in isolation *N. lactamica* over three days records CFU counts of 4.79 x 10^8 cells/mL, 1.39 x 10^8 cells/mL and 1.94 x 10^8 cells/mL respectively. In combination with R022 these counts are 4.39 x 10^8 cells/mL (8.35 % decrease), 2.88 x 10^7 cells/mL (79.14 % decrease) and 1.34 x 10^7 cells/mL (93.09 % decrease) respectively. Day-1 counts are relatively similar between *N. lactamica* in isolation and the number of blue colonies; however there is nearly a log fold difference on Day-2 and a 1.45 log-fold difference by Day-3. The difference in *N. lactamica* in isolation and in co-culture on Day-2 is statistically significant (*P = 0.013 unpaired, non-parametric Mann-Whitney t-test). The Day-3 results are also significant (*P = 0.013 unpaired, non-parametric Mann-Whitney t-test), demonstrating that R022 is having a sustained negative effect on *N. lactamica*. Physical observations noted that post-Day-2 the biofilm does not adhere to the substratum as tightly when compared to both species in isolation. This decrease in viable *N. lactamica* cells in the presence of R022 does show a competition for nutrient resources. The experiment would need to be extended to investigate if *N. lactamica* colonies continue to decrease over time.

5.3.3.3 N. MENINGITIDIS R145

N. meningitidis isolate R145 was identified at weeks 0 and 4 but it was not isolated again throughout the duration of the Lac2 study (total of 28 weeks). During the study *N. lactamica* was not identified in isolation or in combination with R145.

Previous biofilm screening (Section 4.3.2) showed that R145 has a confluent biofilm phenotype similar to that of *N. lactamica*. India ink screening (Section 4.4.1) was unable to confirm whether the isolate expressed capsule, however genetic analysis (Section 4.4.2) demonstrated that R145 does have the necessary genes for capsule production.

In planktonic culture R145 thrived well in TSB (Figure 25 and Figure 26) and this is reflected in biofilm when nutritionally stressed (Table 5).

Figure 31 shows that *N. Lactamica* and R145 did not appear to kill each other outright during the planktonic assay, however when in biofilm the amount of white viable colonies observed rapidly decreases over 3 days. When grown in isolation R145 increases and decreases in number over 3 days from Day 1 = 1.03 x 10^9 cells/mL, Day 2 = 3.08 x 10^7 cells/mL and Day 3= 2.12 x 10^8 cells/mL, although this oscillation is not statistically significant it could allude to the natural biofilm cycle. This oscillation however is partially observed in co-culture with *N. lactamica*. At Day-1 and Day-2 the CFU counts are extremely similar to those observed in isolation (Day-1 in isolation = 1.03 x 10^9 cells/mL, Day-1 in co-culture = 5.18 x 10^8 cells/mL, Day-2 in isolation = 3.08 x 10^7 cells/mL, Day-2 in co-culture = 1.81 x 10^7 cells/mL). However at Day-3 there is a dramatic 4-log fold drop (99.99 % decrease) in white viable colonies when compared to CFU counts in isolation from 2.12 x 10^8 cells/mL in isolation to 1.11 x 10^3 cells/mL in co-culture. This is a statistically significantly reduced number of viable *N. meningitidis* cells **P=0.0022 (Unpaired, non-parametric Mann-Whitney t-test).

In parallel with the decrease in viable white colonies there is a positive increase of the number of blue colonies, 6.39 x 10^7 cells/mL in isolation and 3.28 x 10^8 cells/mL when in co-culture. At Day-1 there is a small increase in the number of blue CFUs when compared *N. lactamica* in isolation, although this increase is not significant it is repeated again at Day-2. This continuing positive increase in blue viable CFUs persists over the 3-day experiment. At Day-3 this increase between *N. lactamica* in isolation and in co-culture is statistically significant **P = 0.0022 (Unpaired, non-parametric Mann-Whitney t-test), indicating that *N. lactamica* has benefitted from being in co-culture with *N. meningitidis* R145.

This is the first physical observation that *N. lactamica* and a *N. meningitidis* isolate have a conflicting relationship when in a biofilm state. The CFU counts for R145 in co-culture at Day-1 and at Day-2 are stable when compared to the CFUs in isolation indicating that there is a change around 48-62 hours to cause such a dramatic decrease in white colonies at Day-3.

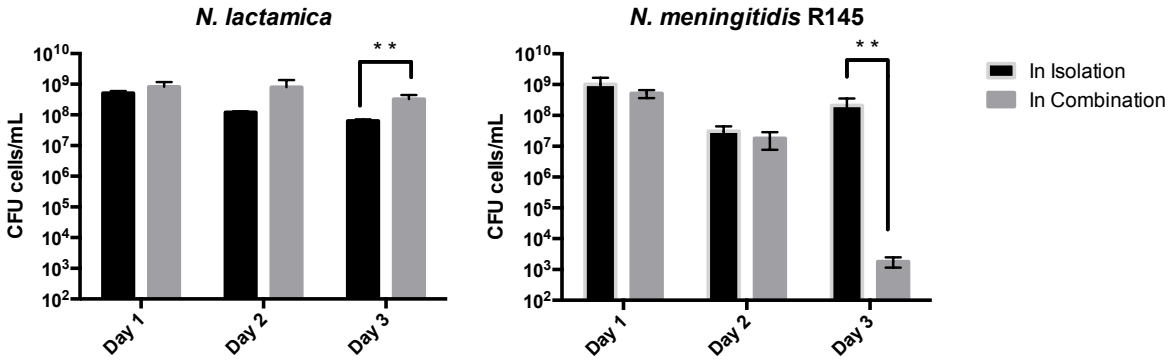

Figure 31 1:1 Challenge experiment for isolate R145 and *N. lactamica* in biofilm.

In co-culture there is a significant increase in the number of *N. lactamica* CFU's at Day-3, this is in contrast with the significant 99.99% decrease in *N. meningitidis* CFU's at Day-3.This result suggests that there is a conflicting relationship between the two. Experiments were performed in 6-well tissue culture plates and repeated in triplicate on separate occasions, n=6. The same master stocks were used in all experiments.

5.3.3.4 N. MENINGITIDIS R242

N. meningitidis R242 like R022 was still identified at week 26 and had been previously identified at several other visits during the Lac2 study (weeks 0, 2, 4 and 16) indicating that long-term carriage is possible for this isolate. R242 produced a dual layer biofilm in the initial screening process; typically it was observed that this biofilm was more durable, especially when compared to the other two biofilm phenotypes. India ink capsule analysis (Section 4.4.1) demonstrated that the isolate is capable of expressing capsule. Genetic analysis (Section 4.4.2) proved that the isolate has the machinery required for capsule production.

In the planktonic assay R242 in isolation (Figure 25) demonstrated a similar growth curve to that of *N. lactamica*, and when in combination and (Figure 26) there was a minor increase in growth, suggesting either a neutral relationship between two or that one had grown successfully whilst the other failed.

During biofilm screening it was identified that in isolation R242 is able to maintain biofilm structure over four days. It was observed that during feeding and washing there was minimal damage to the top air-liquid interface layer. The surface-liquid layer did sustain some damage during feeding and washing but this was not different to the other isolates. The CFU counts for R242 in isolation remained stable; Day-3 counts were 4.74×10^7 cells/mL demonstrating that R242 is able to form a sustainable biofilm.

When in a co-culture biofilm R242 and *N. lactamica* appear to have very little effect on one another at Day-1 and Day-3. Although the counts do vary slightly there is only a minor difference between each species grown in isolation (*N. lactamica*; Day 1 - 7.67×10^8 cells/mL and Day 3 - 1.69×10^8 cells/mL. R242; 2.61×10^8 cells/mL, 1.77×10^7 cells/mL and 4.74×10^7 cells/mL) and in combination (Blue colonies; Day 1 - 1.06×10^9 cells/mL and Day 3 - 3.80×10^8 cells/mL. White colonies; 4.06×10^8 cells/mL, 1.44×10^7 cells/mL and 6.83×10^7 cells/mL over three days).

There is a significant (79.01%) decrease in the number of blue viable colonies at Day 2 (in isolation - 1.51×10^8 cells/mL and in combination – 3.17×10^7 cells/mL). $P = 0.01001$ (Multiple t-test using Holm-Sidak method). The CFU counts quickly recover by Day 3 suggesting the significant decrease may not solely be due to incompatibility. There is no significant change in R245 across the 3 day experiment.

Overall *N. lactamica* has no statistically significant effect on R242, although R242 does have an effect on *N. lactamica*, but only at Day 2 only as no other results are significant.

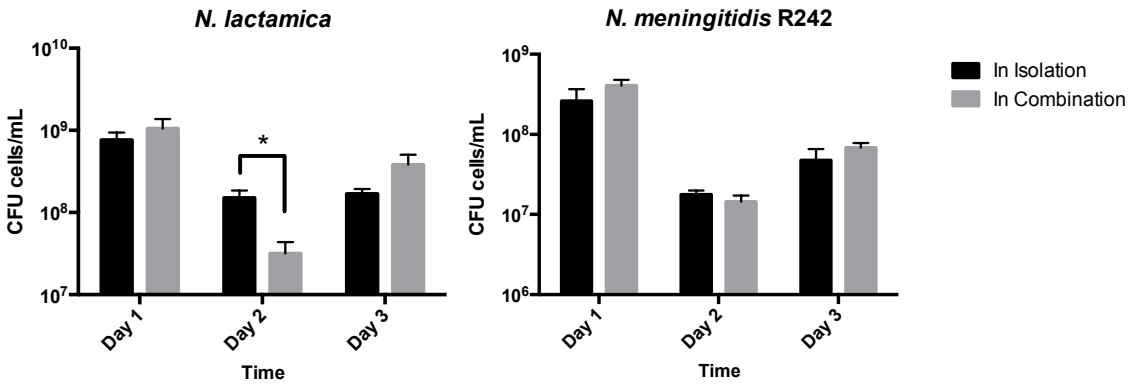

Figure 32 - 1:1 Challenge experiment for isolate R242 and *N. lactamica* in biofilm.

N. Lactamica had no effect in co-culture on R242 over 3 days. There is a significant decrease in CFU's at Day 2 for *N. Lactamica* in co-culture but this is quickly recovered by Day-3. Experiments were performed in 6-well tissue culture plates and repeated in triplicate, n=6. The same master stocks were used in all experiments.

5.4 EXPANSION OF ONE TO ONE CHALLENGE

N. meningitidis isolates MC58, R022, R145 and R242 all demonstrated very different relationships with *N. lactamica* when in biofilm. The difference in results suggested that those isolates which form poor biofilms when in isolation benefit from co-culture with *N. lactamica* in biofilm. Those isolates which are good biofilm-formers in isolation directly compete with *N. lactamica* for resources.

To determine if this was a common theme throughout, all the isolates (listed in Table 7) were screened using the same experimental design as previously used (Section 5.3.1).

5.4.1 PLANKTONIC CO-HABITATION OVER 24 HOURS

Broths containing an equal number of colonies per mL were added to a 96-well plate. Growth of both *N. lactamica* and *N. meningitidis* were observed together and in isolation over 24 hours at 37 ^{o}C and 5 % CO_2 without agitation. Growth was measured using optical density. Readings were taken hourly for the first eight hours and a final reading was taken at 24 hours. This assay also included CFU counts at the 24 hour time point to improve the previous methodology used. Plates were incubated for 20 hours at 5 % CO_2, X-gal solution was used to identify *N. lactamica* colonies. This extra assay gave a quick indicator to which (if any) species was more dominant.

Those isolates which exhibited a negative relationship when in co-culture with *N. lactamica* were of particular interest.

Isolate	OD at 8 hours	OD at 24 hours	*N. men* CFU in isolation (cells/ml)	*N. men* CFU in co-culture (cells/ml)	Biofilm type	Percentage change
R010	0.166	0.366	9.00×10^7	8.00×10^7	Confluent	-11.1 % ↓
R034	0.080	0.157	2.50×10^6	6.67×10^7	Poor/no biofilm	+2568 % ↑
R046	0.092	0.107	0	-	Confluent	-
R049	0.095	0.192	1.50×10^8	1.33×10^8	Confluent	-11.3 % ↓
R054	0.182	0.255	3.00×10^7	3.60×10^7	Confluent	+20.0 % ↑
R059	0.126	0.615	1.65×10^9	3.67×10^9	Dual	+122.4 % ↑
R083	0.082	0.362	2.00×10^8	2.00×10^8	Micro colonies	0.0 %
R104	0.085	0.296	3.50×10^7	3.33×10^6	Micro colonies	-90.5 % ↓
R119	0.083	0.212	3.00×10^8	2.00×10^8	Confluent	-33.3 % ↓
R128	0.107	0.799	8.50×10^8	5.00×10^8	Dual	-41.2 % ↓
R129	0.109	0.243	5.00×10^7	6.67×10^7	Dual	+33.4 % ↑
R136	0.125	0.508	8.50×10^8	6.00×10^8	Confluent	-29.4 % ↓
R191	0.172	0.344	3.00×10^7	Zero white colonies	Dual	-100.0 % ↓
R196	0.118	0.223	2.50×10^8	4.33×10^8	Confluent	+73.2 % ↑
R200	0.146	0.537	5.00×10^8	3.00×10^8	Dual	-40.0 % ↓
R210	0.085	0.595	6.00×10^7	0	Micro-colonies	-100.0 % ↓
R221	0.090	0.181	8.00×10^7	0	Sheet-like layers	-100.0 % ↓
R222	0.091	0.501	5.50×10^8	1.33×10^8	Dual	-75.8 % ↓
R234	0.107	0.370	9.50×10^8	5.67×10^8	Dual	-40.3 % ↓
R263	0.097	0.492	5.50×10^7	1.67×10^7	Confluent	-69.6 % ↓
R279	0.100	0.291	2.00×10^7	0	Confluent	-100.0 % ↓
N. lac	0.207	0.474	1.53×10^8	N/A	Confluent	Control

Table 8 Full planktonic screen of 20 isolates with CFU data.

The full screening experiment collected not only optical density to ensure growth measurements over a 24-hour period but also CFU data of the isolates not only in culture but also in co-culture with *N. lactamica*. Isolates which exhibited a significant decrease in CFU numbers was further investigated. Experiments were repeated in triplicate in separate experiments, using 96-well plates, n=3.

The expansion of the planktonic assay enabled the characterisation of isolates quickly. Isolates could be characterised as following;

- 100 % decrease – Zero white colonies identified.
- 50 %-99.9 % decrease
- 1 % to 49.9 % decrease
- No change
- Positive percentage increase.

The planktonic experiment revealed that of the 20 isolates tested, 7 exhibited a 50-100 % decrease in CFUs and 7 exhibited a 1-49.9 % decrease in CFUs suggesting a negative relationship with *N. lactamica* in co-culture. A single isolate exhibited no change, and 5 isolates exhibited a positive relationship with *N. lactamica*.

5.4.2 BIOFILM CO-HABITATION

From the planktonic extension experiment (Section 5.4.1) a total of 14 isolates could be categorised as exhibiting a negative relationship (1-100 % decrease) with *N. lactamica* in co-culture. To investigate this relationship further only those isolates which saw a 100 % decrease in CFUs when in co-culture with *N. lactamica* were subjected to biofilm investigation. These four isolates (R191, R210, R221 and R279) were 'challenged' in biofilm with *N. lactamica* over a 1 to 3 day period. Isolates were grown in both isolation and in co-culture repeating the methodology used in Section 5.3.3.

5.4.2.1 N. MENINGITIDIS R191

N. meningitidis R191 was isolated at weeks 0 to 4 and again at week 16 but at no other visit post week 16. This suggests that carriage is possible for at least four weeks and possibly up to 16 weeks, alternatively the presence and then 'disappearance' at 8 weeks could suggest that carriage could be re-acquired.

R191 presents a confluent dual layer biofilm when grown in reduced strength media (Section 4.3.2, Table 5). The previous planktonic experiment (Table 8) demonstrated that in isolation R191 increases in growth over 24 hours as would reasonably be expected from OD 0.172 to 0.344 at eight and 24 hours respectively. When *N. meningitidis* R191 and *N. lactamica* are grown together in a planktonic co-culture, x-gal staining of the CFU's demonstrated that only *N. lactamica* thrived and no white colonies could be observed. *N. Lactamica* may be competing with and actively displacing R191 in a planktonic culture.

Figure 33 demonstrates that in biofilm *N. Lactamica* and R191 are able to co-exist successfully over a 3 day period. Although there appears to be a decrease in viable counts at Day-2 when compared to Day-1 this drop is not statistically significant and is likely to be part of the biofilms natural cycle. Viable counts for both species are around the same at each time point in both isolation and in co-culture (Table 9.). This suggests that overall *N. Lactamica* and R191 have a neutral relationship in biofilm. CFU counts for *N. lactamica* at Day 1 are statistically significantly higher in co-culture than in isolation **P = 0.0022 (unpaired, non-parametric Mann-Whitney t-test). This would imply that *N. lactamica* initially benefits from being in co-culture with R191.

Time point	*N. lactamica* in isolation (cells/mL)	*N. meningitidis* in isolation (cells/mL)	*N. lactamica* in co-culture (cells/mL)	*N. meningitidis* in co-culture (cells/mL)
Day 1	8.44×10^7	1.28×10^8	4.44×10^8	8.67×10^7
Day 2	5.71×10^7	1.74×10^7	6.96×10^7	2.19×10^7
Day 3	2.02×10^7	8.06×10^7	7.29×10^7	7.74×10^7

Table 9. Average CFU counts of both *N. lactamica* and *N. meningitidis* over 3 days. Results shown are for both bacteria in isolation and in co-culture. The CFU's show that in both co-culture and in isolation both species exhibit a maintained growth. Experiments were performed in 6-welll tissue culture plates in duplicate and repeated in triplicate on separate occasions, n=6. All experiments used the same master stocks.

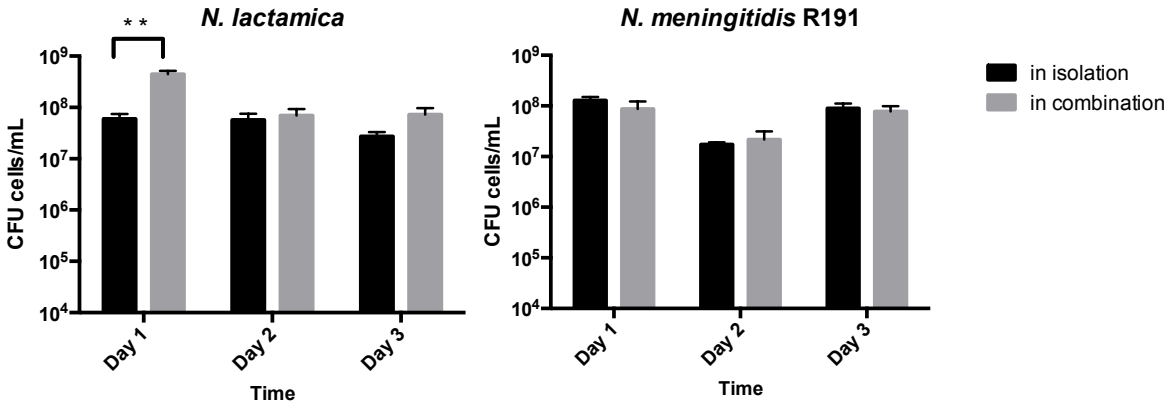

Figure 33. 1:1 challenge experiment for isolate R191 and *N. Lactamica* in biofilm.
After 3 days in co-culture and isolation the results proved to show no negative effect on either *N. lactamica* or R191. On Day 1 there was a statistically significant increase in *N. lactamica* CFUs when grown in combination with R191. Overall the results indicate that the two species have a neutral relationship in biofilm. Experiment performed in 6-well tissue culture plates and repeated in triplicate, n=6.

5.4.2.2 N. MENINGITIDIS R210

N. meningitidis R210 was isolated at weeks 0, 4, 8 and 26 during the Lac2 study. The isolate was not found on swabs at weeks 2 and 16, and was not isolated again post week 26. The results suggest that carriage even for a short period of time is possible. *N. lactamica* was not identified throughout swabbing.
R210 forms a single surface-liquid layer biofilm which presents with small micro-colonies embedded within a thin confluent layer similar to a *N. lactamica* biofilm (Table 5- Section 4.3.2). The micro-colonies increased in number of the 4 day screening process (Table 5).
Genetic analysis (Section 4.4.2) of the isolate demonstrated that R210 is able to produce capsule. The capsule group was positively identified as serogroup X, which has been linked to several meningococcal outbreaks during 2006-2010 in Africa (Xie *et al*.

2013). The India ink screening (Section 4.4.1) demonstrated that the isolate expressed capsule.

From the planktonic experiment (Table 8.) a steady increase in OD was observed from 0.085 at eight hours to 0.595 at 24 hours. CFU counts however showed that like *N. meningitidis* R191 after x-gal staining no white colonies were present after 24 hours. In isolation the CFU count was 6 x 10^7 cells/mL, indicating a significant drop in the viable count.

Figure 34 shows that R210 has no effect on *N. lactamica* in a co-culture biofilm over the 3 day experiment, but *N. lactamica* has a significant effect on R210. This effect is statistically significant at Day-2 – **P= 0.0087 (unpaired, non-parametric Mann-Whitney t-test) resulting in an 85.69 % decrease when compared to R210 in isolation. A similar effect is also seen at Day-3 – *P= 0.0368 (unpaired, non-parametric Mann-Whitney t-test) resulting in an 85.54 % decrease when compared to isolation. This suggests that R210 is in a kind of conflict with *N. lactamica* when in co-culture. The CFU count at Day-2 for R210 in co-culture is 2.19 x 10^7 cells/mL and at Day 3 it is 1.18 x 10^7 cells/mL, a difference of 1.01 x 10^7 cells/mL.

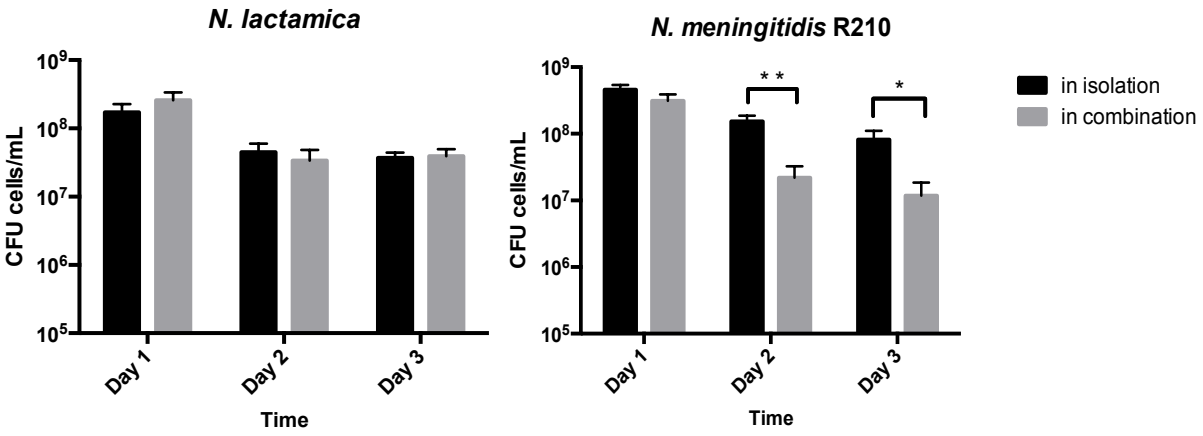

Figure 34. 1:1 challenge experiment for isolate R210 and *N. lactamica* in biofilm. There is no statistically significant effect documented on *N. lactamica*, however there is a significant decrease in viable white colonies on Day-2 and Day-3 on R210 in co-culture. Experiments were performed in 6-well tissue culture plates and repeated in triplicate, n=6.

5.5.2.3 N. MENINGITIDIS R221

N. meningitidis R221 was isolated at weeks 0 and 16. The isolate was not identified post week 16 however at weeks 2, 16 and 26 *N. lactamica* was isolated from the nasal swabs. The identification of both *N. lactamica* and R221 at week 16 could suggest that there is the ability for the two isolates to co-exit.

R221 forms an interesting biofilm, unique among the other isolates observed during this thesis, in sheet-like layers. These layers presented at the air-surface interface and if disturbed would fall to the bottom of the well (Figure 20 Section 4.3.2) only to be quickly replaced over a 24 hour period. It was previously demonstrated that both the top air-liquid interface and the disturbed layers could produce viable CFUs (10^8 cells/mL at Day 3 – Section 4.3.2).

Genetic analysis of R221 (Section 4.4.2) demonstrated that the isolate has the necessary genes for capsule production however the India ink analysis demonstrated that no capsule production was found indicating that the isolate was not expressing

capsule at the time. This isolate could be a prime example of how *N. meningitidis* can adapt to the surrounding environment by varying capsule expression.

The planktonic experiment (Table 8.) revealed a steady increase in OD from 0.090 at 8 hours to 0.181 at 24 hours. As with previous isolates no white colonies were observed after x-gal staining in co-culture; however the CFU counts in isolation were good at 8 x 10^7 cells/mL, with no phenotypic changes and no indication that the isolate could not support growth.

The results show that there is a statistically significant decrease in the number of viable *N. lactamica* CFUs in co-culture with R221 when compared to those in isolation at Day-1 and at Day-2 (Figure 35.). The amount of viable blue colonies was significantly lower, Day-1 *P = 0.0303 and Day-2 **P = 0.0022 (unpaired, non-parametric Mann-Whitney t-test) than those of *N. lactamica* alone (Day 1 = 1.02 x 10^8 cells/mL in isolation 4.50 x 10^7 cells/mL in co-culture. Day-2 = 8.00 x 10^7 cells/mL in isolation and 4.71 x 10^6 cells/mL).

The results demonstrate a 55.88 % decrease in CFU counts when compared to isolation at Day-1 and a 94.11 % decrease at Day-2. During this time period there is not a significant increase in the number of viable CFUs for R221. The results show that in combination there is a negative effect of both species compared to isolation, although this decrease is only statistically significant for *N. lactamica.*

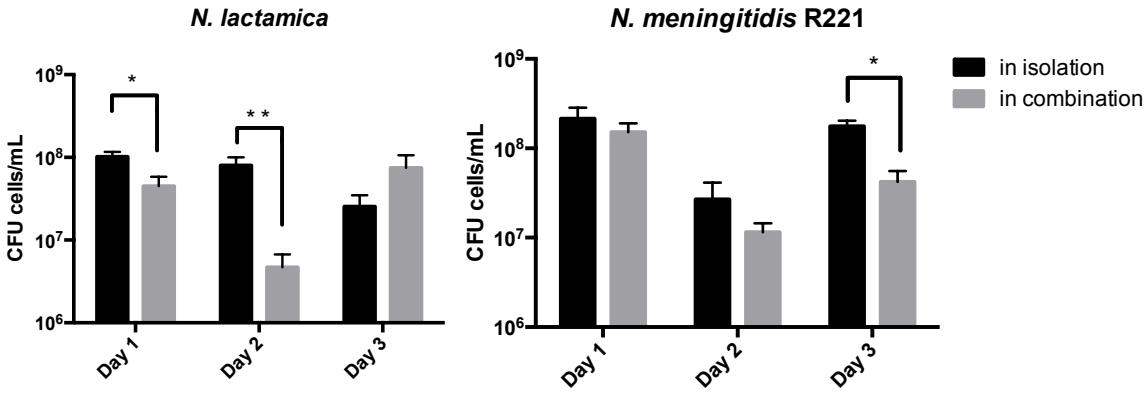

Figure 35. 1:1 challenge experiment for isolate *N. meningitidis* R221 and *N. lactamica* in biofilm.

At Day-1 and Day-2 there is a significant 55.88 % and 94.11% decrease respectively in the viable CFUs of *N. lactamica* when in co-culture, however at Day-3 there is then a significant 76.02 % decrease in the viable CFUs of R221. Experiments were performed in 6-welll tissue culture plates in duplicate and repeated in triplicate on separate occasions, n=6. All experiments used the same master stocks.

At Day-3 however there is a statistically significant (76.02%) decrease in the number of viable cells for R221. The CFU count between R221 in isolation (1.76 x 10^8 cells/mL) and in co-culture (4.22 x 10^7 cells/mL) is nearly a log fold difference. At this same time period *N. lactamica* CFU counts increase from 6.00 x 10^7 cells/mL in isolation to 7.47 x 10^7 cells/mL. Although this is not statistically significant it does suggest a competitive relationship between the two species.

5.4.2.4 N. MENINGITIDIS R279

N. meningitidis was isolated at weeks 0, 2 and 26. The isolate was not found at any other time period but *N. lactamica* was isolated at weeks 2, 8, 16 and 28. The identification of both *N. lactamica* and *N. meningitidis* at week 2 suggests that there is the possibility the two are able to co-exist. R279 then appears to have been lost at weeks 4, 8 and 16 before being identified again at week 26 suggesting that either the

isolate was removed from the host and later re-acquired or that swabbing failed to identify the isolate.

R279 is phenotypically similar to *N. lactamica* in biofilm formation. Previous biofilm screening (Table 5 Section 4.3.2) indicated that R279 didn't form the same strong attachment to the substratum as *N. lactamica,* and on occasion failed to thrive in 50 % media.

The planktonic experiment (Section 5.4.1 Table 8) indicates that growth is steadily maintained from 0.100 at 8 hours to 0.291 at 24 hours, however as with the previous isolates no white colonies were observed after X-gal staining.

Figure 36 demonstrates that when in co-culture *N. lactamica* significantly increases at Day-1 (*P= 0.02, unpaired non-parametric Mann-Whitney t-test). *N. lactamica* CFU numbers remaining constant whilst in combination at Day-2 = 3.96 x 10^7 cells/mL and Day-3 = 2.96 x 10^7 cells/mL.

The effect on *N. meningitidis* R279 whilst in co-culture with *N. lactamica* was unprecedented amongst the other isolates (Figure 37.). *N. meningitidis* R279 at Day-1 did increase from 3.09 x 10^5 cells/mL in isolation to 2.22 x 10^6 cells/mL although this was not significant, and three separate experiments yielded no white colonies at all. At Day-2 CFUs averaged at 1.1 x 10^5 cells/mL but four experiments yielded no white colonies, and by Day-3 five experiments yielded no white colonies with a single experiment CFUs at 1.1 x 10^6 cells/mL. CFU numbers at Day-2, excluding those experiments in which no white colonies were identified, had significantly decreased in combination when compared to isolated growth (**P= 0.002, unpaired non-parametric, Mann-Whitney t-test). By Day-3 no white colonies were identified in five of the six experiments. A single experiment resulted in 1.1 x 10^6 cells/mL, which was a significant decrease compared to *N. meningitidis* R279 in isolation (**P=0.002, unpaired non-parametric, Mann-Whitney t-test).

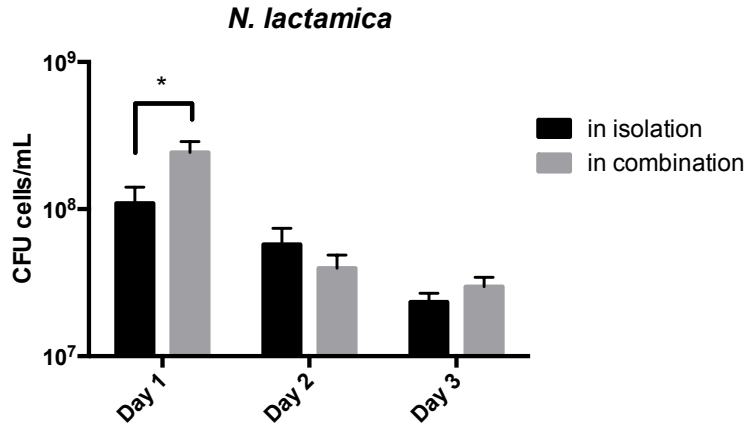

Figure 36. *N. lactamica* when challenged 1:1 with *N. meningitidis* R279.

The CFU counts *for N. lactamica* appear to be stable over the 3-day time period. There is a significant increase in the number of CFUs in combination at Day 1 *P = 0.02 (unpaired, non-parametric Mann-Whitney t-test). Experiments were performed in 6-welll tissue culture plates in duplicate and repeated in triplicate on separate occasions, n=6. All experiments used the same master stocks.

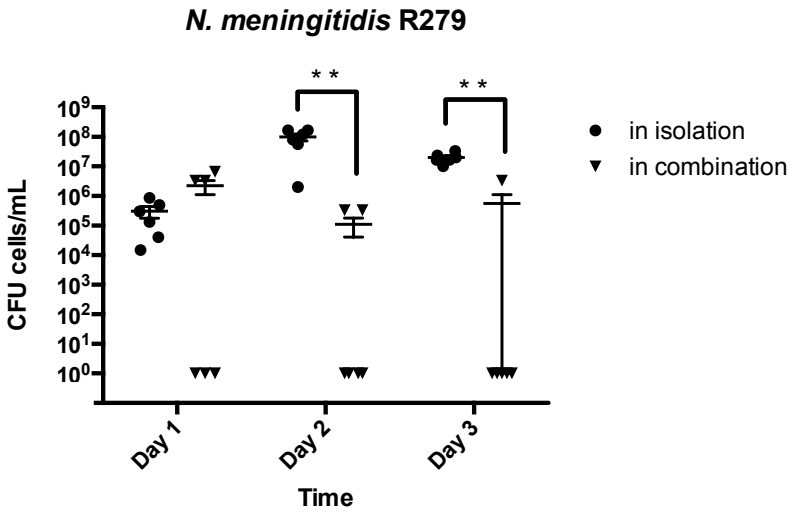

Figure 37. *N. meningitidis* R279 in isolation and co-culture with *N. lactamica*.

At Day-1 three experiments yielded no white colonies, at Day-2 this increased to four experiments and by Day-3 increased to five experiments. This is in contrast *with N. meningitidis* R279 in isolation which is able to thrive. Experiments were performed in 6-welll tissue culture plates in duplicate and repeated in triplicate on separate occasions, n=6. All experiments used the same master stocks.

5.5 CONCLUSIONS AND DISCUSSION

Overall the experiments from this chapter have firstly demonstrated that when in a planktonic co-culture; four *N. meningitidis* carriage isolates are out-competed or killed by *N. lactamica* over a 24 hour time period. This result is very exciting. Further investigation as to why this is happening would be intriguing. It may also be interesting to investigate if this phenomenon occurred with known virulent isolates from patients with disease and not just carriage isolates.

Secondly this thesis has demonstrated that when in biofilm *N. lactamica* has been shown to reduce the viability of 4 out of 8 isolates examined. Survival of all the isolates in isolation demonstrates that this decrease in CFUs is not due to the individual isolate. Survival of 7 of the isolates to at least Day-2 suggests that some persistence is offered by a biofilm state even when in co-culture. Further work would be required to examine if *N. lactamica* reduces viability of the other isolates over a longer time period (post 72 hours).

5.5.1 VIABLE BUT NON-CULTURABLE CELLS

Very few isolates identified at week 0 were consistently isolated at each swabbing visit during the Lac2 study (Section 4.3.3). This could be for a variety of reasons such as swabbing technique, which has previously been shown to be inaccurate (Sim *et al*. 2000), immune clearance of the isolate or simply the isolate is viable but not culturable. The disappearance and then re-appearance of some of the isolates during the Lac2 study is interesting and could suggest that these isolates are in a viable but non-culturable (VBNC) state. A VBNC state occurs when the cells are under high stress, such as a low nutrient environment (Li *et al.* 2014). These cells maintain an intact cell membrane and are not culturable on traditional media. A VBNC state has shown to enhance the longevity of some bacterial species, such as *Vibrio fluvialis* which was shown to be viable after six years of starvation (Amel *et al.* 2008). VBNC cells have

been identified within biofilm, and importantly by a bacterial species known to be invasive to humans (Trevors, 2011). *Staphylococcus aureus* has been shown in a VBNC state in biofilm when there is an antibiotic pressure (Pasquaroli *et al.* 2013).

This thesis has demonstrated that the *N. meningitidis* isolates from the Lac2 study are able to form biofilm which could prolong carriage, should the isolates also be able to enter a VBNC state *in vivo* within the biofilm they swabbing alone could make them unidentifiable.

5.5.2 PLANKTONIC EXPERIMENTS

Prior to the start of the planktonic experiment it was expected that overall growth may be higher in combination as *N. lactamica* grows very quickly in comparison to the *N. meningitidis* isolates (Figure 25.). We hypothesized that the growth curve would be similar to that of *N. lactamica* in isolation. The experiment inoculated each well with the same total number of colonies in both combination and isolation. The results from the combined experiment show that the overall OD measurement of some of the isolates was significantly higher than what had been predicted (Figure 26). Although no CFU analysis was performed at the start, the results appear to suggest that combined growth was beneficial, either for both *N. lactamica* and *N. meningitidis* or for just one. Later CFU analysis demonstrated that when grown in the presence of *N. lactamica*, the majority of the *N. meningitidis* isolates displayed a reduction on CFUs when compared to isolation (Table 8).

5.5.3 BIOFILM EXPERIMENTS

Overall the co-culture biofilm experiments have demonstrated that generally, when in co-culture, *N. lactamica* has a greater effect on *N. meningitidis* than what *N. meningitidis* has on *N. lactamica*. Of the 8 'challenge' experiments conducted only two can be described as - *N. lactamica* having no statistical effect on viability of *N.*

meningitidis, three isolates can be described as having - a negative effect on *N. lactamica* and in four experiments *N. lactamica* had a negative effect on *N. meningitidis*.

This is in comparison with the effect the *N. meningitidis* had on *N. lactamica*. Only three experiments demonstrated that *N. meningitidis* had a positive effect on *N. lactamica,* but only two *N. meningitidis* isolates exhibited a positive when in co-culture with *N. lactamica*.

Isolate Number	*Effect in co-culture on N. lactamica*	*Effect in co-culture on N. meningitidis*
MC58	No effect	Positive – significant at Day-3
R022	Negative – significant at Day-2 and Day-3	Positive – significant at Day-1 and Day-2
R145	Positive – significant at Day-3	Negative – significant at Day-3
R242	Negative – significant at Day-2	No effect
R191	Positive – significant at Day-1	No effect
R210	No effect	Negative – significant at Day-2 and Day-3
R221	Negative – significant at Day-1 and-2	Negative - significant at Day-3.
R279	Positive – significant at Day-1	Negative – significant at Day-3

Table 10. Generalised outcomes of co-culture over 3 days between *N. lactamica* and a *N. meningitidis* isolate.

The difference in data between the planktonic and biofilm experiments suggests that planktonic cultures are able to support rapid growth over a short time period, whereas biofilm cultures are able to support a continued growth for a longer time period when compared to planktonic cultures. Although not all the *N. meningitidis* isolates tested

were successful at biofilm formation the majority could be shown to form some biofilm structure.

5.5.4 *N. meningitidis* isolates negatively affected by *N. lactamica* in Biofilm

Of the eight isolates investigated in biofilm four isolates demonstrated a negative affect when grown in co-culture with *N. lactamica*. *N. meningitidis* R145 was the first isolate investigated which exhibited a significant reduction in viable white colonies when in biofilm with *N. lactamica.* At Day-3 the reduction in *N. meningitidis* CFUs corresponds to a statistically significant increase in *N. lactamica* CFUs (Figure 31).

N. meningitidis R210 demonstrated a negative effect when in co-culture with *N. lactamica* at Day-2 and Day-3, although this affect was more significant at Day-2 than Day-3. There was no effect observed on *N. lactamica*. In isolation *N. meningitidis* R210 CFU counts remain high at around 10^8 cells/mL (Figure 34).

N. meningitidis R221 saw a significant decrease in CFU counts at Day-3 when in co-culture with *N. lactamica*. At Day-1 and Day-2 is was *N. lactamica* which significantly decreased in CFUs in co-culture however by Day-3 *N. lactamica* CFUs in co-culture are similar to CFUs in isolation. This could suggest competition between the two, although the experiment would need to be continued for a longer time period to investigate if the negative trend between the two continued (Figure 35).

Isolate *N. meningitidis* R279 exhibited the most dramatic decrease in CFUs of all the isolates examined when in co-culture with *N. lactamica*. By Day-3 zero white colonies could be identified over five experiments, across several dilution series. Only one experiment at Day-3 yielded any white colonies (Figure 37). We theorised that, due to its weak biofilm structure R279 could easily be displaced by faster growing *N.*

lactamica, suggesting the two are incompatible with one another. There was a significant increase in *N. lactamica* CFUs at Day-1 when in co-culture with *N. meningitidis* R279, *suggesting N. lactamica* benefits from *N. meningitidis* R279 in some way (Figure 36).

All the *N. meningitidis* isolates demonstrated survival in biofilm with *N. lactamica* for at least 24 hours in biofilm. This would suggest that *N. lactamica* is having a negative effect on the *N. meningitidis* isolates after this time period, although the exact mechanism remains unknown. Further investigation into what causes this negative effect on *N. meningitidis* is required. One possible reason for this negative effect post 24 hours could be due to the biofilm cycle of *N. lactamica* and *N. meningitidis*. Garrett *et al.* (2008) demonstrated that at a high cell density a range of signalling molecules are released. These signalling molecules can include enzymes which are able to break down polysaccharide. It is possible that the slower growing *N. meningitidis* isolates may be unintentionally affected by *N. lactamica* as the *N. lactamica* biofilm enters the dispersal phase in the biofilm cycle or from initial cell death, typically seen at Day-2. *P. aeruginosa* has been shown to release alginate lyase which is known to breakdown biofilm matrix (Garrett *et al*. 2008)

These results that *N. lactamica* does appear to have some effect on *N. meningitidis* are extremely encouraging and exciting. Although all would need to be investigated over a longer time period to see if *N. lactamica* was able to completely displace any of the *N. meningitidis* isolates.

5.5.5 *N. meningitidis* isolates Positively affected by *N. lactamica* in Biofilm

Two isolates (MC58 and R022) exhibited a significant increase when in co-culture with *N. lactamica*, and *N. lactamica* demonstrated a positive increase with 3 isolates (R145, R191 and R279).

It was hypothesised that the positive effect on MC58 may be due to the ability of *N. lactamica* to provide a good biofilm matrix in which to survive. MC58 is a poor biofilm-former in isolation; biofilm formation is slow in comparison to other isolates, which is especially noticeable when compared directly to *N. lactamica*. *N. lactamica* expresses a visible biofilm structure after 16-24 hours whereas MC58 requires 48-56 hours for a similar structure to be observed. *N. lactamica* may provide some protective support by creating a matrix structure quickly in which MC58 is able to reside.

It was previously demonstrated that MC58 expresses a capsule (Section 4.4.2) that has been demonstrated to hinder biofilm production (Yi *et al*. 2004). We therefore theorised that MC58 must down-regulate capsule production in order to enable biofilm production and formation. Down regulation of capsule is not instantaneous and may contribute to MC58's inability to quickly produce matrix.

Hey *et al.* (2013) observed that a structure which they termed 'Filopodia'. The production of the 'Filopodia' structure increased over time, along with CFU counts. The group observed that by Day 21 there was an extensive network of filopodium-like protrusions in which individual MC58 bacterial were entwined. This description of 'Filopodia' is reminiscent to a matrix structure previously observed in *N. lactamica* (Chapter 3.3).

The capsule-deficient *N. lactamica* is able form a basic matrix structure quickly, into which MC58 may be able to survive and could then co-exist harmoniously with *N. lactamica*. Neither MC58 nor *N. lactamica* decrease in viable CFU's in the presence of one another, an indication the two can tolerate each other (Figure 29). This tolerance would imply that for MC58 competition is not a factor as to why *N. meningitidis* and *N. lactamica* are rarely isolated together.

To discover if capsule plays a significant role in MC58 biofilm the experiments could be repeated using a capsule-deficient mutant however similar work has already been studied. Lappann *et al* (2006) created several meningococcal mutants, including a

capsule deficient. The group demonstrated that biofilm formation was a trait expressed by those isolates deficient in capsule and further supported those finding by Yi *et al* (2004).

Like MC58, the *N. meningitidis* isolate R022 also increased in CFU numbers when in the presence of *N. lactamica*. The reason for the increase may be the same as MC58, R022 is a slow biofilm-former in comparison to *N. lactamica*, and therefore *N. lactamica* may provide a degree of protection for R022 to establish.

N. lactamica when in biofilm with MC58 did not significantly increase or decrease CFUs, exhibiting a neutral relationship. This was not the case with R022, as *N. lactamica* exhibited a negative relationship for Day-2 and Day-3. Possibly suggesting that *N. lactamica* is incompatible with R022. In isolation R022 decreases in CFUs on Day-2 but increases on Day-3, alluding to the natural biofilm cycle. The decrease in *N. lactamica* CFUs also decreases in co-culture at this time-point; it could be possible that some dispersal machinery used by R022 has a negative effect on *N. lactamica*.

5.5.6 NEUTRAL EFFECTS IN BIOFILM

A possible reason for the neutral relationship seen in the biofilm compared to the negative relationship in planktonic may be due to the methodology of the experiment. Planktonic cultures are used to set up for biofilm, but these cells then become nutritionally stressed, so to maintain a planktonic state 100 %-TSB media was used. In an environment where nutrition is plentiful *N. Lactamica* could have the advantage of rapid replication. The species increases in biomass quickly when compared to the *N. meningitidis* isolates. This exponential growth could negatively impact surrounding species, including *N. meningitidis*. Rapid growth would enable *N. lactamica* a to colonise a small area quickly, however it would be extremely difficult to accurately measure how this growth could be maintained over a long time period without actively removing a proportion of the bacterial culture to enable fresh media to be applied.

The *N. meningitidis* isolates R245 and R191 are both dual layer biofilm-formers, which both exhibited a neutral relationship with *N. lactamica*. This is a novel observation and could indicate a relationship between *N. meningitidis* isolates which are successful in co-habiting with *N. lactamica* and those which struggle. When in the presence of R245, *N. lactamica* exhibited a decrease in CFUs at Day-2 compared to CFUs in isolation. *N. lactamica* CFU counts recover at Day-3 and are higher in combination than in isolation, although it is not statistically significant. At Day-2 the CFUs for R245 naturally decrease in both isolation and in co-culture. This natural decrease may have an effect on *N. lactamica*, temporarily decreasing CFU counts. The experiment requires an extended period of time to analyse if this oscillation of CFUs continues.

We hypothesised that those isolates that which exhibit a dual layer biofilm could have a neutral relationship with *N. lactamica* because of the type of biofilm those isolates produced. It is possible that *N. lactamica* is able to fully colonise the liquid-surface interface successfully with limited opportunity for other species to inhabit, whilst the air-liquid biofilm produced by dual layer biofilm is able to successfully colonise that particular area.

Overall the experiments were designed to investigate if *N. lactamica* was able to displace or compete with any of the *N. meningitidis* isolates. Due to the design of the experiments individual areas of biofilm could not be isolated for investigation. It may be that with some *N. meningitidis* isolates *N. lactamica* is successfully competing with them and even displacing them, but due to the dual nature of the *N. meningitidis* biofilm this is not reflected in the CFU counts.

5.5.7 N. LACTAMICA IN CO-CULTURE WITH N. MENINGITIDIS

When investigating R242 in combination with *N. lactamica* it was difficult to visualise exactly if the two species interacted or combined with one another. *N. lactamica* produces a surface-liquid biofilm which is smooth and confluent in appearance,

whereas R242 expresses a dual biofilm which includes a surface-liquid biofilm. If there was competition for space between the two it would have only been with a single layer of R242, and it seems unlikely that the *N. Lactamica* biofilm would interact with the air-liquid layer of R242. A further experiment would need to be conducted to separate the two layers of R242. Fewer *N. meningitidis* white colonies identified in a bottom surface-liquid interface would indicate that *N. lactamica* has inhibited *N. meningitidis* growth by competition. However dual layer biofilms are difficult to separate successfully and it is likely that disturbing the top air-liquid layer would disperse some of the biofilm. This dispersal could give an elevated white colony CFU count. The two layer biofilm may also explain why R242 was isolated on several different visits over 26 weeks during the Lac2 study. The ability of R242 to exist in a dual layer biofilm could enhance or maintain its own survival. R242 biofilms were also incredibly physically stable and possessed the ability to 'flex' on the air-liquid media surface preventing damage when spent media was removed. This dual layer may offer some explain why as the R242 biofilm colonised the area there was a significant decrease in *N. lactamica* CFU's.

The decrease in *N. lactamica* CFUs at Day-1 and Day-2 when in co-culture with R221 was somewhat unexpected as the planktonic experiment suggested the reverse was occurring. At Day-3 the significant decrease in the number of viable R221 CFUs in co-culture suggests that the two species have a competitive relationship. At each time point during each significant decrease in CFUs there is no significant increase for the other species. We hypothesised that this was due to the competitive nature of the two. Further experimentation would need to be completed to investigate how quickly R221 can establish a biofilm structure. The majority of isolates observed in this thesis form a recognisable biofilm structure within 24-48 hours with some isolates, *N. meningitidis* R200 for example, forming a biofilm structure slower than *N. lactamica*. It is possible that R221 is able to form a biofilm quickly and therefore actively compete for nutrients and space more effectively than those other isolates previously observed. We also theorised that ultimately the significant drop in CFUs for R221 at Day 3 is due

to *N. lactamica's* ability to form a strong and robust biofilm which is not suited for R221 growth.

Three isolates had a positive effect on the CFUs of *N. lactamica* in biofilm, with the *N. meningitidis* isolates exhibiting either no effect or a negative decrease in CFUs.

When in biofilm *N. lactamica* demonstrated a positive increase in CFUs at Day-3, this in turn was coupled with the decrease in CFUs of isolate R145. Although carriage of R145 during the Lac2 study appears to have been relatively short it is not known how long the isolate had been carried prior to the start of the study, although it is possible that this isolate is only about to maintain carriage for a short period of time. The previous screening experiments (Table 5 Section 4.3.2.) demonstrated that biofilm formation and viability is possible for at least 4 days.

If the 'challenge' experiment (Section 5.3.3.3.) were continued over a longer time period, and the conflicting relationship between the two continued, it could be expected R145 would be unable to survive. The increase in *N. lactamica* may be an effect of the R145 cells dying and not competing for resources. The significant increase in *N. lactamica* CFUs coupled with the sharp decrease in R145 CFUs could suggest that there is some environmental change. This could be caused by a genetic change in either bacterium, such as a change in capsule production. The exact causative agent for the decrease in R145 CFUs would require further investigation, but the result is intriguing nonetheless.

5.5.8 FURTHER WORK

The reason why some of the *N. meningitidis* isolates exhibited a negative response to co-culture with *N. lactamica* and others a positive response is currently unknown. It may be due to the phenotype of biofilm each isolate produces. Both MC58 and R022 were poor biofilm formers which took a longer time to establish when compared to *N.*

lactamica, which may offer some explanation as to why they appeared compatible. It is possible the slower growing *N. meningitidis* is not directly competing with faster *N. lactamica*. Although it could be hypothesised that *N. lactamica* should displace these isolates quickly, being the faster to establish. The experiments would need to be continued for a longer time period to investigate if any of the negative effects over the 3-day time period continued.

Another area to investigate is which method each isolate uses to forms a biofilm. Meningococcal strains have been described as using either an eDNA-dependent or eDNA-independent biofilm formation (Arenas *et al.* 2013). Perez-Ortega *et al* (2017) demonstrated that *N. lactamica* was sensitive to DNase I when forming biofilm indicating that the species uses an eDNA-dependant strategy. Some of the *N. meningitidis* isolates may use an eDNA-independant strategy but further investigation into the isolates is required to see if this is the case. Using eDNA has been shown to be important in the initial attachment and stabilisation of the biofilm (Perez-Ortega *et al.* 2017). It is theorised that those *N. meningitidis* isolates which are able to successfully colonise are eDNA-dependent, those isolates which have a higher transmission rate but colonise for a short time are more likely to be eDNA-independent (Lappann *et al.* 2010a). Identifying which isolates from the Lac2 study are eDNA-dependent and which are eDNA-independent may also explain why some *N.meningitidis* isolates were not identified early in the study.

Experiments using planktonic cultures demonstrated that *N. lactamica* was able to directly compete with *N. meningitidis* isolates when compared to biofilm. Investigation into the differences between both *N. lactamica* and *N. meningitidis* in planktonic culture and in biofilm would be interesting.

Further experiments to investigate the effect of *N. lactamica* on an established *N. meningitidis* biofilm would be a logical progression. Experiments using different titrations of *N. lactamica* could determine a minimal concentration required for competition and possible displacement.

CHAPTER 5 MULTI-SPECIES BIOFILMS

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N. LACTAMICA BIOFILM

From this thesis there is good evidence to suggest that under nutritional stress *N. lactamica* can form a biofilm *in vitro*. Individually no single experiment can conclusively demonstrate that biofilm formation is possible however in combination, using methodologies previously described in the literature and employed in this thesis, there is strong evidence. The demonstration that *N. lactamica* can form a biofilm perhaps should not be surprising, as many other respiratory and oral flora species have been shown to form biofilm both *in vitro* and *in vivo* (Alhede *et al*. 2012; Archer *et al.* 2011; Domenech *et al.* 2012; Hall-Stoodley *et al.* 2008; Kaplan and Fine, 2002; Luke *et al.* 2007; Moreau-Marquid *et al.* 2000).

The CV experiments demonstrated that biomass is established prior to the 24 hours time point, and under particular conditions is maintained for a prolonged period of time and that this biomass increases and decreases in an oscillation pattern typical of other biofilm-forming species. The experiments also showed that *N. lactamica* was able to adhere and maintain adherence to two different substrata. Adherence has been shown to be a critical for survival factor for respiratory commensals (Trivedi *et al*. 2011).

The CFU counts demonstrate that the biomass seen from the CV experiments are alive and viable, cells are able to survive and thrive in the environment when under nutritional stress, which can be seen in other biofilm forming species (Poole, 2012). The cell numbers show that the biomass is not simply comprised of dead or dying cells, but cells are able to replicate and survive. This is further evidenced by the confocal images using the live/dead stain. The images demonstrated there is a layer of live/dead cells with predominately live cells forming clumps of peaks on top. Similar aggregates are seen in other *N. lactamica* biofilm studies (Perez-Ortega *et al.* 2017).

The SEM images demonstrate that under nutritional stress (50 %-TSB media and 25 %-TSB media) the cells produce a matrix prior to the 24 hour (Day-1) time point.

CHAPTER 6 FINAL CONCLUSIONS

Previously demonstrated as an essential component of a biofilm (Steichen *et al*. 2011), the matrix provides structural support for the cells including protection from the environment (Hall-Stoodley *et al*. 2004). Although the SEM images are not able to analyse what the matrix is comprised of, it does show that cells are able construct a matrix-structure. The matrix is extremely strong, surviving the SEM processing stages. Although inevitably some of the structure was lost, enough remained to view. The cracks in the matrix structure could be from the dehydration steps during SEM processing, however it is theorized that these are water and nutrient channels that have been observed previously (Yi *et al*. 2004).

The antibiotic experiments demonstrate that when compared to a planktonic state, *N. lactamica* biofilms have a higher resistance to antibiotic concentrations. Antibiotics have no effect on the matrix structure of the biofilm and no dispersal was observed. This higher resistance to antibiotics is another common feature observed across many different biofilm-forming species (Greiner *et al*. 2005; Steichen *et al*. 2008).

The combination of all these experiments demonstrates there is extremely strong evidence that *N. lactamica* is a biofilm-forming species. Although there are limitations for any experiment the volume of evidence accrued indicates that *N. lactamica* does produce a biofilm when under nutritional stress.

The ability to form a biofilm could enhance *N. lactamica* carriage (Yi *et al.* 2004). Further experiments to demonstrate that this phenomenon occurs *in vivo* are required. *Ex vivo* investigations using excised adenoid and tonsillar tissue could be used. There are now many commercially available biofilm stains which can be used to stain cells within biofilm, the biofilm matrix or a combination of both in preparation for microscopy. A study identifying *N. lactamica* positive participants prior to adenotonsillectomy surgery could assist with the location and visualization of biofilm *in situ*.

Alternatively using florescence *in situ* hybridization (FISH) has been previously used in identifying biofilm infections caused by *P. aeruginosa* in CF patients (Bjarnsholt *et al.*

2011). Similarly using green fluorescent protein (GFP) *N. lactamica* could be visualised using time-lapse microscopy. Specific genes could be targeted to visualize how the bacterium produces biofilm. This type of visualization has been successfully used in *Steptococcus mutants* biofilms (Yoshida and Kuamitsu, 2002).

Another avenue of enquiry would be to use immortal epithelial cell lines. Experiments with *N. lactamica* and bronchial epithelial cell lines have been investigated before (Grifantini *et al*. 2006; Lui *et al.* 2010; Wong *et al.* 2011). However there is extremely limited literature on *N. lactamica* biofilms grown on cell lines. This would be interesting to explore as there is plenty of research currently investigating *N. meningitidis* grown on cell lines and established methodologies could be replicated with *N. lactamica*.

N. MENINGITIDIS BIOFILM

There is plenty of evidence that *N. meningitidis* is able to form a biofilm using a variety of methodologies in the literature (Arenas *et al*. 2015; Lappann *et al*. 2010; Neil and Apicella. 2009a; van Alen *et al*. 2010; Yi *et al*. 2004). It is therefore unsurprising that several isolates from a prior carriage study did form a biofilm. The *N. lactamica* model previously developed worked well and ensured that the results could be compared from one species to the other. There are limited descriptions on the phenotype of biofilm formed in the literature; this thesis explored the possibility that a particular phenotype was expressed by both capsulated and non-capsulated *N. meningitidis*.

No significance was discovered between biofilm phenotype, length of carriage and capsule expression. The experiments suggest that any phenotype of biofilm formation could be beneficial for long-term carriage, as demonstrated by the antibiotic test (Section 4.5). There is also some basic evidence that some carriage isolates may be able to regulate capsule production to enable biofilm formation. Further experiments investigating capsule deficient mutants of these isolates would be interesting to research. Techniques such as enzyme-linked immunosorbent assay (ELISA) or western blot assay could also be employed to determine if those isolates are regulating

capsule, or potentially identifying any other proteins which may enhance biofilm formation. The ability for several isolates to form biofilm whilst being identified as potentially capsule positive suggests that some of the isolates investigated could regulate their capsule production, the creation of capsule-deficient mutants could further demonstrate capsule regulation *in vitro*. This has been previously noted in MC58, when grown in a sorbarod system, where capsule production was down regulated and outer membrane proteins were upregulated (O'Dwyer *et al*. 2009). Alternatively it may be possible for isolates which express capsule to be able to form biofilm without the need to down-regulate capsule production. These isolates may use capsule components to aid matrix production although this is highly unlikely, previous assays with encapsulated meningococci have demonstrated that only those isolates deficient in capsule were able to form biofilm *in vitro* (Neil and Apicella, 2009).

As with the antibiotic *N. lactamica* biofilm experiment, it was also demonstrated that biofilm formation among the *N. meningitidis* isolates increases antibiotic resistance when compared to planktonic cultures. Again this suggests that regardless of the biofilm phenotype, the ability to form any biofilm is an advantage against non-biofilm-forming planktonic bacteria. Disrupting biofilm formation could assist with antibiotic treatments (Potera, 2010). Although *N. lactamica* is not commonly invasive or causes disease preventing *N. meningitidis* colonization by biofilm disruption may reduce carriage rates. This has been successfully documented in the literature with *in vitro P. aeruginosa* biofilm (Klare *et al*. 2016). Although *N. meningitidis* has been shown to be sensitive to 16 antibiotics (Jorgensen *et al.* 2005), recent research into *N. gonorrhoeae* biofilm formation has shown that biofilm does contribute to antibiotic resistance (Wang *et al.* 2017). This is in addition to problems surrounding current antibiotic resistance and bacterial species which once could be treated are becoming problematic once more (Ventola. 2015).

The confocal images comparing *N. lactamica* to the *N. meningitidis* isolate R083 demonstrated that although both do not possess the machinery to produce capsule, the two *Neisseria* produce two distinctly different biofilms. This may be due to the

amount of eDNA released or to pilus expression. Pilin as described in Chapter 1 (Section 1.3.3) has been shown to play a critical role in cell adherence (Trivedi *et al*. 2011). Non-pilated meningococci have been shown to attach to epithelial cells but only in low numbers (Stephens and McGee, 1981). It has been shown that *N. meningitidis* can expresses class I and II pili whilst *N. lactamica* typically expresses just class II (Wormann *et al.* 2014). High expression of Pilin could cause the clumping phenotype seen in some of the isolates. Although no firm conclusions can be made from two images it does show the variability of different biofilm structures. The variability of structure is not wholly unsurprising as *P.aeruginosa* is known to change the structure of its biofilm in patients with CF (Klare *et al*. 2016)

The difference in biofilm size between *N. meningitidis* R083 and *N. lactamica* is interesting and has been documented in the literature. Perez-Ortega *et al.* (2017) noted that different *N. meningitidis* isolates produced various different cluster sizes when viewed using confocal microscopy. The group also demonstrated that the *N. meningitidis* isolates generally formed smaller clusters when compared to *N. lactamica*.

MULTI-SPECIES BIOFILM

The ability to develop a working model in which both *N. lactamica* and *N. meningitidis* were able to form biofilm successfully enabled a series of experiments investigating whether the two species were compatible. Rarely are *N. meningitidis* and *N. lactamica* identified together in a single host, as it is hypothesized that *N. lactamica* confers some kind of protection against *N. meningitidis* colonisation (Deasy *et al*. 2015). The proposition that *N. lactamica* actively out-competes *N. meningitidis* was tested amongst the Lac2 isolates.

Initially four different biofilm-forming isolates were picked to explore the effect of biofilm formation when in the presence of *N. lactamica*. All four *N. meningitidis* isolates exhibited a different relationship with *N. lactamica*. This varied relationship

demonstrated that whilst *N. lactamica* may out-compete some *N. meningitidis* isolates, it may not be effective across all *N. meningitidis* carriage isolates.

In planktonic culture 13 isolates exhibited some negative effect (decrease in CFU counts) when grown in co-culture with *N. lactamica*, from this experiment a further four isolates were investigated. As all had exhibited a negative relationship with *N. lactamica* in a planktonic state it was hypothesized the same relationship would present in biofilm, however this was not always the case. In co-culture biofilm there was a mixture of both positive increases in CFU counts and negative decreases to both *N. lactamica* and *N. meningitidis*. Some *N. meningitidis* isolates benefitted from *N. lactamica* biofilm formation; however *N. lactamica* was shown to have some effect on three of the four *N. meningitidis* isolates. The biofilm experiments were conducted over a short time period (3 days) but demonstrated that the two species do not possess the machinery to directly kill each other over this time period. The different relationship between *N. lactamica* and the *N. meningitidis* isolates suggest that whilst competition for resources may displace some carriage isolates, the presence of *N. lactamica* alone it is not an effective means of protection. Perez-Ortega *et al* (2017) recently investigated whether *N. meningitidis* strains interact with each other in biofilm. The group studied three different strains which contained either GFP or RFP and visualized them using confocal microscopy. The group demonstrated that GFP isolates interacted with the same isolate with RFP insert, indicating that the fluorescent tag did not influence biofilm formation or interaction. The group noted that the *N. meningitidis* isolates grew in clusters but interacted with one another. Interestingly the group also demonstrated that the majority of *N. meningitidis* clusters did not mix with the *N. lactamica* clusters, with the exception of isolate α14 - a carrier, non-capsulated isolate.

Further experiments, similar to those used by Perez-Ortega *et al.* (2017), could be used to view the interaction by *N. lactamica* and the *N. meningitidis* isolates isolated from the Lac2 study in biofilm. This would visually demonstrate if the two do physically interact or if there are any differences between the phenotype of biofilm expressed.

The gradual displacement of *N. meningitidis* by *N. lactamica* could be shown to be more successful over weeks instead of days, however to investigate this theory the biofilm experiments would need to be extended by a considerable amount of time.

CLOSING COMMENTS

Overall there is good evidence that *N. lactamica* and some of the *N. meningitidis* isolates are able to produce biofilm *in vitro* when under nutritional stress. Survival of both *N. lactamica* and *N. meningitidis* is increased in biofilm, as demonstrated by an antibiotic pressure. This thesis has demonstrated that the ability to form biofilm, regardless of the biofilm phenotype, structure or morphology, does offer an advantage when compared to a planktonic state.

There is evidence in the literature that *N. lactamica* may offer some protection to the host against *N. meningitidis* colonization. This thesis investigated whether this protection was due to direct competition between the two species. The 3-Day biofilm experiments demonstrated that whilst some *N. meningitidis* isolates could be displaced *in vitro* using *N. lactamica* further investigation over a longer time point is needed. Continuing work would include investigating if *N. lactamica* and those isolates investigated in Chapter 5 could form a biofilm on immortal cell lines. If a model could be reliably replicated, the 'challenge' experiments could be repeated. It would also be interesting to create a serious of mutants, of both *N. lactamica* and *N. meningitidis*, to investigate further the mechanisms of biofilm formation. Drawing on various sources from the literature, various different mutants can be screened for biofilm formation. These mutants could also be examined on cell lines for attachment, aggregation and potential invasion of the cells.

In particular the research using *N. lactamica* and isolate R279 would further be investigated. Why of all the isolates used in this thesis did *N. lactamica* have such a negative effect on R279? *N. lactamica* could be secreting toxins which would inhibit growth (Perez-Ortega *et al.* 2017) but why would this only affect one isolate so significantly?

CHAPTER 6 FINAL CONCLUSIONS

Overall there are more investigations required but these preliminary results are interesting nonetheless.

APPENDIX

APPENDIX 1.

Tryptic Soy Broth media

Preparation of a 500mL bottle

500mL distilled water.

15g TSB powder (Sigma-Aldrich T8907-500G)

1g Yeast extract (Fisher Scientific BP1422-100)

Mix until fully dissolved and autoclave for 20 minutes at 126^{o}C

BHI media

Preparation of a 500ml bottle

500ml distilled water

18.5g BHI powder (Oxoid CM1135)

Mix until fully dissolved and autoclave for 20 minutes at 126^{o}C.

APPENDIX

REFERENCES

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