# Understanding mechanisms of pathogenesis and resistance in *Neisseria gonorrhoeae*




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**Understanding mechanisms of pathogenesis and resistance in *Neisseria gonorrhoeae***

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## Abstract

*Neisseria gonorrhoeae* is a leading cause of sexually transmitted disease worldwide. Recently, *N. gonorrhoeae* has become a significant public health concern due to increasing antimicrobial resistance (AMR). Therefore, novel ways to tackle *N. gonorrhoeae* infection are required. My work aimed to address this by further understanding gonococcal interactions with complement and by providing insights into plasmid-mediated AMR.

Previous research demonstrated that *N. gonorrhoeae* binds Complement Factor H (CFH), a negative regulator of the human complement system, to avoid complement killing. Here, I showed that *N. gonorrhoeae* also binds to Complement Factor H Related protein 5 (CFHR5) which does not negatively regulate the complement system. I demonstrated that CFHR5 binds to PorB, a gonococcal surface porin, and that CFHR5 and CFH bind to several of the same external loops of PorB.

The spread of plasmid-mediated AMR in *N. gonorrhoeae* is due to the conjugative plasmid, pConj. Therefore, I investigated the spread and maintenance of pConj. I showed that pConj is largely restricted to *N. gonorrhoeae*, is extremely stable once acquired, and is highly conserved. I investigated the basis of why pConj is mostly found in particular gonococcal lineages, and showed a correlation between prevalence of the plasmid in a lineage, and the ability of strains from that lineage to acquire pConj. A subsequent unbiased genetic screen failed to identify chromosomal genes involved in pConj acquisition.

Finally, I defined the mechanisms of pConj maintenance and showed that both partitioning and toxin-antitoxin (TA) systems are involved in plasmid maintenance; TA

systems on the gonococcal cryptic plasmid could contribute to pConj maintenance through a split TA system. Lastly, I showed that pConj can be cured by interfering with plasmid partitioning.

Overall, my findings provide insights into novel ways of that could be exploited for tackling gonococcal disease through immunotherapy or plasmid destabilisation.

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## Abbreviations

| | |
|---|---|
| **aHUS** | Atypical haemolytic uraemic syndromee |
| **AMR** | Antimicrobial resistance |
| **AP** | Alternative pathway |
| **AUC** | Area under curve |
| **bp** | Base pair |
| **C4bp** | C4b binding protein |
| **CCP** | Complement control protein |
| **CEACAM** | CEA-related cell adhesion molecule |
| **CFH** | Complement factor H |
| **CFHR** | Complement factor H related protein |
| **CFU** | Colony forming unit |
| **CMP-NANA** | Cytidine 5’ mono-phospho-N-acetylneuraminic acid |
| **CP** | Classical pathway |
| **CR1** | Complement receptor 1 |
| **CRISPR** | Clustered Regularly Interspaced Short Palindromic Repeats |
| **DNA** | Deoxyribonucleic acid |
| **DUS** | DNA uptake sequence |
| **EB** | Elution buffer |
| **ESBL** | extended spectrum beta lactamase |
| **FB** | Fastidious broth |
| **fHbp** | factor H binding protein |
| **GCB** | Gonococcal agar |
| **GCBL** | Liquid gonococcal media (GCB without agar) |
| **GFP** | Green fluorescent protein |
| **GL** | Genetic load region |
| **IncP** | Incompatibility P |
| **kb** | Kilobase |

| | |
|---|---|
| **KDO** | Keto-deoxyoctanoate residue |
| **LB** | Luria-Bertani |
| **LNT** | Lacto-N-neotetraose (LNT) |
| **LOD** | Limit of detection |
| **logCPM** | $Log_2$ of number of reads per million |
| **logFC** | Log fold change |
| **LOS** | Lipooligosaccharide |
| **LP** | Lectin pathway |
| **Lst** | LOS-specific α-2,3-sialyltransferase |
| **MAC** | Membrane attack complex |
| **MBL** | Mannose-binding lectins |
| **MLEE** | Multilocus enzyme electrophoresis |
| **MLST** | Multilocus sequence typing |
| **NET** | Neutrophil extracellular traps |
| **NG-STAR** | *N. gonorrhoeae* sequence typing for antimicrobial resistance |
| **NHS** | Normal human serum |
| **NOD** | Nucleotide-binding Oligomerisation Domain-containing proteins |
| **$OD_{600}$** | Optical density measured at absorbance of 600nm |
| **OMV** | Outer membrane vesicle |
| **P:C** | Plasmid:chromosome |
| **pbla** | Beta-lactamase plasmid |
| **PBS** | Phosphate-buffered saline |
| **pConj** | Conjugative plasmid in *N. gonorrhoeae* |
| **PCR** | Polymerase chain reaction |
| **pCryp** | Cryptic plasmid |
| **PSK** | Post-segregational killing |
| **RMS** | Restriction modification systems |

| | |
|---|---|
| **ROS** | Reactuve oxygen species |
| **SPR** | Surface Plasma Resonance |
| **STI** | Sexually transmitted infection |
| **T4P** | Type IV pili |
| **TA** | Toxin-antitoxin |
| **TLR** | Toll-like receptor |
| **Tn-Seq** | Transposon insertion sequencing |
| **TP** | Terminal pathway |
| **UIS** | Unique insertion sites |
| **UNAG** | UDP-N-acetylglucosamine |
| **v/v** | Volume per volume |
| **w/v** | Weight per volume |
| **WGS** | Whole genome sequences |

# 1 Introduction

## 1.1 *Neisseria gonorrhoeae*

### 1.1.1 *Neisseria* spp.

*Neisseria* spp. is a genus of Gram-negative, non-spore-forming bacteria. Over 20 *Neisseria* species have been identified to date (Liu *et al.*, 2015). In humans, most *Neisseria* spp., including *Neisseria elongata* (Bovre & Holten, 1970), *Neisseria cinerea* (Knapp & Hook, 1988) and *Neisseria lactamica*, colonise the upper respiratory tract. Only two species are pathogenic in non-immunocompromised hosts, *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *N. meningitidis* causes meningitis and can lead to complications such as hearing loss and brain damage (Stephens *et al.*, 2007). On the other hand*, N. gonorrhoeae* causes the sexually transmitted infection (STI) gonorrhoea, with serious sequelae including disseminated gonococcal infection (DGI), pelvic inflammatory disease and infertility (Lewis, 2020).

### 1.1.2 Microbiology

*N. gonorrhoeae* has an outer membrane containing lipooligosaccharide (LOS), which consists of short chains of saccharides attached to Lipid A (Piekarowicz & Stein, 2002). In the presence of sialic acid, gonococcal LOS can be sialylated (Parsons *et al.*, 1988; Mandrell *et al.*, 1990).

*N. gonorrhoeae* is naturally transformable and has a genome of approximately 2 Mb. *N. gonorrhoeae* expresses type IV pili (T4P) that facilitate DNA uptake (Wolfgang *et al.*, 1998, 1999). DNA containing the DNA Uptake Sequence (DUS) 5´-GCCGTCTGAA-3´ is more likely to bind to the minor pilin ComP (Cehovin *et al.*, 2013; Hughes-Games *et al.*, 2022) and be taken up compared to DNA without the sequence (Goodman & Scocca, 1988). Following uptake, homologous recombination with chromosomal DNA (Stohl & Seifert, 2001; Skaar *et al.*, 2002) results in transformation.

### 1.1.3 *N. gonorrhoeae* population structure

Due to high rates of recombination, the population structure of *N. gonorrhoeae* cannot be easily determined by examining a few genes. Early attempts to establish the *N. gonorrhoeae* population structure relied on multilocus typing of several enzymes. Examination of 227 isolates using multilocus enzyme electrophoresis (MLEE) showed that alleles of neighbouring loci were not always co-inherited, indicating frequent recombination (O'Rourke & Stevens, 1993). Indeed, analysis of variation in genes additional to those used in MLEE revealed isolates were increasingly sub-divided when more genes were considered (O'Rourke & Spratt, 1994).

With the advent of nucleotide sequencing, multilocus sequence typing (MLST) was attempted using seven genes: *abcZ, adk, aroE, fumC, gdh, pdhC* and *pgm* (Bennett *et al.*, 2007). MLST aids in determining the relationship between *N. gonorrhoeae* and other *Neisseria* species. However, recombination in housekeeping genes makes it difficult to reliably determine the gonococcal population structure using only seven genes (Harrison *et al.*, 2020).

The *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) scheme was established, using the sequences of *penA, mtrR, porB, ponA, gyrA, parC* and 23S rRNA, which are associated with antimicrobial resistance (AMR) (Demczuk *et al.*, 2017). While this was useful in monitoring dissemination and distribution of AMR strains, NG-STAR does not provide the population structure of *N. gonorrhoeae*. Instead, the core genome MLST, using genes found in 98% of the gonococcal population, has been employed. As a result, 1668 loci were found to comprise the core gonococcal genome, with a difference ≤ 400 loci used to define lineages (Ng_cgc_400 classification) (Harrison *et al.*, 2020) (**Figure 1.1**, with metadata of the dataset used in **Appendix Table 7.1**).

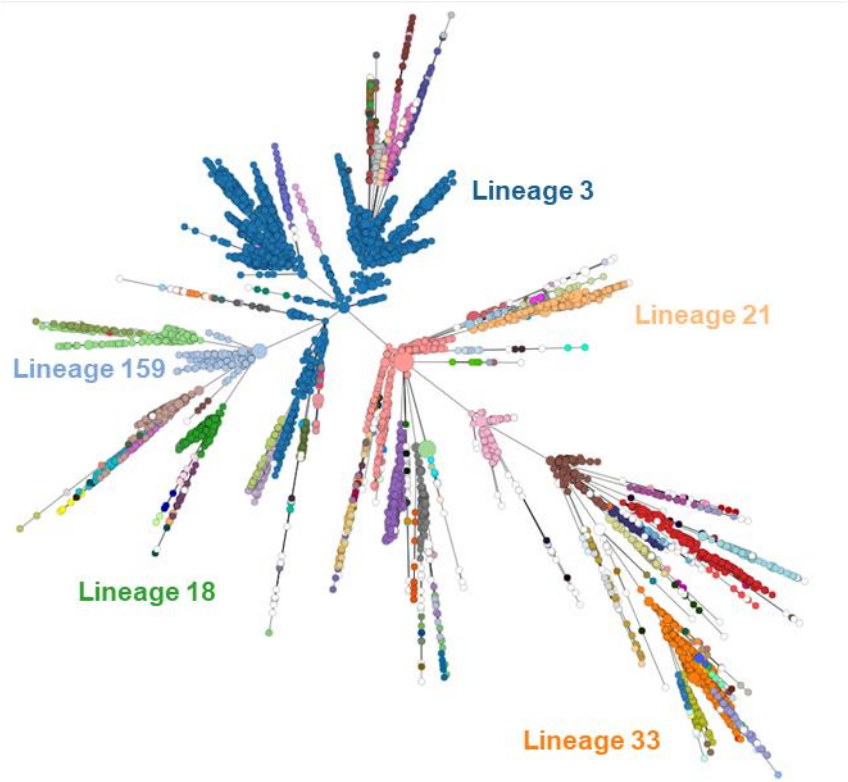



**Figure 1.1 Population structure of *N. gonorrhoeae* with lineages identified using Ng_cgc_400 schematic.** Isolates (n = 9,618) from PubMLST are coloured according to lineage. Isolates are selected based on sequence bin > 1 Mb with contigs ≤ 200 to ensure sequencing quality. Metadata for isolates used in this analysis is in **Appendix Table 7.1**. The most prevalent lineages, 3, 18, 21, 33 and 159, are labelled according to the colours of the lineage as shown.

### 1.1.4 Epidemiology

*N. gonorrhoeae* is a human-specific pathogen first isolated in 1878 by Albert Neisser (Ligon, 2005). Since the 2000s, *N. gonorrhoeae* infection has risen worldwide (World Health Organisation, 2008). In Europe, the number of gonorrhoea cases increased by 17% since 2016 (Day *et al.*, 2017). In the USA, the rate of gonorrhoea increased by 5.7% between 2018 – 2019 and 2019 – 2020, making it the second most common STI in the USA (Centers for Disease Control and Prevention, 2020). In Africa, *N. gonorrhoeae* prevalence among women was 1.9%, with rates as high as 8.2% in East Africa amongst 15 – 24 year olds (Torrone *et al.*, 2018). In Thailand, gonorrhoea prevalence in 15 – 24 year olds was 68.1 per 100,000 in 2017 (Meesaeng *et al.*, 2021), whilst in China, the overall rate was 9.59 cases per 100,000, with more males infected than females (Yue *et al.*, 2019).

### 1.1.5 *N. gonorrhoeae* transmission and disease manifestation

*N. gonorrhoeae* is most frequently transmitted sexually. In men, *N. gonorrhoeae* often infects the urethra, causing urethritis and discharge. In women, *N. gonorrhoeae* infects the cervix, resulting in cervicitis. However, infection is often asymptomatic (Sweet & Walker, 2011). Left untreated, *N. gonorrhoeae* can ascend the reproductive tract and infect the uterus and fallopian tubes, causing pelvic inflammatory disease. This can result in chronic pain, ectopic pregnancy and infertility (Weström *et al.*, 1992). Apart from the reproductive tract, *N. gonorrhoeae* can also infect the oropharynx in adults. Rarely, *N. gonorrhoeae* enters the bloodstream and causes DGI, which can result in arthritis, endocarditis and even death (Lewis, 2020). Newborns can also acquire *N. gonorrhoeae* in the delivery canal, resulting in ocular infection (Lewis, 2020).

### 1.1.6 *Neisseria* pathogenesis

#### 1.1.6.1 Adhesion and invasion of epithelial cells

Infection with *N. gonorrhoeae* begins with bacterial adhesion to host epithelial cells in the reproductive tract. The mucosal surface varies significantly, consisting of stratified, squamous epithelial cells to single layer columnar cells. Experiments using human cervical tissue explants showed that T4P are crucial for infection and penetration in all regions of the cervix (Yu *et al.*, 2019). Furthermore, *N. gonorrhoeae* can form microcolonies *via* T4P which aid in infecting the cervical epithelium (Higashi *et al.*, 2007). In addition to T4P, *N. gonorrhoeae* surface proteins such as opacity proteins (Opas) bind to host receptors of the CEA-related cell adhesion molecule (CEACAM) family (Merz & So, 1997). Specifically, invasion of the transition zone and endocervical epithelium is dependent on Opa expression (Yu *et al.*, 2019).

#### 1.1.6.2 Nutrient acquisition

The human host produces compounds such as calprotectin and enterobactin that sequester nutrients from invading pathogens. To overcome the lack of essential ions, *N. gonorrhoeae* expresses TonB-dependent receptors in its outer membrane that can extract metals from host metalloproteins. For example, TbpA extracts iron from human transferrin (Cornelissen *et al.*, 1992), with the TbpB lipoprotein increasing efficiency of transferrin binding to TbpA (Anderson *et al.*, 1994). Iron is then internalised by a TonB-dependent mechanism. FetA, on the other hand, is a receptor for enterobactin (Carson *et al.*, 1999) and is highly expressed under iron-limited conditions (Thomas & Sparling, 1996).

*N. gonorrhoeae* acquires zinc *via* its transport system ZnuABC (C.-Y. Chen & Morse, 2001). Two TonB-dependent transporters in the outer membrane, TdfH and TdfJ, are essential for gonococcal growth in zinc-sequestered media (Ray *et al.*, 2022). Specifically, TdfH binds to calprotectin (Kammerman *et al.*, 2020), while TdfJ binds to S100A7, a host protein that sequesters zinc (Maurakis *et al.*, 2019). Overall, *N.*

*gonorrhoeae* carries genes important for iron and zinc acquisition when these essential metals are in low abundance.

### 1.1.6.3 Role of neutrophils

While the gonococcus colonises the epithelium, it releases factors such as LOS, outer membrane vesicles (OMVs) and peptidoglycan. LOS and peptidoglycan can trigger innate immune responses *via* Toll-like receptor (TLR) 2, TLR4 and Nucleotide-binding Oligomerisation Domain-containing proteins, NOD1 and NOD2 (Quillin & Seifert, 2018). The detection of *N. gonorrhoeae* by immune cells such as macrophages results in release of pro-inflammatory cytokines such as IL-6 and IL-8. These cytokines subsequently recruit neutrophils to the site of infection (Stevens & Criss, 2018).

Opas expressed on the gonococcal surface binds to CEACAM receptors on neutrophils, mediating *N. gonorrhoeae* phagocytosis (Alcott *et al.*, 2022). The expression of different Opas by *N. gonorrhoeae* influences phagocytosis by neutrophils (Alcott *et al.*, 2022) (**Figure 1.2**). Unlike many pathogens, *N. gonorrhoeae* can survive inside neutrophils: over 50% of gonococci internalised by human neutrophils remain viable six hours after uptake (Simons *et al.*, 2005), and the bacterium can replicate in neutrophils (**Figure 1.2**). Reactive oxygen species (ROS) produced by neutrophils have little impact against gonococcal invasion, as treatment of neutrophils with NADPH oxidase inhibitors did not affect the survival of gonococcal strains which were more susceptible to ROS (Criss *et al.*, 2009). Neutrophils also produce neutrophil extracellular traps (NETs), which can capture bacterial pathogens including *Pseudomonas aeruginosa* (Schultz *et al.*, 2022); however, *N. gonorrhoeae* produces a thermonuclease that degrades NETs (Juneau *et al.*, 2015). This suggests that despite intense inflammatory response towards *N. gonorrhoeae* at the site of infection, the bacterium is not killed. Finally, *N. gonorrhoeae* is also resistant to antimicrobial peptides such as LL-37 (Hu *et al.*, 2022). Therefore, it is not known whether neutrophil recruitment protects against *N. gonorrhoeae*, or if this contributes towards pathogenesis.

### 1.1.6.4 Manipulation of cellular immunity

Apart from neutrophils, *N. gonorrhoeae* can suppress CD4 T cell responses (Liu *et al.*, 2012) by inducing production of IL-10 (Liu *et al.*, 2014). Specifically, CD4 Th1 and Th2 cells are inhibited by *N. gonorrhoeae* Opa proteins, while CD4 Th17 T cells were activated (Liu *et al.*, 2012); serum levels of IL-17, produced by Th17 T cells, are elevated in patients with gonorrhoea (Gagliardi *et al.*, 2011). *N. gonorrhoeae* is thus able to manipulate T cells to its benefit (Russell *et al.*, 2019). Indeed, the lack of immunological memory is a common theme in gonococcal infection, in part due to suppression of B cells by Opa proteins (Pantelic *et al.*, 2005). Consequently, natural infection with *N. gonorrhoeae* does not lead to protective immunity.

Apart from T cells, *N. gonorrhoeae* also influences macrophage polarisation. Macrophages can exhibit an M1 or M2 profile, the latter involved in reducing inflammation. *N. gonorrhoeae* polarises macrophages to an M2 profile (Ortiz *et al.*, 2015) and replicates in these cells (Ivanov *et al.*, 2021). *N. gonorrhoeae* can also induce secretion of both pro-inflammatory (IL-1, IL-6 and TNF-α) and anti-inflammatory (including IL-10) cytokines in primary macrophages and in cell lines (Château & Seifert, 2016). Therefore, whether macrophages contribute to pathogenicity or immunity requires further research.

Overall, the ability of *N. gonorrhoeae* to invade epithelial cells and subvert host cellular immunity results in its success as a human pathogen.

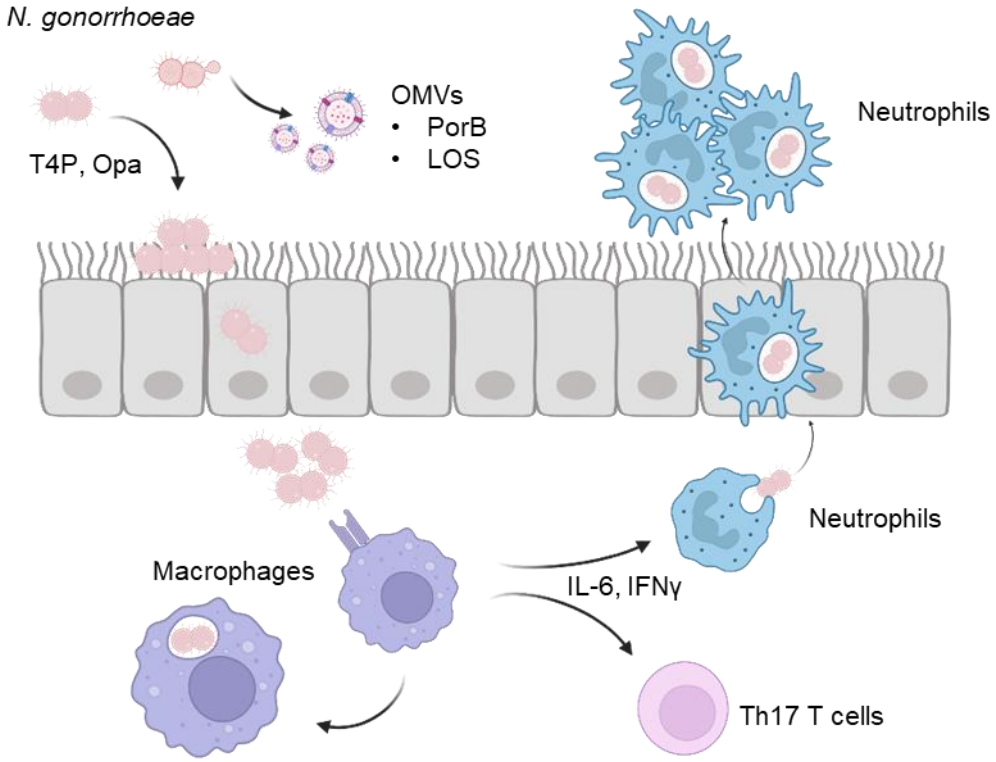



**Figure 1.2 *N. gonorrhoeae* pathogenesis.** *N. gonorrhoeae* reaches the epithelium of the genito-urinary tract. Opa proteins and type IV pili (T4P) aid gonococcal adherence to and invasion into epithelial cells. *N. gonorrhoeae* releases outer membrane vesicles (OMVs) which contain PorB and lipooligosaccharide (LOS), leading to macrophage recruitment. Macrophages then release cytokines such as IL-6 and IFNγ, which recruit neutrophils and Th17 T cells. *N. gonorrhoeae* can survive in both macrophages and neutrophils. As a result, there is persistent pathogenic inflammation at the infection site without resolution. Figure adapted from Quillin and Seifert, 2018 and constructed using BioRender.

### 1.1.7 *N. gonorrhoeae* virulence factors

#### 1.1.7.1 PorB

*N. gonorrhoeae* expresses one of two types of PorB, P.IA and P.IB, which share 65 - 80% amino acid similarity (Judd, 1989). PorB is a trimeric integral outer membrane protein on the gonococcal surface and stabilised by another outer membrane protein, RmpM. Each PorB monomer forms a 16-stranded β-barrel, with eight surface exposed loops on the extracellular aspect of the protein. One of the PorB loops, an α-helix in loop 3, folds within the centre of the β-barrel. Unlike other non-selective channels, PorB exhibits an electronegative charge on its extracellular side and an electropositive charge on its periplasmic side (Zeth *et al.*, 2013).

PorB is a highly expressed and essential protein that is involved in ion exchange (Zeth *et al.*, 2013); consequently, PorB is essential for gonococcal survival (Remmele *et al.*, 2014). Furthermore, co-crystallisation of P.IA and ATP suggests that PorB might play a role in ATP translocation, although the physiological importance of this has not been determined (Zeth *et al.*, 2013). PorB is also permeable to antibiotics as substitutions of amino acids lining the substrate translocation channel, G103 and D104, decrease conductance and the probability of PorB being in an 'open' conformation. This impairs permeability of *N. gonorrhoeae* to antibiotics, resulting in resistance (Olesky *et al.*, 2002).

PorB is important in resistance against the complement system (Chen & Seifert, 2013). Strains expressing P.IA display sensitivity to host complement killing (Schoolnik *et al.*, 1976; Ram *et al.*, 1998), and specific loops on P.IB have been identified to interact with complement factor H (CFH). The interaction of the gonococcus and complement is described in more detail below.

Furthermore, PorB has been shown to influence cellular immunity. For example, PorB has been associated with a delay in oxidative killing of *N. gonorrhoeae* in both macrophages and neutrophils (Lorenzen *et al.*, 2000; Mosleh *et al.*, 1998), and

implicated in inhibiting dendritic cell stimulation of T cell proliferation (Zhu *et al.*, 2018). P.IA also mediates increased invasion of epithelial cells compared to P.IB expressing strains (van Putten, 1993). PorB is thus important at many stages of gonococcal infection.

### 1.1.7.2 Lipooligosaccharide

Gonococcal LOS consists of short oligosaccharide chains attached to Lipid A. RfaC, a heptosyl transferase, catalyses the addition of heptose I (HepI) to the keto-deoxyoctanoate residue (KDO), which is covalently bound to Lipid A; the α, β and γ chains extend from HepI. The structure of LOS is dependent on *lgt* genes (consisting of the *lgt* operon *lgtABCDE*, *lgtF* and *lgtG*) (Gotschlich, 1994). Phase variation in *lgtA, lgtC*, *lgtD* is mediated by polyG nucleotide tracts (Yang & Gotschlich, 1996), while *lgtG* phase variation is mediated by a polyC tract (Banerjee *et al.*, 1998). Phase variation in these four genes alters LOS structure (Shafer *et al.*, 2002; Banerjee *et al.*, 1998).

Apart from phase variation, LOS can also be modified by addition of other groups. LptA, for example, adds a phosphoethanolamine to the phosphate group of Lipid A (Lewis *et al.*, 2009). Lpxl1, on the other hand, is involved in hexa-acylating Lipid A which aids in invading epithelial cells (Post *et al.*, 2002). Additionally, LOS can be sialylated with exogenous sialic acid. The LOS-specific α-2,3-sialyltransferase (Lst), located on the outer membrane (Shell *et al.*, 2002), is responsible for sialylation. Lst sialylates primarily the terminal galactose (Gal) of the lacto-N-neotetraose (LNT) moiety on the α chain of the LOS (Mandrell *et al.*, 1990) through an α-2,3 linkage. However, other sialylation sites have been described, such as sialylation of Gal on both the alternative α and β chains *via* an α-2,6 linkage (Gulati *et al.*, 2019). Furthermore, Lst might transfer KDO instead of sialic acid onto LNT (Jen *et al.*, 2021), although this has not been confirmed by others.

LOS contributes to defence against the complement system; in particular, sialylation of LNT increases resistance against complement (to be discussed later) (Ram *et al.*, 1998; Chen & Seifert, 2013). In addition to LOS sialylation, loss of *lgtD* decreases susceptibility to complement killing compared to wild-type (Balthazar *et al.*, 2011), potentially due to

decreased recognition by host IgM (Griffiss *et al.*, 1991). On the other hand, Δ*lptA* mutants exhibited increased complement susceptibility (Balthazar *et al.*, 2011) through an unknown mechanism.

LOS is also implicated in cellular immune responses. Specifically, LOS sialylation decreases uptake by neutrophils (Rest & Frangipane, 1992), while loss of *lptA* increases sensitivity to neutrophil killing (Handing & Criss, 2015). Furthermore, although changes in the gonococcal LOS structure did not elicit different cytokine profiles, gonococci with sialylated LOS upregulate secretion of the chemokine, MCP-2, which attracts immune cells including monocytes (Patrone & Stein, 2007).

Finally, research suggests that *N. gonorrhoeae* is desialylated in cervicovaginal secretions (Ketterer *et al.*, 2016). Desialylated *N. gonorrhoeae* can then infect men effectively (Schneider *et al.*, 1995). Men then transmit sialylated *N. gonorrhoeae* (Apicella *et al.*, 1990), which can infect women, thus perpetuating the cycle of infection.

#### 1.1.7.3 Type IV pili

T4P are important in many biological functions in *N. gonorrhoeae* including transformation and host cell interactions (Meyer, 1982). The main T4P pilin, PilE, is synthesised as a prepilin that is secreted through the inner membrane before being processed by PilD (Freitag *et al.*, 1995). Upon cleavage, mature PilE forms the main body of the pilin. The PilT ATPase drives pilus retraction (Merz *et al.*, 2000). *N. gonorrhoeae* transformation is dependent on a functional T4P, as Δ*pilT* and Δ*pilE* mutants cannot be transformed (Wolfgang *et al.*, 1998). Interestingly, mutants with truncated PilE can still be transformed, as the N-terminal domain of PilE can be cleaved to release a soluble form of pilin, S-pilin. The exact mechanism of transformation by S-pilin is not yet known (Obergfell & Seifert, 2016). In addition to genes involved in T4P biogenesis, *comP* encodes a minor pilin essential for transformation (Hughes-Games *et al.*, 2022).

T4P undergo antigenic variation which contributes to immune evasion. During antigenic variation, a portion of one of the multiple *pilS* sequences replaces part of the *pilE* gene in a RecA-dependent process. Other genes mediating *pilE* recombination include *recG, recJ, recO, recQ,* and *recR* (Rotman *et al.*, 2016). Recombination is dependent on a G4 motif upstream of *pilE*. The G4 motif forms a guanine quadruplex DNA loop which is crucial for antigenic variation (Cahoon & Seifert, 2009), as mutations disrupting the G4 structure affect pilin antigenic variation (Prister *et al.*, 2020). RecA binds directly to the G4 motif to mediate DNA strand exchange and thus antigenic variation (Kuryavyi *et al.*, 2012). Additionally, *pilE* recombination is also influenced by a G4-associated sRNA, *gar*, as altered transcription impacts antigenic variation (Prister *et al.*, 2019).

#### 1.1.7.4 Opa proteins

*N. gonorrhoeae* can express multiple Opa proteins on its surface. Opa expression is phase variable (Stern *et al.*, 1966) and dependent on promoter sequences. Opas are β-barrel proteins with four surface exposed, hypervariable, loops (de Jonge *et al.*, 2002); the combination of loops confers binding to CEACAMs (Bos *et al.*, 2002). *N. gonorrhoeae* expressing Opa proteins can bind CEACAM-3 on non-immune cells such as neurones (Sarantis & Gray-Owen, 2012). Finally, a subset of Opa proteins can bind to cell surface heparan sulphate proteoglycan (HSPG) (Sadarangani *et al.*, 2011), inducing cytoskeletal changes that result in bacterial uptake (Dehio *et al.*, 1998).

## 1.2 The complement system

The complement system is the first line of defence against many pathogens and can be activated by three pathways: the classical (CP), lectin (LP) and the alternative (AP) pathways (Noris & Remuzzi, 2013) (**Figure 1.3**). All the pathways generate C3 convertases which cleave C3 into its active components, C3a and C3b.

The CP is activated when C1q of C1qrs complex binds to the Fc region of IgG or IgM (Kojouharova *et al.*, 2010). This activates the serine protease C1s which cleaves C4 and C2 to form a C3 convertase, C4bC2b (Mortensen *et al.*, 2017).

The LP is initiated when mannose-binding lectins (MBL) or ficolins bind to carbohydrates on microbial surfaces. This triggers a change in conformation of MASP-1 and MASP-2 bound to MBL/ficolins. Activated MASP-2 cleaves C4 and C2 to form C4bC2b (Héja *et al.*, 2012). C4bC2b from both CP and LP cleaves C3 to generate C3a and C3b.

The AP amplifies complement activation in the presence of C3b (Thurman & Holers, 2006). C3b and Factor B forms another C3 convertase, C3bBb, which hydrolyses factor C3 to generate more C3b, leading to a positive feedback loop. The deposition of C3b on microbes leads to the formation of C5 convertase, C3bBbC3b. C3b can also form a complex with C4bC2b to form C4bC2bC3b, another C5 convertase. Cleavage of C5 by the C5 convertase initiates the terminal pathway (TP), leading to the formation of the membrane attack complex (MAC) on pathogen surfaces (Heesterbeek *et al.*, 2019), resulting in lysis (**Figure 1.3**).

In addition to lysis, complement opsonises pathogens by C3b deposition. C3b then binds to receptors on phagocytes such as complement receptor 1 (CR1) to promote pathogen uptake (Vandendriessche *et al.*, 2021). In addition, C3a and C5a are anaphylatoxins which attract leukocytes (Vandendriessche *et al.*, 2021). Therefore, the complement system integrates the innate immunity system with the adaptive immunity system to clear pathogens from the host.

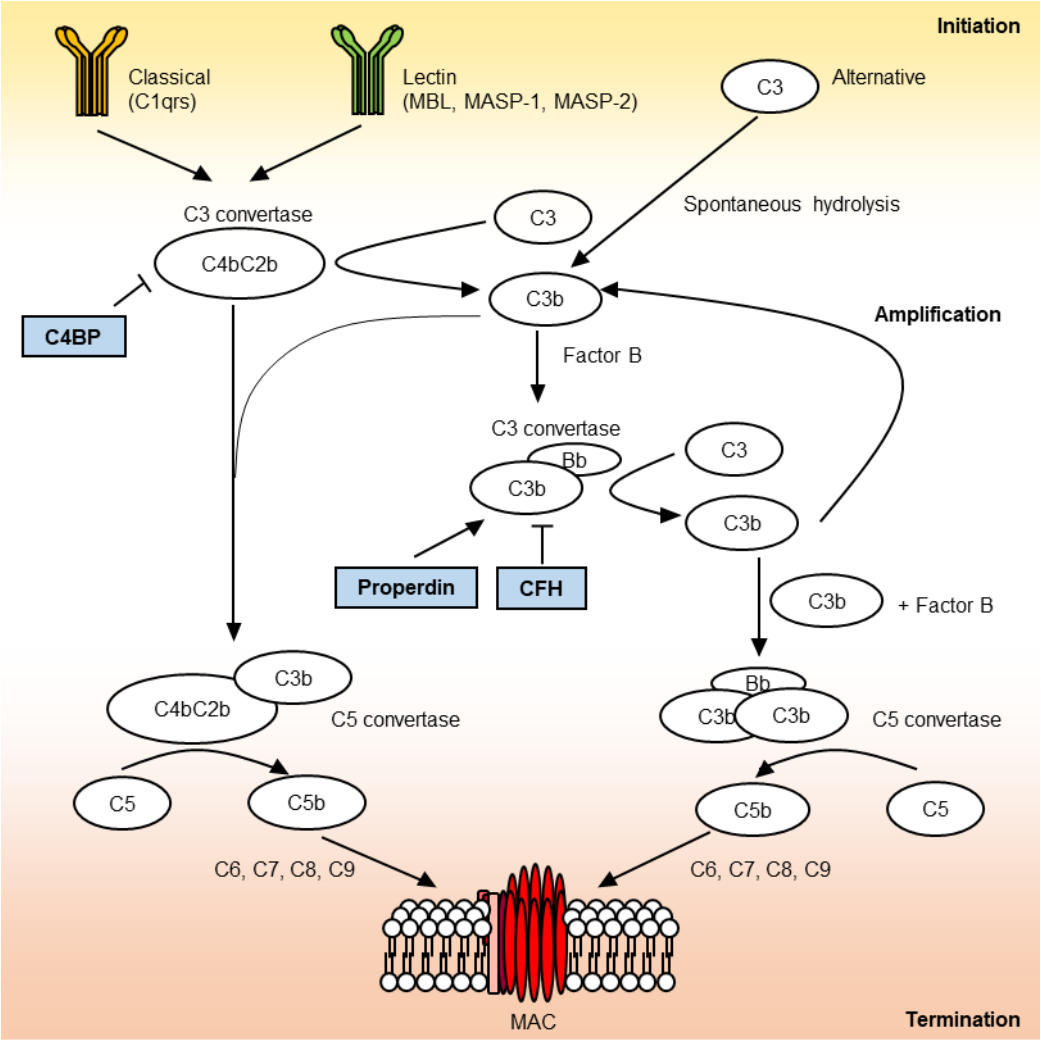



**Figure 1.3 The complement system**. The complement system can be activated by the classical (CP), lectin (LP) or alternative (AP) pathways. All three pathways result in the formation of a C3 convertase, which hydrolyses C3 to C3a (not shown) and C3b. This results in the formation of C3bBb, a C3 convertase, resulting in amplification. The formation of C3b also leads to the formation of C5 convertases, which hydrolyses C5 into C5a (not shown) and C5b. C5b then recruits C6 – 9, generating the membrane attack complex (MAC). The complement pathway can be inhibited by C4 binding protein (C4BP) and complement factor H (CFH).

### 1.2.1 Control of complement system

Inappropriate activation of the complement system leads to various autoimmune diseases such as atypical haemolytic uraemic syndrome (aHUS) and C3 glomerulopathy (Józsi & Zipfel, 2008). However, a lack of complement can increase susceptibility to infection (Ram *et al.*, 2010; Miller *et al.*, 2012). Therefore, the complement system must be carefully regulated. One of the major activators of the complement system is properdin. Properdin stabilises and acts as a platform for assembly of C3 convertases such as C3bBb (Hourcade, 2006) (**Figure 1.3**). Thus, when bound to a surface, properdin can serve as a focal point for complement amplification, thereby activating the complement system (Spitzer *et al.*, 2007).

In contrast to properdin, C4b binding protein (C4BP) is a soluble complement inhibitor which binds C4b (**Figure 1.3**), preventing the formation of C3 convertase C4bC2b (Blom *et al.*, 2004). Furthermore, C4BP is a cofactor for a protease, Factor I, which cleaves C3b (Blom *et al.*, 1999).

CFH is the main inhibitor of the AP. CFH is a 155 kDa protein which consists of 20 complement control protein modules (CCPs) linked by 3 – 8 amino acids (Ripoche *et al.*, 1988). CCPs are cysteine-rich globular domains of approximately 60 amino acids (Ripoche *et al.*, 1986). The first four CCPs of CFH ($CFH_{(1-4)}$) bind C3b and accelerate degradation of the C3 convertase by interacting with Factor I (Gordon *et al.*, 1995). $CFH_{(1-4)}$ also displaces factor B from the C3 convertase, accelerating the decay of this complex (Wu *et al.*, 2010) (**Figure 1.3**). $CFH_{(6-8)}$ and $CFH_{(18-20)}$ bind to host surfaces such as heparin and other glycosaminoglycans (Clark *et al.*, 2013; Schmidt *et al.*, 2008) (**Figure 1.3**).

Many pathogens recruit CFH to evade the complement system. For example, *Streptococcus pyogenes* Scl (Reuter *et al.*, 2010) and Tuf of *P. aeruginosa* (Kunert *et al.*, 2007) bind CFH. *Candida albicans* can also bind CFH *via* $CFH_{(6-7)}$ or $CFH_{(19-20)}$ (Meri *et al.*, 2002). Overall, recruitment of CFH allows pathogens to evade serum killing.

### 1.2.2 Complement interaction with *Neisseria* spp.

*N. meningitidis* and *N. gonorrhoeae* interact with the complement system and bind CFH through different mechanisms. *N. meningitidis* expresses a 27 kDa lipoprotein known as factor H binding protein (fHbp) which binds $CFH_{(6,7)}$ with nanomolar affinity (Schneider *et al.*, 2009). A complement factor H-related protein, CFHR3, binds to fHbp, with increasing CFHR3 concentration competing with CFH binding to fHbp (Caesar *et al.*, 2014) (**Figure 1.4**). NspA and PorB also bind to CFH in the absence of fHbp but to far lesser extent (Giuntini *et al.*, 2015).

In contrast, *N. gonorrhoeae* does not express fHbp on its surface (Jongerius *et al.*, 2013). Instead, *N gonorrhoeae* binds CFH *via* PorB. Loop 5 of P.IA PorB interacts with CFH, while P.IB loops 3 – 7 have been found to be important in interacting with CFH (Ram *et al.*, 1998; Chen & Seifert, 2013) (**Figure 1.4**). This interaction is specific for human CFH (Ngampasutadol *et al.*, 2008).

Apart from PorB, LOS sialylation has also been implicated in binding negative regulators of the complement system. Specifically, sialylation of the LOS LNT moiety enhances binding of CFH to P.IA (Madico *et al.*, 2007) and is essential for CFH and C4BP binding to P.IB (Chen & Seifert, 2013) (**Figure 1.4**).

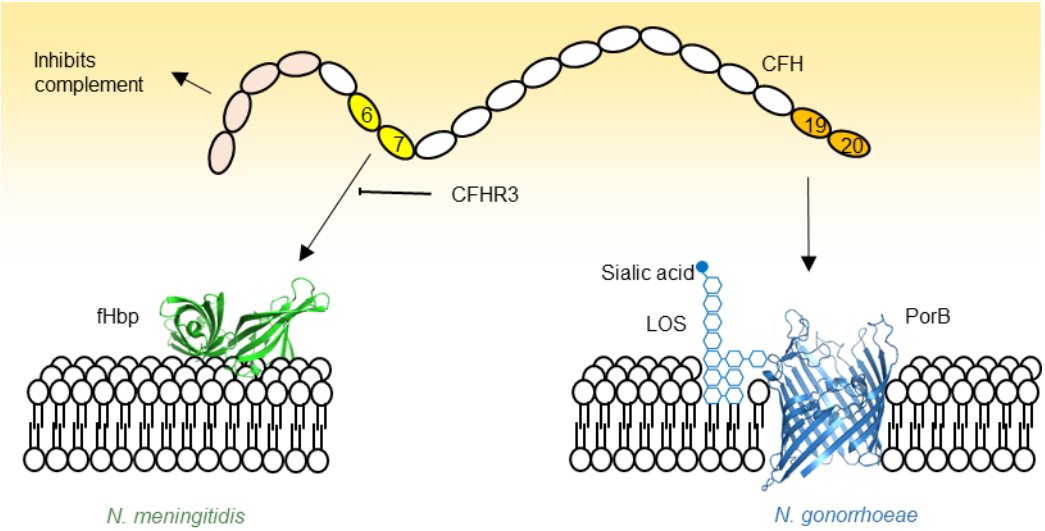



**Figure 1.4 CFH binding to pathogenic *Neisseria*.** Complement factor H (CFH) binds to factor H binding protein (fHbp) on *N. meningitidis via* $CFH_{(6-7)}$. Complement factor H related protein 3 (CFHR3) competes with CFH for binding to fHbp. CFH binds to gonococcal PorB in the presence of sialylated lipooligosaccharide (LOS) *via* $CFH_{(18-20)}$.

### 1.2.3 Complement Factor H Related proteins, CFHRs

CFHRs share high sequence identity with CFH but contain fewer CCPs (**Figure 1.5**). Genes encoding CFHRs (CFHR1 – 5) are located downstream of the *cfh* gene on chromosome 1 (Díaz-Guillén *et al.*, 1999). Mutations affecting the CFHRs leading to duplications or deletions can result in autoimmune disease (Zipfel *et al.*, 2020).

Importantly, none of the CFHRs contain CCPs homologous to $CFH_{(1-4)}$. Therefore, CFHRs cannot negatively regulate complement (**Figure 1.5**). Instead, they activate the complement pathway either by competing with CFH (Caesar *et al.*, 2014), or by recruiting other factors of the complement pathway such as properdin (Chen *et al.*, 2016).

CFHRs 1, 2 and 5 have a dimerisation domain in their N-termini (Goicoechea de Jorge *et al.*, 2013) (**Figure 1.5**). Duplication of the dimerisation domain results in competition between CFHRs and CFH, causing disease. For instance, duplication of the CFHR5 dimerisation domain is associated with familial C3 glomerulopathy (Goicoechea de Jorge *et al.*, 2013). Although CFHRs 3 and 4 do not dimerise, they have been implicated in disease. For example, certain polymorphisms of CFHR3 have been associated with risk to *N. meningitidis* (Davila *et al.*, 2010), while increased CFHR4 levels are associated with age-related macular degeneration (Cipriani *et al.*, 2020).

All CFHRs contain domains related to $CFH_{(19-20)}$, the surface binding domains of CFH. Therefore, CFHRs can bind to both host and pathogen surfaces. For example, CFHR2 and CFHR5 bind the CRASPs from *Borrelia* spp. (Siegel *et al.*, 2010). Furthermore, CFHR1 binds to both *C. albicans* and *S. pneumoniae* (Reuter *et al.*, 2010; Meri *et al.*, 2002). Due to high structural homology, CFHR1 is also thought to bind to *N. gonorrhoeae* (Agarwal *et al.*, 2010), although the implication of this is unknown. It is not known if other CFHRs bind to *N. gonorrhoeae.* This is investigated in Chapter 3 in this thesis.

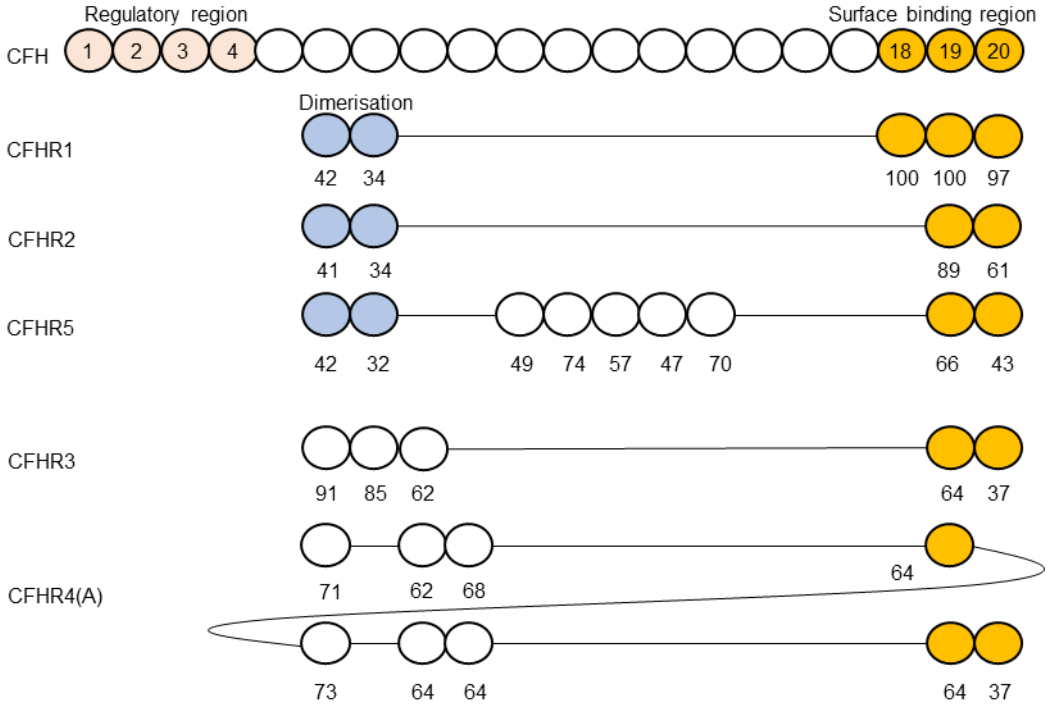



**Figure 1.5 CFHR and CFH comparison.** Complement control proteins (CCPs) of complement factor H related proteins (CFHRs) aligned with related CCPs in complement factor H (CFH). All CFHRs have domains with homology to the surface binding region of CFH (orange). Only CFHRs 1, 2 and 5 have a dimerisation domain (blue). The percentage amino acid identity of CCPs in CFHRs with the corresponding CCPs in CFH is shown.

## 1.3 Antimicrobial resistance in *N. gonorrhoeae*

### 1.3.1 Emergence of *N. gonorrhoeae* resistance

*N. gonorrhoeae* has become increasingly difficult to treat due to a rise in AMR. Penicillin resistance developed following *penA* mutations and the spread of a plasmid, p*bla* (Unemo & Shafer, 2014). Subsequently, tetracycline treatment was discontinued due to acquisition of tetracycline resistance gene encoded by a conjugative plasmid, pConj (Morse *et al.*, 1986). Ciprofloxacin and other fluoroquinolones were used for gonococcal infection for a few years before decreased susceptibility to fluoroquinolones was detected worldwide due to mutations in *gyrAB* and *parC* (Centers for Disease Control and Prevention (CDC), 2007; Starnino *et al.*, 2010). The rapid development of AMR has resulted in many changes in treatment regime (Unemo & Shafer, 2014). Currently, while patients with symptoms of STIs are treated with doxycycline in low/middle income countries (LMICs) (Cehovin *et al.*, 2018), *N. gonorrhoeae* treatment in wealthy countries consists of a dual therapy with ceftriaxone and azithromycin (St Cyr *et al.*, 2020). However, isolates resistant to ceftriaxone have been recorded (Unemo *et al.*, 2012). AMR in *N. gonorrhoeae* can thus affect the efficacy of contact tracing and render gonorrhoea untreatable in the future.

### 1.3.2 *N. gonorrhoeae* resistance mechanisms

*N. gonorrhoeae* can develop AMR through chromosomal mutations. These mutations occur either spontaneously or as a result of horizontal gene transfer from other *Neisseria* spp. (Goytia *et al.*, 2021).

Resistance to antibiotics can arise through increasing antibiotic export, such as through the overexpression of the Mtr efflux pump which extrudes penicillins and tetracyclines. *N. gonorrhoeae* can also limit antibiotic uptake through amino acid substitutions in PorB (Olesky *et al.*, 2006). Finally, mutations in *parC* and *gyrA* prevent ciprofloxacin from binding to its target, thereby conferring resistance (Lindbäck *et al.*, 2002) while mutations

in the 16S and 5S rRNA (Ilina *et al.*, 2013; Galimand *et al.*, 2000) prevent binding of spectinomycin to ribosomes.

Apart from chromosomal mutations, bacteria can develop AMR through plasmid acquisition. Unlike other species, few plasmids have been described in *N. gonorrhoeae*. Only three plasmids have been identified to date (Cehovin *et al.*, 2020), the conjugative plasmid (pConj), beta lactamase plasmid (p*bla*) and cryptic plasmid (pCryp).

### 1.3.3 The β-lactamase plasmid, p*bla*

As the name suggests, p*bla* carries a β-lactamase gene. It carries a replicase 1 gene, and some variants carry mobilisation genes *mobA* and *mobC* (Pagotto *et al.*, 2000). p*bla* is thought to be a multicopy number plasmid (Yan *et al.*, 2019). There are 63 alleles of the β-lactamase gene in *N. gonorrhoeae* so far: worryingly, the TEM-135 allele is only one mutation away from becoming an extended spectrum beta lactamase (ESBL); isolates carrying the $bla_{\text{TEM-135}}$ have been identified worldwide (Ohnishi *et al.*, 2010; Gianecini *et al.*, 2015; Yan *et al.*, 2019). Several variants of p*bla* have been identified, with sizes ranging from 3.2 kb (p*bla*.Au, isolated from Australia) to 9.3 kb (p*bla*.Nz, isolated from New Zealand) (Cehovin *et al.*, 2020). Consistent with their small size, p*bla* does not encode for a functional conjugation system. As such, it relies on the conjugation system encoded on pConj which can mobilise p*bla* (Genco *et al.*, 1984; Roberts & Knapp, 1988a). It is thus unclear whether this mobilisation is by integration of p*bla* into pConj or through other means.

### 1.3.4 The cryptic plasmid, pCryp

pCryp is a small, 4.2 kb plasmid found in > 80% of all *N. gonorrhoeae* strains that encodes its own replicase (*repA*) and replication initiation protein (Cehovin *et al.*, 2020). It also carries genes involved in mobilisation, *mobB* and *mobC* (Sarandopoulos & Davies, 1993). It is not known when pCryp was acquired, as the first recorded gonococcal isolate in 1922 already carried pCryp. Previous research suggests that it is a high copy number plasmid, with approximately 20 – 30 copies (Mayer *et al.*, 1974). Despite the high

prevalence of pCryp, it is not found in lineage 15 strains (Cehovin *et al.*, 2020), and its role is unknown. While pCryp was previously hypothesised to have a role in metabolism (Dillon & Pauze, 1981), these were shown not to be true (Biswas *et al.*, 1986). Of note, the expression of pCryp increases under anaerobic conditions (Isabella & Clark, 2011).

One of the important genes pCryp harbours is a *vapXD* toxin-antitoxin (TA) system (Korch *et al.*, 1985) which exact role is not known. However, the introduction of pCryp *vapX* into *Escherichia coli* increased transfer of pConj into *E. coli* from the gonococcus, suggesting that pCryp VapX may be able to neutralise an orphan putative VapD toxin encoded by pConj (Pachulec & van der Does, 2010).

### 1.3.5 pConj

#### 1.3.5.1 pConj prevalence

pConj was first reported in the literature in 1970s as a 39 kb plasmid which did not confer AMR (markerless). Most markerless pConj were isolated from Southeast Asia or Europe (Roberts *et al.*, 1979). Subsequently, *N. gonorrhoeae* strains with a related 42 kb plasmid conferring tetracycline resistance was described in the 1980s (Waugh *et al.*, 1988; Roberts & Knapp, 1988b). Tetracycline resistance was conferred by one of two *tetM* elements leading to the designation of 'American' and 'Dutch' pConj (Gascoyne-Binzi *et al.*, 1993). Interestingly, a slight decrease in proportion of highly tetracycline resistant *N. gonorrhoeae* (and therefore *tetM*$^{+}$ pConj prevalence) was observed in different countries following the introduction of fluoroquinolones as a treatment for gonorrhoea (Kam *et al.*, 1995; Fox *et al.*, 1997).

The most recent analysis suggests that the prevalence of *tetM*-carrying pConj is higher in LMICs, some of which use doxycycline for treatment of STIs. This prompted the hypothesis that use of tetracyclines drives the persistence and spread of pConj in gonococcal populations (Cehovin *et al.*, 2020).

### 1.3.5.2 Structure of pConj

pConj can been divided into four regions, encoding for i) plasmid replication and partitioning (brown), ii) the mating bridge (*trb* genes, blue) iii) conjugative transfer (*tra* genes, light blue), and iv) the genetic load region (**Figure 1.6**) (Pachulec & van der Does, 2010). Analysis of the *trfA*, *traI*, *traG* and *trbE* which are involved in the replication and conjugation of other plasmids (Pansegrau *et al.*, 1994) indicates that pConj is related to plasmids of the incompatibility P (IncP-1) group. Members of the IncP-1 plasmid group are generally considered to have a broad host range as they can replicate in many different bacterial species (Popowska & Krawczyk-Balska, 2013). Analysis of backbone genes of IncP-1 plasmids, which consists of genes involved in the replication and conjugation, suggests that it can be further divided into five subfamilies, α, β, γ, δ, and ε; however, pConj appears to be phylogenetically divergent from all these subfamilies (Pachulec & van der Does, 2010).

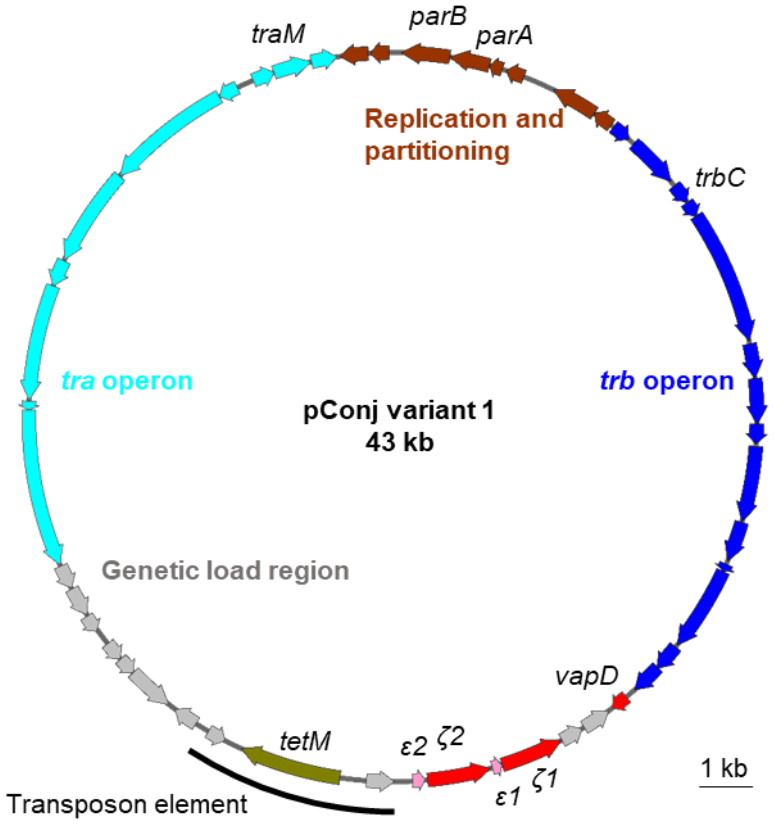



**Figure 1.6 Genetic map of pConj, using pConj variant 1 as a representative.** There are four regions on the conjugative plasmid (pConj): the replication and partitioning genes (brown), genes involved in conjugation (*trb*, blue and *tra*, light blue) and genetic load region (grey). TA-associated genes (toxin, red and antitoxin, pink) and *tetM* (green) are found in the genetic load region. The site of the streptococcal transposon element insertion is as indicated.

#### 1.3.5.2.1 Replication and partitioning systems on pConj

For plasmid replication, TrfA and Ssb bind to the *oriV* origin of replication of plasmids such as the RK2 plasmid (Pinkney *et al.*, 1988). On pConj, bioinformatic analysis shows that TrfA shares 47% amino acid similarity to the TrfA replication initiation protein encoded by *Nitrosomonas eutropha* C91 while Ssb shares 37% amino acid identity to the Ssb of *Xylella fastidiosa* M23 single stranded binding protein; TrfA and Ssb from both species were automatically annotated on the NCBI database due to their similarity to characterised TrfA and Ssb amino acid sequences respectively (Stein *et al.*, 2007; Young *et al.*, 1983). Therefore, replication of pConj is hypothesised to be dependent on *trfA* and *ssb* (Pachulec & van der Does, 2010). While the exact *oriV* on pConj has not been determined by experimental means, it is thought to be the 400 bp region containing eight 5′-GCATGTGTAAATCCG-3′ repeats adjacent to *trfA* (Pachulec & van der Does, 2010). Interestingly, this repeat motif is different from other known TrfA binding sequences.

There are six genes on pConj potentially involved in partitioning: *kfrC, kfrB, korB, incC, kleE* and *korC*. Of these, the amino acid sequence encoded by *incC* and *korB* share 54% and 48% amino acid identity to characterised IncC2 of the Birmingham IncPα plasmid (Pansegrau *et al.*, 1994) and ParB of *X. fastidiosa* (Rogers & Stenger, 2012) respectively (Pachulec & van der Does, 2010). These correspond to *parA* and *parB*, which makes up the type I partitioning system characterised in other plasmids such as the RK2 plasmid in *E. coli* (Williams *et al.*, 1998). In Type 1 partitioning systems, *incC/parA* encodes for a nucleotide triphosphatase (NTPase) while *korB/parB* encodes for a centromere-binding protein (CBP). In RK2, both *parA* and *parB* are expressed under the same promoter, with the ParB binding site adjacent to *parB* (Kolatka *et al.*, 2010). *incC* (which encodes for a ParA homologue) is expressed in two forms, IncC1 and IncC2, with IncC2 a truncated version of IncC1. Both are able to polymerise into filaments (Batt *et al.*, 2009) and have ATPase activity but only IncC1 enhances ParB binding to DNA and has minimal contact with DNA (Jagura-Burdzy *et al.*, 1999). ParB binds to a 13 bp inverted repeat sequence 5′-TTTAGCCGCTAAA-3′ on the RK2 plasmid, and this sequence is also found six times

on pConj, one of which is in the genetic load region (Pachulec & van der Does, 2010). It is not known whether the corresponding genes on pConj are involved in plasmid partitioning as observed in other IncP plasmids.

#### 1.3.5.2.2 *tra* and *trb* operons

In IncP plasmids such as pConj, the *tra* and *trb* operons flank replication genes. The *tra* region consists of two operons (*traCEDFGIJ* and *traKLM*) transcribed in opposite directions (Pachulec & van der Does, 2010). Genes in this region are often involved in DNA processing and conjugation (reviewed (Zatyka & Thomas, 1998)). Of these, TraJ and TraI form part of the relaxosome complex and are involved in binding directly to the plasmid *oriT* and nicking the plasmid to facilitate transfer (Pansegrau *et al.*, 1988, 1990). Other proteins such as TraM are then involved in transfer of IncP plasmids (Fürste *et al.*, 1989).

The *trb* operon consists of 14 genes (*trbA-N*) and encodes for proteins that form the structure of the mating bridge (Pachulec & van der Does, 2010), a type IV secretion system through which plasmids are transferred from the donor to the recipient. In the RK2 plasmid, expression of the *trb* operon is controlled by repressors TrbA, which is encoded by the first gene in the operon, and KorB, a ParB homologue (Bingle *et al.*, 2003). Although pConj carries both *trbA* and *korB* genes, the genetic structure of pConj is different to other plasmids, as genes such as *kfrB* and *kfrC* are found within the replication and partitioning region on pConj (Pachulec & van der Does, 2010); therefore, regulation of *trb* operon expression in pConj remains to be determined.

The structural components encoded by the *trb* operon in pConj are homologous to those encoded by other IncP plasmids. Specifically, the prepilin subunit, encoded by *trbC*, shares 72% amino acid similarity with the prepilin subunit TrbC in the IncP pB3 plasmid (Heuer *et al.*, 2004; Pachulec & van der Does, 2010). In the IncP RP4 plasmid found in *E. coli*, TrbC is first processed by a signal peptidase (Haase & Lanka, 1997) then cyclised by TraF (Eisenbrandt *et al.*, 2000, 1999). Of note, pConj TrbK, which putatively encodes for an entry exclusion protein that prevents the acquisition of similar plasmids, shares

very little homology to other TrbK proteins (Pachulec & van der Does, 2010). Therefore, it is unknown if TrbK is functional.

#### 1.3.5.2.3 Genetic load region

The genetic load consists of genes *neis2203* – *vapD*. Of the genes within the genetic load region, seven of them encode for proteins of unknown function. *neis2208*/*ngonSK11395* is predicted to encode a DNA modification methylase (Pachulec & van der Does, 2010). Some pConj variants carry *tetM*, which confers tetracycline resistance (Cehovin *et al.*, 2020); *tetM* was acquired horizontally from a transposon Tn*916* originally identified in *Streptococcus* (Morse *et al.*, 1986). In *E. coli,* TetM contributes to tetracycline resistance by interacting directly with the tetracycline binding site on the ribosome, preventing tetracycline binding (Dönhöfer *et al.*, 2012). Additionally, pConj carries genes encoding for resolvase and toxin-antitoxin systems

### TA systems

TA systems can be found both on the bacterial chromosome and plasmids. There are eight types of TA systems (Types I-VIII), each of which has a unique antitoxin-toxin neutralisation mechanism (Song & Wood, 2020). In type II TA systems, the protein antitoxin binds to and neutralises the toxin. TAs are involved in a variety of functions such as persister formation (Helaine *et al.*, 2014), biofilm formation (González Barrios *et al.*, 2006) and protection against foreign DNA (LeRoux *et al.*, 2022).

Additionally, plasmid-encoded TA systems were originally described as addiction systems that prevent plasmid loss *via* post-segregational killing (PSK) (Gerdes & Rasmussen, 1986) (**Figure 1.7A**). In PSK, during replication and division, a bacterial daughter cell may not inherit plasmid. In cells that do not have the plasmid, the unstable antitoxin is degraded by proteases belonging to the Lon or Clp family (van Melderen *et al.*, 1994; Diago-Navarro *et al.*, 2013; Brzozowska & Zielenkiewicz, 2014). In the absence of the antitoxin, the stable toxin causes cell death or growth arrest. In this manner, cells which lose the plasmid will be eliminated from a population of growing bacteria, thereby

ensuring stable vertical transmission of the plasmid. The importance of TA systems in plasmid maintenance has been recorded in many plasmids of clinically important species such as *Shigella* spp. (McVicker & Tang, 2016) and *P. aeruginosa* (Hernández-Ramírez *et al.*, 2017).

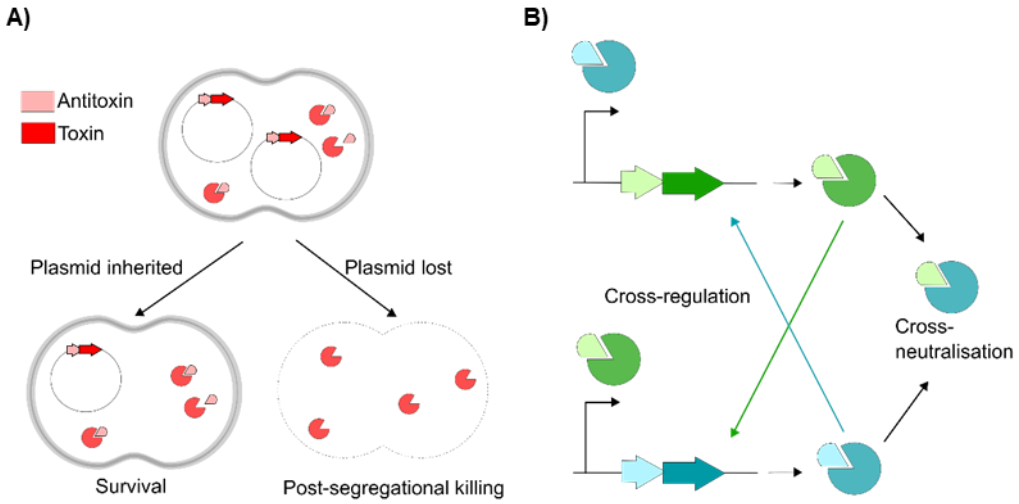



**Figure 1.7 Role of TA systems in plasmid stability and regulation of TA systems. A)** Toxin-antitoxin (TA) systems have been implicated in post segregational killing. Plasmids encode Type II TA, where both the toxin and the antitoxin are proteins. When the plasmid is inherited, the antitoxin is expressed, thus neutralising the toxin. When the plasmid is not inherited, the unstable antitoxin is degraded, causing the toxin to kill the cell. **B)** TA systems can influence the expression of non-endogenous TA systems. For example, TA complex can bind to promoters of other TA loci, resulting in cross-regulation. Antitoxin from other TA systems can also neutralise another toxin, resulting in cross-neutralisation.

## Regulation of TA systems

The genetic architecture of type II TA operons generally consists of a promoter/operator followed by a gene encoding an antitoxin then the toxin (Harms *et al.*, 2018). In some cases such as *mqsRA*, the architecture is flipped, with the toxin downstream of the promoter followed by the antitoxin. Often, this results in different levels of TA proteins being expressed after translation, usually with levels of antitoxin higher than toxins. When toxin and antitoxin levels are in the correct stoichiometry, TA complexes can bind to their own promoter and repress their own expression (Overgaard *et al.*, 2008).

### Cross regulation

When multiple TA systems are present in the bacterial genome, there is potential for different TA systems to cross-regulate each other. One form of TA system regulation is autoregulation, where a TA complex binds its own promoter and represses expression. Interestingly, non-cognate TA complexes have been shown to be able to bind different promoters, suggesting possible cross-regulation. For example, three *relBE* modules in *Mycobacterium tuberculosis* can cross regulate each other (Yang *et al.*, 2010). Additionally, overexpression of a toxin can influence the expression of other TA systems. An example of this is that overexpression of endoribonuclease toxins such as MazF and MqsR activates *relBE* TA system in *E. coli* (Kasari *et al.*, 2013). This evidence indicates that multiple TA systems can cross-regulate each other's expression (**Figure 1.7B**).

### Cross neutralisation

In most cases, TA systems cannot cross neutralise each other. For example, the three RelBE type II TA systems in *E. coli* (Fiebig *et al.*, 2010) and the five *aapA*/IsoA type I TA systems in *Helicobacter pylori* are specific and do not cross neutralise (Arnion *et al.*, 2017).

In *M. tuberculosis*, however, different TAs can cross neutralise*. M. tuberculosis* carries more than 80 TA systems. Of these, three RelBs can neutralise their non-cognate RelEs (Yang *et al.*, 2010). Furthermore, VapB from the *vapBC* TA system and MazF from the

*mazEF* TA system in *M. tuberculosis* have been shown to interact with each other (Zhu *et al.*, 2010) (**Figure 1.7B**).

## TA systems on pConj

### Epsilon:Zeta (*ε:ζ*) TA

There are two *ε:ζ* TA system loci on pConj. One of the *ε:ζ* locus carries *ε:ζ1* while the other locus carries either *ε:ζ2* or *ε:ζ3.* The *ε:ζ* TA system was first identified in the *S. pyogenes* plasmid pSM19035 (Cegłowski *et al.*, 1993; Camacho *et al.*, 2002). Unlike other TA systems, the *ω-ε-ζ* is regulated by *ω*, while *ε* and *ζ* encode the antitoxin and toxin, respectively. Streptococcal ζ interferes with peptidoglycan cell wall synthesis by phosphorylating a cell wall precursor, UDP-N-acetylglucosamine (UNAG) (Mutschler *et al.*, 2011). The ε antitoxin has a shorter half-life than the ζ toxin (Camacho *et al.*, 2002) and is degraded by ClpXP (Brzozowska & Zielenkiewicz, 2014). Deletion of the *ω-ε-ζ* operon and overproduction of ε both decreased stability of plasmids (Zielenkiewicz & Cegłowski, 2005). Since then, the *ε:ζ* TA system and homologues have been found in other AMR plasmids in *Enterococcus faecalis* (Sletvold *et al.*, 2008).

Of the three *ε:ζ* TA systems, *ε:ζ2* and *ε:ζ3* have approximately 40% amino acid sequence identity to the streptococcal *ε:ζ* system (Rocker *et al.*, 2018). The other TA system, *ε:ζ1* (annotated as ngε_1/ngζ_1 in the paper), is functional in *E. coli* (Rocker *et al.*, 2018). ngζ_1 phosphorylates a larger variety of substrates including UDP-muramic acid, UDP-glucose and UNAG, all of which are precursors for cell wall synthesis. Specifically, the lysine residue of ngζ_1 in position 115 is responsible for catalysing the phosphorylation; other residues at the active site such as S87 and H79 form hydrogen bonds to stabilise the substrate (Rocker *et al.*, 2018). However, it is unknown whether any *ε:ζ* TA systems are functional in *N. gonorrhoeae*, and if so, whether they have a role in pConj maintenance.

**VapXD TA**

pConj carries an orphan *vapD* gene. VapD was first identified in *Haemophilus influenza*e, and is one of four virulence-associated proteins (Vap) A-D. *H. influenzae* VapD has structural homology to the Cas2 protein, was shown to dimerise (Daines *et al.*, 2004) and displayed ribonuclease activity (Ren *et al.*, 2012). The upstream gene encodes an antitoxin that counteracts the toxic effect of VapD in *E. coli* (Daines *et al.*, 2004). Rather than maintaining plasmids, disruption of *vapXD* decreased bacterial survival in human cell models (Ren *et al.*, 2012; Daines *et al.*, 2004). However, VapD is not always part of a TA system: in *H. pylori*, VapD has endoribonuclease activity (Kwon *et al.*, 2012) but does not have an obvious antitoxin.

The orphan pConj VapD shares the catalytic residues in *H. influenzae* VapD (Asp24 and Asp95 on pConj VapD corresponds to Asp7 and Asp76 on *H. influenzae* VapD) (Kwon *et al.*, 2012). Although an antitoxin is not present on pConj, VapD can potentially be neutralised by VapX encoded by pCryp (Pachulec & van der Does, 2010). Thus, the roles of the TA systems on pConj need to be determined.

### 1.3.5.3 pConj variants and distribution

The earliest description of pConj was a markerless plasmid in *N. gonorrhoeae*. Subsequently, analysis of whole genome sequences (WGS) identified seven pConj variants, variants 1 – 7. pConj variants 1, 3 and 5 make up majority of the pConj plasmids. Of note, pConj variant 1 carries the American *tetM* allele, pConj variant 3 carries the Dutch *tetM* allele while pConj variant 5 does not carry *tetM*.

Subsequent studies identified *tetM*$^{+}$ pConj in related species *Eikenella* spp. and *Kingella* spp. (Knapp *et al.*, 1988) which exhibited high level tetracycline resistance. Subsequent conjugation assays suggest that while *N. gonorrhoeae* pConj can be transferred to other *Neisseria* spp. and related *Neisseriaceae* family, pConj cannot be transferred into *Branhamella catarrhalis* (Roberts & Knapp, 1988b). Furthermore, pConj was shown to be transferred but not maintained in *E. coli*, as exemplified by loss of tetracycline resistance during passage of transconjugants (Pachulec & van der Does, 2010). Overall, this suggests that despite being of the IncP family of plasmids, both epidemiological and experimental data suggests that pConj does not exhibit broad host range.

Of the *Neisseria* spp., pConj has been most studied in *N. gonorrhoeae*. Analysis of *N. gonorrhoeae* WGS showed that distribution of pConj and other plasmids is uneven across gonococcal lineages. Lineage 3, for example, carries only pCryp, while most isolates of lineage 21 carry pConj and pCryp (Cehovin *et al.*, 2020). Further analysis of pConj carrying strains revealed that pConj variants are found in specific lineages. For example, pConj variant 1 is mostly found in lineage 21 while variants 2, 4 and 7 are found exclusively in lineages 151, 34 and 78, respectively (Cehovin *et al.*, 2020).

## 1.4 Project aims

The overall aim of work in this thesis is to understand the resistance of *N. gonorrhoeae* to host immunity and antimicrobials.

i) I aimed to determine if *N. gonorrhoeae* interacts with CFHRs, and determine the binding sites of CFHR to *N. gonorrhoeae*. This would suggest that the immune system could be harnessed in the future to treat gonococcal infections.

ii) With the rise in *N. gonorrhoeae* AMR, I aimed to determine the prevalence of pConj in *Neisseria* spp. as well as to determine the reason for the uneven distribution of pConj in different *N. gonorrhoeae* lineages (Cehovin *et al.*, 2020).

iii) As pConj is crucial in the spread of both itself and p*bla*, I aimed to investigate the mechanisms of pConj maintenance and show proof of concept of potential strategies to cure pConj.

Overall, by investigating host-pathogen interaction of and plasmid-mediated resistance in *N. gonorrhoeae*, this thesis aims to define new ways to treat AMR in gonococcus in the future through modulating the immune system and pConj elimination.

# 2 Materials and methods

## 2.1 Bacterial strains and growth

The bacterial strains used in this study are listed below. *N. gonorrhoeae* was grown on gonococcal agar (GCB) (1.5% w/v Proteose peptone #3, Thermofisher Scientific; 0.1% w/v Cornstarch, Sigma; 22.9 mM $K_2HPO_4$, Sigma; 7.35 mM $KH_2PO_4$, Sigma and 17.1 mM NaCl, VWR International), supplemented with 1% vitox (Oxoid) and 1.13% w/v Agar No. 1 and incubated for 16 – 22 h at 37°C with 5% $CO_2$. For liquid growth, *N. gonorrhoeae* was initially grown overnight on GCB agar before harvesting and inoculating into either GCBL (*i.e.* GCB without agar) or fastidious broth (FB), both supplemented with 1% vitox (Oxoid). FB consists of 1% w/v Casein Peptone (Sigma), 0.8% w/v Meat Peptone (Sigma), 85.6 mM NaCl (VWR International), 0.5% w/v Yeast Extract (Sigma), 18.1 mM Tris-HCL (Sigma), 13.9 mM Dextrose (Sigma), 6.85 mM Trizma Base (Sigma), 0.07% w/v Agarose (Invitrogen), 0.569 mM L-Cysteine HCl (Sigma), 0.388 mM sodium citrate (Sigma), 0.415 mM $Mg_2SO_4 \cdot 7H_2O$ (Sigma), 0.015% w/v Hematin (Sigma), 43.2 µM $FeSO_4 \cdot 7H_2O$ (Scientific Laboratory Supplies). After autoclaving, 35.9 µM Pyridoxal (Sigma), 0.05% v/v Polysorbate 80 (VWR International) and 22.6 µM NAD (Roche and Sigma) were sterile filtered and added to the media before storing at 4°C. The $OD_{600}$ measured the density of bacterial cultures. Liquid cultures were incubated at 37°C, 5% $CO_2$ with shaking at 180 r.p.m. *E. coli* was grown on Luria-Bertani (LB) agar (2% w/v LB broth, Invitrogen and 3% w/v Agar No. 1, Oxoid) or in 5 ml LB with shaking at 180 r.p.m., overnight at 37°C.

Supplements were added as required at the following concentrations: for *N. gonorrhoeae*, kanamycin, tetracycline and erythromycin (all from Sigma) were used at 50 µg/ml, 2 µg/ml and 2 µg/ml, respectively; media for counter selection contained 6.4% (w/v) 2-deoxy-galactose (2-DOG) (Sigma). For *E. coli*, 50 µg/ml kanamycin (Sigma), 20 µg/ml chloramphenicol (Sigma), and 40 µg/ml X-gal was used for selection.

### 2.1.1 Strains used in this study

| ***N. gonorrhoeae*** | | | |
|---|---|---|---|
| **Strains for CFHR experiments** | | **Background** | **Reference** |
| L1$^{43\text{-}48\rightarrow Ala}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L2$^{83\text{-}88\rightarrow Ala}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L3$^{116\text{-}121\rightarrow Ala}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L3$^{134\text{-}139\rightarrow Ala}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L4$^{\Delta 171\text{-}176}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L5$^{\Delta 203\text{-}224}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L6$^{L253M}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L6$^{254\text{-}259\rightarrow Ala}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| L7$^{290\text{-}295\rightarrow Ala}$ | | FA1090-G4 | (Chen & Seifert, 2013) |
| FA1090Δ*lst* | | FA1090-G4 | This study |
| **Lab and clinical strains** | **Lineage** | **Description** | **Reference** |
| FA1090-WT | 286 | Strain from Professor Ann Jerse, used in the pConj experiments. | Gift from Professor Ann Jerse |
| FA1090-G4 | 286 | Strain from Professor Hank Seifert, used in the CFHR experiments. G4 motif has been mutated to prevent pilin antigenic variation | (Seifert *et al.*, 1994) |
| WHO K | 8 | World Health Organisation control strain | (Unemo *et al.*, 2009) |
| NG104 | 3 | Clinical isolate from the UK | (Cehovin *et al.*, 2020) |
| NG106 | 3 | Clinical isolate from the UK | (Cehovin *et al.*, 2020) |
| NG115 | 159 | Clinical isolate from the UK | (Cehovin *et al.*, 2020) |
| NG116 | 159 | Clinical isolate from the UK | (Cehovin *et al.*, 2020) |
| NM8365 | 3 | Clinical isolate | (Cehovin *et al.*, 2020) |
| 43305 | 151 (now 364) | Clinical isolate from Africa | (Cehovin *et al.*, 2020) |
| 55496 | 34 | Clinical isolate from Africa | (Cehovin *et al.*, 2020) |
| 56327 | 21 | Clinical isolate from Africa | (Cehovin *et al.*, 2020) |
| 60755 | 21 | Clinical isolate from Africa | (Cehovin *et al.*, 2020) |
| NG102 | 296 | Clinical isolate from the UK | (Cehovin *et al.*, 2020) |
| NG118 | 296 | Clinical isolate from the UK | (Cehovin *et al.*, 2020) |
| 65737 | 366 | Clinical isolate from Africa | (Cehovin *et al.*, 2020) |

| **Δ*pilD*::*aph(3)-III* strains as recipients for conjugation** | **Background** | **Reference** |
|---|---|---|
| FA1090-WTΔ*pilD* | FA1090-WT | This study |
| FA1090-G4Δ*pilD* | FA1090-G4 | This study |
| WHO KΔ*pilD* | WHO K | This study |
| NG104Δ*pilD* | NG104 | This study |
| NG106Δ*pilD* | NG106 | This study |
| NG115Δ*pilD* | NG115 | This study |
| NG116Δ*pilD* | NG116 | This study |
| NM8365Δ*pilD* | NM8365 | This study |
| 55496Δ*pilD* | 55496 | This study |
| 56327Δ*pilD* | 56327 | This study |
| 60755Δ*pilD* | 60755 | This study |
| 43305Δ*pilD* | 43305 | This study |
| **pConj carrying strains** | **Background** | **Reference** |
| FA1090:pConj | FA1090-WT | This study |
| WHO K:pConj | WHO K | This study |
| NM8365:pConj | NM8365 | This study |
| **Strains used for pConj maintenance** | **Background** | **Reference** |
| $pC^{GFP}$ | FA1090-WT | This study |
| pCΔGL | FA1090-WT | This study |
| pCt | FA1090-WT | This study |
| pCtΔ*vapD* | FA1090-WT | This study |
| pCt:$\zeta 1^{K115A}$ | FA1090-WT | This study |
| pCtΔ*ζ2* | FA1090-WT | This study |
| pCtΔ*vapD*:*ζ1* | FA1090-WT | This study |
| pCtΔTA | FA1090-WT | This study |
| pCtΔ*parB* | FA1090-WT | This study |
| pCtΔ*parB*ΔTA | FA1090-WT | This study |
| pCΔ*parB*ΔTA | FA1090-WT | This study |
| ***E. coli*** | | |
| DH5α pBAD | | (Custodio *et al.*, 2021) |
| DH5α pBAD:$vapD^{pConj}$ | | This study |
| DH5α pBAD:$vapD^{pConj}vapX^{pCryp}$ | | This study |
| DH5α pBAD:$vapD^{pConj}vapX^{Nm}$ | | This study |

**Table 2.1 List of strains constructed in this study**

### 2.1.2 Plasmids used in this study

| Name | Backbone | Description | Reference |
|---|---|---|---|
| **Intermediate plasmids for *porB* mutation** | | | |
| pBlue_L1 | pBluescript | FA1090-G4 carrying alanine substitutions of amino acids 43-48 in loop 1 of PorB | (Chen & Seifert, 2013) |
| pBlue_L2 | | FA1090-G4 carrying alanine substitutions of amino acids 83-88 in loop 2 of PorB | (Chen & Seifert, 2013) |
| pBlue_L3A | | FA1090-G4 carrying alanine substitutions of amino acids 116-121 in loop 3 of PorB | (Chen & Seifert, 2013) |
| pBlue_L3B | | FA1090-G4 carrying alanine substitutions of amino acids 134-139 in loop 3 of PorB | (Chen & Seifert, 2013) |
| pBlue_L4 | | FA1090-G4 carrying deletion of amino acids 171-176 in loop 4 of PorB | (Chen & Seifert, 2013) |
| pBlue_L5 | | FA1090-G4 carrying deletion of amino acids 203-224 in loop 5 of PorB | (Chen & Seifert, 2013) |
| pBlue_L6 | | FA1090-G4 carrying alanine substitutions of amino acids 254-259 in loop 6 of PorB | (Chen & Seifert, 2013) |
| pBlue_L6253M | | FA1090-G4 carrying leucine to methionine substitute of amino acid 253 in loop 6 of PorB | (Chen & Seifert, 2013) |
| pBlue_L7 | | FA1090-G4 carrying alanine substitutions of amino acids 290-295 in loop 7 of PorB | (Chen & Seifert, 2013) |
| pBlue_lst | | For *lst* deletion in FA1090-G4 | This study |
| | | | |
| **Intermediate plasmids for pConj manipulation** | | | |
| ptRNA | | Low copy plasmid harbouring *fMet* gene in the cloning site from Dr Guangyu Liu; used as vector for cloning. | Tang Lab, unpublished |

| | | | |
|---|---|---|---|
| pT101 132-133 | | Low copy plasmid containing fragments to insert *gfp:kan* into pConj | This study |
| pT101 153-158 | | Low copy plasmid containing fragment amplified by primers 153/158 to delete genetic load region on pConj | This study |
| pT101 Δ*trbC::galK:ery* | | Low copy plasmid containing fragment amplified by primers 159 and 162 to delete *trbC* by *galK:ery* cassette | This study |
| pT101 Δ*trbC*$^{clean}$ | ptRNA | Low copy plasmid containing fragment amplified by primers 159 and 162 to generate markerless deletion of *trbC* | This study |
| pT101 Δtoxins | | Low copy plasmid to delete individual/ multiple toxin genes on pConj | This study |
| pT101 ζ1$^{K115}$ | | Low copy plasmid for ζ$^{K115}$ cloning | This study |
| pT101 Δζ2 | | Low copy plasmid to delete ζ2 only on pConj | This study |
| pT101 *parB* | | Low copy plasmid to delete *parB* on pConj | This study |
| pCR™2.1-TOPO™ -*pilD* | pCR™2.1-TOPO™ | TOPO plasmid containing Δ*pilD* construct | This study |
| | | | |
| **Plasmids for transposon insertion sequencing** | | | |
| pCE005 | | Plasmid with kanamycin resistance in the backbone that contains erythromycin cassette flanked by transposon inversion repeat sequences from Dr Cara Ellison | Tang Lab, unpublished |
| pHsk1 | pCE005 | Plasmid with erythromycin resistance in the backbone that contains kanamycin cassette flanked by transposon inversion repeat sequences | This study |
| | | | |
| **Plasmids for qPCR** | | | |
| TOPO-*recA* | pCR™2.1-TOPO™ | Control plasmid to measure primer efficiency | This study |
| TOPO-*res* | | | This study |

| | | |
|---|---|---|
| **Plasmids for toxicity assays** | | |
| pBAD33 empty | pBAD33 | (Custodio *et al.*, 2021) |
| pBAD:*vapD*$^{pConj}$ | | This study |
| pBAD:*vapD*$^{pConj}$*vapX*$^{pCryp}$ | | This study |
| pBAD:*vapD*$^{pConj}$*vapX*$^{Nm}$ | | This study |

**Table 2.2 List of plasmids constructed in this study**

## 2.2 Genetic methods

### 2.2.1 DNA extraction

Genomic DNA extraction was carried out using the Wizard Genomic DNA Purification Kit (Promega) as per the manufacturer's protocol. Strains were grown overnight on GCB. A loop of bacteria (10 µl) was collected from the plate and resuspended in 600 µl Nuclei Lysis solution and incubated for 5 m at 80°C. RNase (3 µl) was then added to the suspension and incubated for 30 m at 37°C before protein precipitation solution (200 µl) was added and mixed by vortexing for 20 s. After an incubation at 4°C for at least 5 m, bacteria were centrifuged at 14000 *x g*. The supernatant was collected, and DNA precipitated by the addition of isopropanol; DNA pellets were collected by centrifugation and washed twice with 70% ethanol. DNA was then reconstituted in Elution Buffer (EB) (10 mM Tris-HCl pH 8.5) and diluted to 30 µg/ml before sending for sequencing if required (MicrobesNG).

DNA was extracted from agarose gels using the Wizard PCR Clean up system (Promega) according to the manufacturer's instructions. Membrane binding solution was added to dissolve the gel at 90°C before transferring to a column. For purification of polymerase chain reaction (PCR) products, the same volume of membrane binding solution was added to each completed PCR reaction before transferring to a column. DNA was then washed with wash solution, eluted with nuclease free water (Amersham) and sent for sequencing if required (Source Bioscience).

Plasmids were purified using the Miniprep Kit (Sigma-Aldrich). Briefly, *N. gonorrhoeae* was grown overnight in 5 ml GCB + 1% vitox. Alternatively, *E. coli* was grown in 5 ml LB overnight. Overnight cultures were then pelleted and resuspended in 200 µl resuspension solution before lysing with 200 µl lysis solution. After neutralisation, cell debris was pelleted by centrifugation at 14000 *x g.* The supernatant was then loaded onto columns provided before washing with 750 µl Wash Solution. Plasmid DNA was eluted in 50 µl nuclease free water.

### 2.2.2 TOPO cloning

To clone DNA fragments into pCR™2.1-TOPO™, polyA tails were added to PCR products (7 µl) by incubation in 1 µl 10x Taq buffer (Sigma), 1 µl of 20 mM dATP (Bioline) and 0.2 µl of Taq polymerase (Sigma) at 70°C for 20 m. DNA fragments were cloned according to the manufacturer's instructions (Thermofisher). Briefly, an aliquot of the reaction (4 µl) was added to 1 µl of salt solution consisting of 1.2 M NaCl and 0.06 M $MgCl_2$, 0.5 µl of the vector, and dd$H_2O$ added to a total volume of 10 µl. The ligation mixture was incubated at room temperature for 20 m before transforming into *E. coli* DH5α. Colonies were selected on kanamycin + X-gal LB plates.

### 2.2.3 Gibson assembly

Gibson assembly was carried out using the NEB Builder HiFi DNA assembly kit (New England Biolabs) as per the manufacturer's instructions. Briefly, amplified fragments containing 30 bp sequence overlap were mixed with Gibson assembly mastermix containing DNA ligase, DNA polymerase and 5′ exonuclease T5. Gibson reactions were incubated at 50°C for 1 h. The resulting product was used to transform *E. coli* DH5α.

### 2.2.4 Agarose gel electrophoresis

For agarose gels, 1% (w/v) agarose (Invitrogen) was dissolved in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 0.01% SYBR Safe DNA dye (ThermoFisher). Samples were mixed with loading dye (30% v/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF) before loading into gels. HyperLadder (1 kb, Bioline) was used as a size reference. Samples were separated by electrophoresis at 110 – 140 V before visualising using GelDoc (Biorad gel Doc XR+).

### 2.2.5 Bacterial transformation

Plasmid DNA (100 ng) was added to 50 µl of chemically competent *E. coli* DH5α and incubated on ice for 30 m. Bacteria were then subjected to heat shock at 42°C for 45 s, followed by 90 s on ice. For recovery, 1 ml of LB was added and bacteria cells were

incubated for 1 h at 37°C with shaking at 180 r.p.m. Bacteria were then pelleted and resuspended in 50 µl LB before plating onto selective media.

For gonococcal transformation, between 100 ng and 1000 ng of PCR product or linearised plasmid DNA was spotted onto GCB plates and allowed to dry. *N. gonorrhoeae* grown on GCB plates for 16 – 20 h at 37°C in 5% $CO_2$ was streaked over dried DNA and incubated for 8 – 24 h. Transformants were selected by harvesting bacteria and plating to GCB containing appropriate antibiotics.

### 2.2.6 Strain construction

The genetic region of interest was amplified using Taq polymerase (Sigma), Herculase II Fusion polymerase (Agilent) or PrimeSTAR GXL DNA Polymerase (TaKaRa Bio) according to the manufacturers' instructions. All primers are as listed in **Appendix Table 7.2**. For *N. gonorrhoeae*, constructs included approximately 1 kb of sequence upstream and downstream of genes of interest. If the construct did not contain a DUS sequence, the sequence was included at the 5′ end of the primer. Sequences were amplified by PCR with the following conditions: 95°C for 7 m, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 2 m 30 s and a final extension of 4 m at 72°C. Fragments were ligated by Gibson assembly, and the resulting plasmids were then transformed into *E. coli* DH5α and verified by sequencing. The plasmids were then used as a template to amplify the construct by PCR for transformation.

#### 2.2.6.1 Generation of *N. gonorrhoeae porB* mutants

Plasmids for modifying *porB* were kindly provided by Professor Seifert (Chen & Seifert, 2013) in pBluescript vectors. Plasmid DNA was linearised by digestion with *Bsa*I and *Sca*I (New England Biolabs) in CutSmart buffer for 1 h at 37°C. Digested products were purified by gel extraction and used to transform *N. gonorrhoeae* strain FA1090-G4.

#### 2.2.6.2 Generation of *N. gonorrhoeae* Δ*lst* strains

Fragments upstream and downstream of *lst* was amplified with primers 26/27 and 30/31 respectively using DNA from strain FA1090-G4. The erythromycin resistance gene

(*ermC*) was amplified from pNCC1 from the lab (Wörmann *et al.*, 2016) with primers EryF/EryR. pBluscript vector was amplified using primers 16/25. The four fragments were assembled by Gibson assembly and transformed into *E. coli* DH5α. Plasmid DNA was then linearised by digestion with *Bsa*I and *Sca*I (New England Biolabs) in CutSmart buffer for 1 h at 37°C, purified and used to transform *N. gonorrhoeae* strain FA1090-G4 as above .

### 2.2.6.3 Generation of Δ*pilD* strains as recipient strains for conjugation

FA1090-G4Δ*pilD* (generated by Dr Ana Cehovin), has *pilD* replaced with a kanamycin resistance cassette *aph(3)-III*. The Δ*pilD* construct was amplified from FA1090-G4Δ*pilD* with primers pilDupF and pilDdownR. The resulting fragment was purified and ligated into pCR™2.1-TOPO™ and transformed into *E. coli* DH5α to generate pCR™2.1-TOPO™-Δ*pilD*. The insert was amplified with pilDupF and pilDdownR, and the purified PCR product used to transform *N. gonorrhoeae*. Kanamycin resistant colonies were analysed by PCR using primers pilDupF and pilDdownR and verified by sequencing (Source Bioscience).

### 2.2.6.4 Generation of pConj carrying strains by conjugation

Donor (isolate 60755, a Kenyan isolate that naturally carries pConj variant 1) and recipient *N. gonorrhoeae* strains were grown overnight on solid GCB media. Bacteria were then harvested and mixed by plating onto GCB agar and left to incubate at 37°C, 5% $CO_2$ for 20 – 24 h. Bacteria were collected then plated on GCB agar containing appropriate antibiotics, either 2 µg/ml tetracycline (FA1090, NM8365) or 8 µg/ml ciprofloxacin + 2 µg/ml tetracycline (WHO K) to select for transconjugants. Individual colonies were then analysed by PCR with FwdPor and RevPor2 before sending for sequencing to confirm PorB sequences (Source Bioscience).

### 2.2.6.5 Generation of pC$^{GFP}$-carrying strains

Fragments containing *vapD* and *res* were amplified with primers 123/132 and 122/133, respectively. The 1.9 kb $P_{opaB}$*:gfp:kan* fragment was constructed by amplifying a 225 bp fragment of the *opaB* promoter ($P_{opaB}$) with primers S2/92 (Ramsey *et al.*, 2012), *gfp* (encoding superfolder GFP) from pNCC1-sfGFP (Wörmann *et al.*, 2016) with primers S3/S4 and the kanamycin resistance cassette *aph(3)-Ia* from *N. gonorrhoeae* PorB mutants previously generated, with primers KanF/KanR. The three fragments were assembled with the backbone of ptRNA plasmid (from Dr Guangyu Liu), a low copy number plasmid with pSC101 origin, which was amplified with primers 139/142, by Gibson assembly and transformed into *E. coli* DH5α. Plasmids containing the construct were used as a template to amplify the desired fragment with primers 120/121.

### 2.2.6.6 Generation of pCΔGL-carrying strains

A fragment upstream of the genetic load ("GL") region was amplified with primers 153/154 while the downstream region was amplified with primers 157/158 using DNA from strain 60755 as template. $P_{opaB}$*:gfp:kan* was amplified from plasmid DNA purified from *N. gonorrhoeae* FA1090:pC$^{GFP}$ using primers 155/156. The backbone of ptRNA was amplified with primers 139/142 and included with other fragments in a Gibson reaction; the product was used to transform *E. coli* DH5α. The insert was then amplified with primers 153/158 for *N. gonorrhoeae* transformation.

Using FA1090:pCΔGL as the donor, pCΔGL was introduced into recipient strains which carry pConj, selecting with 50 µg/ml kanamycin, followed by passaging on 200 µg/ml kanamycin. Tetracycline-sensitive, GFP-positive transconjugants were selected.

### 2.2.6.7 Generation of Δ*trbC* strain

Upstream and downstream regions flanking *trbC* were amplified with primer pairs 159/160 and 161/162, respectively. $P_{opaB}$*:galK* was amplified with primers 92/165 using FA1090 *fetA::aph(3)-galK* genomic DNA from Dr Ana Cehovin as template (Jones *et al.*, 2022), while the erythromycin resistance gene (*ermC*) was amplified from pNCC1 from

the lab (Wörmann *et al.*, 2016) with primers EryF2/EryR2. The $P_{opaB}$*:galK:ermC* cassette was constructed by including $P_{opaB}$*:galK* and *ermC* in an overlap PCR with primers 92/EryR2. The plasmid backbone of ptRNA was amplified with primers 139/142. The resulting fragments were joined by Gibson assembly and used to transform *E. coli* DH5α. Plasmids were then used as the template to amplify the fragment with primers 159/162. Purified PCR product was then used to transform *N. gonorrhoeae*. Colonies that grew on 2 µg/ml erythromycin were selected and passaged on 8 µg/ml erythromycin.

For markerless deletion, PCR was performed with primers 159/168 and 169/162 with pConj from strain 60755 as the template. Fragments were cloned into ptRNA using Gibson assembly and transformed into *E. coli* DH5α. The resulting plasmid was used as a template to amplify the desired fragment with primers 159/162, which was then used to transform *N. gonorrhoeae*. Transformants were selected on 6.4% (w/v) 2-DOG with tetracycline and passaged once on media containing 6.4% (w/v) 2-DOG. Colonies were then checked for tetracycline and erythromycin resistance.

### 2.2.6.8 Generation of single/ multiple toxin deletion on $pC^{GFP}$

Fragments flanking *vapD* were first amplified with primers 253/254 and S2/261. Primers 92/255 were then used to amplify the *gfp:kan* cassette and a fragment downstream of *ζ1*. Overlap PCR of these two fragments was performed using primers 253/255. The region between *ζ1* and *ζ2* was amplified with primers 256/257, and the fragment upstream of *ζ2* was amplified with primers 258/259. The subsequent overlap PCR was then carried out with primers 256/259. Fragments 253/255 and 256/259 were then joined with 139/142 ptRNA backbone.

For single inactivation of ζ toxin, $pC^{GFP}$ was used as the template. Fragments upstream and downstream of $\zeta^{K115}$ were amplified using primers 259/284 and 253/283, respectively. These primers introduce a K115A substitution (AAA to GCC at 343 – 345 bp of *ζ1*). For pCΔ*ζ2,* fragments flanking *ζ2* were amplified with primers 253/257 and 258/259, respectively. Fragments for each construct were ligated into ptRNA.

Transformed *N. gonorrhoeae* strains were checked using primers 266/Seq017 or Seq018/Seq017.

### 2.2.6.9 Generation of Δ*parB* strains

Sequences upstream and downstream of *parB* were amplified with primers 249/250 and 251/252, respectively using pConj as template. Separately, *ermC* was amplified with primers EryF2/EryR2 as previously. All three fragments were joined with ptRNA (amplified by primers 139/142) using Gibson assembly prior to transformation of *N. gonorrhoeae*.

### 2.2.6.10 Generation of constructs for toxicity assays

The backbone of pBAD33 was first amplified with primers 357/358 to introduce a ribosomal binding site. To construct pBAD-$vapD^{pConj}$, *vapD* was amplified with primers 359/362. Primers 359/349 were used to amplify *vapD* in both pBAD-$vapD^{pConj}vapX^{pCryp}$ and pBAD-$vapD^{pConj}vapX^{Nm}$ construct; however, for the former, $vapX^{pCryp}$ was amplified with primers 350/361. The $vapX^{Nm}$ gene, on the other hand, was constructed using primers 374/375 and 376/361 using $vapX^{pCryp}$ as template. All fragments were joined using NEB HiFi assembly to 357/358 and transformed into DH5α.

## 2.2.7 SDS-PAGE and Western blot analysis

*N. gonorrhoeae* was grown overnight and bacteria ($1 \times 10^9$ CFU) were resuspended in 100 µl 1 x SDS-PAGE loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) with 7.5% *β*-mercaptoethanol and boiled for 10 m. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels before staining with Coomassie Brilliant Blue or transferring to nitrocellulose membranes (Hybond-C extra, Amersham) using the Trans-Blot SD semi-dry transfer system (Bio-Rad, USA). Blots were blocked for 1 h in 3% w/v dry milk powder in PBS at 4°C. This was followed by incubation with α-PorB H5.2 supernatant (1:1,000 dilution, gift from Professor Marcia Hobbs) or α-Lst pAb (1:20,000 dilution, gift from Professor Richard Rest). Membranes were washed three times as before prior to labelling with goat α-rabbit HRP (Santa Cruz

Biotechnology, USA) at 1:10,000 dilution and subjected to additional washing before visualising with ECL (GE Healthcare). All antibodies were incubated for 1 h at 4°C in PBS + 5% milk + 0.1% Tween.

## 2.3 Flow cytometry

For detecting CFH and CFHR binding to *N. gonorrhoeae,* bacteria were grown on solid media prior to subculturing in 5 ml GCBL with or without 2 µg/ml cytidine 5' mono-phospho-N-acetylneuraminic acid (CMP-NANA, Sigma). At $OD_{600}$ 0.6 – 0.8, bacteria ($10^9$ CFU) were fixed in 1 ml 3% paraformaldehyde for 15 m, before washing with phosphate buffer saline (PBS) supplemented with 1 mM $MgCl_2$ and 1.2 mM $CaCl_2$ (supplemented PBS/$PBS^+$, Sigma). Samples were stored at -20°C prior to flow cytometry.

To detect CFH or CFHR binding, $10^8$ CFU of fixed bacteria were re-suspended in 40 µl of normal human serum (NHS) (H6914, Merck), recombinant CFHR5 (0.65 µM, R&D), or purified CFH (0.65 µM, Sigma) diluted in $PBS^+$ for 30 m at room temperature. After two washes in $PBS^+$, bacteria were incubated in 100 µl of an α-CFHR mAb cell supernatant, purified OX-24 (10 µg/ml), or purified α-CFHR5 mAb (1 µg/ml) in 1% (w/v) bovine serum albumin (BSA) in $PBS^+$ for 30 m at room temperature. All wash steps were performed with $PBS^+$. To visualise binding, cells were resuspended in 100 µl of goat anti-mouse IgG-Alexa fluor® 647 conjugate pAb (#A-21235, Molecular Probes, Life Technologies) or goat α-mouse IgM Alexa Fluor 488-conjugated pAb (#A-21042, Molecular Probes, Life Technologies) for CFHR4 and incubated for 30 m at 4°C. Samples were run on a FACSCalibur (BD Biosciences), and at least $10^4$ events recorded. Results were analysed by calculating the geometric mean fluorescence (FL-4) in FlowJo V10.8.1 software (Tree Star).

To detect PorB on *N. gonorrhoeae*, bacteria were incubated with H5.2 supernatant (1:200 dilution) in 1% (w/v) BSA/$PBS^+$ for 30 m at room temperature (primary antibody) before visualising with a goat α-mouse IgG Alexa Fluor 647-conjugated pAb (#A-21235, Molecular Probes, Life Technologies) as above. Separately, to detect sialylation on *N.*

*gonorrhoeae*, bacteria were incubated either with neat 3F11 supernatant or 1:100 dilution of 3F11 antibody (AB_2617191, DSHB, USA) in 1% (w/v) BSA/PBS$^{+}$ for 30 m at room temperature (primary antibody) before visualising with goat α-mouse IgM Alexa Fluor 647-conjugated pAb (#A-21238, Molecular Probes, Life Technologies) as described above.

## 2.4 Conjugation assays

Strains were grown on GCB agar overnight, harvested then used to inoculate 5 ml GCBL with 1% vitox (Oxoid, v/v) at $OD_{600}$ 0.1 in and incubated at 37°C in 5% $CO_2$ with shaking at 180 r.p.m. until cultures reached an $OD_{600}$ 0.6 – 1.0. To investigate the effect of the recipient, 5 x $10^5$ CFU of strain 60755 were used as the donor and mixed with 5 x $10^4$ CFU of each recipient in 10 µl spots. To investigate the effect of donors, 5 x $10^4$ CFU of donor were added to 5 x $10^4$ CFU of the recipient. Conjugation spots were left to dry, and bacteria were incubated for 23 – 25 h before they were harvested and resuspended into 1 ml GCBL, and plated to GCB media containing appropriate antibiotics. Conjugation frequencies were calculated as the percentage of recipients that were transconjugants at the end of the experiment:

$$\text{Conjugation frequency} = \frac{Colonies\ on\ Kan{+}Tet\ (transconjugants\ at\ the\ end\ of\ the\ experiment)}{Colonies\ on\ Kan\ (recipients\ at\ the\ end\ of\ the\ experiment)} \times\ 100\%$$

## 2.5 qPCR

To determine the copy number of *recA* and *res*, fragments of *recA* and *res* were amplified using primers Q7/Q8 and 121/119 for *recA* and *res*, respectively and cloned into pCR™2.1-TOPO™. Standard curves were calculated by determining the linear regression of cycle threshold (Ct) to the DNA template concentration using the equation y = Bx + A, where y is the Ct value and x is the DNA concentration, and A and B are constants to be determined. Primer efficiency was determined by the following formula:

$$\text{Primer efficiency (\%)} = \left(10^{\frac{-1}{Slope}} - 1\right) \times 100$$

Bacteria were grown in GCBL with 1% vitox to an $OD_{600}$ 0.5 – 0.8, and two 10 µl aliquotes were washed in $H_2O$, then resuspended in 100 µl $H_2O$ and boiled for 5 m at 95°C. The purified plasmid or lysate (1 µl) was then added to 19 µl of qPCR Power SYBR green PCR mastermix (Applied Biosystems) containing 10 µl of 2 x SYBR, 1 µl of 10 µM forward and reverse primers each and 7 µl of DNase-free water. All reactions were run on a 96-well plate on the QuantStudio Real-Time PCR System (Applied Biosystems) with thermal cycling conditions as follows: 1) Hold stage: 50°C for 2 m followed by 95°C, 10 m; 2) PCR stage consisting of 40 cycles of 95°C for 15 s before 60°C for 1 m; 3) Melt curve stage consisting of 95°C for 15 s, 60°C for 1 m and finally 95°C, 15 s. Ct values obtained for individual colonies were compared to the standard curve previously obtained with purified TOPO plasmid. Plasmid copy number ratio were determined as the ratio between *res* (pConj) and *recA* (chromosome).

## 2.6 Fitness cost assays

Strains were grown on solid media overnight. Liquid cultures of GCBL + 1% vitox was inoculated at $OD_{600}$ 0.1. Liquid cultures (200 µl) were incubated in 37°C, 5% $CO_2$, shaking at 180 – 200 r.p.m., and $OD_{600}$ measurements were taken every 20 m for 8 h using a FLUOstar Omega Microplate Reader (BMG Labtech). To determine fitness cost during long term growth, 15 ml FB + 1% vitox were inoculated with 5 x $10^4$ CFU/ml bacteria and incubated at 37°C in 5% $CO_2$ with shaking at 180 r.p.m. The number of viable bacteria was measured at each time point.

## 2.7 Competition assays

Liquid cultures (200 µl) were prepared and grown as above, but where plasmid-carrying and plasmid-free strains were mixed at a 1:1 ratio at the start of the experiment. Cultures were incubated for 8 h at 180 – 200 r.p.m. to reduce conjugation. Exact proportions of plasmid-carrying and plasmid-free strains before and after 8 h were determined by plating to solid media and assaying 50 – 300 colonies. At the end of experiments, at least 10 white and 10 green colonies were picked and replica plated onto GCB with

tetracycline. Serial dilutions were also carried out to determine CFU at 0 h and 8 h. Fitness was determined as per previous (San Millan *et al.*, 2014b), as follows:

$$Fitness = \frac{\ln(\frac{Nfp}{Nip})}{\ln(\frac{Nf}{Ni})}$$

where *Nfp* is the number of plasmid-carrying bacteria at the end of experiments, *Nip* is the number of plasmid-carrying bacteria at the start of experiments, *Nf* is the number of plasmid-free bacteria at the end of experiments, *Ni* is the number of plasmid-free bacteria at the start of experiments. Fitness > 1 indicates that plasmid-carrying strains have a fitness advantage.

## 2.8 Plasmid maintenance assays

Bacteria were grown overnight on GCB agar containing antibiotics to select the plasmid, then used to inoculate into 5 ml FB + 1% vitox at $OD_{600}$ 0.1. Cultures were then incubated at 37°C, 5% $CO_2$, shaking at 180 r.p.m. and passaged every 20 h as required (30 – 40 generations). Alternatively, two individual colonies were picked and inoculated in 5 ml FB + 1% vitox before growing for 20 h. To detect plasmid loss, cultures were serially diluted and plated to solid media. Between 100 – 1000 colonies were visualised with a UV transilluminator (Invitrogen), and the number of green and white colonies counted. In every experiment, all white colonies (up to 20) and 20 green colonies were picked and replica plated onto GCB with tetracycline to confirm the presence or absence of pConj.

## 2.9 Minimal Inhibitory Concentration (MIC) assays

MICs were determined as previously (World Health Organization, 2013); briefly, strains were first grown overnight on GCB agar. Bacteria were harvested in GCBL, and then spotted ($10^4$ in 5 µl) onto GCB plates containing a range of ciprofloxacin or spectinomycin concentrations. The lowest concentration of antibiotics where no growth was observed was determined to be the MIC.

## 2.10 Plasmid curing

Bacteria were grown overnight on GCB agar, then used to inoculate into 5 ml FB + 1% vitox at 5 x $10^4$ CFU/ml. Cultures were then incubated at 37°C, 5% $CO_2$ without shaking for 48 h with 0.5 MIC of ciprofloxacin or spectinomycin. Cultures were then serially diluted and 100 – 1000 colonies were plated and assessed for plasmid loss as above.

## 2.11 Toxicity/ antitoxicity assays

*E. coli* strains were grown overnight (22 – 24 h) in 5 ml LB + chloramphenicol + 0.2% (w/v) glucose to repress expression of toxins. Bacteria were then subcultured into 5 ml LB + 0.2% glucose at $OD_{600}$ 0.01 and grown till $OD_{600}$ 0.1. Cultures were then centrifuged and washed with 1 ml LB + 0.2% (w/v) arabinose before incubating in 5 ml LB + 0.2% arabinose at 37°C, 180 r.p.m. Samples were taken at 0, 30 m, 1 h, 3 h and 6 h time points. Serial dilution of the samples was then carried out and spotted onto LB + 0.2% glucose + chloramphenicol plates for CFU counting.

## 2.12 Tn-Seq

### 2.12.1 Generating pHsk1 for transposon mutagenesis

pCE005 (gift from Dr Cara Ellison) contains *ermC* flanked by inverted repeats recognised by the HsmarI transposase, with a kanamycin resistance cassette in the plasmid backbone. pHsk1 was generated from this plasmid, and consists of the kanamycin resistance gene (*aph(3)-Ia*) flanked by the Hsmar inverted repeats, with *ermC* in the plasmid backbone. To generate pHsk1, four fragments were amplified: the kanamycin (*aph(3)-Ia*) and erythromycin (*ermC*) resistance genes, amplified with primers KanF/KanR, and EryF2/EryR2 respectively, and two regions of the plasmid backbone, amplified by primer pairs H1/H4, and H2/H3. All fragments were included in a Gibson assembly reaction (New England Biolabs), which was used to transform *E. coli* DH5α.

### 2.12.2 Transposon library construction

A library of *N. gonorrhoeae* FA1090 transposon mutants was generated by *in vitro* mutagenesis. In each 100 µl transposition reaction, 30 µg genomic DNA was subjected to mutagenesis with 125 nM Hsmar transposase with 5 µg pHsk1 in reaction buffer (20 mM Tris-HCl pH 8, 100 mM NaCl, 10% glycerol, 2 mM DTT, 2.5 mM $MgCl_2$) for 18 h at 30°C. Eight transposition reactions were performed, and the products were precipitated by the addition of 10 µl 3 M sodium acetate pH 5.2 and 800 µl isopropanol and incubation on ice for 20 m; the DNA pellet was collected by centrifuging at 18,000 x *g* for 5 m. The DNA pellet was washed three times with 70% ethanol and finally resuspended in 30 µl $ddH_2O$. To fill and repair single-stranded regions following transposon insertion, the resulting DNA was then added to ligation and repair mix consisting of T4 DNA ligase buffer (Invitrogen), 50 mM NaCl, 0.5 mM dNTPs, 10 µl T4 DNA polymerase (50U, Thermofisher) and 10 µl T4 DNA ligase (10U, Invitrogen). The mixture was incubated at room temperature for 90 m.

*N. gonorrhoeae* FA1090 was grown overnight on GCB agar plates. The next day, repaired DNA (375 ng in 25 µl) was spotted onto GCB plates for each transformation, before *N. gonorrhoeae* FA1090 was streaked over. Eighty-one transformations were performed, before plating onto solid media containing 80 µg/ml kanamycin. Genomic DNA of bacteria in each experiment were extracted using Wizard Genomic DNA Purification kit (Promega, USA). Equimolar of genomic DNA from each experiment were then pooled and sent for sequencing with Quadram Institute (**Figure 2.1A**).

### 2.12.3 Investigating chromosomal genes involved in pConj biology

The mutant library was used as the recipient in conjugation with FA1090:pConj as the donor. The library and donor were grown separately from an $OD_{600}$ 0.1 to $OD_{600}$ 0.6 – 0.8 in 15 ml GCBL, by shaking at 180 r.p.m. in 37°C, 5% $CO_2$. Equal numbers of the recipient and donor were mixed, and $10^5$ CFU in 10 µl were added as spots onto GCB

plates and incubated for 24 h at 37°C in 5% $CO_2$; a total of 72 conjugations spots were performed. After incubation, bacteria were harvested and plated on GCB containing 50 µg/ml kanamycin (to select for the recipient) or GCB containing 50 µg/ml kanamycin and 2 µg/ml tetracycline (to select for transconjugants). After 40 h, bacteria were harvested with pre-warmed GCB. Genomic DNA was extracted from $10^{10}$ CFU of the recipient or transconjugants. Equimolar of genomic DNA from recipient and transconjugants from each experiment were then pooled and sent for sequencing with Quadram Institute.

Following conjugation, bacteria recovered on plates containing kanamycin and tetracycline (to select for pConj transfer) were grown in GCB with 1% vitox for 20 h starting at $OD_{600}$ 0.3 at 180 r.p.m., 5% $CO_2$, 37°C for recovery. The library containing pConj was grown at 150 r.p.m., 5% $CO_2$, 37°C and passaged every 12 h starting at $OD_{600}$ 0.1 in 15 ml FB + 1% vitox with or without tetracycline. Cultures were performed in duplicate; kanamycin was added to select for the presence of the transposon. A total of 14 passages (approx. 50 generations) were performed over 7 days; the tetracycline concentration was increased to 10 µg/ml on the last passage. Genomic DNA from the $2^{nd}$, $4^{th}$ and $14^{th}$ passage ($8^{th}$, $16^{th}$ and $56^{th}$ generation respectively) was sent for sequencing with Quadram Institute (**Figure 2.1B**).

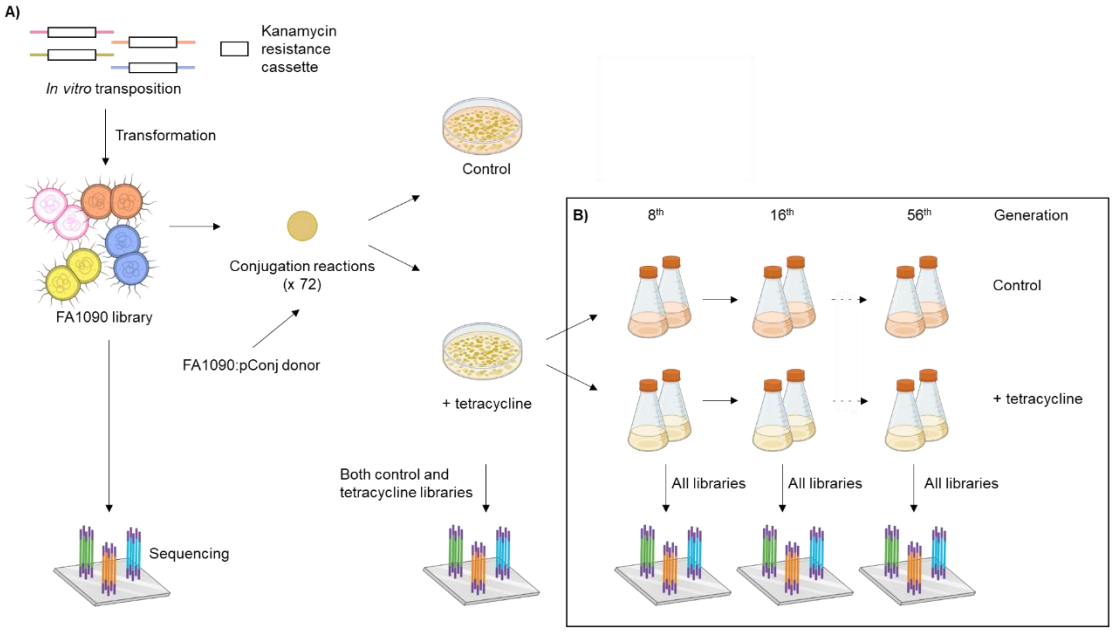



**Figure 2.1 Tn-Seq workflow. A)** *N. gonorrhoeae* FA1090 mutant library is first generated using *in vitro* transposition followed by transformation before pConj was introduced into the library. Bacteria were selected on kanamycin (control) and kanamycin + tetracycline to select for pConj transfer. **B)** pConj-carrying FA1090 library was then passaged over 56 generations in the presence/absence of tetracycline to investigate genes involved in pConj maintenance. Figure was constructed with BioRender.

## 2.13 Bioinformatic analysis

### 2.13.1 Genetic and amino acid sequences

*N. gonorrhoeae* genome sequences were obtained from the *Neisseria* genome database at PubMLST (http://pubmlst.org/neisseria/) (Jolley *et al.*, 2018). Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches were conducted on the NCBI database. Nucleotide sequences were translated using Transseq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Nucleotide or protein sequences were aligned using ClustalOmega (Sievers *et al.*, 2011) and visualised with Jalview (Waterhouse *et al.*, 2009). Snapgene (GSL Biotech) was used to construct genetic maps, annotate features automatically and design primers. Alignments of *Eikenella exigua* and *Inoviridae* spp. with pConj were carried out with EasyFig (Sullivan *et al.*, 2011).

For data analysis involving pConj, pCryp and p*bla* carriage in *Neisseria* spp., all isolates were confirmed to have a sequence bin > 1 Mb and contigs ≤ 200 on the PubMLST database to ensure that sequences available were complete and well sequenced. Presence of *trbC* or *traM* denoted pConj carriage (as above), while presence of *repA* or *mobC* denoted pCryp presence. Metadata of all gonococcal isolates are in **Appendix Table 7.1**; metadata of all other *Neisseria* spp. isolates are in **Appendix Table 7.3**.

For analysis and visualisation of Tn-Seq data, the Bio-TRADIS (Barquist *et al.*, 2016) package was used. Insertions were separately visualised on Artemis (Carver *et al.*, 2012).

### 2.13.2 Protein structure

Protein structure prediction was analysed with Phyre v 2.0 (Kelley *et al.*, 2015), HHpred (Söding *et al.*, 2005) and AlphaFold (Jumper *et al.*, 2021). Predicted structures were visualised using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrodinger, LLC.).

### 2.13.3 Construction of phylogenetic tree

For generation of minimum-spanning tree, allelic profiles of *N. gonorrhoeae* isolates (n = 9,618; metadata in **Appendix Table 7.1**) were first selected on PubMLST. The phylogenetic tree was then constructed and visualised with the GrapeTree plugin on PubMLST using the cgMLST v1.0 scheme.

For generation of pConj maximum likelihood tree, genetic sequences from 126 isolates (metadata in **Appendix Table 7.4**) were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) on the Galaxy platform (https://usegalaxy.org). Alignments were subjected to RAXML (Stamatakis, 2014) using the GTRGAMMA model of nucleotide substitution and 100 bootstrap replicates as per previously described (Sánchez-Busó *et al.*, 2019a), and further checked using RAXML-NG with the default parameters, which combines the starting phylogenetic tree search and bootstrapping analysis (Kozlov *et al.*, 2019). Separately, the FASTA alignment was converted to PhyLIP format using R version 4.2.0. The resulting PhyLIP interleaved file was then input into PhyML (Guindon *et al.*, 2010) and subsequently ClonalFrameML (Didelot & Wilson, 2015) for tree construction. All trees are visualised using the MEGA (Kumar *et al.*, 2018) and FigTree (http://tree.bio.ed.ac.uk/software/figtree/) softwares.

### 2.13.4 Statistical analyses

Statistical analyses of experiments, including linear regression, student *t*-test, Fisher's exact test, area under curve, one-way ANOVA and two-way ANOVA with multiple comparisons were carried out using PRISM v 8.0. For analysis of Tn-Seq data, the Bio-TRADIS package was used to determine any statistically significant hits ($q$-value < 0.01).

# 3 Characterising interactions between *N. gonorrhoeae* and complement

*N. gonorrhoeae* is a human specific pathogen that must evade killing by the immune system, including the complement system (Quillin & Seifert, 2018). Specifically, *N. gonorrhoeae* binds to CFH and C4BP, both of which are negative regulators of the complement system (Blom *et al.*, 2004; Gordon *et al.*, 1995). CFH prevents the formation of C3 convertase of the AP (C3bBb) and accelerates its decay, while C4BP reduces activation of the CP (Noris & Remuzzi, 2013). *N. gonorrhoeae* interacts with CFH *via* an essential porin, PorB, when its LOS is sialylated (Chen & Seifert, 2013; Ram *et al.*, 1998).

In this chapter, I investigated whether *N. gonorrhoeae* binds to other components of the complement system. In addition to CFH, humans express five CFHRs, CFHR1 to 5. CFHRs are structurally related to CFH although they do not have the regulatory domains present in $CFH_{(1\text{-}4)}$ (Goicoechea de Jorge *et al.*, 2013). Therefore, CFHRs are unable to negatively regulate the complement system. However, it has been shown that CFHRs have a role in complement-mediated immunity, as alterations in genes encoding CFHRs lead to complement diseases such as aHUS and C3 glomerulopathy (Zipfel *et al.*, 2020).

CFHRs consist of four (CFHR2) to nine (CFHR4A and 5) CCPs (**Figure 1.5**). Previously, CFHR3 has been shown to compete for CFH binding to fHbp on *N. meningitidis* (Caesar *et al.*, 2014). CFHR3 acts as a competitor of CFH due to amino acid similarity between $CFHR3_{(1,2)}$ and $CFH_{(6,7)}$, which both exhibit high affinity for fHbp (Schneider *et al.*, 2009; Caesar *et al.*, 2014). Similarly, CFHR1 binds to *N. gonorrhoeae* due to high amino acid sequence similarity with $CFH_{(19\text{-}20)}$ (Agarwal *et al.*, 2010). Given the sequence similarity between CFH and CFHRs, we decided to investigate if other CFHRs can also bind to *N. gonorrhoeae*. This work has been published in Infection and Immunity (Yee *et al.*, 2022).

## 3.1 *N. gonorrhoeae* binds to CFHR5 in the presence of sialylated LOS, but not other CFHRs tested

$CFH_{(19-20)}$ was previously shown to be responsible for interacting with *N. gonorrhoeae* strains 252 (which expresses P.IA porin) and sialylated F62 (P.IB porin strain) (Ngampasutadol *et al.*, 2008). Alignment of CFHR sequences with CFH revealed that $CFHR2_{(3,4)}$ and $CFHR5_{(8,9)}$ show similarity with $CFH_{(19-20)}$ ($CFHR2_{(3,4)}$, 89% and 61%; $CFHR5_{(8,9)}$: 66% and 43% similarity to $CFH_{(19,20)}$, respectively) (Goicoechea de Jorge *et al.*, 2013). Therefore, we aimed to determine if these CFHRs bind to *N. gonorrhoeae*.

To determine whether CFHRs bind to *N. gonorrhoeae*, strain FA1090 was grown in GCBL with or without 2 µM CMP-NANA, and then fixed in 3% PFA as previously described (Chen & Seifert, 2013). The FA1090-G4 strain (from Professor Hank Seifert) was used for all experiments in this chapter as it was the only FA1090 strain available. This FA1090 derivative carries a mutation in the G4 locus, resulting in constitutive pilin expression (Cahoon & Seifert, 2009). mAb 3F11 (DSHB, USA) was used in flow cytometry to determine whether *N. gonorrhoeae* grown in the presence of CMP-NANA expresses sialylated LOS. mAb 3F11 binds to the unsialylated Galβ1-4GlcNac epitope found on LOS (Apicella *et al.*, 1981); sialylation thus abolishes 3F11 binding (Mandrell *et al.*, 1988). All fixed *N. gonorrhoeae* samples were checked for sialylation prior to experiments (**Appendix Figure 7.1**).

Bacteria were incubated in neat NHS as the source of CFH and CFHRs as CFHRs are present in human sera (Skerka *et al.*, 2013). Use of NHS ensures that all CFHRs are post translationally modified (Abarrategui-Garrido *et al.*, 2009). Binding was detected using mAbs specific for CFH (OX24, gift from Professor Robert Sim) or for CFHRs 2 – 5 (generated by Dr. Hayley Lavender), and detected by flow cytometry. Of note, all mAbs are IgGs, except for the α-CFHR4 mAb, which is an IgM. The specificity of the antibodies was established by Western blot analysis of NHS by Dr Hayley Lavender (Yee *et al.*, 2022). Results show that there was no detectable binding of CFHRs 2, 3 or 4 to either

sialylated or non sialylated bacteria (**Figure 3.1A**). In contrast, similar to CFH, CFHR5 binding to bacteria was observed when their LOS was sialylated (**Figure 3.1A**).

To confirm that binding of CFH and CFHR5 is specific, bacteria were incubated in serial dilutions of NHS (**Figure 3.1B**). Results show that binding of CFHR5 and CFH exhibits dose-dependency, and saturation is reached at neat concentrations. This suggests that binding of CFH and CFHR5 is specific. To provide further evidence that CFHR5 and CFH binding was specific and not due to other proteins in serum, *N. gonorrhoeae* was incubated in 0.65 µM of purified CFHR5 (R&D) or CFH (Sigma) instead of NHS. Binding was detected by flow cytometry as above. Results demonstrate that purified CFH and CFHR5 bind to *N. gonorrhoeae* only occurred when LOS was sialylated (**Figures 3.1C – D**). These prove that CFH and CFHR5 binding to *N. gonorrhoeae* is specific.

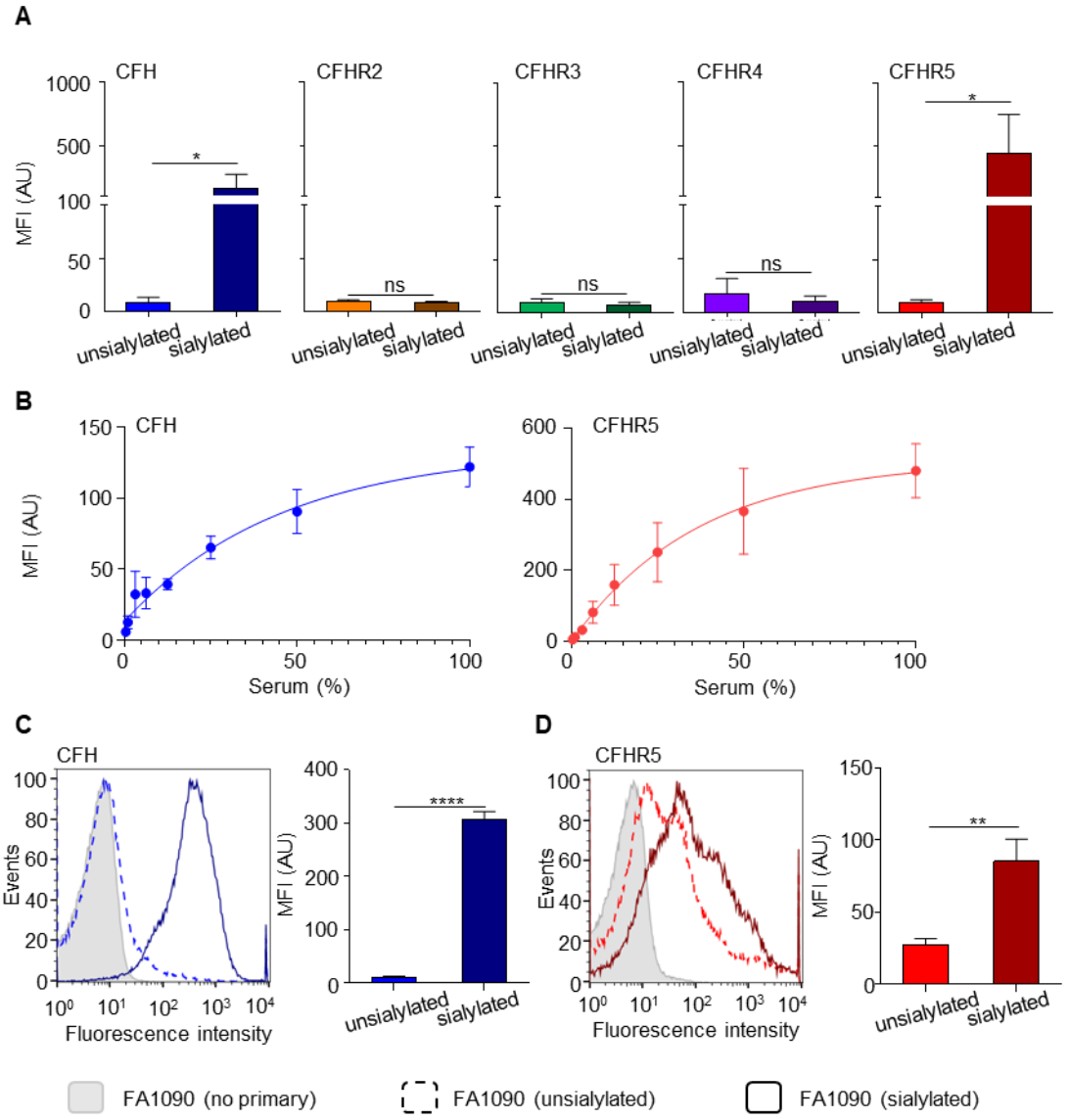



**Figure 3.1 CFHR5 binds to FA1090. A)** *N. gonorrhoeae* FA1090 was incubated in neat normal human serum (NHS) as the source of complement factor H (CFH) (blue) and other complement factor H related proteins (CFHRs), in particular CFHR2 (orange), CFHR3 (green), CFHR4 (purple) and CFHR5 (red). Binding of CFH and CFHRs to sialylated or unsialylated bacteria detected with specific mAbs. **B)** Normal human serum (NHS) was serially diluted in PBS before incubation with *N. gonorrhoeae*. To test specificity of binding, FA1090 was incubated with purified **C)** CFH or **D)** CFHR5 and binding detected as before. For all experiments, mean fluorescence intensity (MFI) in arbitrary units (AU) and s.d. of independent biological repeats (n $\geqslant$ 3) are indicated. Values (in arbitrary units, AU) were analysed with unpaired *t*-test. Significant binding was observed for CFH and CFHR5. ns, $p \geqslant 0.05$; *, $p \leqslant 0.05$, **, $p \leqslant 0.01$, ****, $p \leqslant 0.0001$

Sialylation of LOS requires an exogenous source of sialic acid and expression of the 43 kDa Lst (Shell *et al.*, 2002; Mandrell *et al.*, 1990); deletion of *lst* results in loss of LOS sialyation (Lewis *et al.*, 2015). To confirm that CFHR5 binding to the gonococcus is dependent on LOS sialylation, an *lst* mutant was constructed. The *lst* open reading frame was replaced with an erythromycin resistance gene (*ermC*) in *N. gonorrhoeae* FA1090 (**Figure 3.2A**). Western blot analysis with an α-Lst pAb (gift from Professor Richard Rest) comparing wild-type FA1090 and FA1090Δ*lst* confirmed that Lst is not expressed in FA1090Δ*lst* (**Figure 3.2B**). Results show that FA1090Δ*lst* does not bind CFHR5 (provided in NHS) even when the strain was grown in CMP-NANA (**Figure 3.2C**). This confirms that CFHR5 binding requires a functional Lst.

Overall, these experiments demonstrate that CFHR5 binds to *N. gonorrhoeae*, with this interaction dependent on LOS sialylation by Lst.

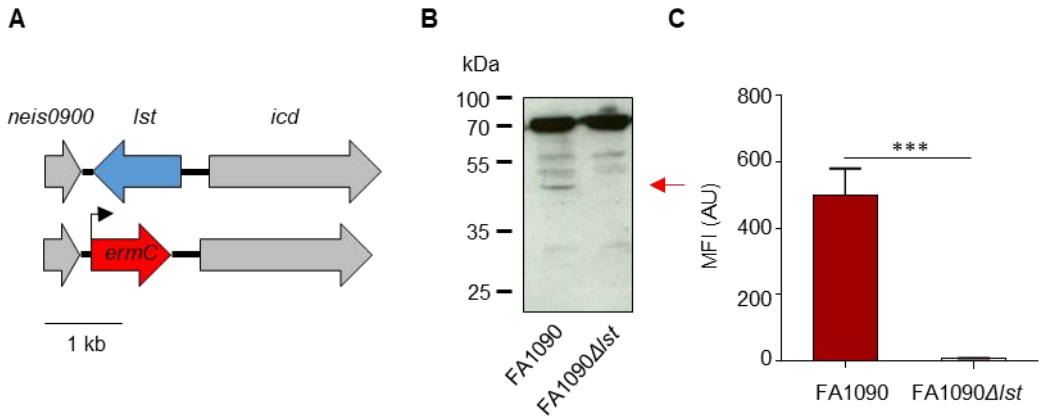



**Figure 3.2 Sialylation of LOS *via* Lst is required for CFHR5 binding. A)** Schematic of the deletion of LOS-specific α-2,3-sialyltransferase gene, *lst*, in FA1090. *neis0900* encodes a putative C-type cytochrome and *icd* encodes isocitrate dehydrogenase, as shown. The *ermC* cassette carries its own promoter but has no terminator. **B)** Western blot analysis of *N. gonorrhoeae* FA1090 and FA1090Δ*lst* using α-Lst pAbs revealed that no Lst was detected in FA1090Δ*lst* (red arrow). Lst is approximately 43 kDa. **C)** Flow cytometry shows that FA1090, but not FA1090Δ*lst*, binds serum-derived CFHR5 when grown in the presence of cytidine 5' mono-phospho-N-acetylneuraminic acid (CMP-NANA). Values (in arbitrary units, AU) of mean fluorescence intensity (MFI) in arbitrary units (AU) and s.d. of independent flow cytometry experiments ($n \geqslant 3$) are shown; significance was analysed by unpaired *t*-test. ***, $p \leqslant 0.01$

## 3.2 *N. gonorrhoeae* binds to CFHR5 and CFH via loops 3-7

As CFHR5 binds to *N. gonorrhoeae* when LOS is sialylated, we hypothesised that CFHR5 also binds to *N. gonorrhoeae* PorB similar to CFH (Chen & Seifert, 2013). As *porB* is essential, the external loops of PorB were modified as previously carried out to characterise CFH and C4BP binding, to determine PorB interactions with CFHR5 (Chen & Seifert, 2013).

Constructs encoding for PorB modification were obtained from Professor Hank Seifert and linearised before transforming FA1090 and selecting for transformants. *porB* sequences of transformants were verified by PCR and sequencing. The FA1090 *porB* mutants (**Figure 3.3**) were confirmed to carry either alanine substitutions or deletions in loops (denoted as $Ln^{xxx \rightarrow Ala}$, with Ln representing the PorB loop and x representing the number(s) of the substituted (→) or deleted (Δ) amino acid(s)).

**A**

| Mutant | Amino acid sequence of modified PorB |
|---|---|
| $L1^{43-48 \rightarrow Ala}$ | KVSKVE to AAAAAA |
| $L2^{83-88 \rightarrow Ala}$ | SVAGTN to AAAAAA |
| $L3^{116-121 \rightarrow Ala}$ | LKNTG to AAAAA |
| $L3^{134-139 \rightarrow Ala}$ | NVLEIS to AAAAAA |
| $L4^{\Delta 171-176}$ | Deletion of DNSGSN |
| $L5^{\Delta 203-224}$ | Deletion of GEGKKIEYDGQTYSIPSLFVE |
| $L6^{L253M}$ | L253M |
| $L6^{254-259 \rightarrow Ala}$ | YGAMSG to AAAAAA |
| $L7^{290-295 \rightarrow Ala}$ | KGTVDS to AAAAAA |

**B**

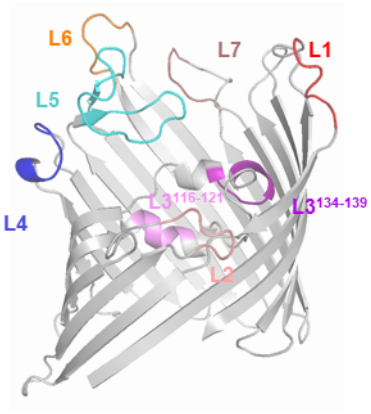



**Figure 3.3 PorB mutants generated in FA1090 A)** Table of *N. gonorrhoeae* FA1090 mutants with the sequence of the modified PorB, with sites of modification shown on **B)** the predicted structure of *N. gonorrhoeae* PorB, modelled using Phyre V 2.0 with the meningococcal PorB (PDB 3VZT) as the template.

The alteration of PorB might affect the level of PorB expressed by bacteria. As a result, changes in CFH or CFHR5 binding observed could be due to PorB levels instead of loop modifications. Therefore, to determine the level of PorB expressed by strains, mAb H5.2 (also referred to as H5; gift from Professor Marcia Hobbs), which binds to FA1090 PorB (Olesky *et al.*, 2002) was used to detect PorB levels in the mutants. Mutants with modified PorB loops were confirmed to express similar levels of PorB as wild-type FA1090 by Coomassie staining of cell lysates, Western blot analysis and flow cytometry (**Figure 3.4A-C**). $L5^{\Delta 203-224}$ could not be detected by mAb H5.2, suggesting that the antibody binds to an epitope in PorB loop 5. Therefore, the PorB expression level of the $L5^{\Delta 203-224}$ mutant was confirmed only by Coomassie staining. Additionally, no difference in growth rate of the strains was seen in GCBL over four hours (**Figure 3.4D**).

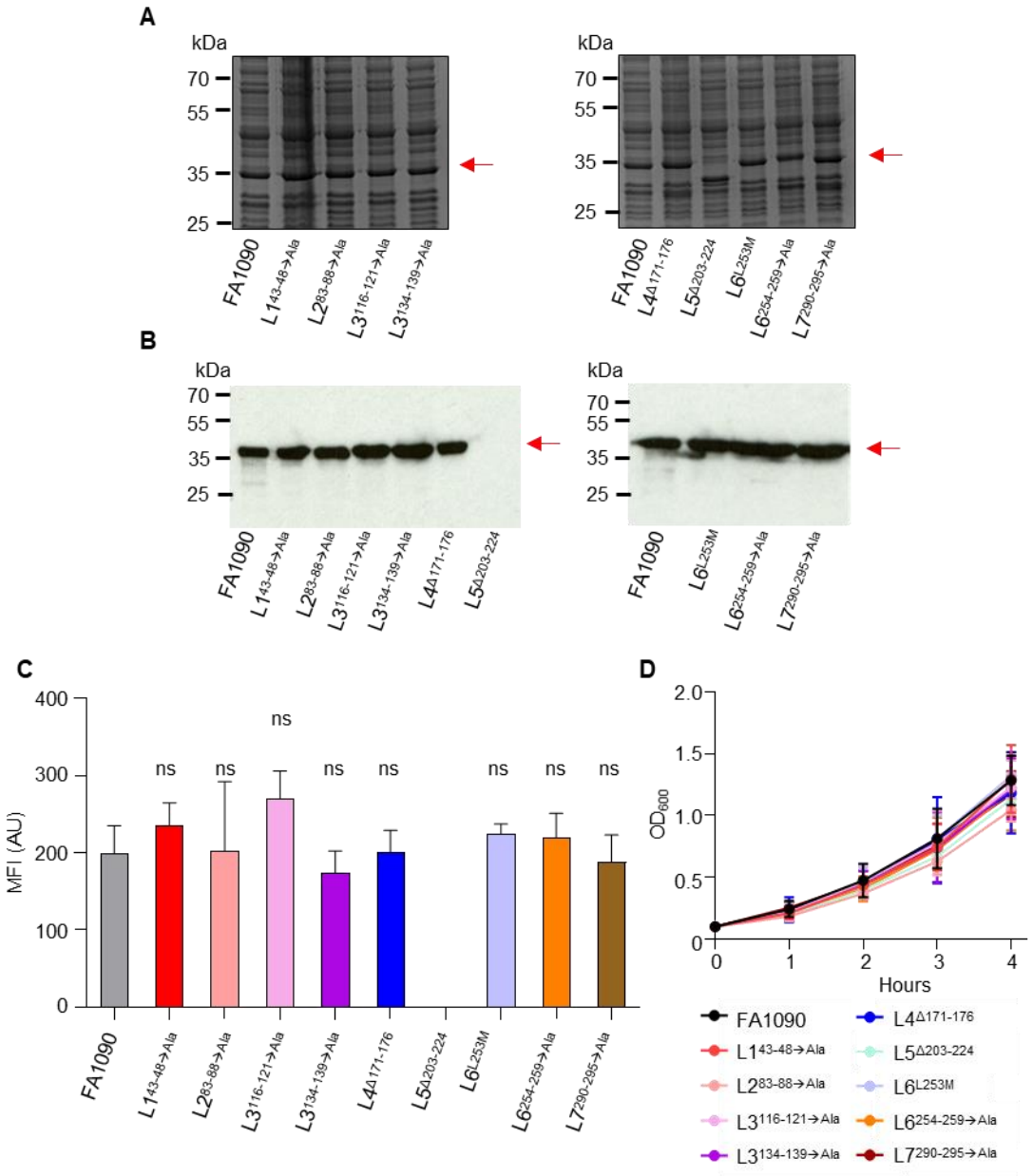



**Figure 3.4 PorB mutants do not show differences in PorB expression or growth.** Analysis of PorB (predicted molecular weight 36 kDa) expression by strains with **A)** Coomassie Blue staining, **B)** by Western blot analysis and **C)** Flow cytometry, where values are shown as mean fluorescence intensity (MFI) in arbitrary units (AU). The H5.2 mAb was used for western blot analysis and flow cytometry. **D)** There are no differences in growth of loop mutants over four hours. Significance was analysed using one-way ANOVA with Dunnett's multiple comparisons (flow cytometry) or two-way ANOVA (growth curve), with wild-type *N. gonorrhoeae* FA1090 as the control. All graphs (n ⩾ 3) show mean±s.d. ns, $p \geq 0.05$

To assess CFH and CFHR5 binding to the *porB* mutants, bacteria were first incubated in neat NHS before binding was detected using OX-24 or the α-CFHR5 mAb, respectively, by flow cytometry. There was no detectable binding of CFH or CFHR5 binding to strains $L3^{116\text{-}121\rightarrow Ala}$, $L4^{\Delta 171\text{-}176}$, $L5^{\Delta 203\text{-}224}$ and $L7^{290\text{-}295\rightarrow Ala}$ (**Figure 3.5**, $p \leq 0.0001$, compared to wild-type FA1090, one-way ANOVA with Dunnett's multiple comparisons). In contrast, there was no change in CFH or CFHR5 binding to *N. gonorrhoeae* expressing $L2^{83\text{-}88\rightarrow Ala}$ and $L3^{134\text{-}139\rightarrow Ala}$ compared with the wild-type strain (**Figure 3.5**, $p \geq 0.05$, one-way ANOVA with Dunnett's multiple comparisons). Of note, $L6^{254\text{-}259\rightarrow Ala}$ showed a significant decrease in CFH binding compared with wild-type bacteria; CFHR5 binding to this strain was undetectable ($p \leq 0.0001$, one-way ANOVA with Dunnett's multiple comparisons). The single amino acid substitution $L6^{L253M}$ did not affect CFH binding, but resulted in a significant decrease in CFHR5 binding ($p \leqslant 0.05$ using one-way ANOVA with Dunnett's multiple comparisons). In contrast, strain $L1^{43\text{-}48\rightarrow Ala}$ exhibited a significant decrease in CFH binding ($p \leq 0.01$, one-way ANOVA with Dunnett's multiple comparisons), while CFHR5 binding was unchanged (**Figure 3.5**). Overall, results suggest that while most PorB modifications have similar effects on CFHR5 and CFH binding, there were significant differences between CFHR5 and CFH binding to strains with changes in loops 1 and 6 of PorB.

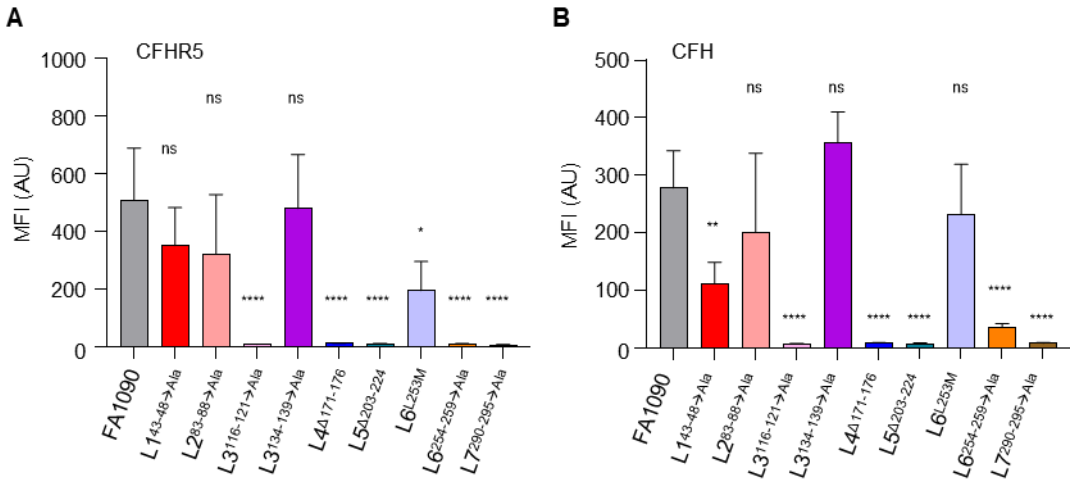



**Figure 3.5 *N. gonorrhoeae* FA1090 with mutations in loops 1 and 6 exhibit differential binding to CFH and CFHR5.** *N. gonorrhoeae* FA1090 was first fixed and incubated in neat normal human serum (NHS). Binding of **A)** Complement factor H related protein 5 (CFHR5) and **B)** Complement factor H (CFH) to FA1090 loop mutants was determined by flow cytometry using α-CFHR5 and OX-24 respectively. Results are shown as mean fluorescence intensity (MFI) in arbitrary units (AU) with s.d. (n ≥ 3). Significance was analysed using one-way ANOVA with Dunnett's multiple comparisons, with wild-type *N. gonorrhoeae* FA1090 as the control. ns, $p \geqslant 0.05$; *, $p \leqslant 0.05$, **, $p \leqslant 0.01$, ****, $p \leqslant 0.0001$.

## 3.3 Clinical *N. gonorrhoeae* isolates with similar loop sequences as FA1090 bind CFHR5 and CFH

Having determined which PorB loops contribute to CFHR5 binding, I examined clinical isolates expressing different PorBs based on their available WGS from the PubMLST Neisseria BIGSdb database (https://pubmlst.org). There are two types of PorB, P.IA and P.IB expressed by *N. gonorrhoeae* (Knapp *et al.*, 1984); FA1090 carries P.IB. Representatives of P.IA (isolates 60755 and 55496) and P.IB (isolates NG102, NG104, NG118 and 65737) expressing strains were selected to determine if both PorB types bind CFHR5. Of the P.IB strains, isolates NG102 and NG118 were selected as they carry double or single amino acid substitutions respectively in loop 5 compared to FA1090. 65737 and NG104 were also chosen as they exhibit 99% and 92% amino acid identity compared to FA1090 PorB. (**Appendix Figure 7.2**). Detection of CFH and CFHR5 binding was performed as described previously, with NHS as the source of complement proteins. All strains were confirmed to be sialylated (**Appendix Figure 7.3**). Results demonstrate that NG104, which expresses a P.IB porin (with substitutions in loops 1, 3 and 6 and an insertion of two amino acids in loop 5 in PorB), exhibits reduced CFH binding and does not bind CFHR5. Strain 65737 with amino acid substitutions in loops 1, 3 and 6 showed reduced CFHR5 and CFH binding, while strains NG102 and NG118 with less than three amino acid substitutions in loop 5 compared with FA1090 bind CFHR5 but exhibited significantly reduced CFH binding (**Figure 3.6**). This suggests that residues in loop 5 and loop 6 can exhibit differential binding to CFH and CFHR5. Of note, both isolates expressing P.IA PorB do not bind CFHR5 under the conditions tested despite being sialylated (**Figure 3.7**).

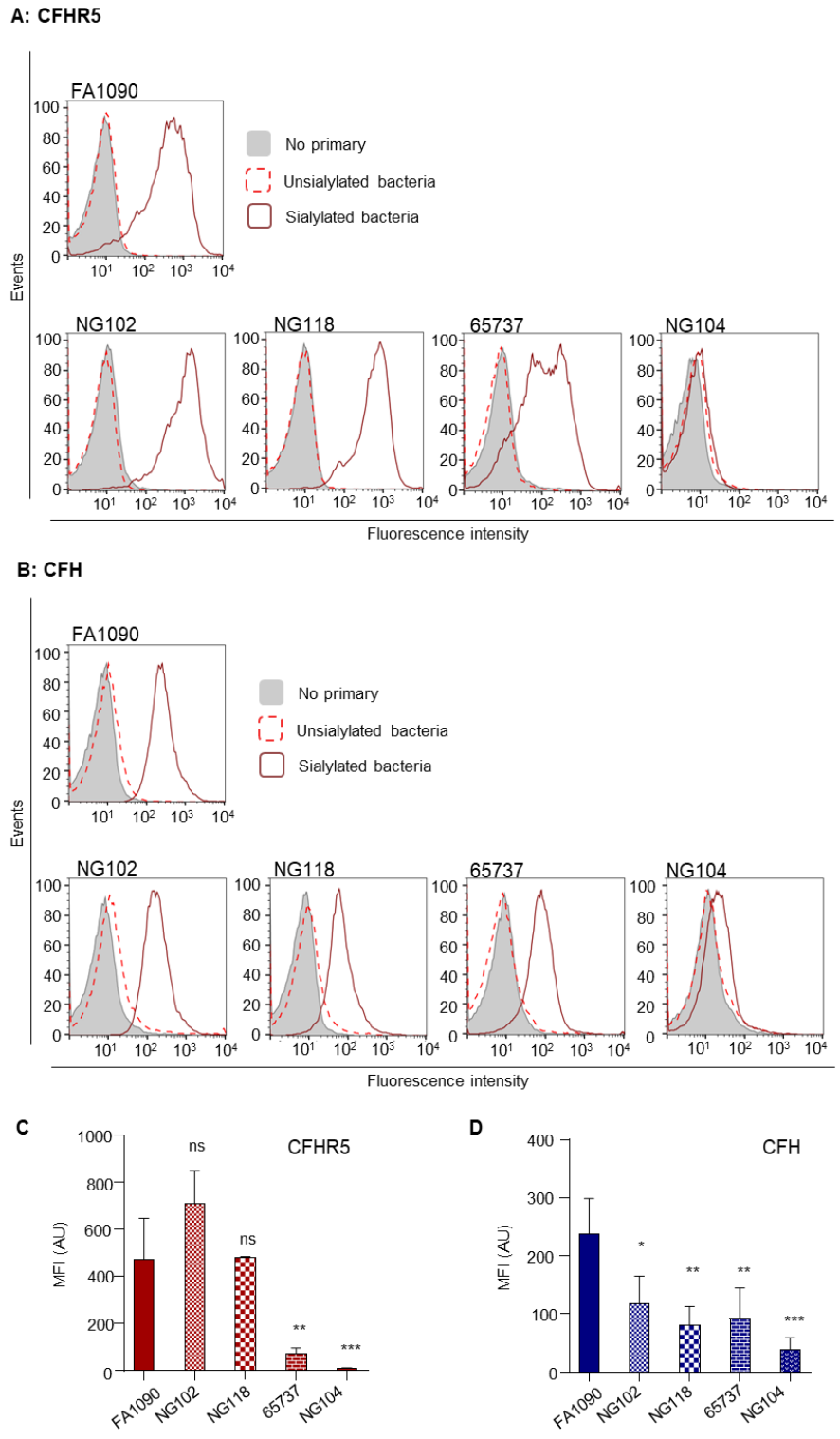



**Figure 3.6 Clinical gonococcal isolates bind CFH and CFHR5 to varying extents.** *N. gonorrhoeae* FA1090 was first fixed and incubated in neat normal human serum as the source of complement factor H (CFH) and complement factor H related protein 5 (CFHR5) before binding of CFHR5 and CFH was detected by α-CFHR5 mAb and OX-24 respectively before visualising with flow cytometry. Representative histograms of isolates expressing P.IB PorB binding to **A)** CFHR5 or **B)** CFH are shown. **C)** CFHR5 binding and **D)** CFH binding are shown as mean fluorescence intensity (MFI) in arbitrary units (AU) with s.d. (n $\geqslant$ 3 independent assays). Significance was analysed with unpaired *t*-test, with wild-type FA1090 as the control. ns, $p \geqslant 0.05$; *, $p \leqslant 0.05$, **, $p \leqslant 0.01$, ***, $p \leqslant 0.001$.

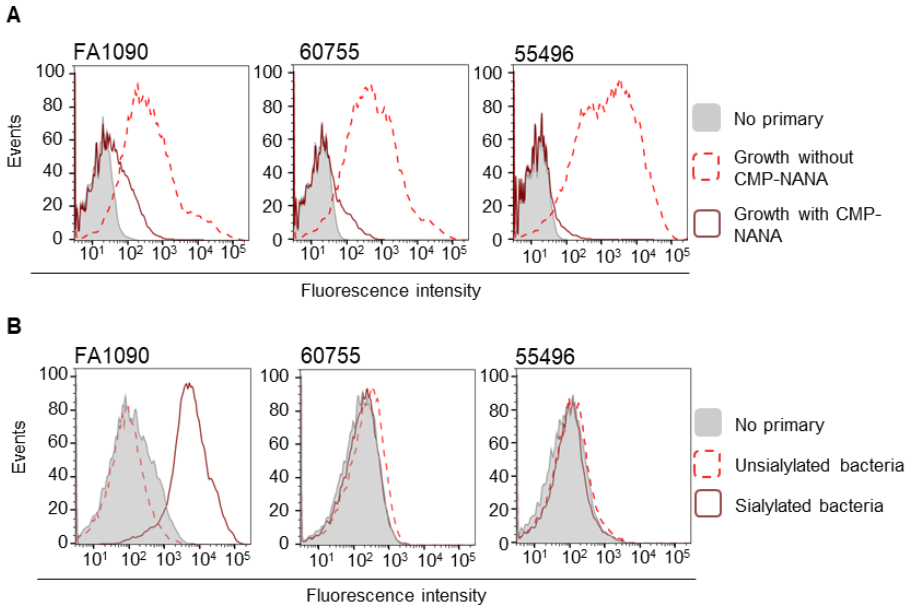



**Figure 3.7 P.IA isolates do not bind CFHR5.** *N. gonorrhoeae* strains was first grown in the presence/absence of cytidine 5' mono-phospho-N-acetylneuraminic acid (CMP-NANA), fixed and incubated in neat normal human serum as the source of complement factor H related protein 5 (CFHR5). Representative histograms of **A)** sialylation and **B)** CFHR5 binding to the P.IA-expressing isolates 60755 and 55496 are as shown. Binding of CFHR5 was detected by α-CFHR5 mAb while sialylation was detected by 3F11 mAb before visualising with flow cytometry.

## 3.4 Summary

Many pathogens including both pathogenic *Neisseria* spp. recruit CFH to evade killing by complement (Reuter *et al.*, 2010; Chen & Seifert, 2013; Schneider *et al.*, 2009). Previously, CFH has been shown to bind to both types of PorB in the presence of sialylated LOS, resulting in decreased serum sensitivity (Chen & Seifert, 2013; Ram *et al.*, 1998). CFHRs show high amino acid sequence similarity to CFH; indeed, $CFHR1_{(4,5)}$, which has 100% and 97% amino acid homology to $CFH_{(19,20)}$ respectively, binds to *N. gonorrhoeae* (Agarwal *et al.*, 2010; Ngampasutadol *et al.*, 2008). Before the studies described in this chapter, it was not known whether any other CFHR binds to *N. gonorrhoeae*.

Here, I show that CFHR5, but not CFHRs 2-4, bind to *N. gonorrhoeae* PorB when LOS is sialylated (**Figures 3.1 – 3.2**). The binding of CFHR5 to PorB is hypothesised to be *via* $CFHR5_{(8,9)}$ as $CFHR5_{(8,9)}$ share 66% and 43% amino acid similarity with $CFH_{(19,20)}$, which is the binding site to PorB. However, no binding of CFHR2 to *N. gonorrhoeae* was detected despite > 60% amino acid similarity between $CFHR2_{(3,4)}$ and $CFH_{(19,20)}$. This is potentially due to low serum levels of CFHR2 and heterodimers of CFHR1 and 2 (0.7 ±0.4, 5.8 ±2.4 µg/mL, respectively) (van Beek *et al.*, 2017) which might limit detection when NHS is used as the source of CFHRs. Furthermore, CFHR1-CFHR2 heterodimers (Goicoechea de Jorge *et al.*, 2013) could impair access of binding sites to bacteria. To circumvent this, purified recombinant CFHR2 could be used in the future for binding assays. Of note, we were unable to determine CFHR1 binding in our experiments as we were unable to generate an antibody that binds specifically to CFHR1.

As both CFH and CFHR5 bind to *N. gonorrhoeae* in the presence of sialylated LOS (**Figure 3.1**), it would be interesting to determine if CFHR5 could compete with CFH for binding to *N. gonorrhoeae*. To do so, I conducted competition assays by adding increasing amounts of purified CFHR5 to serum (n = 1, data not shown). No changes in CFH nor CFHR5 binding were observed under these conditions, suggesting either that

CFH and CFHR5 binding were already saturated with NHS incubation, or that the purified CFHR5, when added to serum, was not functional, for example if it aggregated. Future experiments include repeating the assay with known amounts of purified CFH and CFHR5 to prevent saturation. Aggregation of CFHR5 can also be prevented in these experiments by generating CFHR5 without the dimerisation domains CCPs 1 and 2 (Goicoechea de Jorge *et al.*, 2013). Separately, biophysical quantification of CFH-PorB and CFHR5-PorB interactions could also be carried out. Of note, as sialylated LOS is required for CFH/CFHR5 interactions with gonococcal PorB, any quantitative measurement of binding kinetics such as surface plasma resonance (SPR) would need to be done in the presence of sialylated LOS, which would be technically challenging. Finally, CFH/CFHR5-PorB binding can also be simulated using molecular dynamics to identify crucial interactions between CFH/CFHR5, PorB and LOS mediating binding. These simulations could also show the role of LOS sialylation in mediating CFH/CFHR5-PorB interactions. One hypothesis is that the addition of sialylation changes the charges on the membrane surface which may affect the conformation of loops on PorB, thus allowing CFH and CFHR5 to bind.

I further identified the surface exposed loops on PorB which may be important in both CFH and CFHR5 binding (**Figure 3.5**). Data obtained for loops important for CFH binding was consistent with previous work, with the exception of L1: previous experiment with FA1090 showed that L1 is not important to CFH binding (Chen & Seifert, 2013). However, the role of CFH binding to L1 is disputed, as experiments with F62 suggested L1 plays a role in CFH binding (Madico *et al.*, 2007). Of note, while I observed CFH binding to FA1090 as reported (Chen & Seifert, 2013), I was unable to observe CFH binding to F62 (Madico *et al.*, 2007). This could be due to differences in methodology, or varying levels of CFH in serum used. Results obtained with *porB* loop mutants suggest that the binding of CFH and CFHR5 to *N. gonorrhoeae* PorB is similar, with some differences mediated by loops 1 and 6. The exact binding interface between CFH and PorB, and CFHR5 and PorB, could be determined by co-crystallisation of PorB with CFH or CFHR5 respectively,

or *via* cryo electron microscopy in the presence of sialylated LOS. Amino acids identified through this process could be mutated to confirm their role in the interaction. Structural methods should help to identify the precise biochemical interactions between host CFH/CFHR5 and PorB and potentially explain why only human CFH binds PorB (Ngampasutadol *et al.*, 2008). This could explain why *N. gonorrhoeae* infects only humans as it specifically evades human complement killing, and give mechanistic evidence for the potential role of CFHR5 levels in gonococcal infection.

Using clinical isolates, I determined that it is the precise sequence of loops 5 and 6, rather than the porin type, that is important for CFH and CFHR5 binding (**Figure 3.6**). Consistent with this, strains expressing P.IA PorB do not bind CFHR5 (**Figure 3.7**). It would therefore be interesting to determine in future whether different strains with specific differences in loops 5 and 6 show differential abilities to infect their host.

Overall, I characterised interactions between CFHRs and *N. gonorrhoeae* PorB. As CFHRs have been implicated in increasing serum killing in the closely related pathogen *N. meningitidis* (Caesar *et al.*, 2014), future work would be to determine the physiological importance of CFHR5 binding to *N. gonorrhoeae*, especially as CFHR5 has been shown to recruit the complement activator properdin (Chen *et al.*, 2016). A postdoc in our lab, Dr Hayley Lavender, has carried out serum killing assays for *N. gonorrhoeae in vitro* and showed that pre-treatment of *N. gonorrhoeae* with CFHR5 increased complement-mediated killing, suggesting that CFHR5 may have a physiological role in gonococcal infection (Yee *et al.*, 2022). Levels of properdin and other complement factors on the gonococcal surface can be measured in the future to determine the mechanism in which CFHR5 increases complement-mediated killing. Alternatively, *in vivo* work investigating the role of CFHRs in *N. gonorrhoeae* survival in mouse models could also be carried out; however, transgenic mice with humanised CFHRs would be required as mice CFHRs are not homologous to human CFHRs (Pouw *et al.*, 2015). Furthermore, genomic studies could be carried out to determine if certain single nucleotide polymorphisms (SNPs) in the host CFHR locus increases severity of gonococcal infections as seen previously for

*N. meningitidis* (Davila *et al.*, 2010). These experiments will provide insights into the clinical and physiological role of CFHRs in gonococcal infection.

Overall, I have shown here that *N. gonorrhoeae* binds to CFHR5 at a similar site which recruits CFH. Separately, our paper showed that preincubation of *N. gonorrhoeae* in 4 μM CFHR5 decreased bacteria survival in serum by approximately 40% ($p < 0.001$, not shown) (Yee *et al.*, 2022). This suggests that modulating CFHR5 levels, such as by increasing local levels of CFHR5 at the infection site, could render *N. gonorrhoeae* more susceptible to complement killing. Therefore, understanding the interactions between CFHRs, CFH and *N. gonorrhoeae* provide insights into host:pathogen interactions and highlight the possibility of harnessing the immune system for gonorrhoea treatment in the future.

# 4 Understanding the evolution and distribution of pConj

In the previous chapter, I characterised the interaction between *N. gonorrhoeae* and CFHRs, in particular CFHR5. Identifying novel interactions between *N. gonorrhoeae* and the immune system can aid in harnessing the host immune system against gonococcal infection in the future. In the next two chapters I study the ability of the gonococcus to resist killing by antibiotics conferred by a plasmid, pConj.

Plasmids are critical in disseminating novel traits in bacteria including resistance against antibiotics. *N. gonorrhoeae* contains up to three plasmids, including pConj (39 – 42 kb in size), and p*bla* (3.2 - 9.3 kb) which can confer resistance to tetracyclines and penicillins respectively, and pCryp, a plasmid of unknown function (4.2 kb) (Cehovin *et al.*, 2020). pConj is a conjugative plasmid responsible for the spread of both itself and p*bla* (Roberts & Falkow, 1977). pConj can be classified into seven variants based on the presence of *tetM*, and differences in ε:ζ TA systems and genes encoding the conjugation machinery (Cehovin *et al.*, 2020). Specifically, pConj variants 1 – 4 carry *tetM* (*tetM*$^{+}$ pConj) while variants 5 – 7 do not (markerless pConj) (Cehovin *et al.*, 2020). pConj is not uniformly distributed across lineages: pConj variants 2, 4 and 7 are restricted to lineages 151, 34 and 78 respectively, while variants 1, 3 and 5 are found across the gonococcal population (Cehovin *et al.*, 2020). In addition, pConj is not found in several lineages including lineage 3 and 16 (Cehovin *et al.*, 2020).

In this chapter, I aimed to determine the host range of pConj, its evolution, and the reasons underlying its uneven distribution in the gonococcal population.

## 4.1 pConj epidemiology

### 4.1.1 pConj is restricted to *Neisseriaceae*

Previous research has shown that pConj is prevalent in *N. gonorrhoeae*, with limited reports of pConj in other species including other members of the *Neisseriaceae* family

such as *Kingella denitrificans* and *Eikenella corrodens* (Knapp *et al.*, 1988). Previous work has shown that *tetM*$^{+}$ pConj can be transferred to other *Neisseria* species, but not to *B. catarrhalis* (Roberts & Knapp, 1988b). To determine pConj prevalence in other species, *trb* and *tra* sequences from pConj were used to perform nucleotide BLAST of the NCBI database with *N. gonorrhoeae* sequences excluded. At the same time, the PubMLST database (https://pubmlst.org/) was used to determine pConj presence in *Neisseria* spp.. Only *Neisseria* species with n $\geqslant$ 40 isolates were considered. Results from the NCBI database show that partial sequences of pConj were found only in *Inoviridae* spp. as well as an *E. exigua* plasmid by nucleotide BLAST; alignments of the *Inoviridae* and *Eikenella* plasmid sequences to pConj by ClustalOmega (Sievers *et al.*, 2011) showed overall nucleotide identity of 45% and 60% respectively with pConj (**Appendix Figure 7.4**). pConj was not identified in other non-*Neisseria* species. Results from PubMLST indicate that pConj is found in *N. gonorrhoeae* (found in 2,952 of 9,618 isolates, 30.7%) and in the closely related species *N. meningitidis* (found in 81 of 16,912 isolates, 0.5%), *Neisseria polysaccharea* (found in 3 of 61 isolates, 4.9%) and *N. lactamica* (found in 4 of 551 isolates, 0.7%) (**Figure 4.1**). However, only *N. gonorrhoeae* carries *tetM*$^{+}$ pConj. No pConj sequences were identified in *Neisseria bergeri*, *N. cinerea* and *Neisseria subflava* (**Figure 4.1**), although WGS is available for only a limited number of isolates of these species (n = 60, 40 and 53, respectively). Overall, this indicates that pConj is restricted to *Neisseria* spp. and is only prevalent in *N. gonorrhoeae.*

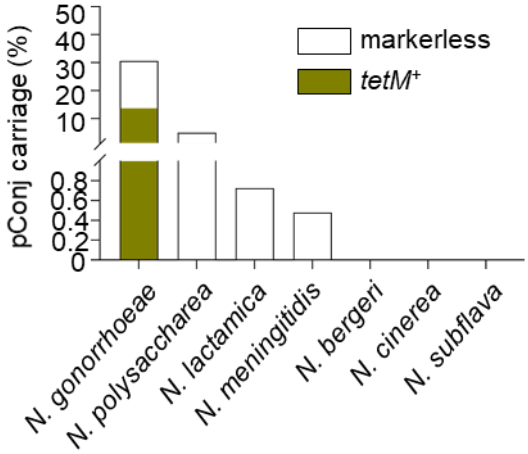



**Figure 4.1 pConj is found in four *Neisseria* spp.** *Neisseria* whole genome sequences (n ≥ 40 for each species) were obtained from PubMLST and examined for presence of pConj *(trbC* and *traM*). pConj were further analysed for presence of *tetM*. The metadata for the dataset is in **Appendix Tables 7.1 and 7.3**.

### 4.1.2 pConj is highly conserved in *N. gonorrhoeae*

As pConj appears to be largely restricted to *N. gonorrhoeae*, I next determined how pConj has evolved over time. Examination of isolates on PubMLST show that the first pConj-carrying isolate was recorded in 1940, decades earlier than reported in the literature (Roberts *et al.*, 1979). Since then, pConj has been classified into seven variants (Cehovin *et al.*, 2020); however, the evolutionary relationships between the variants are unknown.

Representative pConj sequences (n = 126; metadata in **Appendix Table 7.4**) including all seven variants from 1940 to 2022 from different gonococcal lineages and countries were selected. pConj sequences were aligned using a multiple sequence alignment programme MAFFT (Katoh *et al.*, 2002), which aligns sequences by maximising the number of similarities between multiple sequences and allows for accurate and fast computation (Katoh *et al.*, 2002). Phylogenetic trees were subsequently built independently using two programmes, RAXML (Stamatakis, 2014) and ClonalFrameML (Didelot & Wilson, 2015) which uses PhyML to generate a starting tree (Guindon *et al.*, 2010). While RAXML has been previously used for building *N. gonorrhoeae* phylogenetic trees (Sánchez-Busó *et al.*, 2019a), ClonalFrameML is built to account for recombination (Didelot & Wilson, 2015), which is common in the gonococcus as it is naturally transformable (Biswas *et al.*, 1977). Results from both phylogenetic analyses are consistent with each other and show that the seven variants can be divided into two main groups. Group I pConj includes variants 4 – 7 and carries an ε:ζ3 TA system, while group II pConj includes variants 1 – 3 and carries an ε:ζ2 TA system. Further analysis of group I pConj showed that pConj isolated in 1940s are similar to pConj isolated in 2000s (**Figure 4.2**). *tetM1* ("Dutch" allele) also appears to be have been introduced at least twice – once within Group I plasmids which includes markerless pConj, and another together with the acquisition of the ε:ζ2 TA system. *tetM2* ("American" allele) was then acquired within Group II. There was also a substantial change within the *trbJ/K/L* genes

in Group II pConj, resulting in variant 2 plasmids. Apart from these changes, phylogenetic analyses showed that pConj has been highly conserved over 80 years despite being found in many gonococcal lineages.

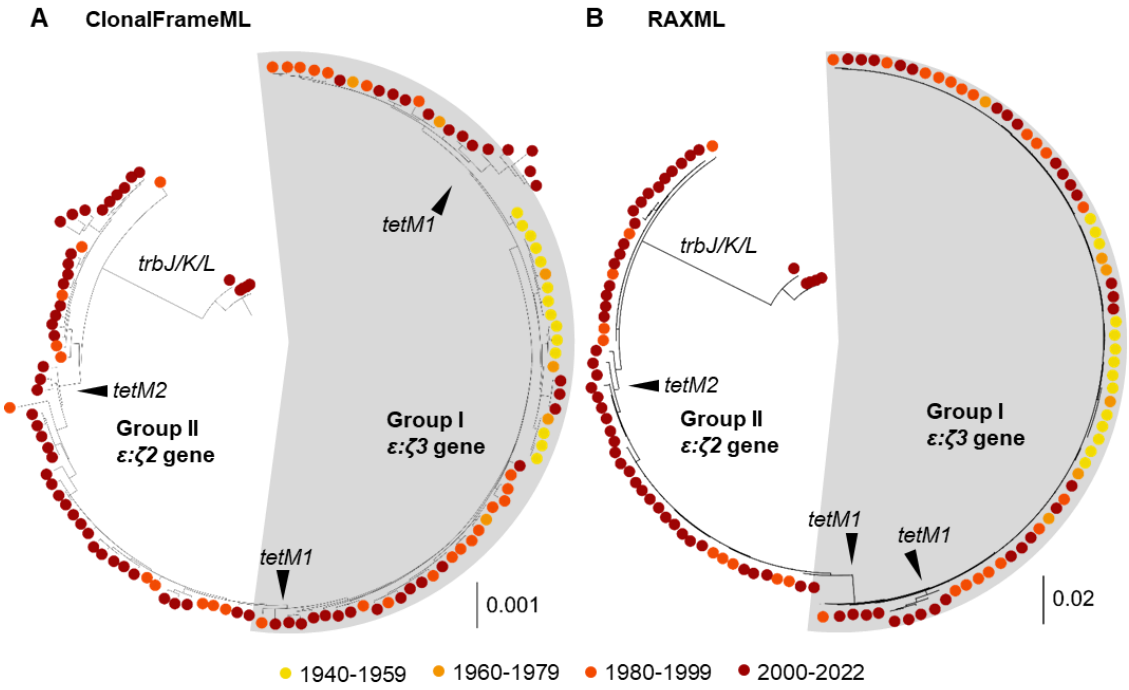



**Figure 4.2 pConj has been conserved over 80 years and can be divided into two phylogenetic groups.** pConj sequences (n = 126) including all seven pConj variants were aligned using MAFFT prior to phylogenetic reconstruction with **A)** PhyML followed by ClonalFrameML and **B)** RAXML with three independent seeds. Isolates were selected to ensure representation across different pConj variants, different years and countries (metadata in **Appendix Table 7.4**). Each dot represents an isolate, colour coded according to year of isolation. Both trees show that pConj falls into two groups.

### 4.1.3 pConj remains prevalent despite changes in treatment

Tetracyclines were used to treat gonococcal infection before the spread of resistance due to *tetM*$^{+}$ pConj. Currently, tetracyclines are not used as a first line treatment for gonorrhoea in the USA and UK (St Cyr *et al.*, 2020; Bignell, 2004). However, tetracyclines (including doxycycline) are still used to treat STIs syndromically in LMICs, potentially contributing to the high *tetM*$^{+}$ pConj prevalence in these countries (Cehovin *et al.*, 2020); markerless pConj is not associated with isolates from LMICs. As such, we wanted to determine whether the prevalence of pConj has fallen in countries (*i.e.* USA and UK) since tetracyclines were discontinued for treating gonococcal infection.

Gonococcal WGS with sequence bin > 1 Mb in the PubMLST database from strains isolated in the USA and UK were examined as the treatment guidelines for these two countries are well documented. In the USA, doxycycline was phased out from the 1980s but reinstated as part of dual therapy between 2010 – 2014 (Centers for Disease Control and Prevention, 2012). As such, analysis of isolates for USA included 2010 – 2019 (n = 2,517 isolates), which encompasses five years during and after doxycycline use (Centers for Disease Control and Prevention, 2012; Workoswki & Bolan, 2015). pConj was identified by the presence of *traM* and/or *trbC*. In the USA, the prevalence of *tetM*$^{+}$ pConj in *N. gonorrhoeae* during (2010 – 2014) and after (2015 – 2019) doxycycline treatment remained steady ($p$ = 0.4547, unpaired $t$-test **Figure 4.3**). Carriage of markerless pConj was also comparable when doxycycline was in use and afterwards ($p$ = 0.7846, **Figure 4.3A**). In UK, pConj has remained prevalent from 2007 – 2016 (n = 3,240) even though doxycycline was discontinued for gonorrhoea treatment in 2004 (Bignell, 2004) (**Figure 4.3B**). Therefore, analysis from these countries demonstrate that *tetM*$^{+}$ and markerless pConj have persisted in *N. gonorrhoeae* in the absence of selective pressure imposed by tetracycline treatment for gonococcal infection. In summary, pConj has remained

conserved over 80 years, is largely restricted to *N. gonorrhoeae*, and has persisted in gonococcal populations following changes in treatment regimes.

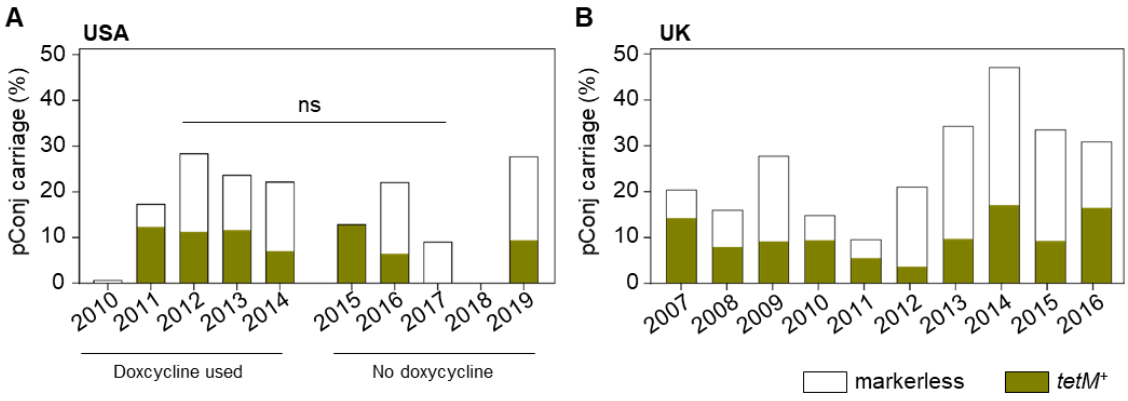



**Figure 4.3 pConj has remained prevalent in the absence of tetracycline treatment for gonococcal infection.** Whole genome sequences from **A)** the USA and **B)** UK were first obtained from PubMLST. Analysis of pConj prevalence of isolates in the USA and UK over the years indicated indicates that pConj carriage, including markerless plasmids, has remained constant during and after tetracycline treatment (n = 2,517 isolates for US, and n = 3,240 for UK). Results were analysed by unpaired *t*-test. ns, $p > 0.05$.

## 4.2 Differences in pConj prevalence in gonococcal lineages

pConj is not uniformly distributed in *N. gonorrhoeae* (Cehovin *et al.*, 2020). This could be due to several factors including differences in copy number, fitness costs, or conjugation frequencies. I next examined whether any of these factors account for the prevalence of pConj in some lineages, but not in others.

As pConj variant 1 is found in different lineages globally (Cehovin *et al.*, 2020), I decided to use pConj variant 1 for subsequent experiments.

### 4.2.1 pConj has plasmid:chromosome copy number of approximately five regardless of strain background

The entry of plasmids into a new host can result in changes in copy number during the co-evolution of the host and plasmid (Dionisio *et al.*, 2005; Yano *et al.*, 2016). As *N. gonorrhoeae* is a polyploid bacterium with estimated three copies of its chromosome (Tobiason & Seifert, 2010), the plasmid:chromosome ratio (P:C ratio) was calculated using qPCR. I first validated the efficiencies of primers for qPCR. Primer pairs were designed to have a melting temperature between 55 – 60°C and bind sequences conserved between different isolates or across pConj variants. Control plasmids carrying sequences to which primers anneal were generated in pCR™2.1-TOPO™ background and were serially diluted before being used in qPCRs. Of the primers designed, Q7/Q8 for *recA* (160 bp product) and Q21/Q22 for *res* (130 bp product) have primer efficiencies of 102.4% and 90.1%, respectively (**Figure 4.4A**) and were thus used to determine the P:C ratio in different genetic backgrounds.

To generate different strains carrying pConj, pConj variant 1 from strain 60755 was introduced into three strains which do not carry pConj: WHO K, NM8365 and the lab isolate FA1090-WT. WHO K and NM8365 are from lineages 8 and 3 respectively which

have 0.6% and 0.1% pConj carriage respectively; FA1090-WT (from Professor Ann Jerse) is a laboratory isolate that does not naturally carry pConj. To measure copy number, bacteria were first grown in liquid culture to mid-log phase before harvesting cells, and DNA extracted. The P:C ratio was calculated by first calculating the DNA concentrations of the plasmid (with *res*) and chromosome (with *recA*) separately, taking into account the individual primer efficiency, by qPCR. The relative levels of *res* (on the plasmid) and *recA* (on the chromosome) was then determined (**Figure 4.4A**). Results demonstrate that the P:C ratio was not significantly different between all four strains tested: 60755 (lineage 21, 81.2% pConj carriage, P:C ratio 6.30 ±1.90), which naturally carries pConj, and strains into which pConj had been introduced: FA1090 (lineage 286, 0% pConj carriage, P:C ratio 4.60 ±1.63), WHO K (P:C ratio 6.71 ±2.87) and NM8365 (P:C ratio 7.48 ±4.18) ($p > 0.05$, one-way ANOVA with Kruskal Wallis multiple comparisons) (**Figure 4.4B**).

Overall, this suggests that pConj has a P:C ratio of approximately five in *N. gonorrhoeae* and the copy number is not significantly different in the four lineages tested.

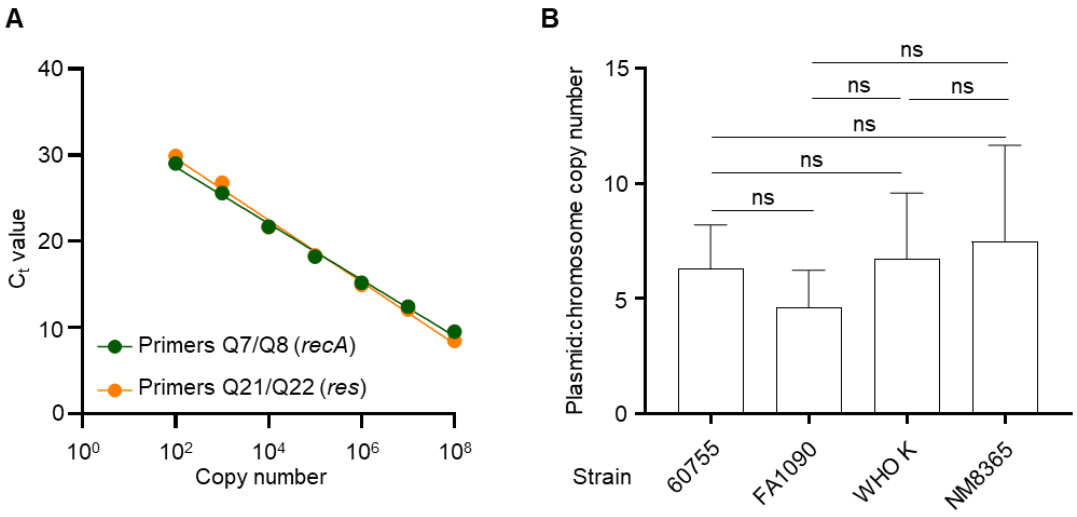



**Figure 4.4 pConj has a plasmid:chromosome ratio of approximately five. A)** Efficiency of primers Q7/Q8 and Q21/Q22 using control plasmids as targets. The plasmid concentration of plasmids was initially measured by Qubit. Aliquots (1µl) of serially diluted plasmid were subjected to qPCR, Ct values plotted against copy number. Linear regression was calculated using GraphPad Prism v 8.0. The gradient of the slope was used to calculate primer efficiency. **B)** The ratio of the pConj to chromosome copy number was determined by qPCR of *res* and *recA*, respectively, taking into account primer efficiencies. There was no significant difference in plasmid:chromosome ratio in the isolates tested. Values (n ⩾ 3) were analysed by Kruskal-Wallis test and shown as mean ± s.d. ns, $p > 0.05$

### 4.2.2 pConj does not impose fitness costs in isolates from different lineages

Plasmids can impose a significant fitness cost on their host due to a variety of factors including expression of plasmid-encoded genes and use of host enzymes for plasmid replication (San Millan & MacLean, 2017). Plasmid fitness costs can be strain dependent, with some strains exhibiting higher plasmid fitness costs having lower plasmid prevalence (Alonso-del Valle *et al.*, 2021). Therefore, I next examined whether pConj carriage adversely affects growth of *N. gonorrhoeae*. Strains 60755, FA1090, WHO K and NM8365 with or without pConj were grown in GCBL + 1% vitox for 8 h, and $OD_{600}$ readings taken every 20 m. No significant differences in the growth rate of strains with or without pConj were seen ($p > 0.05$, two-way ANOVA with Sidak's multiple comparisons**, Figure 4.5A**). The growth of all four strains was also assessed over 24 h in FB media, which supports *N. gonorrhoeae* growth for longer durations. Again no significant difference in growth rates was observed in bacterial strains with or without pConj ($p > 0.05$, **Figure 4.5B**). Results indicate that pConj does not affect bacterial growth in the conditions tested as assessed by recovering viable bacteria in FB or measuring $OD_{600}$ of cultures in GCBL (**Figure 4.5B**).

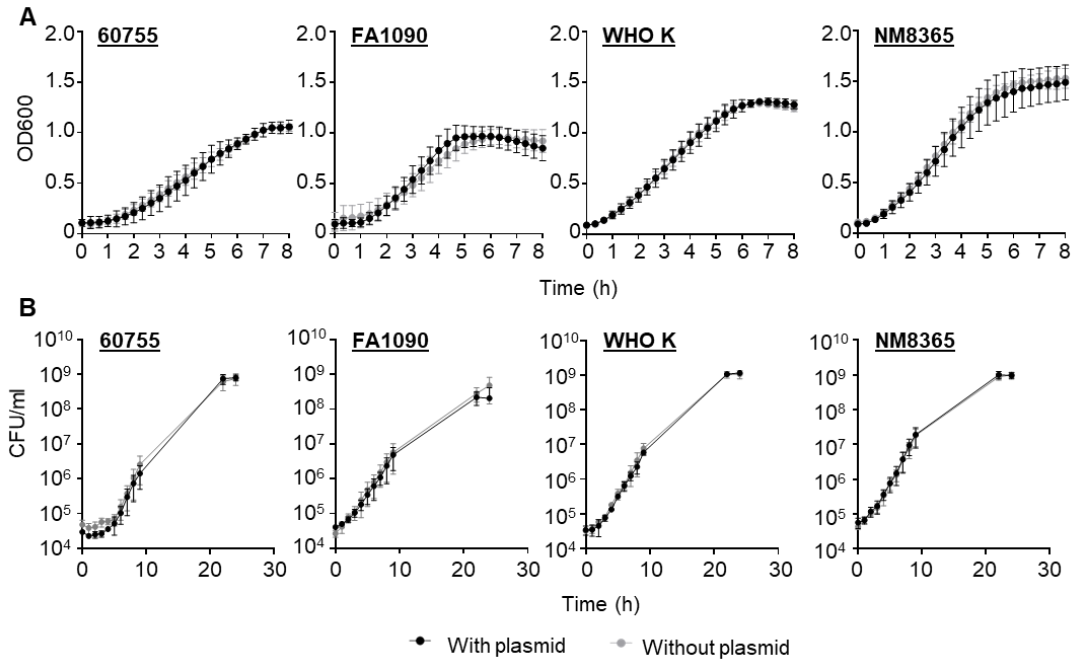



**Figure 4.5 pConj does not impose significant fitness costs on *N. gonorrhoeae*.** *N. gonorrhoeae* strains 60755, FA1090, WHO K and NM8365 with or without pConj were grown for **A)** 8 h in gonococcal broth (GCBL) or **B)** 24 h in fastidious broth (FB). Liquid cultures were prepared in GCBL (200 µl) at $OD_{600}$ 0.1 or FB (15 ml) with 5 x $10^4$ CFU/ml. For growth in GCBL, the $OD_{600}$ was measured over 8 h. For growth in FB, samples were plated to solid media and colony forming units (CFU/ml) were counted. Values ($n \geqslant 3$) were analysed with two-way ANOVA with Sidak's multiple comparisons and shown as mean±s.d. ns, $p > 0.05$

### 4.2.3 pConj prevalence in lineages correlates with conjugation frequencies.

Next, I examined whether the uneven distribution of pConj in the gonococcus could be explained by different conjugation frequencies. To do so, the conjugation setup, such as amount of starting bacteria, ratio of donor to recipient and duration of incubation, must first be determined. This was done using an isogenic conjugation between FA1090:pConj as the donor and FA1090-WT as recipient. Previous literature determining pConj conjugation frequencies relied on either filter matings (200 µl to 3 ml) (Roberts & Knapp, 1988b; Genco *et al.*, 1984) or mating on solid agar (spotting 100 µl to plates) (Pachulec & van der Does, 2010). Briefly, both donor and recipient were first cultured in gonococcal medium base + 1% Kellogg's supplement for a duration ranging from 4 h to overnight. Donor:recipient ratios ranging from 1:1 to 1:10 were used (Genco *et al.*, 1984) at $10^7$ – $10^8$ CFU starting bacteria (approximately $10^8$ CFU/ml). Conjugation reactions for pConj transfer were incubated overnight (16 – 24 h) (Genco *et al.*, 1984; Roberts & Knapp, 1988b; Pachulec & van der Does, 2010) and conjugation frequency was calculated as the number of transconjugants per recipient (Roberts & Knapp, 1988b).

Therefore, to determine parameters for conjugation assays, I first grew donor and recipients to mid-log phase in 5 ml GCBL + 1% vitox prior to conjugation. I examined donor:recipient ratios ranging from 1:1 to 10:1, and varied the initial total number of bacteria from $10^3$ to $10^6$ CFU. To facilitate high throughput conjugation assays, 10 µl conjugation reactions were spotted onto plates, and incubated for 24 h before bacteria were harvested and plated to selective media. The total number of recipients (including transconjugants) and the number of transconjugants were measured at the end of the assay, and conjugation frequencies were calculated as the percentage of recipients that had acquired pConj at the end of the assay similar to previous literature (Roberts & Knapp, 1988b).

Of note, with higher numbers of bacteria (*i.e.* initially > $10^4$) and the donor in excess (*e.g.* donor:recipient ratio of 10), fewer recipients (including transconjugants) were recovered at the end of conjugation than expected (**Figure 4.6A**). This suggests that survival of the recipient is compromised by a high density of the donor. However, when initial bacterial population was around or below $10^4$ CFU, the proportion of recipients, which includes transconjugants, recovered at the end of the conjugation was similar to the input. Of note, at starting bacteria of approximately $10^3$ CFU, individual colonies could be observed in the conjugation spots after 24 h incubation.

In addition, conjugation frequencies were determined. Results show that when there were > $10^5$ CFU initially (**Figure 4.6**), the conjugation frequency was high for all donor:recipient ratios. In contrast, decreasing the number of starting bacteria below $10^5$ CFU decreased the conjugation frequency by more than an order of magnitude compared to when the initial population was more than $10^5$ CFU (**Figure 4.6B**), suggesting that conjugation frequencies could be limited by the bacterial density.

Therefore, all subsequent conjugation assays carried out in this thesis first grew bacteria to mid-log as above. Conjugations (10 µl) included $10^4$ – $10^5$ CFU initially, then incubated for 24 h before bacteria were harvested, and conjugation frequencies were assessed by plating onto selective media.

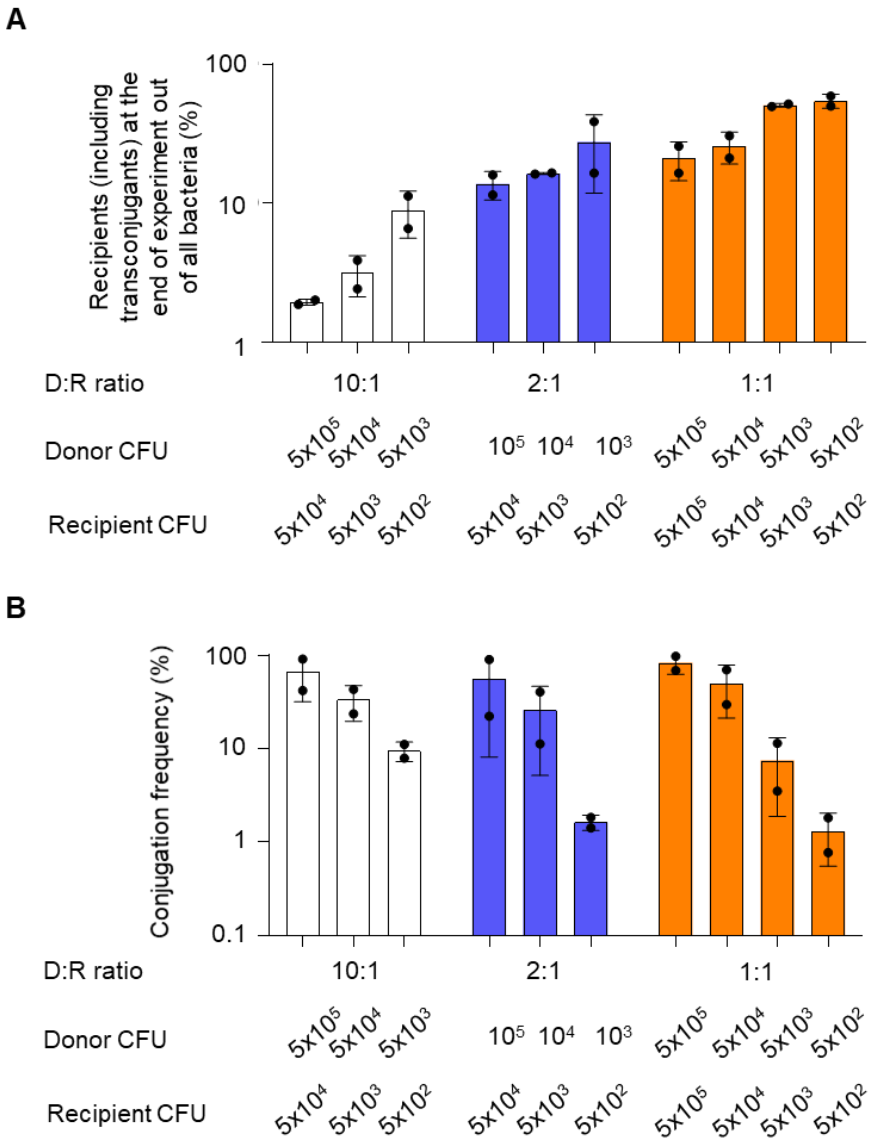



**Figure 4.6 Conjugation assays with initial starting bacteria of approximately $10^5$ CFU or more have similar conjugation frequencies.** Isogenic FA1090 donor and recipients were grown in liquid to mid-log then mixed at the ratio (D:R) and concentration (determined using colony forming units, CFU) as indicated, before 10 µl was spotted onto plain gonococcal agar (GCB). Plates were incubated for 24 h before harvesting and plating to selective media (n = 2). **A)** Recipients (including transconjugants) at the end of the experiment were quantified as a percentage of total CFU obtained on non-selective media. **B)** The conjugation frequency is quantified and defined as the percentage of recipients that acquired pConj at the end of the experiment.

As *N. gonorrhoeae* is naturally competent (Sparling, 1966), I next confirmed that the assay measures the transfer of *tetM*$^{+}$ pConj by conjugation and not by transformation. To do so, I first heated the donor to 100°C for 10 m, then added heat killed donor together with live recipient (not shown). This was similarly done for heat killed recipient and live donor. Mating was then carried out in a 1:1 donor to recipient ratio, starting at $10^5$ CFU. No colonies resistant to both kanamycin and tetracycline were observed, suggesting that pConj is not transferred by transformation.

Furthermore, I constructed a control strain by deleting *trbC* from pConj using the markerless method (Jones *et al.*, 2022); *trbC* is predicted to encode the conjugative pilin (Pachulec & van der Does, 2010). FA1090 carrying pConj with or without *trbC* was mated with FA1090 recipient using the same set up described above with a 1:1 donor to recipient ratio. The recipient FA1090 is non-piliated as it harbours a kanamycin resistance cassette *aph(3)-III* (to allow selection of transconjugants) in *pilD* (Δ*pilD*::*aph(3)-III*). As *pilD* is involved in processing the prepilin subunit for T4P formation, deletion of *pilD* gene prevents acquisition of pConj by transformation (Biswas *et al.*, 1977). As expected, Δ*trbC* had a significant decrease (> $10^3$ fold) in conjugation frequency compared to wild-type pConj ($p$ = 0.0005, unpaired $t$-test **Figure 4.7**). However, some colonies resistant to both kanamycin and tetracycline were observed at very low levels.

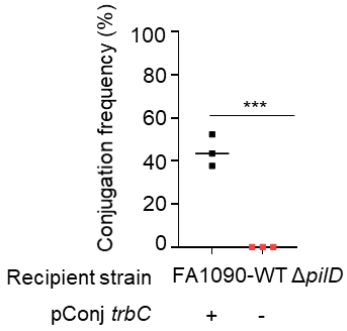



**Figure 4.7 pConj is transferred by conjugation**. Isogenic FA1090 donor and recipients were grown in liquid to mid-log then mixed at a ratio of 1:1. $10^5$ colony forming units of bacteria was spotted onto gonococcal agar and incubated for 24 h. Results (n ≥ 3) were analysed by unpaired $t$-test. ***, $p < 0.001$

To determine whether these kanamycin and tetracycline resistant colonies arose from transfer of pConj or Δ*pilD*::*aph(3)-III*, I carried out the same conjugation assay as above using FA1090 carrying pConjΔ*trbC* as the donor, with a non-isogenic recipient strain, 60755Δ*pilD* (not shown). Again, a few kanamycin and tetracycline resistant colonies were observed; sequencing of *porB* indicated that these colonies were from the FA1090 donor which had acquired Δ*pilD*::*aph(3)-III*. However, transformation of the donor occurs at a much lower level than conjugation (**Figure 4.7**), so transformation will not significantly affect the conjugation frequencies measured with the assay I established.

Having determined conditions for conjugation assays, I examined whether conjugation frequencies are affected by the identity of the recipient. I selected isolates from lineages which have low or high pConj carriage, and constructed Δ*pilD* mutants in each of them. Specifically, lineage 3 (0.1% pConj carriage, three isolates) and lineage 159 (5.2% pConj carriage two isolates) were selected from lineages with low pConj carriage, while lineage 21 (81.2% pConj carriage, two isolates), lineage 34 (86.1% pConj carriage, one isolate) and lineage 364 (100% pConj carriage, one isolate) were selected from lineages with high pConj carriage. Conjugations were conducted as previously except 60755, which naturally carries pConj and belongs to lineage 21 was used as the donor. The donor:recipient ratio was 10:1 to ensure that the abundance of the donor did not limit conjugation frequencies. Results show that with strain 60755 as the donor, conjugation frequencies were different for different recipients (**Figure 4.8A**); specifically, conjugation frequencies correlate with the prevalence of pConj in the recipient lineage ($R^2$ = 0.83, **Figure 4.8B**). This indicates that pConj prevalence in different lineages may be due to differences in their ability to acquire pConj by conjugation.

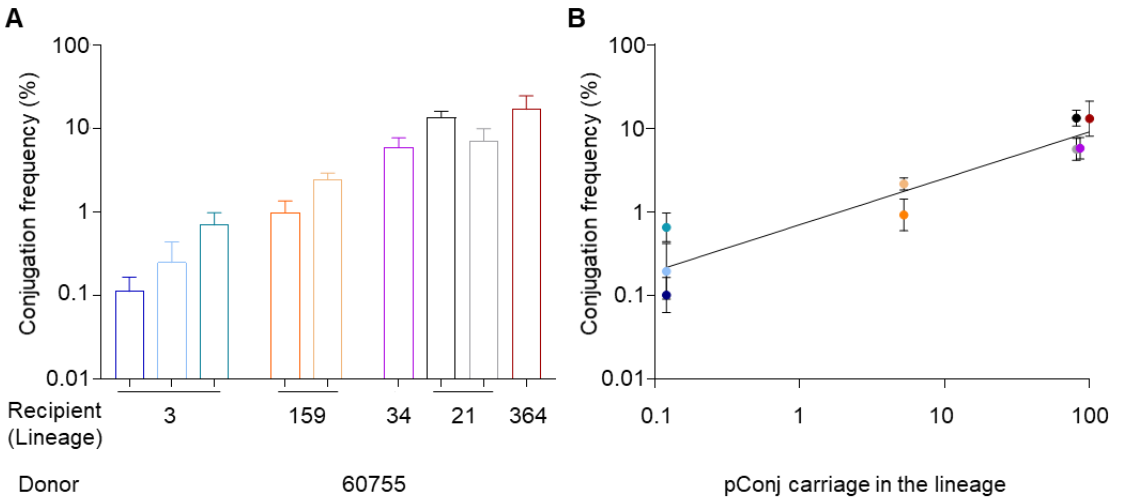



**Figure 4.8 Different recipients exhibit different conjugation frequencies**. The donor (strain 60755) and all recipients were mixed at 10:1 ratio and spotted onto plain gonococcal agar then incubated for 24 h before harvesting and plating to selective media. **A)** At least three independent conjugations were carried out for each recipient and **B)** used to determine if conjugation frequencies correlate with pConj carriage in the lineage. The dots correspond to the same-coloured recipient in **A**. Results show mean±s.d., and linear regression ($R^2$) was calculated between log transformed values of conjugation frequencies and pConj carriage in the recipient lineage. $R^2 = 0.83$

## 4.3 Factors contributing to differences in conjugation frequencies

### 4.3.1 Differences in conjugation frequencies in isolates of different lineages are due to the recipient rather than the donor

As data in **Figure 4.8** was generated with only one donor, further assays were performed with different donors as well as recipients to determine if the observations also apply to different donors. Three strains were used in these experiments, 60755, NM8365 and WHO K. NM8365 and WHO K belong to lineages 3 and 8, with 0.1% and 0.6% pConj carriage respectively, while 60755 is from lineage 21, with 81.2% pConj carriage. The 60755 recipient was generated by curing the strain of pConj and generating a Δ*pilD* mutant. *pilD* deletions were also introduced into WHO K and NM8365 so they could be used as recipients. WHO K and NM8365 donors were generated by conjugation with wild-type 60755 which contains *tetM*$^{+}$ variant 1 pConj. Therefore, all donors harboured the same pConj. Results demonstrate that the conjugation frequency is significantly different with 60755, NM8365 and WHO K as the recipient when 60755 is used as the donor ($p < 0.0001$, normalisation followed by one-way ANOVA with Tukey's multiple comparisons, **Figure 4.9A**). Similar trends were seen when NM8365 and WHO K were used as the donor. Of note, changing the donor did not impact the conjugation frequency in most instances ($p > 0.05$, **Figure 4.9B**); the exception is the low conjugation frequency seen when mating WHO K with itself ($p = 0.0024$, **Figure 4.9**). Furthermore, conjugation between isogenic strains resulted in different conjugation frequencies, with the highest being between 60755 strains and the lowest between WHO K strains ($p < 0.0001$, **Figure 4.9**). Similar to results in **Figure 4.8**, conjugation frequencies with 60755 as the recipient (irrespective of the donor) were higher than those between WHO K and itself in an isogenic mating. Overall, these results confirm that differences in conjugation

frequencies are seen with recipients from different lineages and can be higher than isogenic matings.

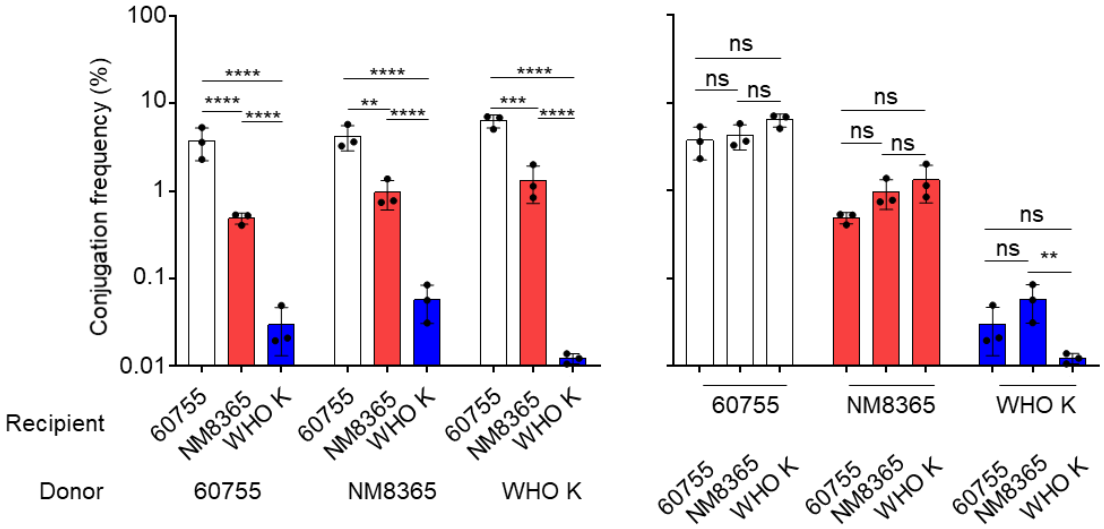



**Figure 4.9 Conjugation frequencies are dependent on the recipient.** Donor and recipients strains as indicated in the figure were mixed at a 1:1 ratio then spotted onto plain gonococcal agar. Plates were incubated over 24 h before harvesting, then plated to selective media to determine conjugation frequencies. Statistical analysis of conjugation frequencies was done after log transformation, followed by one-way ANOVA with Tukey's multiple comparisons. Results (n = 3) show mean±s.d. ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

### 4.3.2 Identification of potential genetic factors responsible for pConj acquisition

Having determined that differences in recipients result in different rates of pConj acquisition, I next aimed to define chromosomal factors which are required for pConj acquisition.

Unbiased genetic screens are a powerful approach to identify genes involved in a particular process. Therefore, I applied Tn-Seq to define gonococcal genes that contribute to the acquisition of pConj. This was to provide candidate genes which might be the basis for the different conjugation frequencies exhibited by different lineages (**Figure 4.9**). Tn-Seq first requires construction of a library with transposons inserted randomly in the genome. The library is then subjected to a control and a test condition and the resulting libraries are sequenced, thus identifying the sites of transposon insertion. In this way, genes essential for the test condition can be determined (Cain *et al.*, 2020).

FA1090 was selected for this work as the strain exhibits a high rate of pConj acquisition, is genetically tractable (Dillard, 2011), and both Illumina and PacBio genome sequences were available. I constructed a library of FA1090 mutants by *in vitro* transposon mutagenesis of FA1090 chromosomal DNA using HsmarI transposase (kind gift from Professor Ronald Chalmers) and pHsk1 plasmid carrying a transposon with a kanamycin resistance gene (**Appendix Figure 7.5**). The mutagenised DNA was introduced into FA1090 *via* transformation. The resulting library was selected on 80 µg/ml kanamycin and had 189,314 unique insertion sites (UIS).

Next, to determine chromosomal genes that contribute to pConj acquisition, FA1090:pConj was used as the donor with the mutant library as a recipient. Conjugation reactions (n = 72, 10 µl, donor:recipient ratio of 1:1, starting at $10^5$ CFU) were spotted onto GCB, dried and left to incubate for 24 h before plating to media containing

kanamycin only (to select for recipients), and on kanamycin + tetracycline for transconjugants. Mutants with insertions within genes involved in conjugation would be recovered on media containing kanamycin only, but not on media which includes tetracycline. Genomic DNA was extracted from initial library, pooled and subjected to Illumina sequencing on the NextSeq 550 at the Quadram Institute. The libraries were sequenced twice. The control condition had 172,913 UIS while the test condition had 183,919 UIS. Comparison of control and test libraries was then carried out with BioTRADIS, which gives the following parameters: log fold change (logFC), defined as the $\log_2$ of fold change in number of insertions between control and test libraries, logCPM, which is the $\log_2$ of number of reads per million and *q* value, which is the *p* value corrected for multiple testing (Page *et al.*, 2020).

A list of genes statistically associated with failure to acquire pConj (logFC < 0, $q < 0.00001$) was generated. The hits with the lowest *q* values, logFC of < -3.3 (*i.e.* > 10 times decreased under test conditions) and logCPM > 3.3 (*i.e.* > 10 reads) are shown in **Table 4.1**. Results indicate that membrane proteins NEIS0092, NEIS0377 and AbcZ may have a role in pConj acquisition. Hypothetical proteins NEIS0603, NEIS2649 and NEIS1931 were also shortlisted.

| *gene_name* | *Function* | *logFC* | *logCPM* | *q.value* |
|---|---|---|---|---|
| *neis0092* | putative inner membrane protein | -8.68129 | 5.777548 | 3.49E-36 |
| *neis0377* | putative integral membrane protein | -4.72585 | 5.629585 | 1.80E-37 |
| *neis0603* | hypothetical protein | -4.06544 | 4.603198 | 1.10E-15 |
| *neis2649* | hypothetical protein | -3.97551 | 5.786345 | 2.07E-28 |
| *neis3039* | transcriptional regulator | -3.56492 | 6.421317 | 1.98E-23 |
| *neis1016* | DedA protein orthologue | -3.48354 | 5.024991 | 2.52E-20 |
| *neis1931* | hypothetical protein | -3.36809 | 7.331647 | 8.92E-44 |
| *abcZ* | | -3.24774 | 9.061859 | 1.86E-61 |

**Table 4.1 List of genes shortlisted from Tn-Seq.** FA1090 transposon library was first constructed using *in vitro* transposon insertion. The library was then used as recipients in conjugation assay, with FA1090-WT pConj as donor. Library was then selected with kanamycin ("No Tet"; all recipients) or kanamycin and tetracycline ("+Tet"; transconjugants). Genomic DNA was extracted from both conditions and sequenced with NextSeq 550 before analysing with Bio-TRADIS and visualised with Artemis. The hits with the lowest *q* values, $\log_2$ fold change of < -3.3 between the two conditions (logFC, > 10 times decreased under test conditions) and $\log_2$ counts per million > 3.3 (logCPM, > 10 reads) are as shown.

To determine the putative function of the three hypothetical proteins, the amino acid sequences were analysed by HHpred (Söding *et al.*, 2005). HHpred detects homologous regions of proteins and predicts protein domains using pairwise comparisons of the query amino acid sequence and those available on the UniProt database. Results show that NEIS0603 and NEIS2649 were predicted to consist of domains homologous to the L9 ribosomal protein and cytochrome c oxidase respectively; however, the E-value, which indicates the expected number of false-positives per search, for these predictions were poor (> 1). In contrast, NEIS2649 is predicted to be a thioesterase with an E-value of 1.8 x $10^{-22}$, suggesting that *neis2649* encodes for a thioesterase.

To elucidate the potential roles of the identified genes in **Table 4.1**, the genetic location of the eight genes was also examined (**Figure 4.10**). Results show that genes *neis1016*, *abcZ*, *neis1931* and *neis3039* are in the vicinity of genes encoding outer membrane

proteins and transport proteins, suggesting that these genes may be involved in export or import of molecules. *abcZ* encodes a putative ATP-binding cassette transporter of unknown function (Vollan *et al.*, 2022). In contrast, *neis0092* encodes for an inner membrane protein and is next to a gene involved in bacteriocin resistance, suggesting that it may be involved in membrane integrity. Apart from membrane-related genes, *neis2649* is next to a gene encoding for aspartate ammonia-lyase, while *neis0377* is between a predicted nucleotidyl transferase and a ligase, suggesting that both genes may encode for proteins involved in metabolic pathways. Finally, the potential role of *neis0603* is unknown as it is within an operon of other hypothetical genes; their roles also could not be identified by HHpred with the exception of *neis0604*/*ngo1391*, which is homologous to the suppressor of fused family protein (Das *et al.*, 2010). Its function is unknown.

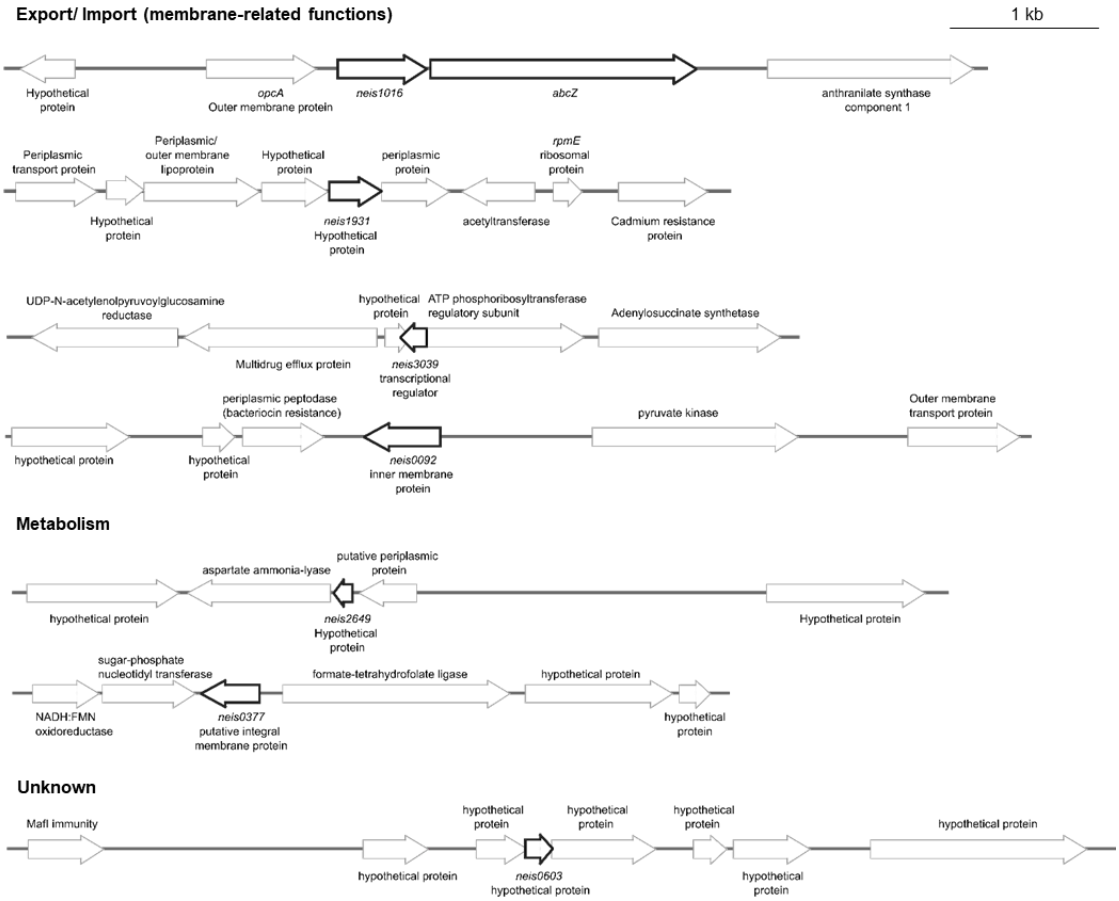



**Figure 4.10 Representation of genetic loci of genes identified by Tn-Seq.** The genes identified by Tn-Seq as significant in Table 4.1 are shown with black arrows; the surrounding genes are shown in grey. All genes are annotated with the associated function

Of note, as inspection of insertion plots (**Figure 4.11**) suggests that significance was due to differences in a small number of insertions per gene which is characteristic of false-positive hits (Mahmutovic *et al.*, 2020), the genes were not investigated further experimentally.

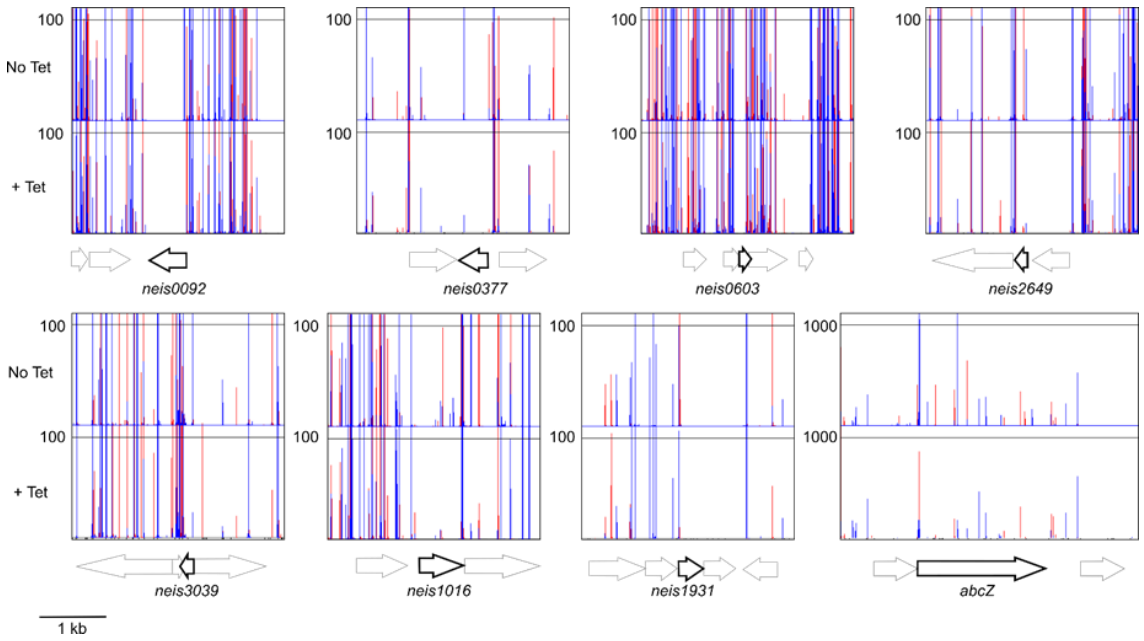



**Figure 4.11 Significant hits involved in pConj transfer, viewed on Artemis.** FA1090 transposon library was used as recipients in conjugation assay, with FA1090-WT pConj as donor. Library was then selected with kanamycin (all recipients) or kanamycin and tetracycline (transconjugants). Genomic DNA was extracted from both conditions and sequenced with NextSeq 550 before analysing with Bio-TRADIS and visualised with Artemis. The hits with the lowest *q* values (adjusted *p* values), $\log_2$ fold change of < -3.3 (i.e. > 10 times decreased under test conditions) between the two conditions and $\log_2$ counts per million > 3.3 (i.e. > 10 reads) are shown here. Red indicates transposon insertion in the forward direction while blue indicates insertion in the reverse direction. The genes identified as significant in Table 4.1 are shown in black and annotated; the surrounding genes are shown in grey. The y-axis indicates the number of insertions in each location.

## 4.4 Summary

In this chapter, I show that pConj is restricted to *Neisseria* spp. and is predominantly found in *N. gonorrhoeae*. Of note, pConj may be more widespread in other *Neisseria* spp. than my work suggests (**Figure 4.1**) due to limited WGS available for these *Neisseria* spp. and because the PubMLST database is not exhaustive. Therefore, increased sampling of commensal species and other members of the *Neisseriaceae* family will be important in understanding the distribution of pConj, especially as *tetM*$^{+}$ pConj has been previously described in *Eikenella corrodens* and *Kingella denitrificans* (Knapp *et al.*, 1988). These were not identified in my bioinformatics searches as the sequences of these isolates are not on the NCBI database. Of note, pConj was not detected in the 78 *Kingella* spp. (five *K. denitrificans*) or 40 *Eikenella* spp. (22 *E. corrodens*) assembled genome sequences on the NCBI database.

Phylogenetic analysis demonstrates that pConj has remained remarkably conserved, with plasmids isolated in 2000s virtually identical to those isolated in 1940s (**Figure 4.2**). Similar phylogenetic trees were obtained with two different tree building methods, increasing the robustness of conclusions. However, future work could include building phylogenetic trees with a larger number of isolates, which I was unable to do as our current bioinformatics platform ran out of computational memory during alignments. It would also be interesting to examine phylogenetic relationships of pConj in *N. meningitidis* and compare those with pConj in *N. gonorrhoeae*. This may provide insights into the acquisition and evolution of pConj in *N. meningitidis*. Finally, using the 126 pConj sequences, I was unable to estimate a date for the first introduction of this plasmid into *N. gonorrhoeae* prior to its first isolation in 1940s due to the remarkable conservation of the plasmid. However, by including a larger number of pConj sequences from both *N. gonorrhoeae* and *N. meningitidis*, it might be possible to estimate when pConj was first introduced into *Neisseria* spp.

Additionally, I found that the seven pConj variants can be categorised into two groups, with the main difference between the groups being the *ε:ζ* TA genes. The significance of the different *ε:ζ* TA system remains to be determined, especially as *tetM*$^{+}$ pConj with *ε:ζ2* has spread worldwide but not *tetM*$^{+}$ pConj with *ε:ζ3*. One potential explanation is that the *ε:ζ3* locus imposes a higher fitness cost than *ε:ζ2*. Toxicity and antitoxicity assays of ε:ζ2 and ε:ζ3 in *N. gonorrhoeae* could thus be performed to verify this hypothesis.

Previous research shows that *tetM*$^{+}$ pConj carriage is more common in LMICs, where doxycycline is often prescribed for STIs (Cehovin *et al.*, 2020). Of note, pConj prevalence in higher income countries has not fallen despite tetracycline no longer being used as treatment for gonorrhoea (**Figure 4.3**). However, it should be noted that in the USA doxycycline was part of dual therapy until 2014; doxycycline monotherapy stopped in 1980s (USA) or 2004 (UK) following the emergence of *tetM*$^{+}$ pConj. As such, our results might not accurately reflect the effect of doxycycline on pConj prevalence. Furthermore, doxycycline is still used as a therapy for *Chlamydia trachomatis* worldwide (World Health Organization, 2016), providing a potential selective advantage for strains with *tetM*$^{+}$ pConj (St Cyr *et al.*, 2020). However, the persistence of markerless pConj, which does not confer tetracycline resistance, suggests that pConj is not easily lost in the absence of antibiotics.

pConj is associated with different gonococcal lineages (Cehovin *et al.*, 2020). Using pConj variant 1, I showed that the introduction of pConj into different lineages did not result in fitness costs in two conditions (**Figure 4.5**). Previous research has suggested that the fitness cost of plasmids may only be apparent under nutrient limited conditions (Smith & Bidochka, 1998). In the future, I could determine if growth in limited media results in fitness cost for pConj carriage. It would also be interesting to determine if pConj imposes fitness costs *in vivo*. These experiments would involve infecting and determining the survival of strains with or without pConj in mouse models (Jerse *et al.*, 2011). Competition experiments, by co-infecting mice with strains with or without pConj, could

also reveal insights into pConj dynamics *in vivo*. Results from these experiments might therefore more accurately reflect pConj fitness costs in real world conditions. Finally, our results are only based on pConj variant 1; we would need to determine if similar results may be obtained for other pConj variants. Therefore, further experiments using another pConj variant from Group I would be of interest.

Experiments demonstrate that the conjugation frequency is associated with pConj prevalence in a lineage; recipients from lineages with low pConj prevalence exhibited lower conjugation frequencies than recipients from lineages with high pConj prevalence (**Figure 4.6**). It would thus be interesting to determine if this phenomenon is observed at a species level – specifically, is the conjugation frequency with *N. gonorrhoeae* as a recipient higher with *N. meningitidis* or *N. lactamica* as recipients? Previous research suggests that while conjugation frequencies vary at a species level, conjugation frequencies were not species specific, as some *N. meningitidis* and *N. cinerea* strains exhibited higher conjugation frequencies than *N. gonorrhoeae* when different donors were used (Roberts & Knapp, 1988b). However, it is also possible that mechanisms for the differences in distribution of pConj within *N. gonorrhoeae* do not explain differences in pConj presence between species. Furthermore, the conjugation frequencies observed previously were lower (Roberts & Knapp, 1988b) than that observed here. It would be interesting to determine whether we observe similar rates for conjugation into other *Neisseria* species as what was previously described.

Acquisition of foreign DNA by conjugation can be prevented by restriction modification systems (RMS) in recipient bacteria (Rocha & Bikard, 2022). RMS cleave incoming DNA which has a different methylation profile from the host bacterium. Also, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas systems can recognise certain genetic sequences and cleave incoming DNA. CRISPR systems can prevent phage infection (Rocha & Bikard, 2022). While *N. gonorrhoeae* does not carry any CRISPR-Cas systems (Zhang, 2017), *N. gonorrhoeae* has 15 RMS (Sánchez-Busó *et al.*, 2019b). Therefore, it is possible that RMS are involved in restricting incoming

plasmids. However, we show here that conjugation frequencies are not highest between isogenic strains, but are instead recipient dependent, with non-isogenic recipients sometimes having higher conjugation frequencies than isogenic recipients. Therefore, RMS are unlikely to be the sole explanation the differences in conjugation frequencies observed here.

To identify chromosomal genes involved in pConj acquisition, I carried out Tn-Seq. Tn-Seq identified many chromosomal genes potentially involved in conjugation. No potential surface molecule was identified which could act as a candidate involved in stabilising the mating bridge (**Figure 4.10**), such as *Klebsiella pneumoniae* OmpK36 which interacts with pKpQIL TrbN (Low *et al.*, 2022). Tn-Seq suggested that genes involved in membrane functions are involved in pConj transfer, consistent with observations in other species (Johnson & Grossman, 2014). However, it should be noted that false-positives are frequently identified in Tn-Seq (Johnson & Grossman, 2014). The metabolic genes identified could contribute to pConj acquisition, as disruption of recipient's metabolism occur during acquisition of RP4 plasmid (Prensky *et al.*, 2021). Therefore, it would be necessary to knock out these genes individually and determine their effects on acquiring pConj. Furthermore, as Tn-Seq is unable to define the effect of multiple genes, it would also be interesting to determine if these genes affect conjugation frequencies only when multiple genes are deleted together.

pCryp has been hypothesised to be important in the ability to acquire pConj as introduction of VapX from pCryp into *E. coli* increased conjugation frequencies (Pachulec & van der Does, 2010). Here, I was unable to assay the effect of pCryp due to its high copy number, which Illumina sequencing suggests was approximately 100 (not shown); Tn-Seq would not inactivate all copies of pCryp. Thus, it would be interesting to determine if any genes on pCryp have a role in pConj acquisition. Future work could include deleting or suppressing expression of *vapXD* on pCryp using CRISPR-Cas9. CRISPR-Cas has been previously used to construct genetic knockouts, even in multiple loci (Vo *et al.*, 2021). On the other hand CRISPRi, which uses a non-functional version

of Cas9, has been used to downregulate gene expression in other bacteria (Depardieu & Bikard, 2020) and can therefore be repurposed to determine the effect of *vapXD* on pConj acquisition.

Overall, I established that pConj has a P:C copy number of approximately five and is highly conserved in the gonococcus. The uneven distribution of pConj in different lineages may be due to differences in the strains' ability to acquire pConj, which potentially facilitates entry of pConj into a lineage, allowing for its spread and maintenance in a population by conjugation. However, I was unable to identify any single chromosomal gene important in pConj acquisition, similar to findings with other mobile genetic elements (Pérez-Mendoza & de la Cruz, 2009).

# 5 Mechanisms of pConj maintenance

In Chapter 4, I showed that differences in pConj prevalence in lineages may be due to differences in the ability of bacteria to acquire the plasmid. Additionally, I demonstrated that pConj is a highly conserved plasmid which is largely restricted to and persisted in *N. gonorrhoeae*. Thus, in this chapter, I aimed to determine the mechanisms responsible for pConj maintenance.

## 5.1 Use of fluorescence to detect pConj loss

Initial experiments with pConj indicate that the plasmid is lost infrequently during growth in the laboratory. Therefore sensitive methods to detect pConj loss are required as loss cannot be accurately detected by replica plating bacteria on media with or without tetracycline. In *E. coli*, negative selection markers such as *sacB* or *pheS* (Kast & Henneckef, 1991; Li *et al.*, 2013) can be used to identify bacteria that have lost plasmids. Recently, our lab described the use of *galK* from *E. coli* as a negative selection marker; as such, *galK* was cloned into pConj as a marker for bacteria losing pConj. The *galK:kan* cassette under the *opaB* promoter was inserted in the intergenic region between *vapD* and *res* on pConj. However, insertion of *galK* into pConj resulted in decreased growth of bacteria (Jones *et al.*, 2022), suggesting that *galK* is not suitable for plasmid loss assays.

Therefore, a fluorescence-based method was developed to detect the presence of pConj. $pC^{GFP}$ was constructed by inserting a *gfp*:*kan* cassette with an *opaB* promoter into the intergenic region between *vapD* and *res* on pConj (**Figure 5.1A**). The 88 bp intergenic region with a predicted terminator was duplicated. $pC^{GFP}$ was introduced into FA1090, and colonies with the plasmid appeared green when observed under a UV transilluminator. Initial experiments showed that using GFP fluorescence to determine pConj carriage had a sensitivity of 99.4% (1303/1311 colonies that were tetracycline resistant were also GFP-positive) and specificity of 98.7% (593/601 colonies that were tetracycline sensitive were also GFP-negative), suggesting that this technique accurately reflects the presence/absence of pConj. By plating out at least 200 colonies, this method

can detect plasmid loss at a low frequency (< 0.5%). To further ensure that loss of GFP fluorescence was due to plasmid loss rather than potential recombination events within the plasmid, GFP-negative colonies in all remaining experiments were re-streaked onto media with/without tetracycline to confirm plasmid loss.

### 5.1.1 pC$^{GFP}$ and pConj, does not impose a fitness cost

To determine whether insertion of the *gfp:kan* cassette into pConj confers a fitness cost, the growth of FA1090:pConj and FA1090:pC$^{GFP}$ was measured. Liquid cultures (200 µl) of GCBL inoculated at an $OD_{600}$ 0.1 and growth monitored by measuring the $OD_{600}$ every 20 m over 8 h. No significant differences in growth rates nor area under curve (AUC) of growth curves were seen between the strains ($p > 0.05$, two-way ANOVA with multiple comparisons, **Figure 5.1B-C**). Additionally, competition experiments were carried out. Briefly, FA1090 with or without pC$^{GFP}$ were mixed at a 1:1 ratio and grown for 8 h. The proportion of bacteria with or without pConj was determined by plating and examining 50 – 300 colonies for GFP-positivity with a UV transilluminator. Fitness was calculated by measuring the proportion of bacteria with pConj before and after growth, normalised by the number of generations (San Millan *et al.*, 2014b). In the absence of any fitness cost or advantage, the ratio of bacteria with:without pConj would be 1:1, *i.e.* a fitness of 1. Fitness < 1 suggests that pConj acquisition has a fitness cost, while fitness > 1 suggests that pConj acquisition has a benefit. Results indicate that fitness was 1.74 ±0.49, suggesting that pC$^{GFP}$ confers a fitness benefit rather than imposes a fitness cost (**Figure 5.1D**).

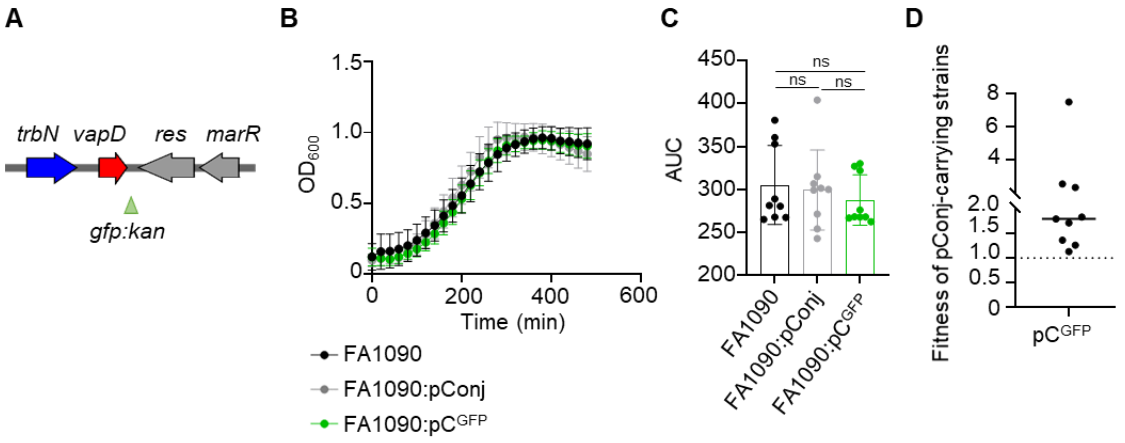



**Figure 5.1 Insertion of *gfp:kan* cassette does not result in fitness cost. A)** Schematic of *gfp:kan* insertion. The intergenic region between *vapD* and *res*, which contains a terminator sequence, is duplicated. No significant differences in **B)** growth rate and **C)** area under curve (AUC) of growth curves of FA1090 with or without pConj, with insertion of *gfp:kan* cassette in $pC^{GFP}$ was observed. The $OD_{600}$ was measured every 20 m over 8 h using Fluostar Omega (BMG Labtech). No plasmid loss was observed at the end of the experiment (shown by replica plating of 50 colonies, not shown). Results ($n \geqslant 3$) were analysed by two-way ANOVA with multiple comparisons; graphs show mean ± s.d. ns, $p > 0.05$. **D)** pConj-carrying and pConj-free bacteria were mixed at a 1:1 ratio and the exact ratio of pConj-carrying to pConj-free bacteria before and after the experiment was determined by plating (n = 8).

### 5.1.2 Introduction of *gfp:kan* does not affect pConj maintenance

Next, I examined whether insertion of *gfp:kan* affects pConj maintenance. FA1090:pConj and FA1090:pC$^{GFP}$ were grown overnight on GCB plates with tetracycline (to maintain the plasmid), then passaged three times in liquid FB without tetracycline for 20 h, before plating onto solid media. For FA1090:pConj, plasmid loss was detected by checking at least 120 colonies of each strain for tetracycline resistance. For FA1090:pC$^{GFP}$, green-white detection revealed no plasmid loss, and all 120 randomly picked colonies of FA1090:pConj remained resistant to tetracycline upon replica plating (not shown). There was also no loss of pConj at the end of experiments.

These results demonstrate that the introduction of *gfp:kan* into pConj does not affect either the fitness cost of the plasmid or its maintenance. Furthermore, GFP expression by colonies can be used to accurately assess pConj presence/absence. Therefore, pC$^{GFP}$ was used in subsequent studies to define genes involved in pConj loss.

## 5.2 pConj is stable over 160 generations

Previous research has shown that some plasmids can be lost in a population after 40 to a few hundred generations of growth onwards (Wein *et al.*, 2019; San Millan *et al.*, 2014b). Thus, it was important to determine if pConj is lost from the gonococcus over time. Having shown that pC$^{GFP}$ neither imposes a fitness cost nor affects pConj maintenance, FA1090:pC$^{GFP}$ was used to determine the stability of pConj over time. FA1090:pC$^{GFP}$ with *trbC* deleted (pCt$^{GFP}$) was also included to determine if pConj maintenance is dependent on a functional conjugation system. Briefly, strains were first grown on solid GCB. Liquid cultures (15 ml) in FB + 1% vitox starting at $OD_{600}$ 0.001 were then set up in the absence of antibiotics. Cultures were passaged every 24 h (approximately 10 generations) at starting $OD_{600}$ 0.001. Cultures were serially diluted and plated onto GCB every 48 h; colonies were then examined for GFP positivity under

the UV transilluminator to determine plasmid loss. Results show that no pConj loss was detected over 160 generations in the presence or absence of a functional conjugation system (not shown) (LOD = 0.3%), suggesting that conjugation does not play a role in maintaining pConj on a population level even in the absence of selection.

## 5.3 Host genes involved in pConj maintenance

pConj is extremely well maintained in *N. gonorrhoeae* and does not impose a fitness cost (Chapter 4). pConj is also largely restricted to *Neisseria* spp, suggesting that genes on the *N. gonorrhoeae* chromosome may be involved in pConj maintenance. To identify host genes involved in pConj maintenance, I set up an unbiased screen for genes involved in plasmid maintenance using Tn-Seq as in Chapter 4.

### 5.3.1 Tn-Seq library complexity decreases with each passage

The pConj-carrying Tn-Seq library generated in chapter 4.3.2 was passaged in duplicate in the presence or absence of tetracycline for 56 generations. Kanamycin was included to ensure selection of mutants in the library. The profile of insertions was determined at generations 0, 8, 16 and 56 using Bio-Tradis (Barquist *et al.*, 2016). Results show that the number of UIS diminished over time, with 183,919 UIS in the initial library and 81,655 to 89,336 UIS and 54,708 to 70,973 UIS after the 8$^{th}$ and 16$^{th}$ generation, respectively. By the 56$^{th}$ generation, there were only between 5,815 and 6,442 UIS in the cultures, too few to identify genes involved in plasmid maintenance. The profile of insertions was highly consistent between all cultures (**Figure 5.2A**).

### 5.3.2 Tn-Seq did not reveal any host genes important for pConj maintenance

Due to the limited number of UIS by the 56$^{th}$ generation, earlier passages were analysed. Transposon insertions were compared in cultures with or without tetracycline after 8 and 16 generations, which are the two earliest passages when the cultures were sampled. After 16 generations, eight significant hits were identified which were enriched in cultures without tetracycline, indicated by logFC < 0, $q$ < 0.01 and were identified after 16 generations (**Figure 5.2B**).

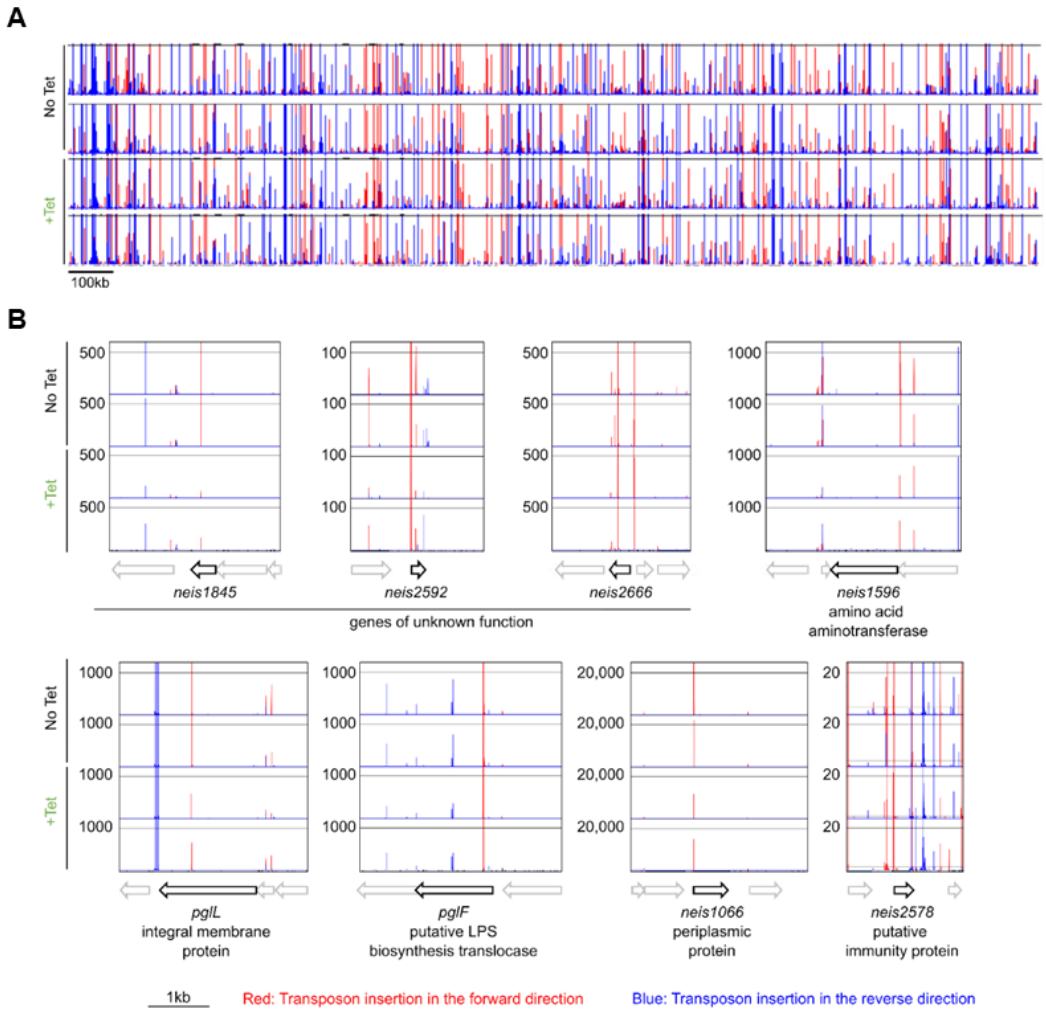



**Figure *5.2* Tn-Seq did not identify chromosomal genes involved in pConj maintenance.** FA1090 transposon library carrying tetM$^{+}$ pConj was passaged over 56 generations under kanamycin only and kanamycin and tetracycline conditions. The library was then sequenced before analysing with Bio-TRADIS and viewed on Artemis. **A)** Representation of insertions in *the* transposon insertion library at the *56*$^{th}$ generation when grown in the absence or presence of tetracycline (n = 2 biological repeats done in parallel). The number of unique insertion sites (UIS) at the 56$^{th}$ *generation was < 10% of the initial library.* ***B)*** *Significant hits identified in the 16*$^{th}$ *generation (log* fold change *< 0,* q *< 0.01) were viewed on Artemis as shown.* The genes identified as significant in Table 4.1 are shown in black and annotated; the surrounding genes are shown in grey. The y-axis indicates the number of insertions in each location.

The genes were further analysed bioinformatically to provide clues about their potential roles in pConj maintenance. Of note, attempts to determine the functions of NEIS1845, NEIS2592 and NEIS2666 using their respective amino acid sequences by both Phyre and HHpred were unsuccessful. Analysis of the surrounding genes revealed that *pglF* is part of the pilin glycosylation pathway (Aas *et al.*, 2007), *neis1066/ngo0834* may be involved in curli fibre formation (Płaczkiewicz *et al.*, 2019), while *pglL/ngo0178* is thought

to be involved in Lipid A synthesis (Kwiatek *et al.*, 2014). Separately, genes *neis1845*, *neis2592*, *neis2666* and *neis1596* are found near genes involved in metabolism, while *neis2578* is found within a *maf* genomic island that encode a toxin-antitoxin system involved in bacterial antagonism in *N. meningitidis* (Jamet *et al.*, 2015) (**Figure 5.3**). Of note, similar to what was observed in Chapter 4, inspection of insertion plots suggests that significance was due to differences in only one or two insertion(s) per gene (**Figure 5.2B**), which is characteristic of false-positive hits (Mahmutovic *et al.*, 2020). Therefore, the genes were not investigated further experimentally.

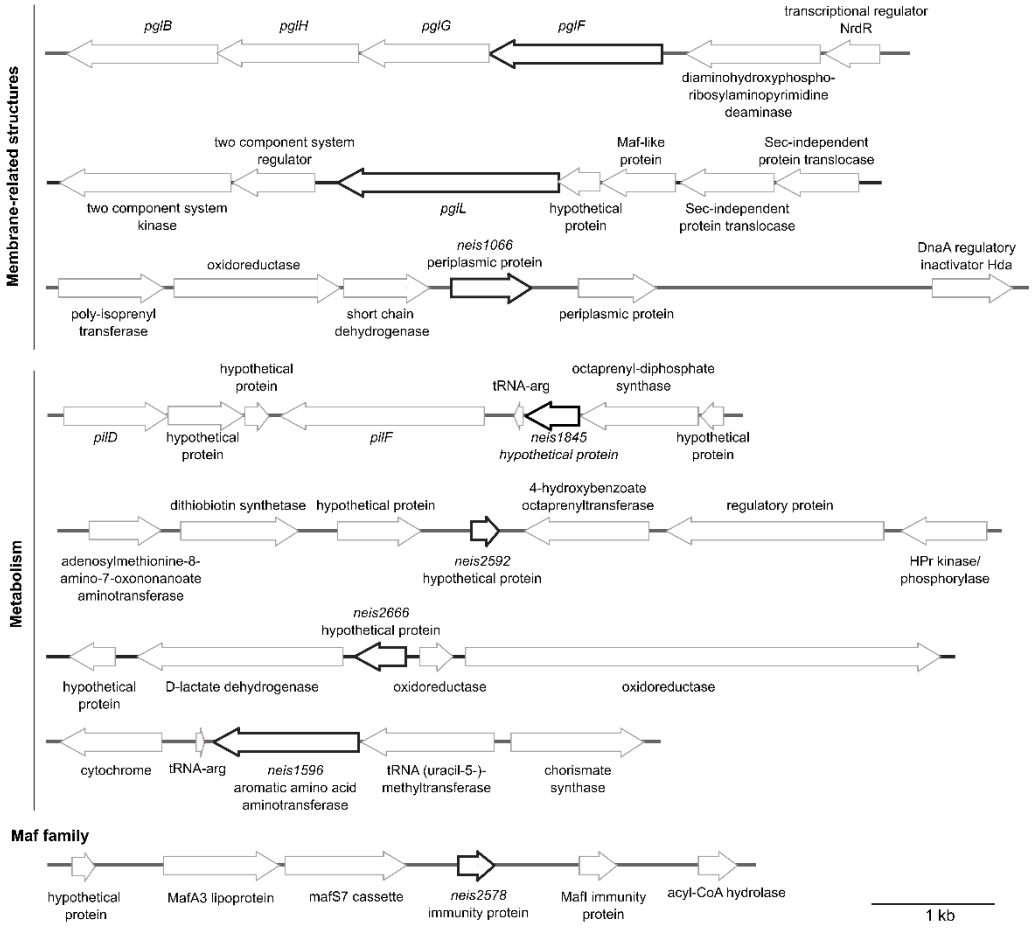



**Figure 5.3 Representation of genetic loci of genes identified in Figure 5.2.** The genes identified as significant in Figure 5.2 are shown here with black arrows; surrounding genes are shown in grey. Genes are grouped based on putative functions

Apart from the hits identified by Tn-Seq, other genes could be involved in pConj maintenance, but were not identified through statistical analysis by BioTradis due to the low number of insertions within the gene, for example, if the genes were essential or important for gonococcal growth. pConj carries type II TA systems including two ε:ζ TA systems and a gene encoding for *vapD* toxin; the function of Type II TA systems (which have a protein antitoxin) which likely require host cell proteases to cleave the antitoxin (Brzozowska & Zielenkiewicz, 2014; Diago-Navarro *et al.*, 2013; van Melderen *et al.*, 1994). Furthermore, in other bacteria, essential host genes such as those encoding helicases (Wahle & Kornberg, 1988) are involved in plasmid replication and partitioning. Therefore, the distribution of transposon insertions in and around 16 genes implicated in plasmid maintenance in other systems were inspected using Artemis. Many of these genes are likely to be essential for growth in FB as they lack transposon insertions. The surrounding insertions were similar in bacteria grown in the presence or absence of tetracycline at the 8$^{th}$ (not shown) and 16$^{th}$ generation (**Figure 5.4**). Therefore, our results indicate that any host gene involved in plasmid maintenance is likely to be essential or redundant.

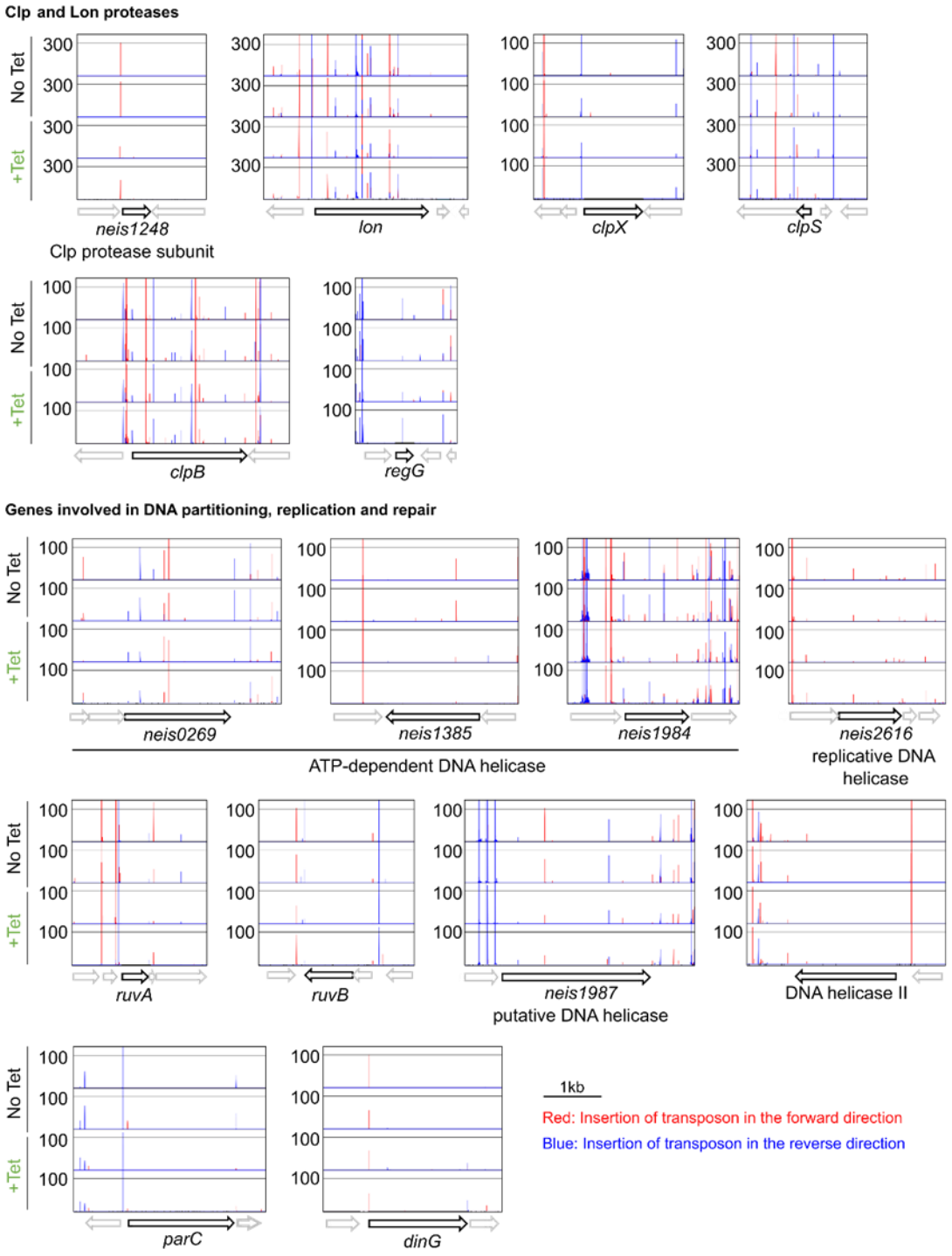



**Figure 5.4 Transposon insertions around potentially essential genes that might be involved in pConj maintenance.** FA1090 transposon library carrying *tetM*$^{+}$ pConj was passaged over 56 generations under kanamycin only ("No Tet") and kanamycin and tetracycline ("+Tet") conditions. The library was sequenced at the 16$^{th}$ generation before analysing with Bio-TRADIS and viewed on Artemis. The profiles of transposon insertions from duplicate cultures with or without tetracycline near genes involved in plasmid maintenance in other bacteria are shown here at the 16$^{th}$ generation. The genes that were hypothesised to be involved in pConj maintenance are shown in black and annotated; the surrounding genes are shown in grey. The y-axis indicates the number of insertions in each location. No significant differences were observed in bacteria grown with or without tetracycline.

## 5.4 Plasmid genes involved in pConj maintenance

As no chromosomal genes were identified for pConj maintenance, I next examined the role of genes on pConj. Plasmid genes encoding partitioning and TA systems are important in plasmid maintenance in other bacteria. Furthermore, interactions between plasmids have been implicated in plasmid maintenance (San Millan *et al.*, 2014a).

### 5.4.1 TA systems are involved in pConj maintenance

The 13 kb genetic load region of pConj contains three TA systems together with several hypothetical genes. None of these genes have been studied previously in the gonococcus. Therefore, to determine if genes in the genetic load region contribute to pConj maintenance, the entire genetic load region was replaced with *gfp*:*kan*, generating pCΔGL (**Figure 5.5A**). Plasmid loss was assessed following three passages of bacteria for 20 h under non-selective conditions (equivalent of 30 – 35 generations) in 5 ml liquid FB cultures; approximately 300 colonies were examined for the presence of GFP after this time (limit of detection, LOD = 0.3%). Results show that while plasmid loss for FA1090:pC$^{GFP}$ was below the LOD, FA1090:pCΔGL exhibited 8.5 ±3.1% plasmid loss (**Figure 5.5B**).

Within the genetic load region, there are three TA-associated genes: two *ε:ζ* TA systems are present in tandem and an orphan *vapD* gene. To determine which genes in the genetic load region contribute to plasmid maintenance, I first deleted *trbC* from pC$^{GFP}$ to prevent strains from re-acquiring pConj by conjugation (pCt$^{GFP}$). I then deleted or inactivated the TA systems individually (yielding pCtΔ*vapD*, pCt:*ζ1*$^{K115A}$, and pCtΔ*ζ2*), in combination (pCtΔ*vapD*:*ζ1*), or all together (pCtΔTA) (**Figure 5.5C**). Of note, the alanine substitution of K115 in ζ1 results in loss of ζ1 catalytic activity and thus toxicity (Rocker *et al.*, 2018). As previously, *gfp:kan* was introduced into these plasmids to allow assessment of plasmid loss by green-white detection, with approximately 300 colonies examined (LOD = 0.3%). Of note, similar to pC$^{GFP}$, no loss of pCt$^{GFP}$, pCtΔ*vapD* or

pCtΔ*ζ2* was observed after three passages. However, inactivation of *ζ1* led to detectable plasmid loss (0.33 ±0.3%), although this was near the LOD. Subsequent removal of *vapD* or all TA systems increased plasmid loss in a stepwise manner (3.2 ±0.4% and 5.6 ±1.6% for pCtΔ*vapD*:*ζ1* and pCtΔTA, respectively, $p = 0.041$ and $p = 0.019$, one-way ANOVA with Sidak's multiple comparisons, **Figure 5.5C**). Therefore, all TA systems contribute to pConj maintenance with the contribution of VapD and ζ2 only seen in the absence of other TA systems.

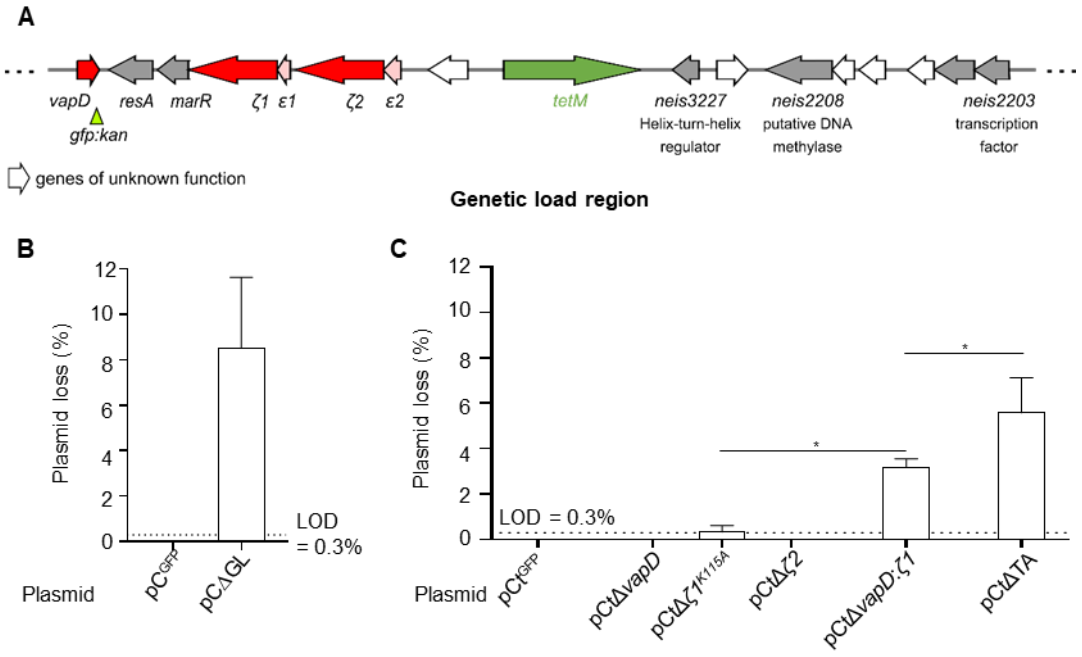



**Figure 5.5 TA systems within the genetic load contribute to pConj maintenance. A)** Schematic of the genetic load region. *gfp:kan* was used to monitor for the presence of pConj, while the *tetM* gene is as indicated. Red = toxins; pink = antitoxins; grey = genes with functions known; white = genes with unknown function. **B)** Deletion of the genetic load region resulted in plasmid loss, and **C)** individual TA systems were deleted to determine their role in pConj maintenance. Plasmid loss was determined for pConj with individual or all TA systems inactivated. Plasmid loss determined after three passages of growth under non-selective conditions; limit of detection (LOD) is as shown. n ⩾ 3, one-way ANOVA with Sidak's multiple comparisons, shown as mean±s.d. * $p < 0.05$

### 5.4.2 The role of VapD on pConj maintenance

#### 5.4.2.1 $VapD^{pConj}$ is toxic and can be neutralised by $VapX^{pCryp}$

Removal of *vapD* reduced pConj stability in the absence of ζ1 (**Figure 5.5C**), suggesting that VapD is part of a TA system without an obvious antitoxin encoded on the plasmid. In *H. influenzae*, VapD forms a TA system with VapX (Ren *et al.*, 2012). Co-crystallisation of VapX with VapD from *H. influenzae* indicates that VapX binds VapD in a 1:2 ratio (Bertelsen *et al.*, 2021). Although no VapX was identified on pConj, a *vapXD* TA system is present on pCryp (Korch *et al.*, 1985), which is carried by > 80% of gonococcal isolates (Cehovin *et al.*, 2020). *vapXD* is also found on the gonococcal chromosome in some gonococcal isolates (Cehovin *et al.*, 2020). Therefore, I aimed to determine whether $VapD^{pConj}$ is toxic, and whether VapX from pCryp or the chromosome can neutralise $VapD^{pConj}$.

$VapD^{pConj}$ shares 34% amino acid similarity with previously characterised VapD toxins and is predicted by Alphafold to dimerise, similar to other functional VapD toxins (Kwon *et al.*, 2012; Bertelsen *et al.*, 2021) (**Figure 5.6A**). These findings suggest that $VapD^{pConj}$ might be toxic.

I next determined whether $VapX^{pCryp}$ is predicted to interact with $VapD^{pConj}$. As *H. influenzae* VapD binds VapX in a 2:1 ratio (Bertelsen *et al.*, 2021), prediction of the $VapD^{pConj}$-$VapX^{pCryp}$ and $VapD^{pCryp}$-$VapX^{pCryp}$ complexes by AlphaFold used the same stoichiometry. Results suggest that while $VapX^{pCryp}$ binds to its cognate $VapD^{pCryp}$ in a similar configuration as *H. influenzae* VapXD, this was not the case for the predicted $VapD^{pConj}$-$VapX^{pCryp}$ complex (**Figure 5.6B**).

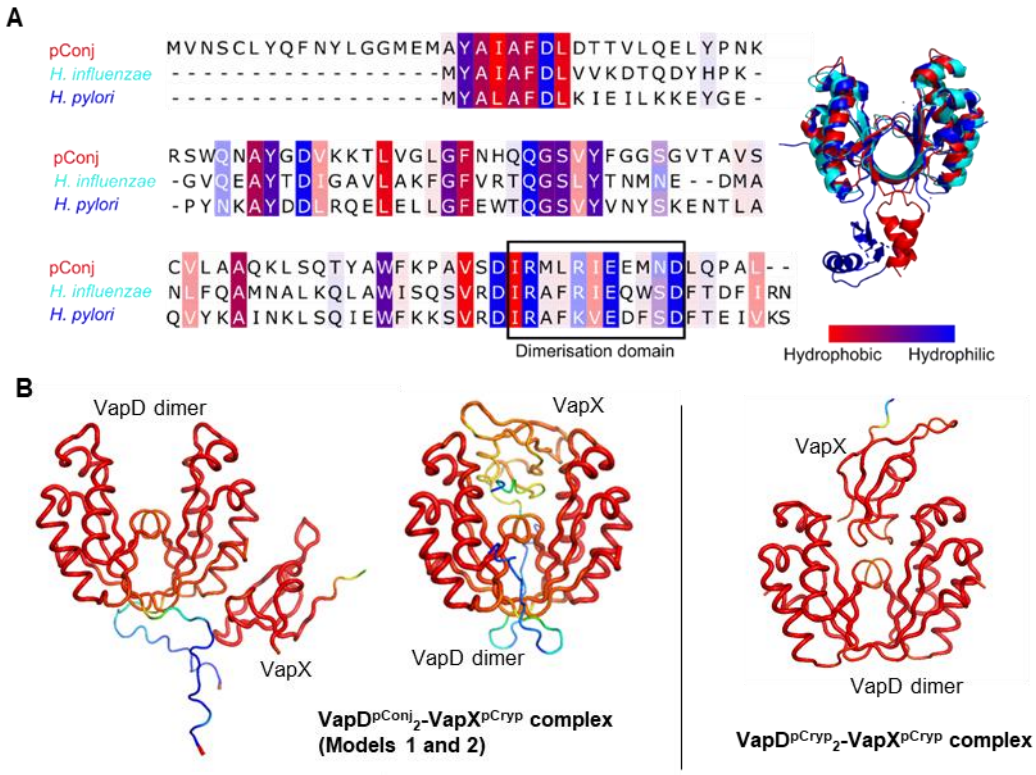



**Figure 5.6 Prediction of VapD and VapD-VapX interactions A)** Amino acid sequence of VapD$^{pConj}$ was aligned with amino acid sequences of VapD from *H. influenzae* and *H. pylori* with ClustalOmega. The predicted structure of VapD$^{pConj}$ (red) obtained from AlphaFold was overlaid with structures of VapD from *H. influenzae* (light blue, PDB 6ZN8) and *H. pylori* (dark blue, PDB 3UI3). **B)** Predicted VapD-VapX binding. VapD$^{pConj}$ and VapX$^{pCryp}$ sequences or VapD$^{pCryp}$ and VapX$^{pCryp}$ sequences were entered into AlphaFold and ran with five independent seeds. All models were predicted using the multimerisation function on Alphafold.

To assess whether VapD$^{pConj}$ is toxic and whether VapX$^{pCryp}$ can neutralise VapD$^{pConj}$, *vapD* or *vapD-vapX* constructs were ligated into pBAD33 under an arabinose inducible promoter and introduced into *E. coli*. After growing bacteria in liquid culture in the presence of glucose to inhibit expression, VapD$^{pConj}$ expression was induced by the addition of 0.2% (w/v) arabinose. VapD$^{pConj}$ expression resulted in decrease in bacterial viability compared to the empty vector control, showing that VapD$^{pConj}$ is toxic (**Figure 5.7A**). Co-expression of VapX$^{pCryp}$ abrogated VapD$^{pConj}$ toxicity ($p < 0.0001$, two-way ANOVA with Tukey's multiple comparisons, **Figure 5.7A**), indicating that VapD$^{pConj}$ potentially forms a 'split' TA system with VapX$^{pCryp}$.

Having established that VapX$^{pCryp}$ can neutralise VapD$^{pConj}$, I determined if other VapX variants can also neutralise VapD$^{pConj}$. Previously (Chapter 4), I showed that pConj is found in a proportion of *N. meningitidis* isolates, some of which carry another *vapX* allele, allele 22. The meningococcal *vapX* (*vapX$^{Nm}$*) encodes for a variant with an insertion of three amino acids near its N terminus; the rest of the protein is identical to VapX$^{pCryp}$ (**Appendix Figure 7.6**). Therefore, to determine if VapX$^{Nm}$ neutralises VapD$^{pConj}$, a neutralisation assay was carried out as above with VapX$^{Nm}$. Results demonstrate that similar to VapX$^{pCryp}$, VapX$^{Nm}$ neutralises the activity of VapD$^{pConj}$ ($p = 0.0013$, two-way ANOVA with Tukey's multiple comparisons, **Figure 5.7B**).

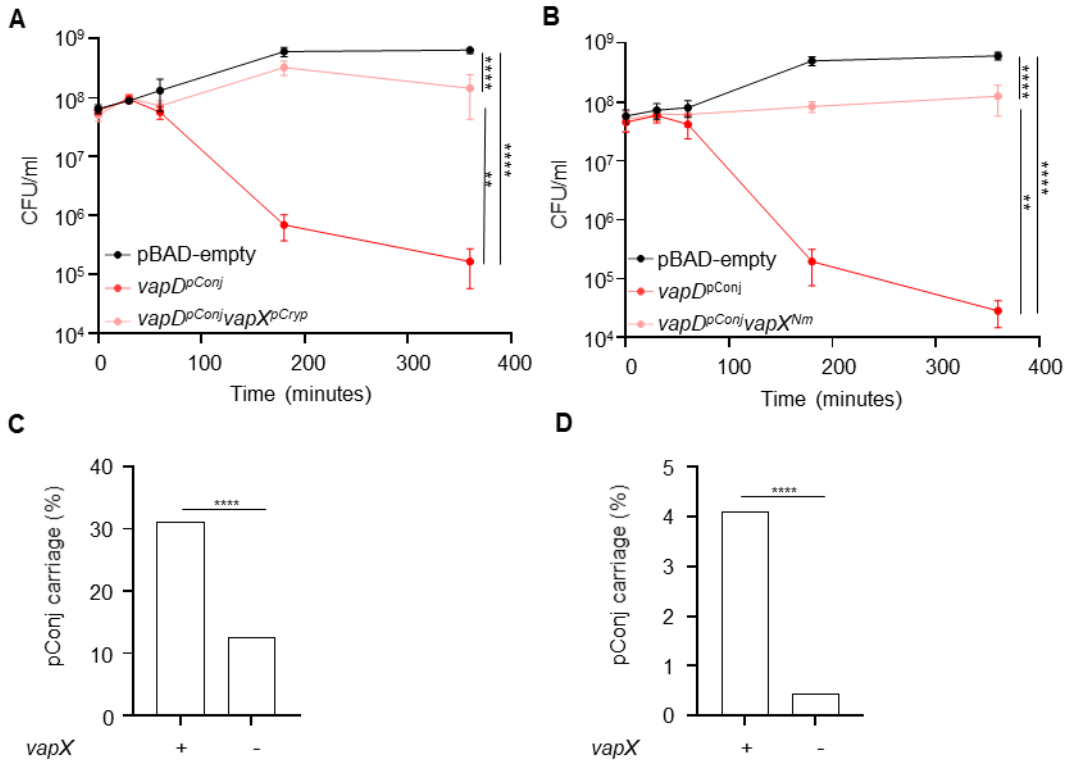



**Figure 5.7 VapX$^{pCryp}$ and VapX$^{Nm}$ can neutralise VapD$^{pConj}$, and pConj carriage is associated with presence of *vapX*.** To determine the toxicity of VapD$^{pConj}$, *vapD$^{pConj}$* was ligated into pBAD under an inducible promoter. Empty pBAD33 was used as a control. Expression was induced with 0.2% of L-arabinose and bacterial growth was monitored by plating and determining colony forming units (CFU/ml). The toxicity of VapD can be abrogated by **A)** VapX$^{pCryp}$ or **B)** VapX$^{Nm}$. Results (n = 9) were analysed by one-way ANOVA with Tukey's multiple comparisons and shown as mean±s.d. pConj is associated with *vapX* in **C)** *N. gonorrhoeae* and **D)** *N. meningitidis*. Results were analysed by Fisher's exact test. **, $p < 0.01$ ****, $p < 0.0001$

### 5.4.2.2 VapX is associated with pConj carriage in both *N. gonorrhoeae* and *N. meningitidis*

Having shown that both pCryp and meningococcal VapX can neutralise $VapD^{pConj}$, I next examined if there is an epidemiological association between VapD and VapX in *N. gonorrhoeae* and *N. meningitidis*. WGS on PubMLST were interrogated, and the presence of pConj detected by presence of *traM* or *trbC*. pCryp is present in the first gonococcal isolate in the database (from 1928, isolate DO371), while the first appearance of pConj (isolate DO17810) was in 1940; the first description of pConj in literature is from the 1970s (Roberts & Falkow, 1977). Furthermore $tetM^{+}$ pConj was reported in 1983 (Knapp *et al.*, 1987) (first isolate on PubMLST is isolate 02_227, in 1990), decades after markerless pConj was identified.

Next, I examined recorded gonococcal isolates to determine if there is an association between *vapD* and *vapX*. Isolates with a sequencing bin > 1 Mb in size and with ≤ 200 contigs were included to ensure that only isolates with sufficient sequence quality were examined. This yielded 9,618 isolates. pConj carriage was associated with presence of *vapX*: 31.2% (2,914/9,316) of *vapX*-carrying strains contain pConj, compared to only 12.5% (38/302) pConj carriage in the *vapX*-negative population ($p < 0.0001$, Fisher's exact test, **Figure 5.7C**). Thus, strains with *vapX* are more likely to carry pConj (odds ratio 3.1, 95% confidence interval 2.2 – 4.4, Woolf logit).

The association of pConj with *vapX* in *N. meningitidis*, a closely-related pathogen was also examined. *vapX* was found in a limited number of meningococcal isolates (1.1%, 194/16,912 isolates) and usually found on a horizontally-acquired island encoding a Type IV secretion system (T4SS, in 169/194, 87.1% of *vapX*-carrying strains, designated $vapX^{Nm}$), and less commonly on pCryp (10.3%, 20/194) (Harrison *et al.*, 2015b). The position of *vapX* in the remaining isolates could not be determined as *vapX* was assembled in a separate contig. pConj is present in 4.1% of meningococci with *vapX* (8/194 isolates) but only in 0.44% of strains lacking *vapX* (73/6,718 isolates, $p < 0.0001$,

odds ratio 9.8, 95% confidence interval 4.7 – 20, Baptista-Pike) (**Figure 5.7D**). Furthermore, unlike in the gonococcus, I found examples of pConj in *N. meningitidis* that lack *vapD*. Specifically, nine meningococcal isolates from different years carried pConj which has a 433 bp sequence in place of *vapD.* The flanking genes, *trbN* and *res,* are intact. Importantly, none of the meningococcal isolates with this pConj carries *vapX*, further emphasising the link between $VapD^{pConj}$ and VapX in pathogenic *Neisseria*.

### 5.4.3 TA and partitioning systems are important in pConj maintenance

ParAB partitioning systems influence the maintenance of large, low copy number plasmids (Funnell, 2016). Bioinformatic analysis suggests that pConj carries a potential ParAB partitioning system (Pachulec & van der Does, 2010), sharing 54% and 48% amino acid identity with IncC2 of Birmingham IncP-α plasmid and ParB from *X. fastidiosa,* respectively. pConj ParB was predicted to bind 5′-AATTTTAGCCGCTAAATAA-3′, although this has not been tested experimentally (Pachulec & van der Does, 2010). Therefore, pConj lacking ParB was generated (pCtΔ*parB*), and the effect on plasmid maintenance was examined. Due to the relatively high rate of loss of these plasmids, plasmid maintenance was examined after bacterial growth in liquid culture for only 20 h (6 – 9 generations). Strikingly, deletion of *parB* resulted in a reduction in plasmid maintenance, with 15 ±15% of bacteria losing pConj, compared to the strain with pConj containing *parB*, which had no detectable pConj loss (**Figure 5.8A**).

As disruption of ParAB led to an increase in plasmid loss, it was possible that the effect of partitioning was compensated for by PSK mediated by the TA systems on pConj. I therefore generated pConj lacking *parB* and the TA systems (pCtΔ*parB*ΔTA). After a single passage in liquid culture as above, almost all bacteria lost pConj when both the partitioning and TA systems were deleted (loss = 98.1 ±3.55%, $p = 0.0022$ compared to pCtΔ*parB* Mann-Whitney test) (**Figure 5.8A**). This demonstrates that TA and partitioning systems on pConj operate in concert to prevent plasmid loss. Therefore, ParAB acts to segregate pConj during replication and division, while the TA systems provide a fail-safe mechanism if partitioning fails, as evidenced by their role in the absence of ParAB (**Figure 5.8A**).

Given the high rate of conjugation observed in Chapter 4, I hypothesised that pConj loss can be partially rescued by conjugation. Therefore, I generated pCΔ*parB*ΔTA with a functional conjugation machinery. Interestingly, conjugation did not reduce pConj loss in FA1090:pCtΔ*parB*ΔTA (**Figure 5.8B**).

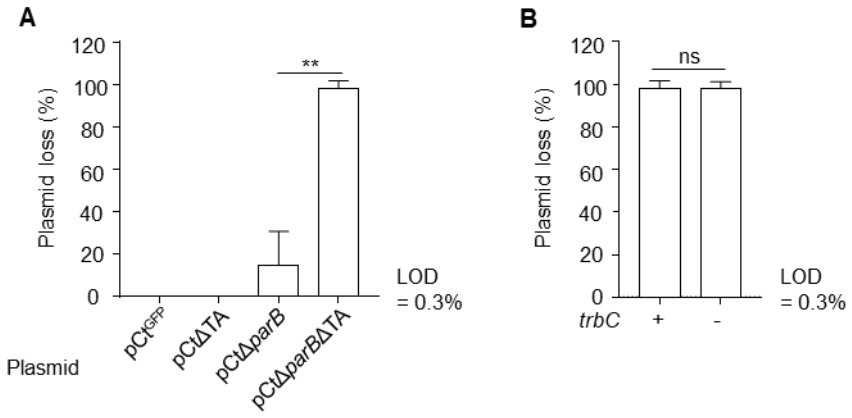



**Figure 5.8 Partitioning and TA systems contribute to pConj maintenance. A)** *parB* was deleted in the presence/absence of TA systems. Strains were grown on GCB kanamycin plates for 24 h, before individual colonies were picked and grown in FB + 1% vitox for 20 h (6 – 9 generations). Bacteria were then examined for the presence of pConj by detecting GFP by UV transilluminator. **B)** FA1090:pCtΔ*parB*ΔTA was grown as in **A)** and plasmid loss in the presence (*trbC*$^{+}$) or absence (*trbC*$^{-}$) of a functional conjugation system was determined. All results (n ⩾ 3) were analysed with Mann-Whitney test; limit of detection (LOD) is as shown. Graphs show mean±s.d. ns $p > 0.05$, ** $p < 0.01$

## 5.5 Ciprofloxacin treatment promotes pConj loss

Ciprofloxacin and other quinolones have been reported to affect plasmid maintenance (Weisser & Wiedemann, 1985). Furthermore, a decrease in plasmid carriage in *N. gonorrhoeae* was reported independently in different countries following the introduction of fluoroquinolones to treat gonococcal infection (Fox *et al.*, 1997; Kam *et al.*, 1995). Therefore, I sought to determine whether epidemiological observations of the effect of ciprofloxacin for treating gonococcal infection could be replicated in the laboratory. Ciprofloxacin affects DNA segregation due to interference with gyrase and topoisomerase (Belland *et al.*, 1994). Since plasmid partitioning, which I found was important in pConj maintenance, first requires proper plasmid segregation, ciprofloxacin was chosen for the experiment. Separately, spectinomycin was selected as a control as it was used in gonorrhoea treatment and affects ribosomal function in the protein synthesis pathway instead of any process directly implicated in plasmid biolgy (Schroeter *et al.*, 1975; Galimand *et al.*, 2000).

Previous research suggests that plasmid curing can occur at sub-MIC ciprofloxacin (Weisser & Wiedemann, 1985). Therefore, to determine concentrations of antibiotics for curing experiments, MIC assays were performed. Briefly, strains were grown for 18 – 22 h before $10^4$ CFU were spotted onto GCB plates containing different antibiotic concentrations. Plates were incubated for 20 – 24 h before visualisation. Results suggest that the MIC of 60755 and FA1090 is 1 µg/ml and 0.002 µg/ml respectively for ciprofloxacin, and 32 µg/ml for both strains for spectinomycin (**Figure 5.9A**). The MIC value for ciprofloxacin in FA1090 are consistent with those previously reported (Bettoni *et al.*, 2021). The MIC for spectinomycin in FA1090 is higher than previously reported partly because the strain is a streptomycin resistant variant selected for other experiments (Professor Ann Jerse, personal communication).

To determine whether ciprofloxacin can induce pConj loss, strains were subjected to 0.5 MIC ciprofloxacin. As a control, 0.5 MIC concentration of spectinomycin was used. To

eliminate effects of PSK by pConj TA systems, strains carrying pCΔGL, which does not carry any TA systems, were first grown on plain GCB overnight. Liquid culture containing 5 x $10^4$ CFU/ml in 5 ml FB was incubated in 37°C, 5% $CO_2$ under static conditions for 48 h (7 – 10 generations for 60755, 10 – 13 generations for FA1090). After 48 h, bacteria were plated onto GCB and plasmid loss was detected by loss of GFP and confirmed by re-streaking colonies to media with or without kanamycin. Results demonstrate that for 60755 and FA1090, ciprofloxacin treatment resulted in a significant increase in plasmid loss compared to spectinomycin (pConj loss of 4.66 ±1.69%, *vs.* 1.45 ±0.79%, $p = 0.0404$ for 60755; 7.61 ±0.24% *vs.* 1.82 ±1.07%, $p = 0.0008$ for FA1090 respectively **Figure 5.9B**).

**A**

| Antibiotics | 60755 | FA1090 |
|---|---|---|
| Ciprofloxacin | 1 µg/ml | 0.002 µg/ml |
| Spectinomycin | 32 µg/ml | 32 µg/ml |

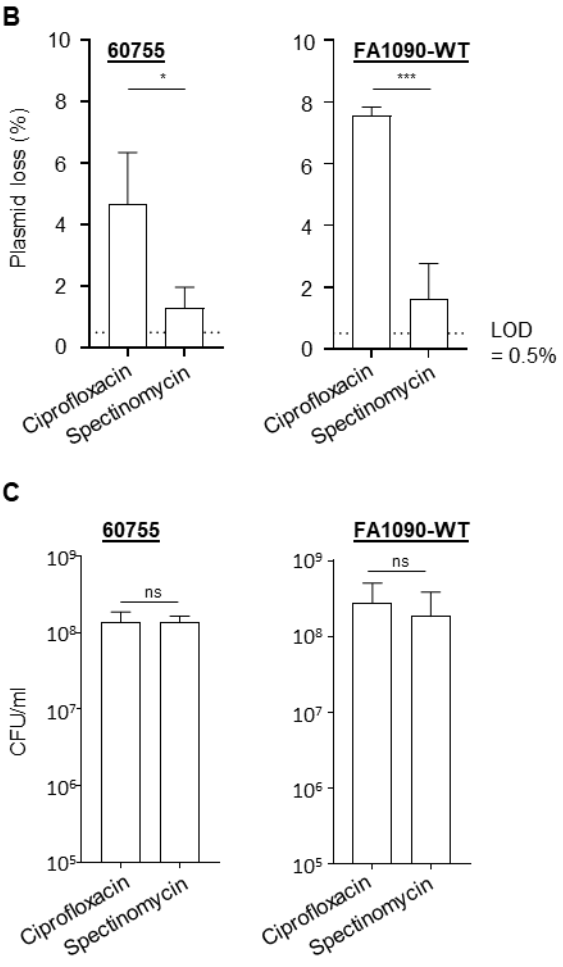



**Figure 5.9 Ciprofloxacin promotes higher pConj loss than spectinomycin. A)** The minimal inhibitory concentration (MIC) of strains 60755 and FA1090 to ciprofloxacin and spectinomycin was first determined using agar dilution **B)** Effect of ciprofloxacin or spectinomycin treatment on pConj loss. Liquid culture (5 ml) of each strain was prepared without shaking. Antibiotics were added to a final culture of 0.5 MIC; plasmid loss was determined using serial dilution and GFP detection using UV transilluminator. **C)** Growth in presence of 0.5 MIC ciprofloxacin or spectinomycin. At the end of the assay, cultures were serially diluted and spotted onto gonococcal agar; colony forming units (CFU/ml) was subsequently determined. Results (n = 3) were analysed with student's unpaired *t*-test and shown as mean±s.d. Limit of detection (LOD) is as shown * $p < 0.05$, *** $p \leq 0.001$

## 5.6 Summary

In this chapter, I aimed to determine how pConj is maintained in *N. gonorrhoeae*. To achieve this, I developed a fluorescence-based method for detecting pConj loss. This allowed assays to be carried out with LOD of 0.3 – 0.5%. Although this was sufficient for my experiments, the LOD is much higher than in systems with negative selection markers such as *sacB* or *galK.* We were unable to use *galK* in our experiments as *galK* on pConj resulted in a fitness cost (Jones *et al.*, 2022), potentially due to toxicity as pConj is present in multiple copies. As such, it would be helpful to develop additional negative selection markers for *N. gonorrhoeae* to detect pConj loss.

Using $pC^{GFP}$, I showed that pConj is extremely stable in *N. gonorrhoeae*, with no loss seen at 160 generations in the absence of antibiotic selection. Results from competition and growth experiments show that pConj does not impose fitness costs (**Figure 5.1**). This suggests that pConj is well-adapted to the gonococcus. However, as the competition and growth experiments ended in the stationary phase, future work would include determining if pConj imposed a fitness cost during the late stationary phase or when strains are competing with each other. Additionally, it would be interesting to determine if pConj is lost after more generations, as some plasmids have been reported to be lost only after 800 generations, although evidence of plasmid loss often occurs earlier (Wein *et al.*, 2019).

Furthermore, all experiments were conducted *in vitro* in liquid culture. Therefore, it would be interesting to determine if pConj is lost or imposes a fitness cost *in vivo*. *N. gonorrhoeae* is known to infect neutrophils and epithelial cells in humans. Thus, cellular infection models for *N. gonorrhoeae* could be established using primary cells as well as cell lines such as HL-60 cells (neutrophil-like) (Pantelic *et al.*, 2004), and human endometrial adenocarcinoma cells (HEC-1-B) (Griffiss *et al.*, 1999). Although these cells have been used to understand gonococcal infection dynamics, they could be repurposed to define the effect of pConj on gonococcal survival. However, it should be noted that

these cell lines do not necessarily reflect processes *in vivo*. Therefore, gonococcal infection models could also be used. These include female BALB/c mice treated with estradiol to mimic the conditions of the reproductive tract in humans (Jerse, 1999). Murine models could be used to determine if pConj contributes to invasion and survival. Human male participants have also been recruited for gonococcal infection studies (Cohen & Cannon, 1999); these would provide the most physiologically relevant model for understanding of pConj biology as the gonococcus is an obligate human pathogen.

To determine if chromosomal genes contribute to pConj stability, I carried out Tn-Seq (**Figures 5.2 – 5.4**). Tn-Seq involves generating a mutant library with transposons randomly inserted into the chromosome. Tn-Seq thus allows for unbiased large scale screening of non-essential genes in the gonococcal genome, with insertions of interest identified by sequencing. Tn-Seq is thus advantageous to chemical mutagenesis, in which identifying individual mutations is more challenging. Data here suggests that pConj maintenance is not reliant on a single, non-essential host gene. While eight genes were identified to have a potential role in pConj maintenance, bioinformatic analysis of their roles, which suggest that they are involved in a variety of functions ranging from pilin glycosylation, general metabolism to part of the *maf* island, do not suggest any obvious individual candidate gene or pathway which might contribute to pConj maintenance. Of note, the role of general metabolism on plasmid stability is consistent with previous research (Ricci & Hernández, 2000). It would be interesting however to determine if the TA systems in the *maf* island contribute to pConj maintenance. On the other hand, the significant hits identified are likely to be false-positive signals as they are due to a small number of insertions. Overall, this suggests that pConj is not reliant on any non-essential host factor.

However, there are also shortcomings to Tn-Seq. Firstly, the complexity of the Tn-Seq library decreased by the 56$^{th}$ generation (**Figure 5.2**). Insertions in the 56$^{th}$ generation were consistent across all cultures both with or without tetracycline, suggesting that the culture has been dominated by mutants with increased fitness when grown in FB +

kanamycin in *vitro*. This limited the sensitivity of the assay for genes contributing to pConj maintenance. Furthermore, Tn-Seq cannot detect for the effects of multiple or essential genes. Other assays such as CRISPRi could be employed to determine if essential genes are involved in pConj maintenance (Rousset *et al.*, 2018; Peters *et al.*, 2016).

I demonstrated that pConj TA and partitioning systems contribute to its maintenance. When genes encoding individual TA systems were deleted, no pConj loss was seen, except for ζ1 inactivation, which led to plasmid loss close to the LOD (**Figure 5.5**). Additional deletion of *vapD* and *ζ2* showed that TAs act synergistically to maintain pConj. ε:ζ1 was shown to be a functional TA system in *E. coli* previously, with its mechanism of action the phosphorylation of sugars involved in peptidoglycan synthesis (Rocker *et al.*, 2018). Future work could determine whether all ε:ζ TA systems are functional in *N. gonorrhoeae* and the biochemical activity of all three TA systems measured to determine the efficacy of each, and whether any cross-regulation might be present.

VapD$^{pConj}$ could affect pConj stability through an interaction with VapX$^{pCryp}$. Although VapD$^{pConj}$ can be neutralised by VapX$^{pCryp}$ in *E. coli*, the exact mechanism is unknown (**Figures 5.6 – 5.7**). AlphaFold predicted that VapX$^{pCryp}$ binds to a different site on VapD$^{pConj}$ than previously characterised *H. influenzae* VapD$_2$-VapX structures. This suggests that either VapX$^{pCryp}$ interacts with VapD$^{pConj}$ at a unique site, or that VapX$^{pCryp}$ neutralises VapD$^{pConj}$ indirectly. Alternatively, the AlphaFold prediction could also be incorrect as only a single VapD-VapX complex has been crystallised and deposited in the database. To determine whether VapX$^{pCryp}$ interacts with VapD$^{pConj}$, VapX$^{pCryp}$ can be crystallised with VapD$^{pConj}$; however, this would not confirm their interaction in *N. gonorrhoeae*. Site-directed mutagenesis could also be carried out to test if the VapD$^{pConj}$ dimerisation domains and active site predicted by AlphaFold are correct. Additionally, previous studies have suggested that VapD is an endonuclease that cleaves mRNA; however, *H. influenzae* VapD is not toxic when expressed in *E. coli* (Kwon *et al.*, 2012) unlike VapD$^{pConj}$. As such, future work would include purifying VapD$^{pConj}$ to determine its mechanism of action by biochemical assays (Kwon *et al.*, 2012).

Furthermore, although I showed that VapX$^{pCryp}$ can neutralise VapD$^{pConj}$ in *E. coli*, it remains to be determined if this is relevant for pConj maintenance. I was unable to determine the effect of pCryp VapXD on pConj maintenance as clinical strains without pCryp could not be obtained or generated. I attempted to delete *vapXD* in WHO F as this strain does not carry pCryp. Instead, WHO F harbours *vapXD* in the chromosome; however, PubMLST sequence of WHO F suggests that it also carries additional genes such as a T4SS, common in gonococcal isolates with chromosomal *vapXD* (Cehovin *et al.*, 2020). Thus, while studies on WHO F might provide insights into the role of *vapXD* on pConj, WHO F would not be an ideal gonococcal isolate for determining pConj-pCryp interactions. It would also be interesting to determine if the pConj-pCryp interaction helps stabilise pConj especially as previous research have suggested that epistasis between co-infecting plasmids can reduce fitness costs of individual plasmids (San Millan *et al.*, 2014a). Therefore, future work could include curing pCryp by CRISPR, or at least inhibiting *vapXD* expression through CRISPRi, as discussed in Chapter 4. This may be challenging as pCryp is predicted to have a high copy number as determined by Illumina sequencing (not shown).

Here, results suggest that pConj is maintained by both the TA and partitioning systems. The ParAB partitioning system appears to be functional and more important than TA systems as pConj loss could be detected when *parAB* was deleted; under the same conditions, plasmid loss was below LOD with the loss of TA systems (**Figure 5.8**). Plasmid loss is exacerbated when both TA and partitioning systems were deleted. Therefore, this suggests that pConj deploys a fail-safe mechanism – under usual circumstances the plasmid is segregated prior to cell division by a functional ParAB partitioning system. However, if partitioning fails, TA systems act *via* PSK to eliminate bacteria which lost the plasmid. Future experiments could include determining the sequence to which ParB binds, and whether any of the other genes in the replication and partitioning locus on pConj are involved in partitioning. Interestingly, conjugation did not contribute to pConj maintenance in our experiments despite a high conjugation

frequency (Chapter 4). It would therefore be interesting to determine the extent to which conjugation aids pConj maintenance in different circumstances, such as in dense bacterial populations.

Given the importance of the ParAB to pConj maintenance, I decided to determine if pConj could be cured by ciprofloxacin. Ciprofloxacin is a fluoroquinolone that can cure plasmids in *E. coli* (Weisser & Wiedemann, 1985). Ciprofloxacin inhibits DNA gyrase and topoisomerase (Belland *et al.*, 1994), suggesting that ciprofloxacin can inhibit plasmid segregation and therefore partitioning. I showed that compared to spectinomycin, exposure to ciprofloxacin results in higher pConj loss (**Figure 5.9**). However, it should be noted that the effect of ciprofloxacin was only seen in the absence of the genetic load region; no loss was seen with the wild-type plasmid. Despite this, the findings provide proof of concept for future work targeting ParAB or/and the TA systems could cure pConj from *N. gonorrhoeae*. Finally, the effect of ciprofloxacin correlates with observations that introduction of this antibiotic for gonorrhoea treatment was associated with a decrease in p*bla* and pConj prevalence (Fox *et al.*, 1997; Kam *et al.*, 1995). Future work would include identifying conditions in which ciprofloxacin has a direct effect on pConj.

Overall, I have shown here that pConj is maintained by the synergistic effect of the TA and partitioning systems. pConj also potentially interacts with pCryp, although understanding pConj-pCryp interactions and their contribution to pConj maintenance would require additional work. Finally, I showed that pConj can be eliminated by ciprofloxacin, suggesting that new generation gyrase inhibitors (Bradford *et al.*, 2020) could be useful in removing pConj from gonococcal populations.

# 6 Discussion

The overall aim of the thesis was to identify new opportunities to target AMR in *N. gonorrhoeae*. This was addressed through two lines of investigation: firstly, identifying novel interactions between complement and *N. gonorrhoeae*, and determining mechanisms underlying the spread and maintenance of a resistance plasmid, pConj.

## 6.1 Novel ways of solving the AMR crisis

*N. gonorrhoeae* is becoming resistant to an increasing number of antibiotics (Unemo & Shafer, 2014). This is a common problem not just for *N. gonorrhoeae*, but also other pathogens such as *K. pneumoniae*, *A. baumanii* and *M. tuberculosis* (Koch *et al.*, 2018; de Oliveira *et al.*, 2020). One approach to circumvent the problem of resistance is to develop vaccines (Micoli *et al.*, 2021). However, vaccination is not always a viable option – for *N. gonorrhoeae*, attempts to develop vaccines leading to long lasting immunity have failed (Edwards *et al.*, 2016). Also, vaccines do not necessarily prevent the development of AMR. For example, multidrug resistant *M. tuberculosis* has arisen despite an available vaccine (World Health Organisation, 2021). Therefore, novel ways of solving the AMR crisis are needed.

The concept of immunotherapy first arose to target cancer, and currently, there are multiple approved immunotherapies ranging from drugs to CAR-T cells against cancer (Waldman *et al.*, 2020). Similar to cancer, pathogens possess mechanisms to evade or suppress the immune system. For example, both *M. tuberculosis* and *Salmonella enterica* can programme macrophage polarization towards an anti-inflammatory phenotype (M2-like) (Benoit *et al.*, 2008). Similarly, *N. gonorrhoeae* survives in neutrophils and evades killing, although the precise mechanism is not well defined (Criss *et al.*, 2021). Currently, there are immunotherapies against a variety of bacterial infections in development. These include checkpoint inhibitors targeting the PD-1/PD-L1 T cell pathway which prevents overactivation of immune response. Pathogens such as *Burkholderia pseudomallei* and *H. pylori* can upregulate PD-1 expression; therefore, this

checkpoint is currently the target for sepsis and *H. pylori* treatments. Apart from checkpoint inhibitors, drugs modulating cytokine levels are also being developed for infectious diseases, such as targeting T cells *via* TGF-β for the treatment of tuberculosis (McCulloch *et al.*, 2022).

Currently, some immunotherapy is being developed against gonococcal infection. Specifically, as *N. gonorrhoeae* binds to $CFH_{(18\text{-}20)}$, $CFH_{(18\text{-}20)}$ fused to human IgG1 antibody has been developed and shown to increase complement-mediated killing of *N. gonorrhoeae* and decrease bacterial load in mouse models (Shaughnessy *et al.*, 2016). Research is currently ongoing to improve these chimeric molecules for therapeutic purposes (Shaughnessy *et al.*, 2020); additional research into host-pathogen interactions in *N. gonorrhoeae* will expand the possibilities of immunotherapies for gonorrhoea in the future. Here, I showed that CFHR5 binds *N. gonorrhoeae* and so could be exploited for immunotherapy: *N. gonorrhoeae* binds CFH to evade complement-mediated immunity (**Chapter 3**) (Chen & Seifert, 2013), and the binding of CFHR5 to PorB suggests that CFHR5 could be a competitive antagonist of CFH, resulting in increased complement killing of *N. gonorrhoeae*. However, it should be noted that gonococcal immunity is not only limited to the complement system as immune cells such as neutrophils and Th17 cells play a role in gonococcal infection (Sarantis & Gray-Owen, 2012; Liu *et al.*, 2012). Future work on the interaction between *N. gonorrhoeae* and immune cells should shed light on how *N. gonorrhoeae* is able to prevent killing by immune cells during infection and inhibit immune memory. Thus, harnessing both the innate and adaptive immunity may be required for the development of immunotherapies against *N. gonorrhoeae*.

Apart from immunotherapy, an alternative way to target AMR is by destabilising plasmids. Plasmids are the main carriers of AMR determinants in *Enterobacteriaceae* (Rozwandowicz *et al.*, 2018). Furthermore, a single plasmid can carry multiple AMR genes: examples include R plasmids that were isolated since 1960s from infected patients (Watanabe, 1963; Suzuki *et al.*, 1978). The presence of multiple AMR genes

on plasmids is facilitated by integrons (Fluit & Schmitz, 2004) and transposons carrying different resistance cassettes. For example, R100 was first isolated in *Shigella flexneri* and carries genes encoding for resistance against sulphonamides and aminoglycosides on Tn*21*, against chloramphenicol on Tn*9*, and against tetracycline on Tn*10* (Nikaido, 2009; Miki *et al.*, 1978). Plasmids of *K. pneumoniae* can carry multiple AMR genes on different transposons (García-Fernández *et al.*, 2012). Thus, the elimination of these plasmids would result in increased susceptibility of bacteria to available antibiotics. Unfortunately, despite plasmids usually imposing a fitness cost, decrease in antibiotic use is often insufficient to reverse plasmid-mediated AMR in a clinical setting (Salyers & Amabile-Cuevas, 1997), as AMR-carrying plasmids can persist through conjugation (Lopatkin *et al.*, 2017) or by mutations which reduce plasmid cost (Hall *et al.*, 2021). Therefore, additional methods targeting plasmid-mediated AMR are required.

Plasmids are stabilised by TA systems and/or partitioning systems. TA systems identified on plasmids are often type II TA systems, in which the antitoxin protein binds to the toxin to neutralise its toxicity. For example, the GmvAT system is important in pINV maintenance in *S. flexneri* (McVicker & Tang, 2016), while the ParDE TA system contributes to plasmid stability in various *Enterobacteriaceae* (Kamruzzaman & Iredell, 2019). Type II TA systems form protein-protein complexes and are thus vulnerable to host proteases such as Lon (McVicker *et al.*, 2019) and Clp (Brzozowska & Zielenkiewicz, 2014), suggesting that expression of these proteases could affect plasmid stability (McVicker *et al.*, 2019). Indeed, TA systems targeted by CRISPR-Cas can kill bacteria by inactivating the antitoxin genes (Citorik *et al.*, 2014).

Apart from TA systems, targeting DNA partitioning machinery can also lead to plasmid curing. In particular, fluoroquinolones such as ciprofloxacin is able to cure plasmids in *E. coli* (Weisser & Wiedemann, 1985). Similarly, novobiocin, an aminocoumarin antibiotic which also interferes with DNA gyrase, was shown to cure plasmids in *Lactobacillus* spp. (Ruiz-Barba *et al.*, 1991) and *E. coli* (McHugh & Swartz, 1977). Thus, drugs can potentially be repurposed to cure plasmids.

Unlike many plasmids in enteric bacteria, *N. gonorrhoeae* plasmids encode for a single or no AMR determinant. Specifically, pConj carries the *tetM* gene which is responsible for high-level tetracycline resistance (Morse *et al.*, 1986), while p*bla* carries $bla_{TEM}$ which is one mutation away from becoming an ESBL (Kandinov *et al.*, 2022). I show that pConj is maintained by the collective effects of TA and partitioning systems (**Chapter 5**). Importantly, conjugation did not seem to be important in pConj maintenance in my experimental conditions, suggesting that compounds inhibiting conjugation such as linoleic acid (Fernandez-Lopez *et al.*, 2005) are unlikely to increase pConj loss. There was increased pConj loss when bacteria carrying pConj were exposed to ciprofloxacin, even in the presence of a functional conjugation machinery. Of note, the genetic load region was deleted in my curing experiments. Thus, strategies targeting TA and partitioning systems could eliminate plasmid-mediated AMR in *N. gonorrhoeae*. It would also be interesting to determine if these strategies could be extended to plasmids in other bacteria.

Apart from plasmid-mediated AMR, chromosomally-mediated AMR is also important in *N. gonorrhoeae*. For example, mutations in *rpsJ*, *mtrR* and *porB* result in tetracycline resistance (> 2 μg/ml) (Hu *et al.*, 2005), and mosaic *penA* alleles contribute to high-level β-lactam resistance (Lindberg *et al.*, 2007). Thus, plasmid curing may not have a large impact on AMR in *N. gonorrhoeae*. However, it should be noted that pConj confers tetracycline resistance of > 10 μg/ml (Morse *et al.*, 1986), which is higher than that conferred by chromosomal mutations, suggesting that pConj elimination could still aid in reintroducing tetracyclines for treating gonorrhoea. Furthermore, given the role of chromosomal genes in AMR, resistance may develop against chemical methods for plasmid curing. Indeed, the use of ciprofloxacin in *N. gonorrhoeae* provides a cautionary tale, as although use of ciprofloxacin coincided with a decrease in plasmid prevalence in *N. gonorrhoeae*, fluoroquinolone resistance ( ≥ 1 μg/ml) quickly emerged in countries where ciprofloxacin was used (Kam *et al.*, 1995; Fox *et al.*, 1997). There is hope, however, that fluoroquinolones can still be used as curing agents despite resistance, as

increased pConj loss was observed in the presence of ciprofloxacin for strain 60755, which has a ciprofloxacin MIC of 1 μg/ml.

Overall, with the increasing resistance of *N. gonorrhoeae* against antibiotics, novel ways of targeting *N. gonorrhoeae* are needed. These could include targeting *N. gonorrhoeae* through the immune system or through plasmid curing.

## 6.2 Species-specific plasmids

Plasmids can be categorised as broad or narrow host range plasmids. Examples of broad host range plasmids include those in enteric bacteria belonging to IncP group (Popowska & Krawczyk-Balska, 2013). As these plasmids often carry multiple AMR determinants and can spread to different species, they are clinically important and thus have been studied extensively (Rozwandowicz *et al.*, 2018). Broad host range plasmids can harbour multiple origins of replication and initiate replication independent of host factors (Jain & Srivastava, 2013). However, these features do not define broad host range plasmids as there are also plasmids with multiple origins that have narrow host range, such as R6K (Rakowski & Filutowicz, 2013). It has also been suggested that narrow host range plasmids are more susceptible to restriction systems compared to broad host range plasmids; however, transfer of broad host range plasmids is also biased towards recipients with similar restriction-modification systems (Dimitriu *et al.*, 2019). Therefore, narrow host range plasmids require more study to define their characteristics.

Despite being an IncP plasmid (Pachulec & van der Does, 2010), pConj is an example of a narrow host range plasmid as it can only be transferred and maintained in *Neisseria* spp. (Roberts & Knapp, 1988b). Analysis of all *Neisseria* spp. revealed that pConj exists mostly in *N. gonorrhoeae* (**Chapter 4**). Here, I show that pConj is exquisitely adapted to *N. gonorrhoeae* as it does not impose a significant fitness cost when introduced into strains which do not naturally carry it. pConj also persists in *N. gonorrhoeae* despite the

absence of antibiotic selection. Thus, the work presented here offers insights into how a narrow host range plasmid behaves in its natural host. It would be interesting to determine how pConj behaves in other related hosts such as *N. meningitidis* or *N. lactamica*. Comparing pConj carriage in *N. meningitidis* or *N. lactamica* with carriage in *N. gonorrhoeae* may shed light on how and why narrow host range plasmids are specific for their hosts. Overall, understanding pConj biology may pave way to understanding other narrow host range plasmids.

## 6.3 Insights into the role of pCryp in *N. gonorrhoeae*

Cryptic plasmids are plasmids with no discernible benefit to the host and are found in many bacteria species, ranging from the clinically important and the environmental species (Challacombe *et al.*, 2017; Hamidian & Hall, 2018). *N. gonorrhoeae* carries a cryptic plasmid which is present in > 80% of all isolates (Cehovin *et al.*, 2020). Despite the prevalence of pCryp, its role in *N. gonorrhoeae* is unknown. Although expression of genes on pCryp is increased under anaerobic conditions, the significance of this finding is not clear (Isabella & Clark, 2011). Here, I show that pCryp may contribute to pConj maintenance, and thus AMR spread.

Additional studies into pCryp may reveal important roles of the plasmid to *N. gonorrhoeae* biology. To study pCryp, isogenic strains with and without pCryp must be generated. Attempts to cure pCryp were unsuccessful (not shown). We managed to obtain a strain, WHO F, which does not carry pCryp but carries *vapXD* on the chromosome. While it is possible to genetically manipulate WHO F, it remains to be determined whether WHO F is a suitable isolate for study as it might have accumulated mutations to compensate for its lack of pCryp. Furthermore, the WHO F sequence on PubMLST shows that the strain harbours two type IV secretion system islands encoded on its chromosome which is very unusual in the gonococcus. Thus, future work could include targeting pCryp with CRISPR-Cas systems to achieve pCryp curing in a pCryp-carrying strain.

## 6.4 Interactions between different genetic elements which stabilise plasmids

The introduction of a plasmid into a host often results in interactions between various genetic elements which influence the fitness cost of the incoming plasmid. This includes both plasmid-plasmid and plasmid-chromosome interactions.

Bacteria can carry multiple plasmids which can stabilise or destabilise each other. The inability of plasmids from the same incompatibility group to coexist in a single bacterium has been well established (Novick, 1987). However, recent research suggests that plasmids of distinct incompatibility groups can also influence each other's fitness. For example, the fitness cost of pOXA plasmids in *E. coli* is influenced by the presence of other plasmids (Lee & Ko, 2021). In this thesis, I hypothesised that pCryp might interact with pConj through a split VapX-VapD system. Previous research provides independent evidence for this, as the presence of pCryp VapX in *E. coli* increased conjugation of pConj from the gonococcus (Pachulec & van der Does, 2010). However, it should be noted that I did not measure the effect of pCryp on pConj fitness or maintenance, as all strains tested carried pCryp. Additional research into the impact of pCryp on pConj should determine if the presence of pCryp prior to pConj introduction contributes to the lack of fitness cost seen for pConj. This is interesting as positive epistasis between large and small plasmids has been demonstrated both experimentally and epidemiologically (San Millan *et al.*, 2014a). If true, this could be another reason why pConj is found almost exclusively in *N. gonorrhoeae*, particularly as pCryp is also only identified in *N. gonorrhoeae*. Of note, I found no evidence that pCryp is mobilised by pConj (not shown) although pCryp encodes its own mobilisation genes (Sarandopoulos & Davies, 1993).

In addition to plasmid-plasmid interactions, plasmids can interact with the chromosome (Vial & Hommais, 2020). Previous research in *E. coli* has suggested that plasmid fitness cost may be due to incompatibility between the plasmid and host genes (Hall *et al.*, 2021) due to transcription or replication conflicts (Wein *et al.*, 2019), although this has recently

been questioned (Rodríguez-Beltrán *et al.*, 2022). Mutations in chromosomal genes, particularly those involved in metabolism, have been associated with increasing plasmid stability in *Pseudomonas fluorescens* (Harrison *et al.*, 2015a). As pConj has only been found associated with *Neisseriaceae*, chromosomal genes may play a role in the acquisition or maintenance of pConj; however, we were unable to identify any through Tn-Seq. Further work, such as by evolving pConj in other species, may reveal genes important in pConj stability.

Finally, it has been suggested that the host range of a plasmid can be increased by evolving a plasmid in a different host (Loftie-Eaton *et al.*, 2016). This has been demonstrated previously between *Pseudomonas putida* and *Stenotrophomonas maltophilia* (de Gelder *et al.*, 2008). In those experiments, plasmids pB10 and pP21, initially extracted from wastewater, were unstable and lost quickly in both species. However, after evolving both plasmids in both *P. putida* and *S. maltophila* separately, the evolved plasmids exhibited increased stability when reintroduced into the two respective species. It would thus be interesting to determine if this would also apply to pConj: specifically, pConj is already present in closely related species such as *N. polysaccharea* and *N. meningitidis* and documented in *Kingella* spp. and *Eikenella* spp. (Knapp *et al.*, 1988). By evolving pConj in these species, it would be interesting to determine if the host range of pConj could be expanded beyond primarily *N. gonorrhoeae*.

## 6.5 Closing remarks

Overall, my work aimed to open new avenues for treatment not just of *N. gonorrhoeae*, but also other bacteria. The identification of CFHR5-*N. gonorrhoeae* interaction (**Chapter 3**) opens the possibility that other bacteria in addition to *B. burgdoferi* may also bind CFHRs (Siegel *et al.*, 2010). It would therefore be interesting to determine the effect of CFHR-pathogen interactions on survival in serum, and whether modulation of the complement system could be exploited for treating infectious diseases.

Separately, I sought a deeper understanding of mechanisms underlying pConj spread and maintenance (**Chapters 4 and 5**). There have been no previous attempts to understand the underlying mechanisms of pConj stability and spread, which were investigated here. I showed that pConj is stabilised by both TA and partitioning systems, and that inhibiting the partitioning system in the absence of TA systems increased pConj loss. The next step thus would be to determine if there are any drugs or technological advances which could be brought to clinical use against pConj, and potentially also p*bla*, in *N. gonorrhoeae*.

The investigation of *N. gonorrhoeae* pConj also constitutes an in-depth study of a narrow host range plasmid within its endogenous host from its first appearance 80 years ago till today. As far as I am aware, this has not been done before for any other plasmid. The investigation has revealed that pConj is extremely stable, does not undergo much genetic change, and imposes no fitness cost. It would therefore be interesting to see if such stable, species-specific plasmids can be found in other species, how these plasmids have evolved, and what their roles are.

# 7. Appendices

## Tables

| Country | Number of isolates |
|---|---|
| Argentina | 72 |
| Armenia | 1 |
| Australia | 174 |
| Austria | 47 |
| Belarus | 22 |
| Belgium | 57 |
| Brazil | 137 |
| Bulgaria | 1 |
| Canada | 298 |
| Chile | 1 |
| China | 44 |
| Cuba | 3 |
| Cyprus | 8 |
| Denmark | 215 |
| Ecuador | 1 |
| Estonia | 15 |
| Finland | 1 |
| France | 65 |
| Germany | 57 |
| Ghana | 56 |
| Greece | 54 |
| Hungary | 50 |
| Iceland | 5 |
| India | 2 |
| Indonesia | 2 |
| Ireland | 43 |
| Italy | 49 |
| Ivory Coast | 2 |
| Jamaica | 2 |
| Japan | 329 |
| Kenya | 125 |
| Latvia | 38 |
| Lithuania | 1 |
| Malaysia | 1 |
| Malta | 20 |
| New Zealand | 3 |
| Norway | 822 |

| | |
|---|---|
| Philippines | 26 |
| Poland | 27 |
| Portugal | 104 |
| Romania | 1 |
| Russia | 16 |
| Slovakia | 35 |
| Slovenia | 75 |
| South Africa | 6 |
| South Korea | 1 |
| Spain | 315 |
| Sweden | 52 |
| Thailand | 10 |
| The Netherlands | 447 |
| Turkey | 4 |
| UK | 3120 |
| USA | 2281 |
| Vietnam | 227 |
| Unknown | 48 |
| **Year** | **Number of isolates** |
| 1920 - 1939 | 10 |
| 1940 - 1959 | 79 |
| 1960 - 1979 | 12 |
| 1980 - 1999 | 333 |
| 2000 - 2019 | 8887 |
| Unknown | 297 |

**Appendix Table 7.1 Metadata of *N. gonorrhoeae* isolates used in WGS analysis.** Metadata of country and year of isolation of each isolate was obtained from PubMLST. Unavailable or unknown data is characterised as unknown.

| Primer | Primer sequence (5′ → 3′) |
|---|---|
| **Porin manipulation and sequence determination** | |
| FwdPor | CGGTAAATGCAAAGCTAAGCGG |
| RevPor2 | CGACAGGCTTTTTGTTGATACCG |
| **Construction of Δ*lst*** | |
| 16 | AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGCACATTTCCCCGAAAAGTGC |
| 25 | AGTTTACTCATATATACTTTAGATTGATTT |
| 26 | ACGGTATCGATAAGCTTGATATCGAATTCAAACCGCACCGCCGCGTTCAAATCTAT |
| 27 | ATCGTCATCGGGGGTATCGGATCCAAGGGCAAAATGGCGGAGTAAGTAAGGCAAAAATCA |
| 28 | GCCCTTGGATCCGATACCCC |
| 29 | GTTTATGCATCCCTTAACTTAC |
| 30 | CTTATCTCTTTTCAATAGCTATAAATTATTTAATAAGTAAGTTAAGGGATGCATAAACATCCCTAAAAACTCCATTCCGAC |
| 31 | GCTTACATAAACAGTAATACAAGGGGTGTTATGTCGGCAGTTTTGACATC |
| **Plasmid copy determination (qPCR)** | |
| 119 | CAGCGCGGTATTAATTAATCCCCGCTCTGCCCTTT |
| 121 | CCTCACTAAAGGGAACAAAAGCTGGAGCTCTGGCCGACATTTTGCACAAC |
| Q7 | GTGTGCGCCTTTGTCGATG |
| Q8 | CTACCATATCTATGCCGCCC |
| Q21 | CGTCCGGGAAATACTGGTT |
| Q22 | CAAAGAGCGTCAAATGGCC |
| ***pilD* deletion** | |
| pilDupF | AATCCGAGGCCAAGTGGAA |
| pilDdownR | AATACCAACGATTAGGTTTGAGAATGAAAATG |
| SeqpilDF | ATGCCATACTGCTGCGTATG |
| SeqpilDR | CTCACGTAGGCTTTTGAGGC |
| **Generation of pC$^{GFP}$** | |
| 92 | GGAATGACGGCGGAAAGATG |
| S2 | AAAATTACTCCTGCTATAGTGGATCCTCT |
| S3 | GAGAGGATCCACTATAGCAGGAGTAATTTTATGAGCAAAGGAGAAGAACTTTTCACT |
| S4 | TTTTGAGACACAATTCATCGATGATGGTTGTCATTTGTAGAGCTCATCCATGCCA |
| S5 | CAACCATCATCGATGAATTGTGTCT |
| S6 | AATGTTAGCGAAAGTACGGTCAAACGGGCTTGCAAGGCAAAGTAGTTTGGTACTTTATCCGGCAACGCC |

| | |
|---|---|
| 120 | ACGGTATCGATAAGCTTGATATCGAATTCCAGCCGTTGCCCAACCC |
| 121 | CCTCACTAAAGGGAACAAAAGCTGGAGCTCTGGCCGACATTTTGCACAAC |
| 122 | TTTCATTTGATGCTCGATGAGTTTTTCTAAAGCGATAGGTATCTTAAGAAAAGGGCA |
| 132 | GAGTCCAAGCGAGCTCTCGAACCCCAGAGTAGCCGTTGCCCAACCC |
| 133 | ATATGAACATGATGAGTGATCGTTAAATTTTGGCCGACATTTTGCACAAC |
| **Generation of pCΔGL** | |
| 153 | GAGTCCAAGCGAGCTCTCGAACCCCAGAGTGCCGTCTGAATATGCCTGTCCACGGGC |
| 154 | GACCGTCGGGCATCTTTCCGCCGTCATTCCCCATGTCAAGGGGGGAAT |
| 157 | GCGCGATGCGGTTTT |
| 158 | ATATGAACATGATGAGTGATCGTTAAATTTGCGACAAAAAAGGCGAGC |
| 155 | GGAATGACGGCGGAAAGA |
| 156 | CGGGCAACAACCCGGAAAACCGCATCGCGCGGCAGCCGATAGGGGGTGTTTTTGGTACTTTATCCGGCAACG |
| 139 | AAATTTAACGATCACTCATCATGTTCATATTTA |
| 142 | GCCCGACTGATACGTTGATT |
| **Generation of Δ*trbC* strain** | |
| 159 | GAGTCCAAGCGAGCTCTCGAACCCCAGAGTGCCGTCTGAACAAACTTGGTTGAAGTGATGTTCAAT |
| 160 | TCATGTTCATTTAATCTTATATTTGTCATATATTTACCTCACAGATATAACGTGGTT |
| 161 | TTCAATAGCTATAAATTATTTAATAAGTAACAGCATTACCTTCTTCCCTTGG |
| 162 | ATATGAACATGATGAGTGATCGTTAAATTTTATGGGCGCAAAGACGGTG |
| 165 | TTGACTGTGTTTTATATTTTTCTCGTTCATAACACCCCTTGTATTACTGTTTATG |
| 168 | TTTCGACGCCAAGGGAAGAAGGTAATGCTGATATTTACCTCACAGATATAACGTGGTTGT |
| 169 | ACAACCACGTTATATCTGTGAGGTAAATATCAGCATTACCTTCTTCCCTTGG |
| **Manipulation of TA systems on pConj** | |
| 253 | GAGTCCAAGCGAGCTCTCGAACCCCAGAGTGCCGTCTGAAGGGAAATGGGTTGACTAATGGTTTATGT |
| 254 | CGCTGCCCTTTTCTTAAGATACCTATCGCTCCATGTCAAGGGGGGAATCAGTTAATCATC |
| 255 | TCCGCTGGCAGAACGTTTCTATC |
| 256 | TTGCTTCCGATAGAAACGTTCTGCCAGCGGATTATTGCTCCTTATTTGCCGCCCAC |
| 257 | GCGGGAAGGAAATGAATTGTCCG |
| 258 | GGTTTAACGGACAATTCATTTCCTTCCCGCTCATTTTACACGTCCAACTGTTTGGTC |

| | |
|---|---|
| 259 | ATATGAACATGATGAGTGATCGTTAAATTTCCTCGGATATGAGTGTTGGAGAATTCAATG |
| 261 | AGCGATAGGTATCTTAAGAAAAGGGCAGCG |
| 266 | ATCGTCATCGGGGGTATCGGATCCAAGGGCAATTCCTCAAACGGATTAAAAACTCTTGC |
| 283 | TCCTGCATCATCATGTTCTTTACTGTCGAAGTCGCTCCCGAACCCGGAATTCCCGT |
| 284 | ACTTCGACAGTAAAGAACATGATGATGC |
| Seq017 | CGAGTTTGTGCTTGTACGCC |
| Seq018 | GCCAACGATAAATTCAAACCCTATAT |
| **Deletion of *parB* on pConj** | |
| 249 | GAGTCCAAGCGAGCTCTCGAACCCCAGAGTGCCGTCTGAACCAACTCCTGTATTTTCGGAACACC |
| 250 | TTCAATAGCTATAAATTATTTAATAAGTAATACTGGGAATTTATTATAATACCCCCCCG |
| 251 | ATCGTCATCGGGGGTATCGGATCCAAGGGCGTTTTCCAAACCCAAATTACTGTTATTCAT |
| 252 | ATATGAACATGATGAGTGATCGTTAAATTTGGTAGGAAAAACGGCTATGTCG |
| **Generation of plasmid for transposon insertion sequencing** | |
| KanF | TTAGAAAAACTCATCGAGCATCAAATGAAACT |
| KanR | ATGAGCCATATTCAACGGGAAACGT |
| EryF2 | ATGAACGAGAAAAATATAAAACACAGTCAAA |
| EryR2 | TTACTTATTAAATAATTTATAGCTATTGAAAAGAGATAAGAATTGT |
| H1 | GCAAGACGTTTCCCGTTGAATATGGCTCATTATAACCCTCTTTATTTTTTCCTCCTTATAAAATTAGT |
| H2 | TTTCATTTGATGCTCGATGAGTTTTTCTAACGATGCCGTCTGAAGGAT |
| H3 | TTGACTGTGTTTTATATTTTTCTCGTTCATGCGAAACGATCCTCATCC |
| H4 | TTCAATAGCTATAAATTATTTAATAAGTAAGCGGGACTCTGGGG |
| **Construction of pBAD vectors for toxicity assays** | |
| 349 | TTATAAAGCGGGCTGCAAATCGTTC |
| 350 | GAAATGAACGATTTGCAGCCCGCTTTATAACCATGCAAACCACTATCCCC |
| 357 | GGTATATCTCCTTCTTTGCATGCCTGCAGGTCGAC |
| 358 | TGGCTGTTTTGGCGGATGAGAG |
| 359 | CCTGCAGGCATGCAAAGAAGGAGATATACCATGGTAAATTCATGTTTATATCAATTCAATTATTTAG |
| 360 | AAAATCTTCTCTCATCCGCCAAAACAGCCATTATAAAGCGGGCTGCAAATCG |
| 361 | AAAATCTTCTCTCATCCGCCAAAACAGCCATTAGGCAGCCTTTGGGTCTTG |
| 374 | GAAATGAACGATTTGCAGCCCGCTTTATAACCATGCAGACCACAGCAAATACCATCCCCACCGGTTCAATCC |

| | |
|---|---|
| 375 | CTCGATATTTACGAGCCAATCGCCGTCAGGAAGCTGTTCGGCAGGC |
| 376 | CCTGACGGCGATTGGCTCGTAAATATCGAGCTGCCGGAAAGCGGCG |
| pBAD_F | ATGCCATAGCATTTTTATCC |
| pBAD_R | GATTTAATCTGTATCAGG |
| **Primers for sequencing library preparation (Tn-Seq)** | |
| Ch-Tn-i5S502 | AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTTCCCTACACGACGCTCTTCCGATCTCATGATGAGTGATCGTTAAATTTG |
| Ch-Tn-i5S503 | AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAACATGATGAGTGATCGTTAAATTTG |
| Ch-Tn-i5S505 | AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTTCCCTACACGACGCTCTTCCGATCTATGACATGATGAGTGATCGTTAAATTTG |
| Ch-Tn-i5S506 | AATGATACGGCGACCACCGAGATCTACACACTGCATAACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTATGACATGATGAGTGATCGTTAAATTTG |

**Appendix Table 7.2 List of primers used in this study**

| ***Neisseria bergeri*** | |
|---|---|
| **Country** | **Number of isolates** |
| France | 3 |
| Germany | 1 |
| Malawi | 20 |
| Mali | 36 |
| | |
| **Year** | **Number of isolates** |
| 1984 | 1 |
| 2006 | 1 |
| 2010 | 11 |
| 2011 | 15 |
| 2012 | 10 |
| 2015 | 1 |
| 2016 | 1 |
| 2017 | 1 |
| Unknown | 19 |
| ***Neisseria cinerea*** | |
| **Country** | **Number of isolates** |
| France | 13 |
| Greece | 1 |
| Ireland | 3 |
| Sweden | 6 |
| UK | 14 |
| Unknown | 3 |
| | |
| **Year** | **Number of isolates** |
| 1977 | 1 |
| 1979 | 1 |

| | |
|---|---|
| 1983 | 1 |
| 1989 | 1 |
| 1991 | 1 |
| 1999 | 1 |
| 2003 | 1 |
| 2006 | 1 |
| 2007 | 1 |
| 2012 | 2 |
| 2014 | 1 |
| 2015 | 8 |
| 2016 | 6 |
| 2019 | 7 |
| 2020 | 1 |
| Unknown | 6 |
| ***Neisseria lactamica*** | |
| **Country** | **Number of isolates** |
| Australia | 6 |
| Brazil | 51 |
| China | 20 |
| Ethiopia | 1 |
| France | 3 |
| Greece | 5 |
| Ireland | 19 |
| South Africa | 2 |
| UK | 441 |
| USA | 1 |
| Unknown | 2 |
| | |
| **Year** | **Number of isolates** |
| 1992 | 4 |
| 1996 | 2 |
| 1997 | 8 |
| 1998 | 3 |
| 1999 | 5 |
| 2000 | 2 |
| 2012 | 1 |
| 2013 | 20 |
| 2014 | 89 |
| 2015 | 331 |
| 2016 | 10 |
| 2017 | 7 |
| 2018 | 1 |
| Unknown | 68 |
| ***Neisseria meningitidis*** | |
| **Country** | **Number of isolates** |
| Algeria | 18 |
| Argentina | 1 |
| Australia | 66 |

| | |
|---|---|
| Austria | 9 |
| Bangladesh | 36 |
| Belarus | 8 |
| Benin | 46 |
| Brazil | 211 |
| Burkina Faso | 1336 |
| Cameroon | 20 |
| Canada | 563 |
| Central African Republic | 31 |
| Chad | 131 |
| Chile | 17 |
| China | 356 |
| Colombia | 116 |
| Congo [DRC] | 1 |
| Croatia | 1 |
| Cuba | 1 |
| Cyprus | 5 |
| Czech Republic | 214 |
| Denmark | 12 |
| Djibouti | 3 |
| Dominican Republic | 3 |
| Ethiopia | 546 |
| Finland | 7 |
| France | 775 |
| Germany | 34 |
| Ghana | 65 |
| Greece | 154 |
| Guinea | 13 |
| Hungary | 2 |
| Iceland | 2 |
| India | 1 |
| Ireland | 609 |
| Isle of Man | 2 |
| Israel | 1 |
| Italy | 449 |
| Ivory Coast | 8 |
| Japan | 90 |
| Kazakhstan | 2 |
| Latvia | 1 |
| Lithuania | 5 |
| Madagascar | 8 |
| Mali | 68 |
| Malta | 114 |
| Mauritius | 3 |
| Morocco | 102 |
| New Zealand | 111 |
| Niger | 236 |

| | |
|---|---|
| Nigeria | 109 |
| Norway | 34 |
| Peru | 5 |
| Poland | 6 |
| Portugal | 87 |
| Romania | 1 |
| Russia | 75 |
| Saudi Arabia | 8 |
| Senegal | 9 |
| Slovakia | 2 |
| Slovenia | 18 |
| South Africa | 201 |
| Spain | 184 |
| Sweden | 656 |
| Switzerland | 136 |
| Taiwan | 53 |
| The Gambia | 2 |
| The Netherlands | 723 |
| Togo | 72 |
| Tunisia | 58 |
| Turkey | 47 |
| UK | 5267 |
| Ukraine | 3 |
| USA | 2296 |
| Venezuela | 1 |
| Vietnam | 9 |
| Unknown | 236 |

| **Year** | **Number of isolates** |
|---|---|
| 1915 | 1 |
| 1940 - 1959 | 2 |
| 1960 - 1979 | 102 |
| 1980 - 1999 | 877 |
| 2000 - 2019 | 14621 |
| Unknown | 1309 |

***Neisseria polysaccharea***

| **Country** | **Number of isolates** |
|---|---|
| Australia | 2 |
| Belgium | 1 |
| France | 5 |
| Germany | 1 |
| Greece | 1 |
| Ireland | 5 |
| Malawi | 10 |
| Mali | 8 |
| Spain | 1 |
| Sweden | 1 |
| The Gambia | 8 |

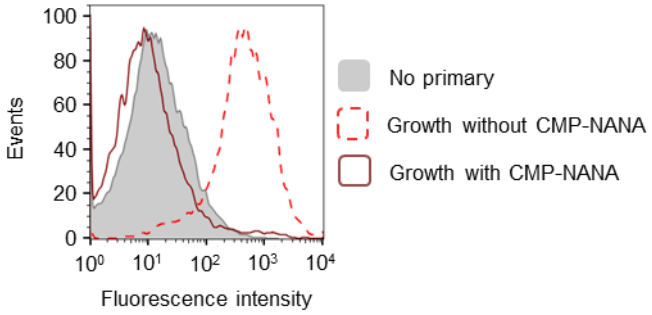
| | |
|---|---|
| The Netherlands | 1 |
| UK | 12 |
| Unknown | 5 |
| | |
| **Year** | **Number of isolates** |
| 1984 | 1 |
| 1997 | 8 |
| 2010 | 8 |
| 2014 | 7 |
| 2015 | 6 |
| 2016 | 4 |
| 2017 | 2 |
| 2019 | 5 |
| 2020 | 3 |
| Unknown | 17 |
| ***Neisseria subflava*** | |
| **Country** | **Number of isolates** |
| Australia | 5 |
| China | 2 |
| France | 5 |
| Ireland | 3 |
| Malaysia | 1 |
| Sweden | 4 |
| UK | 17 |
| USA | 3 |
| Unknown | 13 |
| | |
| **Year** | **Number of isolates** |
| 1979 | 1 |
| 1989 | 2 |
| 1992 | 1 |
| 1999 | 1 |
| 2012 | 2 |
| 2014 | 6 |
| 2015 | 16 |
| 2017 | 1 |
| 2018 | 1 |
| Unknown | 22 |

**Appendix Table 7.3 Metadata of country and year of isolation of other *Neisseria* spp.** Metadata of country and year of isolation of each isolate was obtained from PubMLST. Unavailable or unknown data is characterised as unknown.

| Country | Number of isolates |
|---|---|
| Australia | 2 |
| Austria | 1 |
| Belarus | 1 |
| Belgium | 1 |
| Brazil | 1 |
| Canada | 4 |
| China | 6 |
| Cuba | 2 |
| Denmark | 25 |
| France | 1 |
| Germany | 2 |
| Ghana | 1 |
| India | 2 |
| Indonesia | 2 |
| Jamaica | 1 |
| Japan | 2 |
| Kenya | 14 |
| Norway | 2 |
| Philippines | 5 |
| Portugal | 4 |
| Slovenia | 3 |
| South Africa | 4 |
| Sweden | 1 |
| Thailand | 5 |
| The Netherlands | 3 |
| USA | 10 |
| Vietnam | 2 |
| Unknown | 19 |
| **Year** | **Number of isolates** |
| 1940 - 1959 | 14 |
| 1960 - 1979 | 6 |
| 1980 - 1999 | 30 |
| 2000 - 2019 | 76 |

**Appendix Table 7.4 Metadata of country and year of isolation of 126 isolates used for pConj analysis.** Metadata of country and year of isolation of each isolate was obtained from PubMLST. Unavailable or unknown data is characterised as unknown.

## Figures




**Appendix Figure 7.1 Representative histogram to detect sialylation.** Bacteria (FA1090) were first grown in the presence or absence of cytidine 5' mono-phospho-N-acetylneuraminic acid (CMP-NANA) before fixing. Cells were then stained with mAb 3F11 and subsequently visualised with flow cytometry to determine if bacteria were sialylated. Sialylation was detected by the absence of mAb 3F11 binding.

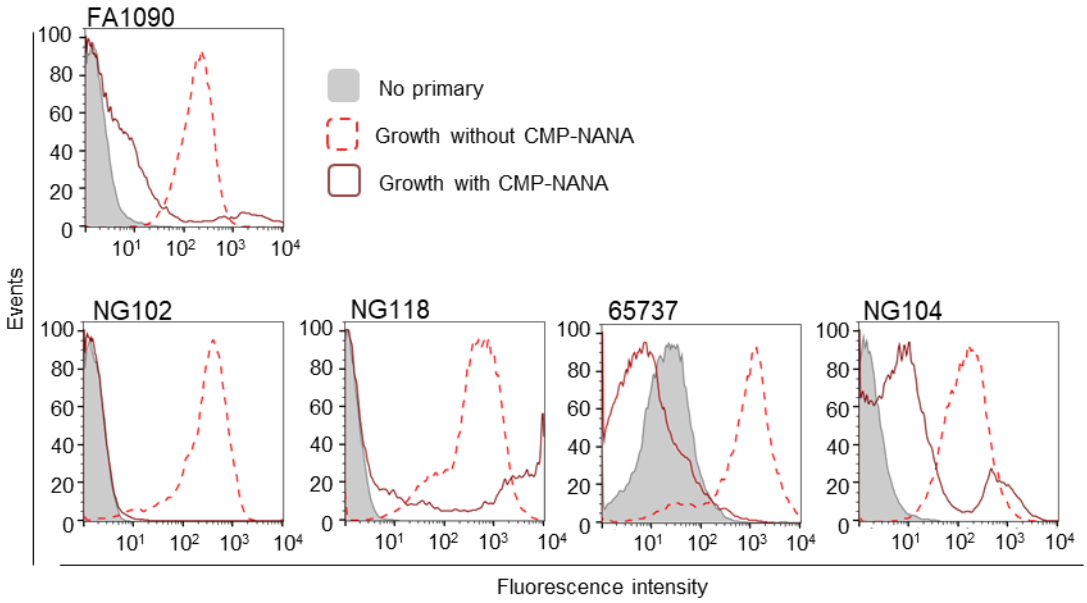
```
FA1090   MKKSLIALTLAALPVAAMADVTLYGAIKAGVQTYRSVEHTDGKVSKVETGSEIA
NG102    MKKSLIALTLAALPVAAMADVTLYGAIKAGVQTYRSVEHTDGKVSKVETGSEIA
NG118    MKKSLIALTLAALPVAAMADVTLYGAIKAGVQTYRSVEHTDGKVSKVETGSEIA
65737    MKKSLIALTLAALPVAAMADVTLYGAIKAGVQTYRSVEHTEGKVSKVETGSEIA
NG104    MKKSLIALTLAALPVAATADVTLYGAIKAGVQTYRSVEHREGKVVGVGTGSEIS

FA1090   DFGSK...IRAGSLNSPLKNTGANVNAWESGKFTGNVLEISGMAQREHRYLSVRYDS
NG102    DFGSK...IRAGSLNSPLKNTGANVNAWESGKFTGNVLEISGMAQREHRYLSVRYDS
NG118    DFGSK...IRAGSLNSPLKNTGANVNAWESGKFTGNVLEISGMAQREHRYLSVRYDS
65737    DFGSK...IRAGSLNSPLKNTGANVNAWESGKFTGNVLEISRMAQREHRYLSVRYDS
NG104    DFGSK...IRVGSLNSPLKNTGANVNAWESGKYTGELLEISKMAGREHRYLSARYDS

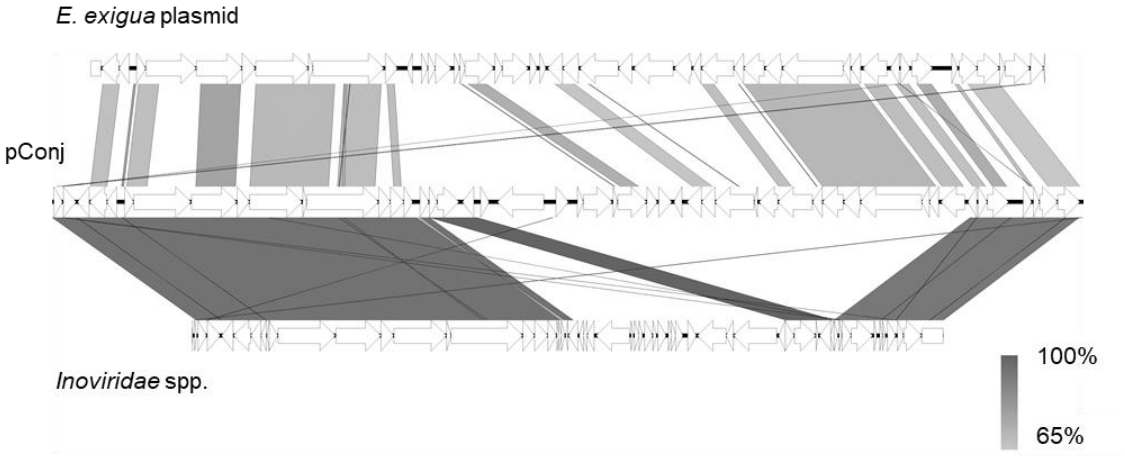
FA1090   PEFAGFSGSVQYAPKDNSGSNGESYHVGLNYQNSGFFAQYAGLFQRYGEGTKKIE
NG102    PEFAGFSGSVQYAPKDNSGSNGESYHVGLNYQNSGFFAQYAGLFQRYGEGTKKIE
NG118    PEFAGFSGSVQYAPKDNSGSNGESYHVGLNYQNSGFFAQYAGLFQRYGEGTKKIE
65737    PEFAGFSGSVQYAPKDNSGSNGESYHVGLNYQNSGFFAQYAGLFQRYGEGTKKIE
NG104    PEFAGFSGSVQYAPKGNSGSNGESYHVGLNYRNSGFFAQYAGLFQRYGEGTKKIE

FA1090   YDG--QTYSIPSLFVEKL...AKLYGAMSGNSHNSQTEVAATAAYRFGNVTPRVSYAHGFKGTVDSAN...
NG102    YDG--QAYSIPTLFVEKL...AKLYGAMSGNSHNSQTEVAATAAYRFGNVTPRVSYAHGFKGTVDSAN...
NG118    YDG--QTYSIPTLFVEKL...AKLYGAMSGNSHNSQTEVAATAAYRFGNVTPRVSYAHGFKGTVDSAN...
65737    YDG--QTYSIPSLFVEKL...AKLYAAMSGNSHNSQTEVAATAAYRFGNVTPRVSYAHGFKGTVDSAN...
NG104    YYDDQTYYSMPSLFVEKL...AKLYGATRVNSHNSQTEVAATAAYRFGNVTPRVSYAHGFKGTVDDAN...
```

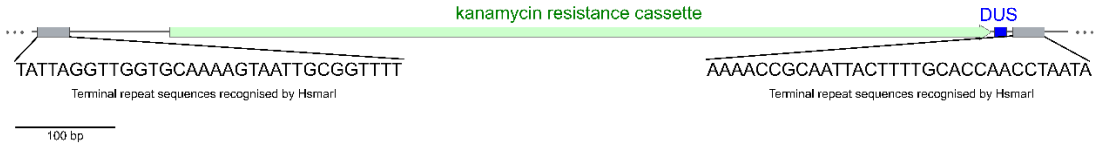
**Appendix Figure 7.2 Alignment of PorB sequence from P.IB-expressing isolates.** PorB sequences from strains FA1090, NG102, NG118, 65737 and NG104 were first obtained from the PubMLST database and translated into the amino acid sequences using Transseq. Sequences were then aligned by Clustal Omega. Amino acids of each PorB that is different to P.IB expressed by FA1090 are highlighted in blue.




**Appendix Figure 7.3 Representative histogram showing LOS sialylation of different P.IB-expressing isolates.** *N. gonorrhoeae* strains FA1090, NG102, NG118, 65737 and NG104 were first grown in the presence or absence of cytidine 5' mono-phospho-N-acetylneuraminic acid (CMP-NANA). Cells were then fixed and stained with mAb 3F11 and subsequently visualised with flow cytometry to determine if bacteria were sialylated. Sialylation was detected by the absence of mAb 3F11 binding.




**Appendix Figure 7.4 Alignment of pConj sequences with *E. exigua* plasmid and *Inoviridae* sequences identified through BLAST.** *E. exigua* plasmid and *Inoviridae* sequences were first obtained from the NCBI database; the sequence of the representative pConj from *N. gonorrhoeae* isolate 60755 was obtained from the PubMLST database. Sequences were then aligned with EasyFig as shown here. The percentage similarity is as shown.




**Appendix Figure 7.5 Schematic of the transposon used in Tn-Seq.** The transposon construct consists of the kanamycin resistance cassette, aph(3), followed by a DNA uptake sequence (DUS) to facilitate uptake of mutagenised DNA. Two terminal inverted repeats flank the kanamycin resistance cassette. The HsmarI transposase used in *the in-vitro* transposition reaction recognises the terminal repeats

VapX$^{pCryp}$ MQT - - - T I P T G S I R T F G D Y G V M Y I V G T P A E Q L P D G D W L V N I E L P E S G E H T Q Y K L S H I V Q D P K A A
VapX$^{Nm}$ MQ T T A N T I P T G S I R T F G D Y G V M Y I V G T P A E Q L P D G D W L V N I E L P E S G E H T Q Y K L S H I V Q D P K A A

**Appendix Figure 7.6 Alignment of VapX$^{pCryp}$ and VapX$^{Nm}$ amino acid sequences.** Nucleotide sequences of VapX$^{pCryp}$ (allele 1 on PubMLST) and VapX$^{Nm}$ (allele 22 on PubMLST) were first obtained from PubMLST database and translated using Transseq. Sequences were then aligned and visualised with ClustalOmega.

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