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Characterisation of the ATP-dependent ligase, Lig E: An investigation into its significance in *Neisseria gonorrhoeae*

A thesis
submitted in fulfilment
of the requirements for the degree
of

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at
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by

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Abstract

The rapid rise in multidrug-resistant *Neisseria gonorrhoeae* isolates has caused major public concern for the treatment of its sexually transmitted disease, gonorrhoea. Previously easily treatable, gonorrhoea is now spreading at alarming rates due to the bacterium's ability to take up DNA from its environment without regulation. With approximately 106 million new cases annually, thorough investigations into novel gonococcal pathways that can be targeted to limit its survival and spread are crucial. One possible target is the disruption of its DNA repair systems, which include ligases that seal breaks in DNA, and would greatly discourage bacterial growth. Interestingly, in addition to its replicative ligase, *N. gonorrhoeae* expresses a minimal ATP-dependent ligase, Ligase E (Lig E), which contains a signal peptide that indicates a likely periplasmic location as opposed to the cytosolic location of genomic DNA. Hence, I hypothesise that Lig E is working on repairing fragmented extracellular DNA which has a role in both biofilm formation and bacterial competence, both of which would be greatly beneficial for the persistence and acquisition of antibiotic resistance genes in *N. gonorrhoeae*.

In this thesis, I focussed on characterising Lig E from *N. gonorrhoeae* (Ngo-Lig E) to determine its contribution to gonococcal phenotype, as well as its structure, function and cellular location. To analyse its biological role, several *ngo-lig E* mutants were created *in vivo* and their growth, stress response, biofilm formation as well as infection and adhesion to a human cell line were studied. I showed that Ngo-Lig E is not essential for normal planktonic *N. gonorrhoeae* growth and survival; however, it is important for its biofilm formation and subsequent adhesion onto human cells. Following this, I further investigated the potential role of Ngo-Lig E on biofilm formation by looking at its morphology via scanning electron microscopy and confocal laser scanning microscopy after growing them under constant shear forces with a continual supply of fresh media. Data from this supported earlier indications that Ngo-Lig E is important for *N. gonorrhoeae* in biofilm formation, as well as the extent of damage the bacterium can cause to human tissue. Taking its natural competence into account, I also investigated the potential role of Ngo-Lig E in the uptake of fragmented DNA during transformation and found a decreased ability of *N. gonorrhoeae* to repair and take up nicked DNA when *ngo-lig E* was absent. Investigations into the potential location of Ngo-Lig E

showed that it is likely transported into both the periplasmic and extracellular space in *N. gonorrhoeae*, where it may perform the aforementioned dual-role in biofilm formation and DNA uptake. Finally, I solved the three-dimensional structure of Ngo-Lig E, highlighting its minimal architecture and lack of complete encirclement around its substrate DNA.

The work presented in this thesis provides the first report of a solved structure of Lig E from a human pathogen, the first report of *in vivo* characterisation of this enzyme, as well as the first report of attempting to characterise its role and location. Collectively, this thesis has allowed for the establishment of important characteristics of Ngo-Lig E that were previously unknown. Although further research into this enzyme is necessary, the results presented here bring to light a potential novel pathway that may be targeted by future drug developments to tackle the emerging threat of multidrug-resistant *N. gonorrhoeae* in our community.

Acknowledgments

How do I even begin. I don't think I can count the number of times I have written and rewritten this section, thinking it would be the easiest part of my thesis to write. Spoiler alert, it turned out to be the hardest section to craft, which makes absolutely no sense as there is no way I would have made it this far on my own. But perhaps that is exactly the reason I struggled to write this; because there is no way I would have made it this far on my own.

Staying home, waking up to write, and going to sleep just so I can have more energy to write has made me realise that in no way can I operate without doing some lab work in the long term. Or perhaps, it was this particular lab, and everyone who was a part of it that has made this so hard. Adele, thank you for taking me under your wing and guiding me throughout this whole PhD. You believed in me much more than I ever have or could, so thank you for not giving up on me. Jo, Emma, and Stacy, thank you for getting me all cultured up on all things gonorrhoea-related. Will, thank you for the much-needed distractions and side projects during my PhD. Geetanjali, sorry for spilling my antics onto the C.2.0.3 floor and potentially messing up the C.2.0.3 lab system. And Erica, thank you for being so supportive and offering to look through my results. Thank you also to David, who is perhaps one of the loveliest person I have ever worked with; thank you for being so welcoming and helping me settle in during my brief stint at Cardiff. And to Abdullah, thanks for sharing my qualms about this finicky bacterium and for putting up with my bothersome presence for three whole months. To the people in the superior C2 office: Meghan, Chelsea, Ally, Avi, Harman, Kate, Ronja, Aakash, Liz and Caitlin, I am so sorry that you guys have had to put up with me and my regular scheduled breakdowns. In saying that, Aakash, Abdullah, Andrew, Emily, Keely, Kyrin, Liz, and Marina, your support has been so much more than I deserve and I struggle to express my immense gratitude for all you lot have done for me because I do not think I can do it any justice; all I know how to do at the moment is to say thank you over and over and over again.

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Etc.

You know what I mean.

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List of Abbreviations

2-D	Two-dimensional
3'OH	3'-Hydroxy
3-D	Three-dimensional
5'PO_4	5'-Phosphate
A	Adenine
AD domain	Adenylation domain
ADLs	ATP-dependent ligases
Ame-Lig E	Lig E from *Alteromonas mediterranea*
ANOVA	Analysis of variance
Arg	Arginine
Asp	Aspartic acid
ATP	Adenine triphosphate
b-ADLs	Bacterial ATP-dependent ligases
BER	Base excision repair
bp	Base pair
BRCT domain	Breast cancer carboxy terminal domain
C	Cytosine
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CFU	Colony forming unit
ChV	*Chlorella* virus
CLSM	Confocal laser scanning microscopy
C-terminus/C-terminal	Carboxy-terminus/Carboxy-terminal
D	Aspartic acid
DNA	Deoxyribose nucleic acid
DSBs	Double-stranded breaks
dsDNA	Double-stranded DNA
DUS	DNA uptake sequence
E	Glutamic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Em	Emission wavelength
EPS	Exopolysaccharides

ESCs	Extended-spectrum cephalosporins
Exc	Excitation wavelength
exDNA	Extracellular DNA
FAM	Carboxyfluorescein
FBS	Foetal bovine serum
G	Guanine
G4 motif	Guanine quadruplex motif
GCB	Gonococcal base medium
GCBL	Gonococcal base liquid
GGI	Gonococcal genetic island
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GO	Gene ontology
h	Hour
H_2O_2	Hydrogen peroxide
Hin-Lig E	Lig E from *Haemophilus influenzae*
His	Histidine
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HtH motif	Helix-turn-helix motif
HU	Histone-like protein
IDT	Integrated DNA Technologies
IHF	Integration host factor
IMAC	Immobilised metal affinity chromatography
K	Lysine
kan^R	Kanamycin resistance gene
kb	Kilobase
kDa	kiloDalton
LDH	Lactate dehydrogenase
Lig E	Ligase E
LOS	Lipooligosccharide
LPSN database	List of Prokaryotic names with Standing in Nomenclature database
Lys	Lysine

MBP	Maltose-binding protein
MDR	Multidrug-resistance
min	Minute
MOI	Multiplicity of infection
mRNA	Messenger RNA
MX	Macromolecular crystallography
NAATs	Nucleic acid amplification tests
NAD^+	Nicotinamide adenine dinucleotide
Nal-acid	Nalidixic acid
NCBI	National Center for Biotechnology Information
NDLs	NAD^+-dependent ligases
NETs	Neutrophil extracellular traps
Ngo-Lig E	Lig E from *Neisseria gonorrhoeae*
NHEJ	Non-homologous end joining
Nme-Lig E	Lig E from *Neisseria meningitidis*
NMN	Nicotinamide mononucleotide
N-terminal/N-terminus	Amino-terminal/amino-terminus
Ntase domain	Nucleotidyltransferase domain
OB domain	Oligonucleotide binding domain
OD_{550}	Optical density at 550 nm
OD_{600}	Optical density at 600 nm
Opa protein	Opacity protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PE domain	Phosphoesterase domain
PID	Pelvic inflammatory disease
pKa	Acid dissociation constant
PPI	Pyrophosphate
PrimPol domain	DNA primase/polymerase domain
Psy-Lig E	Lig E from *Psychromonas* sp.
RAPD analysis	Random amplified polymorphic DNA analysis
rHVE	Reconstituted human vaginal epithelium
RNA	Ribonucleic acid

rRNA	Ribosomal RNA
RT-qPCR	Reverse transcription-quantitative PCR
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
Ser	Serine
sfGFP	Superfolder green fluorescent protein
SSBs	Single-stranded breaks
ssDNA	Single-stranded DNA
STI	Sexually transmitted infection
T4P	Type IV pili
T4SS	Type IV secretion system
TEV protease	Tobacco etch virus protease
T	Thymine
Tyr	Tyrosine
USS	Uptake signal sequence
UV	Ultraviolet
v1	Version 1
v2	Version 2
Vib-Lig E	Lig E from *Aliivibrio salmonicida*
WHO	World Health Organization
WT	Wild-type
XDR	eXtensive Drug Resistance
Y	Tyrosine

1 Chapter One

Introduction

The rapid rise and spread of multidrug-resistant (MDR) strains of *Neisseria gonorrhoeae*, the causative agent for the sexually transmitted infection (STI) gonorrhoea, calls for urgent investigations into new pathways that can be targeted against it. Although this bacterium affects both men and women, symptoms vary greatly, with the potential for dissemination into the upper reproductive tract in females, causing complications like pelvic inflammatory disease (PID) and ectopic pregnancy (Morse & Knapp, 1992; Miller, 2006). Previously easily treatable, *N. gonorrhoeae* has now become resistant to almost all classes of antibiotics that have been used against it, and is now classified as a high-priority pathogen by the World Health Organization (WHO) (Tapsall, 2005). One of the features that allow it to develop resistance so quickly is its unregulated natural competence, or its ability to take up pieces of deoxyribose nucleic acid (DNA) from its environment at all stages of growth, including novel antibiotic resistance genes (Hamilton & Dillard, 2006). However, these pieces of extracellular DNA (exDNA) are most likely heavily fragmented due to the relatively oxidative nature of the reproductive tract which they inhabit. Thus, I predict that there must be a DNA repair mechanism in place to ensure the integrity of these pieces of exDNA before recombination into the genome.

N. gonorrhoeae expresses a minimal adenosine triphosphate (ATP)-dependent ligase, Ligase E (Lig E), in addition to its essential nicotinamide adenine dinucleotide (NAD^+)-dependent ligase, the latter of which is responsible for housekeeping DNA repair, replication and recombination processes. As a DNA ligase, Lig E seals breaks in double-stranded DNA (dsDNA). Interestingly, it has an amino (N)-terminal signal peptide that indicates a likely periplasmic location as opposed to the cytosolic location of genomic DNA (Magnet & Blanchard, 2004). Furthermore, Lig E is found in many other naturally competent human pathogens like *Campylobacter jejuni*, *Haemophilus influenzae* and *Vibrio cholerae* (Williamson *et al.*, 2016). To my knowledge, at the inception of this PhD research, no *in vivo* work had been conducted on this enzyme, and its biological function and cellular location were still uncharacterised. I hypothesised that Lig E may have a

role in competence by sealing breaks in fragmented DNA before homologous recombination, permitting the bacterium to acquire new resistance genes with high efficacies. Furthermore, I predicted that Lig E may also be working on fragmented exDNA to create higher molecular weight DNA that would be beneficial to exDNA-dependent biofilm formation, allowing the bacterium to persist effectively in its environment.

The aim of this doctoral research thesis was thus to understand the biological function of the enigmatic enzyme Lig E from *N. gonorrhoeae* (herein referred to as Ngo-Lig E) and focuses on the work that had been conducted to characterise this protein. As part of this work, I have created different *ngo-lig E* mutants in *N. gonorrhoeae* to study its function and location *in vivo*, as well as expressed and purified recombinant Ngo-Lig E from *Escherichia coli* to solve its structure *in vitro*. This thesis starts with a literature review that is presented in two parts; the first part includes a general introduction to the project and the Ngo-Lig E enzyme, while the second part is presented as a review article manuscript on exDNA-dependent biofilms in *Neisseria* spp. (one of the predicted functions of Ngo-Lig E) that is ready for submission:

Pan, J., & Williamson, A. (2025). The role of extracellular DNA in *Neisseria* biofilms. (*To be submitted*).

Results are presented in this doctoral thesis in three separate chapters: Chapter Three, Chapter Four and Chapter Five. These chapters are presented as manuscripts, with one having been published (Chapter Three, which focuses on the phenotype and structure of Ngo-Lig E), one that has been submitted (Chapter Four, which focuses on its potential function in biofilm formation) and one in preparation (Chapter Five, which looks at its potential function in DNA uptake, and its likely cellular location). A summary of the findings and future work are presented in the final chapter (Chapter Six).

1.1 Research objectives

To characterise the enzyme Ngo-Lig E in *N. gonorrhoeae*, the following objectives were explored in this thesis:

Objective One:

To determine the phenotypic implications of deleting *ngo-lig E* in *N. gonorrhoeae* by studying its deletion mutant *in vivo* and hence if it is essential for *N. gonorrhoeae* growth and survival.

Objective Two:

To determine the structure and activity of Ngo-Lig E by X-ray crystallography and *in vitro* activity assays.

Objective Three:

To determine the role and impact of Ngo-Lig E on extracellular processes in *N. gonorrhoeae* including competence and biofilm formation.

Objective Four:

To determine the cellular location of Ngo-Lig E in *N. gonorrhoeae*.

Results from **Objectives One and Two** are presented together in Chapter Three as a final peer-reviewed publication that focused on the phenotype and structure of Ngo-Lig E:

Pan, J., Singh, A., Hanning, K., Hicks, J., & Williamson, A. (2024). A role for the ATP-dependent DNA ligase Lig E of *Neisseria gonorrhoeae* in biofilm formation. *BMC Microbiology, 24*(1), 29.

Results from **Objective Three** are presented in Chapter Four as a manuscript that has been submitted for publication and is awaiting review:

Pan, J., Albarrak, A., Hicks, J., Williams, D., & Williamson, A. (2025). Influence of the ATP-dependent DNA ligase, Lig E, on *Neisseria gonorrhoeae* microcolony and biofilm formation. (*Submitted*).

Results from **Objectives Three and Four** are presented in Chapter Five as a manuscript in preparation for submission:

Pan, J., Singh, A., Hicks, J., & Williamson, A. (2025). DNA uptake during natural competence? A proposed location and role of the ATP-dependent DNA ligase, Lig E, in *Neisseria gonorrhoeae* (*In preparation*).

Note: Any changes made to the original manuscripts are highlighted in blue in this thesis. These changes were made to ensure consistency between manuscripts in terms of nomenclature, and to add extra information relevant to this thesis.

1.2 Acknowledgement of funds

This research was funded by the Health Research Council of New Zealand Explorer Grant (Grant ID 21/754) awarded to my Chief Supervisor, Dr. Adele Williamson, who was also supported by the Rutherford Discovery Fellowship (20-UOW-004). Financial support during the duration of my PhD was provided by the University of Waikato Doctoral Scholarship. A visit to the Cardiff University School of Dentistry to perform *N. gonorrhoeae* biofilm study and visualisation was funded by the Maurice Wilkins Centre Flexible Research Programme (Category 4), as well as the Cardiff University and the University of Waikato Strategic International Partnership Collaborative Seed Fund. Access to the Australian Synchrotron beamline was funded by the New Zealand Synchrotron Group.

1.3 References

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2 Chapter Two

Literature Review

2.1 Preface

This chapter is divided into two parts; The first section focuses on *N. gonorrhoeae* and the enzyme Lig E from *N. gonorrhoeae* (Ngo-Lig E), which is the focal point of this thesis. The second section is presented as a review manuscript ready for submission and covers the importance of exDNA in *Neisseria* biofilms, which I predict Ngo-Lig E may be acting on and is thus relevant in this section. Due to the nature and structure of the manuscript in Part two, I acknowledge that there may be some overlap in information provided in terms of the introduction to *N. gonorrhoeae* and its characteristics, which would have been provided in Part one to provide context to the work.

2.2 Part one: Background

2.2.1 *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a Gram-negative diplococcus, responsible for the STI gonorrhoea. With an estimated 106 million new cases each year, *N. gonorrhoeae* is continually spreading, especially in developed countries, although it is possible that numbers are underreported elsewhere due to a variety of factors like different socioeconomic status (Tapsall, 2005). This obligate human pathogen infects the mucosal cells of the urogenital tract, causing urethritis in men (pain with urination) and cervicitis in women (vaginal pain and abnormal bleeding) (Burch *et al.*, 1997; Tapsall, 2005; Dillon *et al.*, 2015). Furthermore, *N. gonorrhoeae* infections may ascend up the female reproductive tract, causing pelvic inflammatory disease (PID) that presents as persistent severe pain, and may cause ectopic pregnancy, infertility and first-trimester abortions (Tapsall, 2005). These infections may also be passed onto the newborn during passage through the birth canal, causing conjunctivitis or blindness of the baby (Burch *et al.*, 1997; Tapsall, 2005; Dillon *et al.*, 2015). In addition to this, extragenital infections may occur in the pharynx and rectum of both sexes (Edwards & Apicella, 2004). *N. gonorrhoeae* infections also exacerbate the transmission of the human immunodeficiency virus (HIV) (5-fold increase) due to

the body's inflammatory response towards the bacterium including neutrophil infiltration which may harbour the virus (Cohen, 1998; Tapsall, 2005).

However, a majority of gonococcal infections are asymptomatic (>50% in women, but only <10% in men), especially for extragenital cases, leading to late or no treatment and the underreporting of cases, facilitating further undetected transmission in the community (Barbee, 2014; Dillon *et al.*, 2015). This dampened immune response is associated with the bacterium's ability to form biofilms and carry out anaerobic respiration which allows it to break down host-produced nitrous products during nitrite reduction, leading to weaker host inflammatory responses and hence the progression of symptoms (Falsetta *et al.*, 2009). Clinical diagnosis of gonorrhoea typically begins with the detection of the bacterium in polymorphonuclear leukocytes from patient urine samples, or via nucleic acid amplification tests (NAATs) (Unemo & Shafer, 2014).

Due to its panmictic nature, no vaccines have been developed to treat gonorrhoea. Instead, treatment focuses on prevention, surveillance and control via antibiotics. However, *N. gonorrhoeae* has quickly gained resistance to all classes of antibiotics that have been used against it to date, making it difficult to treat (Suay-García & Pérez-Gracia, 2017). For example, the bacterium quickly became resistant to one of the earliest classes of antibiotics used for treatment, sulphonamides, which were first introduced in the 1930s, and by the 1940s, 90% of strains were resistant (Suay-García & Pérez-Gracia, 2017). In 1943, penicillin became the standard treatment, however, this was stopped in the 1980s after *β-lactamase*-containing plasmids were found in some strains, which provided resistance against beta-lactam antibiotics like penicillin (Suay-García & Pérez-Gracia, 2017). In the 1960s, tetracyclines and aminoglycosides were introduced, although these were rendered ineffective when *tetM* plasmids that provided resistance to these drugs were acquired in the 1980s (Suay-García & Pérez-Gracia, 2017). Third-generation fluoroquinolones including ciproflaxin were later introduced in the 1980s and resistance to these were detected in the 1990s, reaching more than 50% in the Western Pacific Region by 2005 (Tapsall, 2005; Suay-García & Pérez-Gracia, 2017). The currently used medications against *N. gonorrhoeae* include azithromycin (macrolides, a category II drug), as well as ceftriaxone and cefixime (third generation extended-spectrum cephalosporins (ESCs); category I drug) (Lee *et al.*, 2017). The former was

introduced in the 1980s, with resistance developing in the 1990s, while the latter two were first used in the 1990s, with resistance developing in the 2000s (Tapsall, 2005; Dillon *et al.*, 2015; Suay-García & Pérez-Gracia, 2017). These quick-forming resistances led to the classification of *N. gonorrhoeae* as a superbug in 2012, as well as a high priority pathogen by the WHO in 2017.

Currently, dual-therapy with both cephalosporin and azithromycin is the most common strategy used against *N. gonorrhoeae*, as is currently the prescribed combination in New Zealand (Barbee, 2014; Suay-García & Pérez-Gracia, 2018). However, in 2015, eXtremely Drug Resistant (XDR) strains of *N.* gonorrhoeae resistant to two or more antibiotics (resistance to at least two category I and three category II drugs) emerged around the globe, rendering treatment with this dual-therapy unsuccessful in many cases (Dillon *et al.*, 2015; Lee *et al.*, 2017). In fact, the 2017-2018 World Health Organization global antimicrobial resistance surveillance found complete resistance to azithromycin and ciprofloxacin in 51% and 100% of countries respectively, and decreased susceptibility or resistance to ceftriaxone and cefixime in 31% and 47% of countries respectively (Unemo *et al.*, 2021). More specifically, a 2014-2015 New Zealand *N. gonorrhoeae* population study demonstrated decreasing susceptibility of *N. gonorrhoeae* isolates towards the currently used ESCs, while ceftriaxone-resistant strains have been identified in Japan and Argentina, painting a very ominous picture of the possible future of untreatable gonorrhoea across the globe (Ohnishi *et al.*, 2011; Gianecini *et al.*, 2016; Lee *et al.*, 2017).

2.2.1.1 Antigenic and phase variation

One of the characteristics of *N. gonorrhoeae* that allows it to so effectively evade the host immune system is its ability to undergo antigenic and phase variation, creating a highly heterogenous population in the hostile human host environment (Makino *et al.*, 1991). Antigenic variation refers to the bacterium's ability to modify surface proteins important for infection via changes in its alleles that occur during DNA recombination, creating different forms of its surface proteins, and subsequently allowing it to remain undetected by the host immune system (Burch *et al.*, 1997; Hamilton & Dillard, 2006; Cahoon & Seifert, 2009). In *N. gonorrhoeae*, antigenic variation occurs frequently with the PilE pilin protein, the major subunit of the Type 4 pili (T4P) system which is responsible for the attachment of *N.*

gonorrhoeae to human mucosal cells and its subsequent internalisation (Koomey *et al.*, 1987). The pilin is encoded by an expression locus, *pilE*, and multiple copies of the silent storage locus, *pilS*, which do not have a promoter for expression (Koomey *et al.*, 1987; Hill & Davies, 2009). Unidirectional intragenic gene conversion often occurs from the silent *pilS* to *pilE* via homologous recombination in a RecA-dependent manner, creating unique pilin variants that influence the assembly of the T4P system, and hence the piliation state and extent of infection of the host cells, allowing the bacterium to continuously evade the host immune system after cellular internalisation (Figure 2.1) (Hagblom *et al.*, 1985; Koomey *et al.*, 1987; Makino *et al.*, 1991; Kline *et al.*, 2003; Criss *et al.*, 2005; Cahoon & Seifert, 2009; Hill & Davies, 2009; Zöllner *et al.*, 2017). This process is dependent on a cis-acting guanine quadruplex (G4) motif (5′-$G_3TG_3TTG_3TG_3$) upstream of *pilE* that forms a four-stranded structure which RecA binds to (Cahoon & Seifert, 2009; Kuryavyi *et al.*, 2012; Zöllner *et al.*, 2017; Prister *et al.*, 2020). In fact, this particular example of pilin gene conversion in *N. gonorrhoeae* occurs at the highest frequency when compared to other pathogenic antigenic variations that have been observed (Criss *et al.*, 2005).

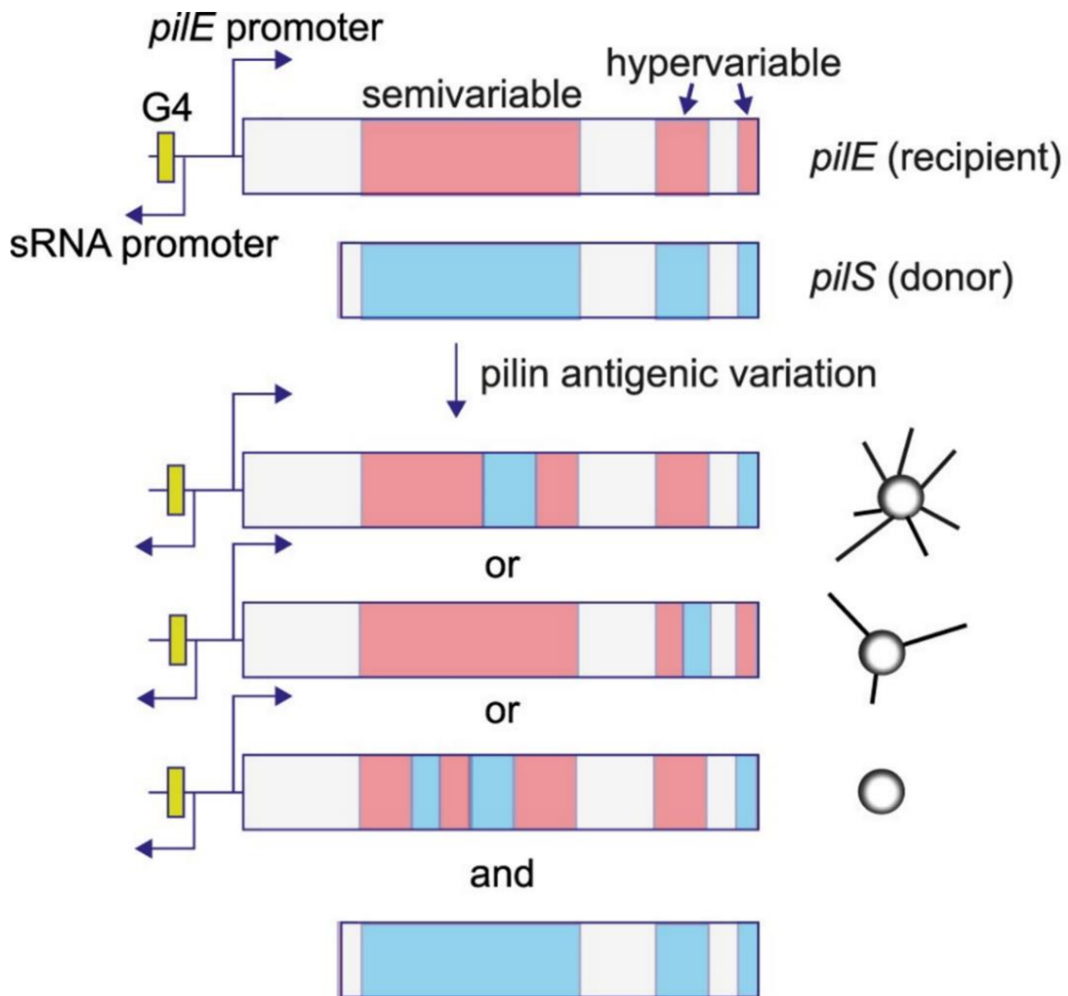

Figure 2.1 Antigenic variation in *Neisseria gonorrhoeae* via unidirectional intragenic gene conversion between *pilS* and *pilE.* This process creates different variants of the pilin protein and affects the piliation status of *N. gonorrhoeae* and its ability to attach to mucosal cells. Figure obtained from Zöllner *et al.*, 2017.

N. gonorrhoeae is also able to switch the expression of the pili on or off via RecA-independent phase variation (Hagblom *et al.*, 1985; Koomey *et al.*, 1987; Long *et al.*, 1998). There are multiple ways this phase variation may occur including non-revertible recombination which leads to deletion, or reversible missense or frameshift mutation via redundant guanine-cytosine (GC) base pairs (Koomey *et al.*, 1987). Perhaps the most common example of phase variation however, occurs with surface opacity (Opa) proteins that affect the opacity of the bacterium and are important for adhesion onto and invasion of host cells (Mayer, 1982). Opa protein phase variation occurs via changes in the C-T (thymine)-C-T-T repeats which affect the open reading frame (ORF) of the gene, leading to the frequent polymorphic nature of gonococcal communities (Stern *et al.*, 1986; Makino *et al.*, 1991).

2.2.1.2 Competence and natural transformation

Another feature that enables *N. gonorrhoeae* to persist so well in the human host is its unregulated natural competence, or its ability to take up DNA and incorporate it into its genome at all phases of growth, especially during its lag and early exponential phases (Sparling, 1966; Biswas *et al.*, 1977; Seifert *et al.*, 1988; Hamilton & Dillard, 2006). This is in contrast to other pathogens like *Haemophilus influenza*, which is only competent during the stationary phase when nutrition is low (Sparling, 1966; Biswas *et al.*, 1977; Seifert *et al.*, 1988; Hamilton & Dillard, 2006). Competence is a natural process in at least 40 other bacterial species and is one of the methods used for DNA exchange in bacteria (the other two being conjugation and transduction). Interestingly, it does not require other genetic elements like bacteriophages and only relies on the competent state of the acceptor cell (Koomey, 1998; Chen & Dubnau, 2004; Hamilton & Dillard, 2006; Seitz & Blokesch, 2013; Veening & Blokesch, 2017). There are multiple theories for the role of DNA uptake in bacteria, the three main ones being to obtain DNA for food, DNA for repair of DNA damage or DNA to increase genetic diversity (Chen & Dubnau, 2004; Hamilton & Dillard, 2006).

Regardless of its role, the general process of DNA transformation is quite similar between bacterial species. The most significant difference however exist between Gram-positive bacteria compared to Gram-negative bacteria as exDNA in the latter must cross two membranes and the periplasmic space, as opposed to just a single cell membrane in Gram-positive bacteria. There are three main steps to natural transformation in *N. gonorrhoeae*: 1) Donation of DNA, 2) DNA binding and uptake, and 3) DNA processing and homologous recombination into the genome (Figure 2.2).

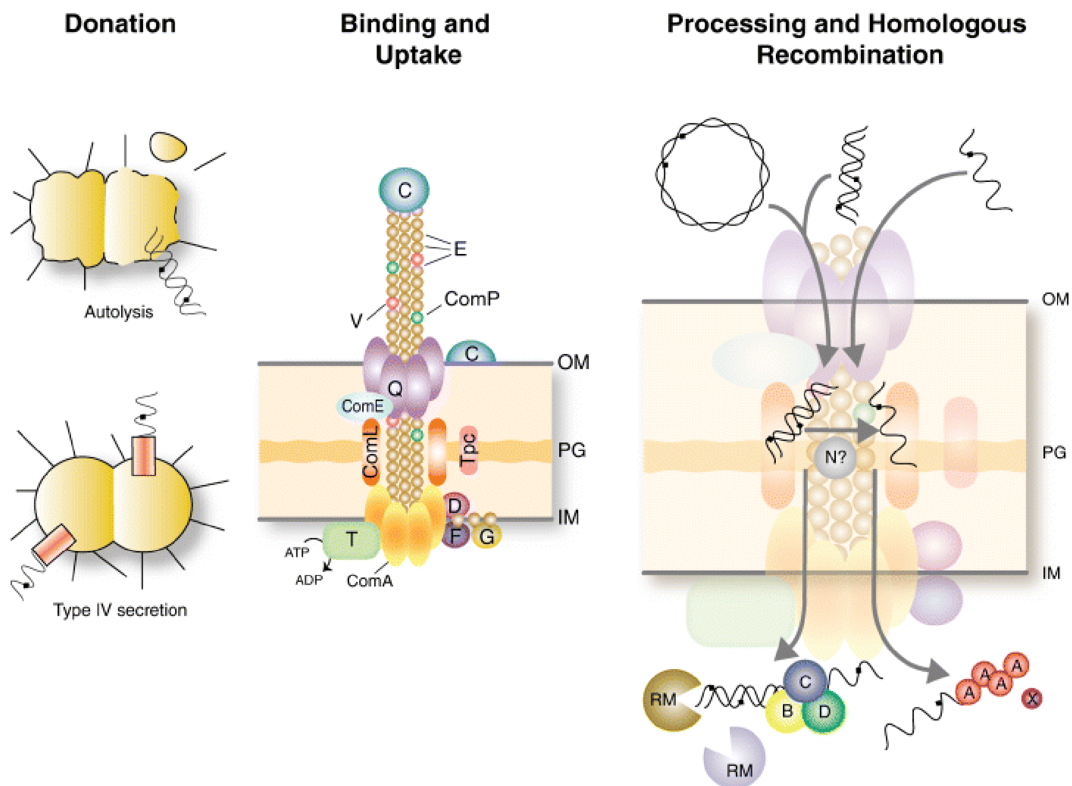

Figure 2.2 The current model for the DNA uptake process in *Neisseria gonorrhoeae* occurs in three separate steps. 1) Donation of DNA through autolysis or the Type IV secretion system. 2) Binding of DNA onto the type IV pili system (made up of Pil proteins E, V, Q, C, T, D, F, G) and uptake of the DNA into the periplasmic space or peptidoglycan layer via Com E. 3) Processing of DNA into single strands in the periplasm and homologous recombination into the genome. Figure obtained from Hamilton & Dillard, 2006. OM – outer membrane, IM – inner membrane, PG – peptidoglycan.

The first step of transformation in *N. gonorrhoeae* is the donation phase, where donor DNA is deposited into the environment. These may originate from dead gonococcal cells that have undergone autolysis during the stationary phase, or DNA that is specifically secreted by other gonococci via their Type IV secretion system (T4SS) (Seifert *et al.*, 1988; Kline *et al.*, 2003; Hamilton & Dillard, 2006). The T4SS is encoded on a gonococcal genetic island (GGI) which is present in ~80% of *N. gonorrhoeae* isolates and allows for the secretion of single-stranded DNA (ssDNA) that is also important for initiating biofilm formation (Hamilton & Dillard, 2006; Zweig *et al.*, 2014).

Specifically, *N. gonorrhoeae* takes up DNA from its environment with a very defined 10 bp DNA uptake sequence (DUS), 5’-GCC GTC TGA A-3’, which is common in the gonococcal genome, occurring every 1096 bp in the FA1090 strain (Graves *et al.*, 1982; Goodman & Scocca, 1988; Kline *et al.*, 2003; Hamilton &

Dillard, 2006; Ambur *et al.*, 2007). The DUS is believed to act as a transcriptional terminator as it occurs as inverted repeats between genes and hence aids with the formation of hairpin structures (Graves *et al.*, 1982; Goodman & Scocca, 1988; Kline *et al.*, 2003; Hamilton & Dillard, 2006; Ambur *et al.*, 2007). The presence of the DUS allows the bacterium to differentiate species-specific donor DNA from other pieces of exDNA that may bind to its surface (Graves *et al.*, 1982; Goodman & Scocca, 1988; Hamilton & Dillard, 2006). In fact, it is believed that some of the gonococcal chromosomal-acquired antibiotic resistance may have originated from some commensal *Neisseria* spp. (e.g. from commensal *Neisseria meningitidis* strains) that cohabit the pharynx and also contain these DUS in its genome (Barbee, 2014). In the laboratory, the presence of DUS in exDNA increases *N. gonorrhoeae* transformation efficacy by 20-fold in the FA1090 strain, and 150-fold in the MS11 strain, although DUS-lacking constructs are still able to be transformed, albeit with very low frequencies (Duffin & Seifert, 2010). It is also hypothesised that the DUS may protect exDNA from cytoplasmic and periplasmic nucleases, both of which are more active in the MS11 strain than the FA1090 strain, the latter of which lacks the GGI and hence the T4SS (Duffin & Seifert, 2010), Furthermore, recent work has identified two nucleotides (A (adenine)-T) at the 5' end of the DUS that further increases transformation efficacy (i.e. DUS12: 5'-ATG CCG TCT GAA-3'), with a majority of the DUS sequences (76%) being extended in this manner (Ambur *et al.*, 2007; Duffin & Seifert, 2010). A similar donor DNA discrimination process is observed in *H. influenzae* as well, which recognises a specific 5'-AAG TGC GGT-3' uptake signal sequence (USS) (Goodman & Scocca, 1988).

The second binding and uptake step of gonococcal DNA transformation strongly relies on the T4P machinery, with DNA exchange increasing by 1000-fold if the bacterium is piliated (Biswas *et al.*, 1977; Seifert *et al.*, 1988; Koomey, 1998; Chen & Gotschlich, 2001; Duffin & Seifert, 2010). After binding to the gonococcal surface, DNA has to be transported across the outer membrane (via the PilQ secretin channel), followed by the periplasm and then the inner membrane (Chen & Dubnau, 2004). Much is still unknown about the exact mechanisms involved in this step including the recognition of the DUS and binding of DNA to the cell surface. However, it is has been established that both Opa and ComP proteins are important for the non-specific binding of exDNA to the gonococcal surface, with the latter recognising the DUS sequence (Hamilton & Dillard, 2006). Another protein that is

important for DNA uptake in *N. gonorrhoeae* is the ComE protein, a homologue of the ComEA receptor protein from *Bacillus subtilis*, which binds, stabilises and pulls the DNA through the channel (Chen & Gotschlich, 2001). Rather than being an integral membrane protein however, ComE possesses a predicted signal peptide and is predicted to be translocated across the inner membrane into the periplasm where it may non-specifically protect pieces of exDNA from nuclease degradation (Chen & Gotschlich, 2001; Hamilton & Dillard, 2006).

Before genomic recombination, dsDNA is subjected to restriction in the periplasm and subsequently forms ssDNA (Hamilton & Dillard, 2006; Duffin & Seifert, 2010). This single-strand passes through the ComA channel in the inner membrane and undergoes homologous recombination with the genome via the RecBCD pathway (Duffin & Seifert, 2010). However, it is also believed that some ssDNA may bypass this pathway and bind directly to RecA for homologous recombination instead (Hamilton & Dillard, 2006; Duffin & Seifert, 2010). Due to the highly oxidising environment of the human body, the pool of exDNA for *N. gonorrhoeae* transformation is likely nicked or otherwise damaged. Hence, we hypothesise that there must be an extra step during or before the processing and homologous recombination phases where pieces of exDNA are repaired in the periplasm by a DNA ligase (for example, Lig E), the process of which would enhance the integrity of DNA before recombination into the genome.

2.2.2 DNA ligases

DNA ligases are enzymes responsible for sealing breaks in DNA via the formation of phosphodiester bonds between 3'-hydroxy (OH) and 5'-phosphate (PO_4) ends during DNA repair, replication and recombination (Zimmerman *et al.*, 1967; Shuman, 2009). These enzymes are part of the nucleotidyl transferase superfamily, which also encompasses ribonucleic acid (RNA) ligases and messenger RNA (mRNA) capping enzymes, all of which require a nucleotide cofactor to function (Tomkinson *et al.*, 2006). At a minimum, DNA ligases consist of two conserved core domains, and many isoforms have additional domains involved in DNA binding and other processes. Of the conserved core domains, the larger adenylation (AD) or nucleotidyl transferase (NTase) domain is connected to the smaller oligonucleotide binding (OB) domain by a flexible linker that facilitates movement between open and closed confirmations of the enzymes (Tomkinson *et al.*, 2006).

The N-terminal AD domain is the site of ligation and binds the adenylate donor which provides energy for catalysis. This domain includes five conserved motifs which are found across the nucleotidyl transferase superfamily (Figure 2.3) (Shuman, 2009). Motif 1 (Lys (lysine)-x-Asp (aspartic acid)-Gly (glycine)-x-Arg (arginine)) contains the catalytic lysine that attacks the cofactor during ligation, initiating the ligation process (Subramanya *et al.*, 1996; Wilkinson *et al.*, 2001; Shuman, 2009). In contrast, the carboxy (C)-terminal OB domain consists of a five-stranded antiparallel beta barrel and an alpha helix, which are important for binding and positioning the DNA substrate which sits in the positively charged concave surface of the domain (Shuman, 2009; Williamson & Leiros, 2020).

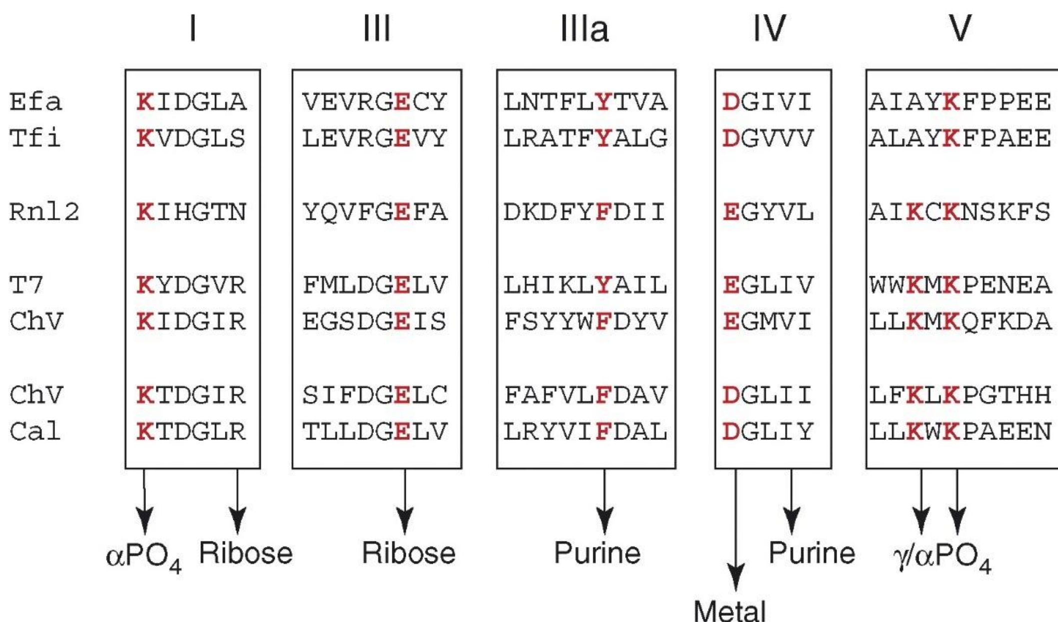

Figure 2.3 The five conserved motifs found in the N-terminal adenylation domain of nucleotidyl transferase enzymes. Contacts made with the nucleotide cofactor are indicated below the motifs (black arrows) and the conserved amino acids are highlighted in red (lysine (K), glutamic acid (E), tyrosine (Y), phenylalanine (F), aspartic acid (D)). Sequences include NAD^+-dependent ligases from *Enterococcus faecalis* (Efa) and *Tiedemannia filiformis* (Tfi), ATP-dependent ligases from the T4 RNA ligase 2 (Rnl2), the T7 bacteriophage (T7) and the *Chlorella* virus (ChV), and GTP-dependent capping enzymes from ChV and *Candida albicans* (Cal). Figure obtained from Shuman & Lima, 2004.

DNA ligation occurs via a ‘ping-pong’ reaction mechanism (Figure 2.4) (Lehman, 1974; Rossi *et al.*, 1997; Cherepanov *et al.*, 2008; Shuman, 2009; Williamson & Leiros, 2020). In the first step, the amino group of the conserved lysine (motif I) performs a nucleophilic attack on the 5’-PO_4 of an adenylate cofactor (either NAD^+ or ATP). This forms a phosphoamide covalent bond between the ligase and the

cofactor, releasing either nicotinamide mononucleotide (NMN) or pyrophosphate (PPi) and forming a ligase-adenylate intermediate (Lehman, 1974; Wilkinson *et al.*, 2001; Tomkinson *et al.*, 2006; Shuman, 2009). During this step, the ligase shifts from an open conformation to a closed one, followed by subsequent reopening to release the leaving groups. Specifically, motif VI of the OB domain orients the PPi leaving group where ATP is involved, whereas the N terminal Ia domain orients the NMN leaving group when NAD^+ is used and the residues of motif IV are in contact with the cofactor (Williamson & Leiros, 2020). This first step is also assisted by a single metal ion for NAD^+-dependent ligases, and more than one metal ion for ATP-dependent ligases. These metal ions lower the acid dissociation constant (pKa) of the lysine which stabilises the intermediate structure, and also helps orient the beta and gamma phosphates of ATP specifically (Williamson & Leiros, 2020).

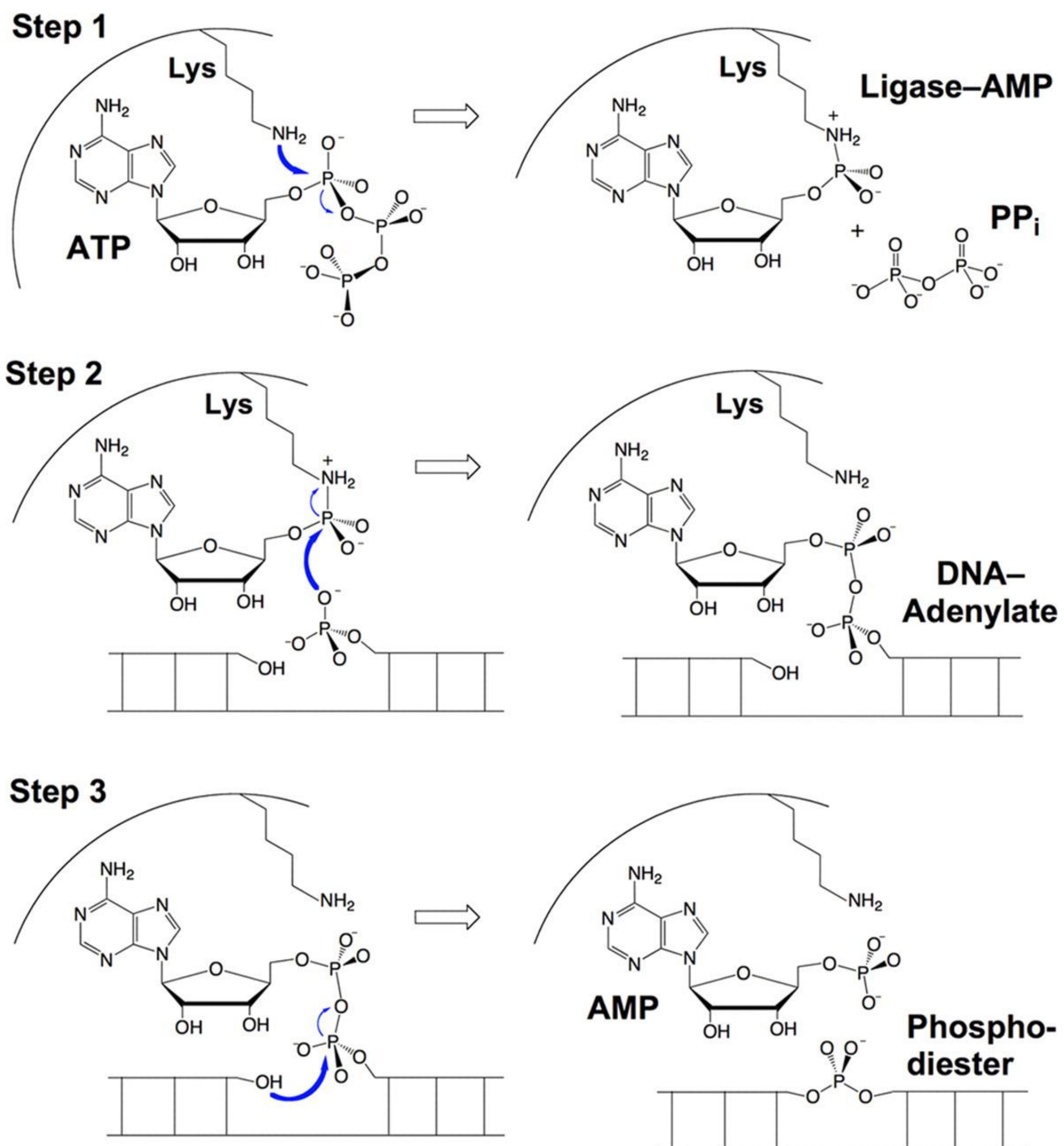

Figure 2.4 DNA ligation occurs in three ping-pong-like steps. In the first step, the catalytic lysine (lys) of the ligase is adenylated by an adenylate donor (ATP in this example), forming a ligase-adenylate intermediate. In the second step, the adenyl group is transferred onto the 5'-phosphate (PO_4) end of the nick, forming a DNA-adenylate intermediate. In the last step, the activated 5'-PO_4 end interacts with the 3'-hydroxy (3'-OH) end of the nick, forming a phosphodiester bond, which is followed by the release of the adenylate by-product. Figure obtained from Shuman, 2009.

In the second step of ligation, the oxygen of the 5'-PO_4 of the adenylyl group attacks the 5'-PO_4 end of the nicked DNA to form a DNA-adenylate intermediate with a pyrophosphate/phosphoanhydride bond, leaving the lysine behind (Lehman, 1974; Wilkinson *et al.*, 2001; Tomkinson *et al.*, 2006; Shuman, 2009). During this step, the nicked DNA strand is bound across the AD domain, while the complement is engaged by the OB domain which sits within the minor groove of the DNA (Williamson & Leiros, 2020). Together, these two domains form a C-shaped clamp

structure, distorting the duplex and facilitating the interaction of the 3'-OH end of the nick with the adenylated lysine (Williamson & Leiros, 2020).

In the final step of ligation, the alpha phosphate of AMP (in the case of ATP-dependent ligases) reorients almost 90° about the diphosphate bond to be in close proximity to the 3'-OH end of the DNA, allowing the activated 5'-PO_4 to attack the 3'-OH end which creates a phosphodiester bond and leaves AMP as a leaving group (Lehman, 1974; Wilkinson *et al.*, 2001; Shuman, 2009; Williamson & Leiros, 2020).

2.2.2.1 NAD$^+$-dependent ligases

In all bacteria, NAD$^+$-dependent ligases (NDLs), also termed Ligase A (Lig A), are essential and carry out the housekeeping functions of DNA repair, replication and recombination processes such as sealing Okazaki fragments in the lagging strand during DNA replication (Williamson *et al.*, 2016). NDLs are also found in some eukaryotic viruses, bacteriophages and archaea, but not in eukaryotes (Shuman, 2009). The structures of NDLs are highly conserved and consist of an N-terminal Ia domain (Pfam database domain architecture: PF22745) which engages with the NAD$^+$ cofactor, the AD (PF01653) and OB (PF03120) domains, and three C-terminal modules including a helix-turn-helix (HtH) motif (PF12826; interacts with both DNA strands across the minor groove for stabilisation around the DNA bend), a zinc finger motif (PF03119; has a structural role of bridging the OB domain and the HhH motif) and the BRCA1 C-terminal (BRCT) domain (PF0053; has a potential role in nick-sensing and stabilisation of the protein complex) (Shuman, 2009; Williamson & Leiros, 2020). These three additional domains aid in complete encirclement and engagement with the substrate DNA for effective ligation activity (Nandakumar *et al.*, 2007).

2.2.2.2 ATP-dependent ligases

ATP-dependent ligases (ADLs) are found in all eukaryotes and archaea and in most eukaryotic viruses and bacteriophages where they carry out the essential housekeeping DNA ligation processes (Tomkinson *et al.*, 2006; Shuman, 2009). Unlike NDLs, ADLs have very diverse structures (Williamson & Leiros, 2020). Furthermore, the OB domain of ADLs includes an additional motif (motif IV) which interacts with the PPi leaving group and hence is not found in NDLs (Shuman, 2009).

In eukaryotes, three families of ADLs have been identified; DNA ligase I (Okazaki fragment sealing, long patch base excision repair (BER)) and DNA ligase IV (non-homology end joining (NHEJ) of double-stranded breaks (DSBs)), both of which are found in all eukaryotes, as well as DNA ligase III (short patch BER, single-stranded breaks (SSBs) and mitochondrial DNA repair) which are only found in vertebrates (Wilkinson *et al.*, 2001; Ellenberger & Tomkinson, 2008; Williamson & Leiros, 2020). These all contain an additional N-terminal DNA binding domain in addition to the two core domains, making their structural architecture very similar to that of archaeal ADLs (Ellenberger & Tomkinson, 2008; Chambers & Patrick, 2015).

In addition to their essential NDLs, some bacteria also express additional ADLs, denoted Lig B, C, D and E (after the NAD^+-dependent 'Lig A') (Figure 2.5). These vary greatly in terms of their sizes and activities, including the presence of additional domains that may aid in complete encirclement of the DNA substrate (Doherty & Wigley, 1999). The presence of ADLs in bacterial genomes is widespread but not ubiquitous and hence they are believed to only act as accessory enzymes. For example, some species possess more than one bacterial ADL (b-ADL), while some express none, and many b-ADLs seem to co-occur with each other, although exceptions do exist (Williamson *et al.*, 2016). These b-ADLs have been deleted from several bacterial species without impacting viability, and as described below, a number of them have been implicated in stationary phase repair processes.

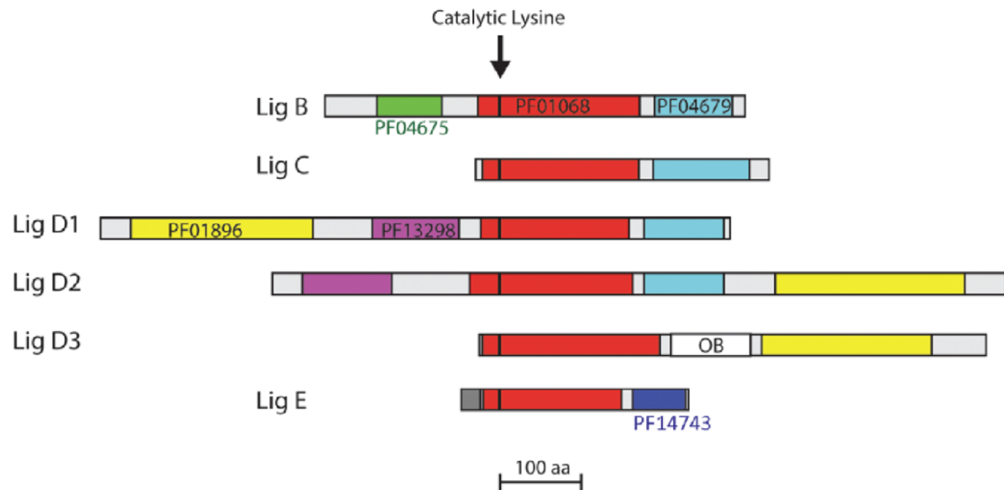

Figure 2.5 Pfam database domain architectures of bacterial ATP-dependent ligases (Lig B, C, D and E). Red (PF01068) illustrates the adenylation domain, and both blue (PF05679; PF14743) and white represent the oligonucleotide-binding domains. Additional domains are illustrated in green (PF04675), yellow (PF01896) and purple (PF13298). Figure from Williamson *et al.*, 2016.

2.2.2.2.1 Lig B

The architecture of the Lig B ligase in bacteria is akin to that of archaeal and eukaryotic ADLs due to the presence of a large DNA binding domain on its N-terminus (PF04675) (Figure 2.5), and it has high nick-sealing activity in the absence of any accessory enzymes (Wilkinson *et al.*, 2001; Williamson & Pedersen, 2014; Williamson & Leiros, 2020). Much is still unknown about its specific repair pathway *in vivo*; however, it occurs in a gene cluster with an Lhr helicase, a binuclear metallophosphoesterase and a putative exonuclease (Williamson & Leiros, 2020). Lig B is believed to be working in concert with these enzymes, where the helicase would unwind the DNA duplex, the metallophosphoesterase would cleave any stem and loop structures, and the exonuclease would work on any interstrand crosslinks (Gong *et al.*, 2004; Williamson & Leiros, 2020).

2.2.2.2.2 Lig C

Lig C is involved in the base excision repair of DNA lesions during the stationary phase of bacterial growth (Płociński *et al.*, 2017). This enzyme only possesses the two core domains, and has poor nick-sealing activity on DNA without any binding partners (Zhu & Shuman, 2007; Williamson *et al.*, 2016). It is found with a PrimPolC DNA polymerase, the DNA glycosylases NTH, IPG, MPG, and the nucleases EndoIV, ExoIII and XthA, which are all important for lesion processing (Williamson & Leiros, 2020). After removal of nucleotides in an abasic site or

lesion to form a gapped substrate, PrimPolC fills the gap with ribonucleotides, making a nicked dsDNA substrate with an RNA 3'-terminus for Lig C to act on (Williamson & Leiros, 2020). Lig C is commonly found in bacteria prone to desiccation and dormancy, including both the *Mycobacterium* and *Bacillus* species, and it often co-occurs with Lig B (Williamson *et al.*, 2016; Williamson & Leiros, 2020).

2.2.2.2.3 Lig D

In addition to its core domains, Lig D also includes a PrimPol (DNA primase/polymerase) enzymatic domain (PF01896) and a PE (phosphoesterase) enzymatic domain (PF13298), although the placement of these in relation to the AD and OB domains differ between species (Lig D1-3) (Figure 2.5) (Weller & Doherty, 2001; Weller *et al.*, 2002). Lig D is responsible for non-homologous end joining of double-stranded breaks (DSBs) during the stationary phase, where its PE domain removes bases from 3'-phosphate ends of 'dirty' breaks before the addition of new nucleotides via its PrimPol domain (at the 3'-OH), and the final ligation via the core domains of the enzyme (Lewis & Resnick, 2000; Zhu & Shuman, 2007; Williamson & Leiros, 2020). Lig D is found alongside a Ku homologue, which is an end binding protein that creates synapses at the double-stranded break site (Weller & Doherty, 2001; Williamson & Leiros, 2020). Like Lig C, Lig D is found in bacteria prone to desiccation and radiation damage where DSBs are common (Williamson & Leiros, 2020). Lig D2 often co-occurs with Lig B and Lig C, although it is interesting to note that the Lig D3 architecture occurs alone in most genomes (Williamson *et al.*, 2016).

2.2.2.2.4 Lig E

Similar to Lig C, Lig E has a minimal structure, consisting only of the core AD and OB domains with no additional accessory domains, making it very small and compact (20.7-32.5 kDa) (Magnet & Blanchard, 2004; Williamson *et al.*, 2016). However, its OB domain (PF14743) differs slightly from the OB domain of other b-ADLs, as it has fewer helical structures, similar to the OB domain from the T7 bacteriophage and *Chlorella* virus (Chv) ligases, making it the most minimal b-ADL (Subramanya *et al.*, 1996; Odell *et al.*, 2000; Shuman, 2009; Williamson *et al.*, 2014). Despite being the first b-ADL characterised in 1997 (from *H. influenzae*), much is still unknown about the function of Lig E *in vivo* (Cheng & Shuman, 1997).

As of now, Lig E from several bacteria including *H. influenzae* (Hin-Lig E) (Cheng & Shuman, 1997), *N. meningitidis* (Nme-Lig E) (Magnet & Blanchard, 2004), *Aliivibrio salmonicida* (Vib-Lig E) (Williamson & Pedersen, 2014), *Psychromonas* sp. (Psy-Lig E) (Williamson *et al.*, 2014) and *Alteromonas mediterranea* (Ame-Lig E) (Williamson *et al.*, 2018) have been recombinantly expressed, and all show high levels of independent single nick-sealing activities, with some able to work on cohesive, blunt-ended, mismatched and gapped DSB substrates, although to a lesser degree. Solved structures of Psy-Lig E (partially open and fully open) and Ame-Lig E (DNA-bound structure) show that due to the absence of additional domains, Lig E is unable to achieve complete encirclement of the DNA substrate (Figure 2.6) (Williamson *et al.*, 2014). Instead, it forms a C-shaped clamp around the DNA with the nick sitting above the AMP binding pocket and relies on strong positive charges from basic residues from both domains to interact with the negative surfaces of DNA via charge-pair interactions (Magnet & Blanchard, 2004; Williamson *et al.*, 2014; Williamson *et al.*, 2018). Although its OB domain closely resembles that of the Chv ligase, it lacks the lysine-rich beta-hairpin loop latch between the 9th and 10th beta sheet of the Chv ligase OB domain that allows for DNA encirclement (Figure 2.6) (Odell *et al.*, 2000; Nair *et al.*, 2007; Shuman, 2009). Instead, Lig E has a Gly-Lys-Gly-Lys-Aromatic motif with two lysines that interact with the nicked strand, leading to tighter enzyme-DNA interactions (Shuman, 2009; Williamson *et al.*, 2018). Unlike the Chv ligase that undergoes an increase in secondary structuring when DNA is bound, there is also a lack of organisation of any unstructured regions when DNA binds to Lig E, indicating that the surface of Lig E is likely preorganised for binding before ligation occurs (Odell *et al.*, 2000; Shuman, 2009; Williamson *et al.*, 2018). Structural analyses of Lig E from different bacteria also predict disulphide bonds forming for Hin-Lig, Nme-Lig and Cje-Lig (Lig E in *C. jejuni*), with the latter predicted to form an additional disulphide bond during the DNA-bound state (Pan *et al.*, 2021).

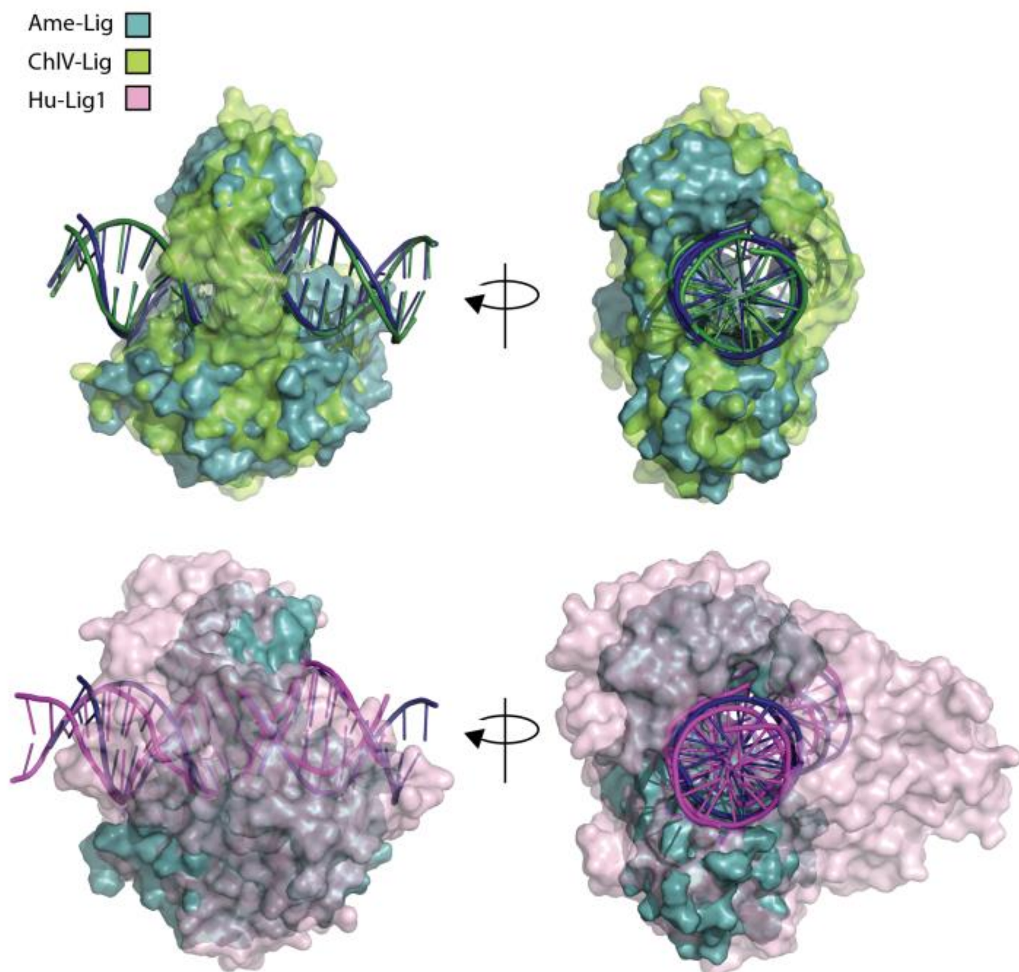

Figure 2.6 Superimposition of Lig E from *Alteromonas mediterranea* (Ame-Lig) with other ligases, highlighting the lack of complete encirclement of DNA by Lig E. The ligase from *Chlorella* virus ligase (referred to here as 'ChIV-Lig') is shown at the top, while the Human Ligase 1 (Hu-Lig1) is shown at the bottom. DNA is shown in dark blue for Ame-Lig, green for the *Chlorella* virus ligase and pink for Hu-Lig 1. Figure obtained from Williamson *et al*., 2018.

Analysis of the genomic context of Lig E found no synteny in the nature of the adjacent genes between Lig E-expressing bacterial species, although a majority of them encode a homologue of the ComEA DNA receptor protein necessary for DNA uptake (Williamson *et al.*, 2016; Williamson & Leiros, 2020; Pan *et al.*, 2021). Specifically, Lig E is found in many naturally transformable Gram-negative proteobacteria including human pathogens like *N. gonorrhoeae* (betaproteobacteria), *H. influenzae* (gammaproteobacterial) and *C. jejuni* (epsilonproteobacteria), as well as bacteria that live in aqueous marine environments (Pan *et al.*, 2021). Phylogenetic analysis place Lig E as a separate group from other b-ADLs with a potential bacteriophage origin (Figure 2.7) (Williamson *et al.*, 2016). Meanwhile, ligases Lig B, Lig C and Lig D are believed to have been inherited together from a common ancestor of possible archaeal

origins due to their structural similarities and frequent co-occurrence in different bacterial species (Williamson *et al.*, 2016; Pan *et al.*, 2021). Further analysis of the 16s ribosomal RNA (rRNA) of Lig E-expressing bacteria suggests a relatively recent horizontal gene transfer to *H. influenzae* due to the absence of Lig E in other *Haemophilus* spp. like *Haemophilus ducreyi*, and how *H. influenzae* clusters close to other betaproteobacteria despite it being a gammaproteobacteria (Williamson *et al.*, 2016). Furthermore, Lig E does not co-occur with other b-ADLs.

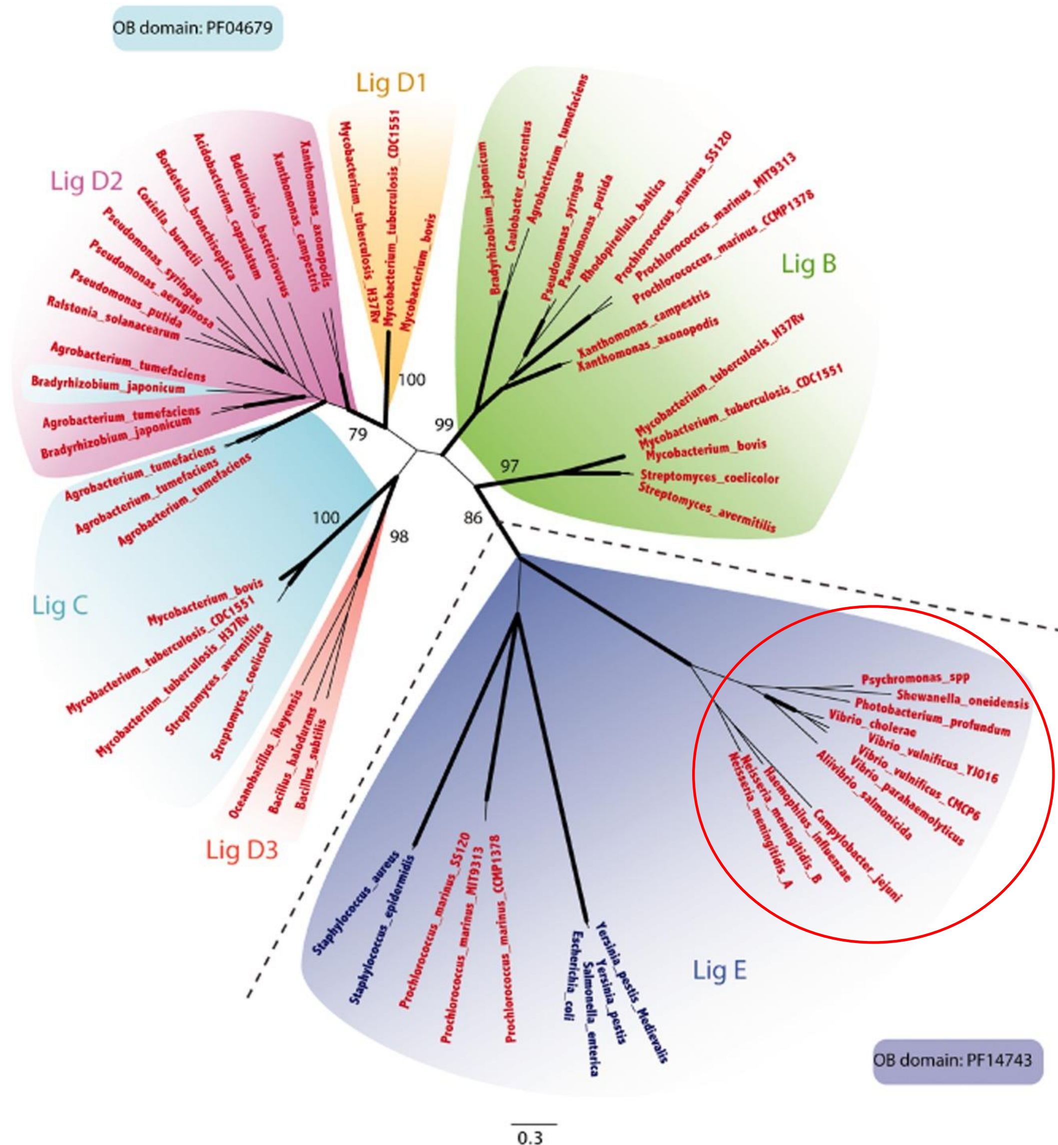

Figure 2.7 Phylogenetic tree of bacterial ATP-dependent ligases based on their domain arrangements and biochemical characteristics, retrieved from Williamson *et al.*, 2016. Lig E (circled) forms a different clade from the other ATP-dependent ligases. The three left branches in the Lig E clade (uncircled) are no longer classified as Lig E due to the presence of additional DNA binding domains that have been identified since.

2.2.2.2.5 Predicted role of Lig E in human pathogens

Perhaps the most enigmatic element of Lig E is the 20-40 amino acid signal peptide on its N-terminus, which when removed increases its ligation activity and stability, supporting its role as a localising sequence outside the cell (Williamson & Pedersen, 2014). This is an interesting feature for a ligase to contain as a bacterium's genetic material is contained within the cytosol. Thus, we hypothesise that Lig E may be working in the periplasmic or extracellular space to seal nicks in exDNA, potentially during competence and uptake, increasing their information content and making them ideal for homologous recombination into the genome (Figure 2.8A) (Chaussee & Hill, 1998). We believe that this process occurs before the formation of ssDNA and its translocation into the cytoplasm. This is particularly relevant considering its occurrence in many naturally competent human pathogens where the acquisition of antibiotic resistance genes is important for their survival, and where the highly oxidative environment of the human body would cause much exDNA to be nicked or fragmented, and hence in need of ligation and repair before incorporation into the genome.

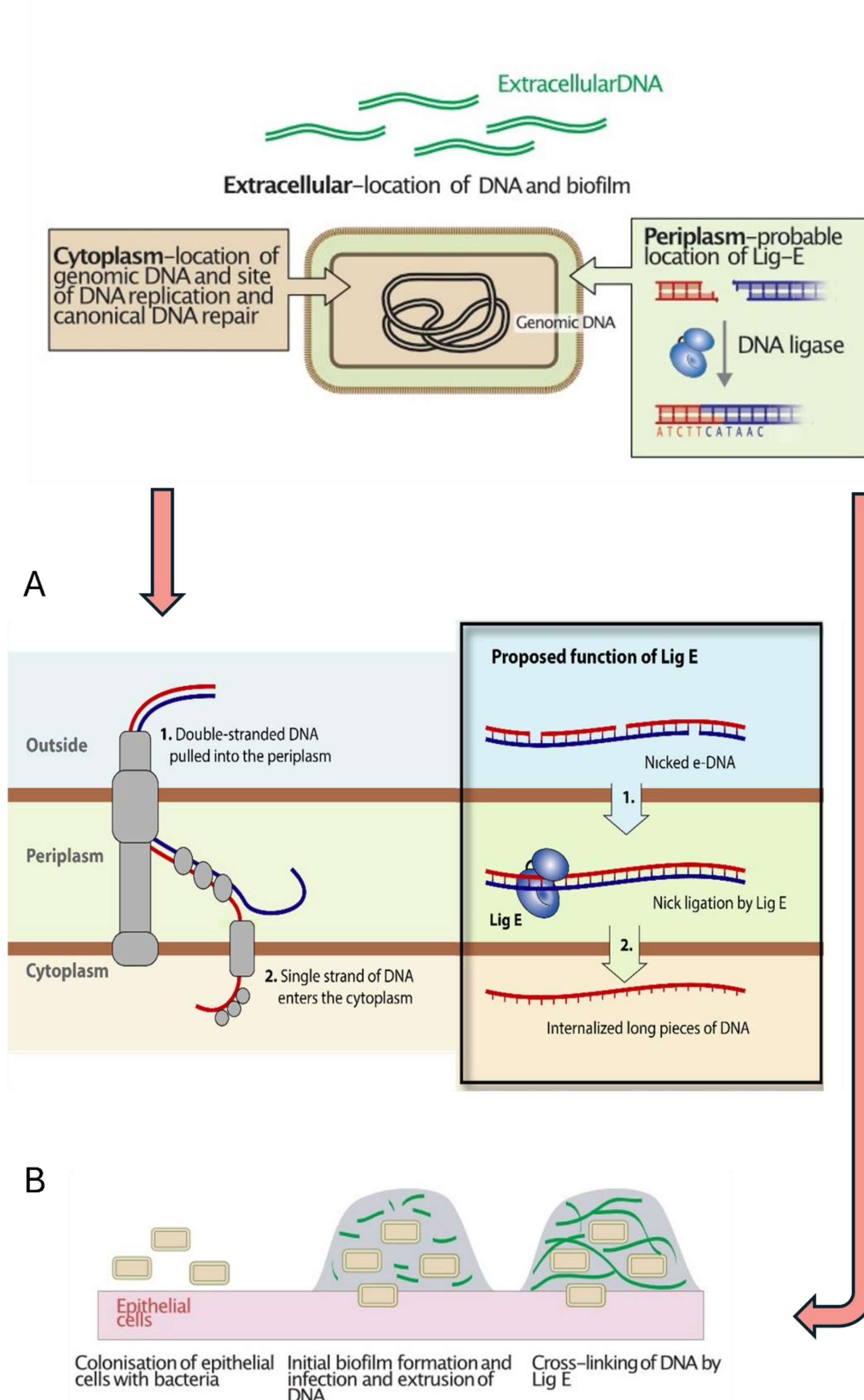

Figure 2.8 Proposed functions of Lig E in Gram-negative bacteria (Williamson, 2021, unpublished). (A) Role in DNA uptake: Lig E may repair nicked double-stranded DNA in the periplasm before generation of single-strands, which increases its integrity for subsequent homologous recombination into the genome. (B) Role in biofilm formation: Lig E may work on fragmented extracellular DNA, creating higher molecular weight substrates that better contribute to the structure and stability of bacterial biofilms as well as its initiation.

We postulate that in a mixed population of bacteria where a subset of bacterial cells have acquired antibiotic resistance, an event like oxidative stress or neutrophil consumption in the human host may kill most of the population but spare some, albeit with some damage to their genomic DNA. The fragmented DNA from lysed bacteria containing antibiotic resistance genes may then be taken up by the surviving, but damaged bacteria. Lig E may function to seal the breaks in these antibiotic resistance-containing fragments, allowing integration and acquisition of these genes into the genome. This hypothesised role of Lig E is supported by the discovery of extracellular ATP that could potentially act as a cofactor for Lig E activity in the periplasm, as well as the presence of homologues of the competence protein, ComEA, in Lig E-expressing bacteria, which hints at their transformability (Mempin *et al.*, 2013; Abbasian *et al.*, 2019). However, Lig E-containing bacteria are not the only naturally competent bacterial species, indicating that if Lig E is important in the DNA uptake process, it works to enhance the DNA transformation process rather than be essential for it. In addition to this, we also predict that Lig E may be working on exDNA present in bacterial biofilms, increasing their lengths and enhancing their ability to contribute to biofilm stability and structure (Figure 2.8B), allowing them to persist effectively in their habitat (more details in Section 2.3).

Considering the number of human pathogens that express Lig E including *H. influenzae, N. meningitidis, N. gonorrhoeae, V. cholerae*, and *C. jejuni*, the characterisation of this enzyme would have important implications as a therapeutic target in clinical settings if it is involved in their survival, resistance or pathogenesis (Williamson *et al.*, 2018). However, to date, no *in vivo* experiments have been conducted to confirm either its potential extracellular or periplasmic location, or its potential role of sealing exDNA during DNA competence and biofilm formation. In particular, studying this minimal enzyme in the potential superbug, *N. gonorrhoeae*, is not only important for gaining a better understanding of horizontal gene transfer across different organisms, but is also critical for finding new targets against the bacterium before it becomes an untreatable epidemic across the globe.

2.2.3 References

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2.3 Part two: Review – The role of extracellular DNA in *Neisseria* biofilms

2.3.1 Preface

In this section, I present an in-depth review on the presence of exDNA in *Neisseria* biofilms, which constitutes their primary polymeric component, unlike many other bacterial biofilms. This review provides a detailed analysis on the potential sources and roles of exDNA in *Neisseria* spp. biofilms, with comparisons made to other bacteria that also form exDNA-dependent biofilms. In particular, it highlights the consistent gap in knowledge in commensal *Neisseria* research and stresses the importance of fully understanding their interactions with pathogenic *Neisseria* spp. We also discuss Ngo-Lig E and how this ligase may contribute to exDNA-dependent biofilm formation in *N. gonorrhoeae* in addition to its potential role in DNA uptake, with references made to the findings presented in Chapter Three. This manuscript is ready for submission:

Pan, J., & Williamson, A. (2025). The role of extracellular DNA in *Neisseria* biofilms. (*To be* s*ubmitted*).

2.3.1.1 Author contributions

The collation of literature, figure making and manuscript writing of all sections were conducted by myself, with editing and feedback provided by my Chief Supervisor, Adele Williamson. The co-authorship form for this manuscript can be found in Appendix D.

Review: The role of extracellular DNA in *Neisseria* biofilms

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2.3.2 Abstract

The *Neisseria* is a genus of Gram-negative bacteria that includes two pathogenic species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, as well as other commensal species that also inhabit the human host. One of the features that aid with host colonisation in *Neisseria* is biofilm formation, which allows the bacteria to persist and evade the human immune system. Extracellular DNA (exDNA) forms a major component of *Neisseria* biofilms and contributes to the initial step in their formation, as well as the structure and stability of the extracellular matrix. In this review, we focus on the potential roles and sources of exDNA in *Neisseria* biofilms and its relevance to both pathogenic and commensal *Neisseria* spp. We highlight the paucity in research on biofilm formation by commensal *Neisseria* and hope that this review will stimulate interest in the role of exDNA in non-pathogenic *Neisseria*, particularly regarding biofilm formation and colonisation of the human host.

2.3.3 Introduction

Named after Albert Ludwig Neisser who first isolated *Neisseria gonorrhoeae* from patient-derived neutrophils in 1879, the genus *Neisseria* is a group of Gram-negative β-proteobacteria that colonises different mucosal surfaces in animals, including humans (Neisser, 1879; Elias *et al.*, 2015). These bacteria are characterised by their distinctive lack of flagella and diplococci morphology, although exceptions do exist (i.e. singular cocci during conditions of stress, or the rod-shape of *Neisseria elongata*) (Wolfgang *et al.*, 2011; Elias *et al.*, 2015). Of the 38 species identified and listed on the List of Prokaryotic names with Standing in Nomenclature (LPSN) database (https://lpsn.dsmz.de/search?word=neisseria), approximately 17 species can be isolated from humans, of which only two, *Neisseria meningitidis* and *N. gonorrhoeae*, are classified as pathogenic. The remainder are classified as commensal, forming part of the normal human microbiota. Much is yet to be discovered about these remaining *Neisseria* spp., with

most research to date having been focused on the two pathogenic strains. In addition, species categorisation of *Neisseria* is constantly being updated as new genome-focused methodologies are being utilised to study members of this genus (Bennett *et al.*, 2012).

Members of the *Neisseria* spp. are naturally and constitutively competent, which means that they are able to undergo genetic transformation at any stage of growth (Biswas *et al.*, 1977; Hamilton & Dillard, 2006). This natural competence is highly beneficial for pathogenic *Neisseria,* particularly by enabling acquisition of new antibiotic resistance genes to survive antibiotic treatment in the human host, as well as facilitating adaptation to stressors in the environment for commensal *Neisseria* spp. In fact, the amount of genetic exchange that occurs amongst different *Neisseria* spp. is thought to be quite extensive, as evidenced by the number of virulence genes found in commensal *Neisseria* of pathogenic *Neisseria* origin (Marri *et al.*, 2010). Another feature common to *Neisseria* is their ability to form biofilms. Interestingly, *Neisseria* spp. lack the genes necessary to produce exopolysaccharides (EPS), a material common in the extracellular matrix (ECM) of many bacterial biofilms (Ferretti *et al.*, 1997; Steichen *et al.*, 2011). While rare, this lack of EPS genes is not unique to *Neisseria*; for example, some *Mycobacteria* spp. also lack this ability and instead rely on lipids such as mycolic acids and lipopeptides for biofilm stability and structure (Zambrano & Kolter, 2005; Rose *et al.*, 2015). Similarly, the biofilms of *Neisseria* consist of other substrates like outer membrane vesicles and extracellular DNA (exDNA) instead (Steichen *et al.*, 2011). In this report, we review the evidence of a functional role of exDNA in *Neisseria* biofilms with a focus on its potential origin(s) and incorporation. We also highlight the lack of research on biofilm formation by commensal *Neisseria* and indicate that this area of research requires more study.

2.3.4 The genus *Neisseria*

2.3.4.1 Pathogenic *Neisseria* spp.

The two most widely studied species of *Neisseria* are the human pathogens *N. meningitidis* and *N. gonorrhoeae*. Both species can undergo extensive phase and antigenic variation, which allow them to evade the host immune response (Hagblom

et al., 1985; Zöllner *et al.*, 2017). The resultant heterogeneity from both processes makes it difficult to design effective vaccines against both species.

N. meningitidis is an opportunistic facultative human pathogen that invades the nasopharyngeal tract of the human host, where it attaches to the microvilli of mucosal cells before its engulfment and subsequent transversion across the epithelial layer (Stephens *et al.*, 1983; Rosenstein *et al.*, 2001). *N. meningitidis* typically spreads via aerosols or secretions with very high ranges of transmission by carriers (~8-50%) (Neil & Apicella, 2009; Elias *et al.*, 2015). Despite this, a majority of meningococcal colonisations are asymptomatic, with approximately 10% of healthy individuals predicted to carry the bacterium (Yazdankhah & Caugant, 2004). However, when symptomatic (0.5-5 cases per 100,000 annually for meningococcal disease), infections are severe with high mortality rates that result from bacterial meningitis where the meninges or membranes lining the brain and spinal cord are inflamed, causing symptoms like fever and nausea (Lappann & Vogel, 2010). Cases are most common in children and if left untreated, infections may lead to paralysis, seizures and developmental impairment, as well as other conditions like pneumonia, urethritis and arthritis (Tzeng & Stephens, 2000; Ferguson *et al.*, 2002; Yazdankhah & Caugant, 2004). Furthermore, the bacterium may cross into the bloodstream resulting in meningococcal septicaemia, which if left untreated, may block blood vessels, leading to the loss of limbs (Tzeng & Stephens, 2000; Ferguson *et al.*, 2002).

N. meningitidis isolates can be categorised into different clonal complexes based on their genetic lineage, with healthy individuals harbouring more genetically diverse isolates compared to those with the disease phenotype (Claus *et al.*, 2005; Brehony *et al.*, 2007). Strains that constitutively express a polysaccharide capsule are more invasive due to the protective role of the capsular layer against external stressors like desiccation or the host immune response (Frosch *et al.*, 1989; Spinosa *et al.*, 2007; Stephens, 2007). In fact, isolates obtained from patients with invasive meningococcal disease are often encapsulated, while those isolated from carriers are often unencapsulated (Johswich *et al.*, 2012). Based on the different genes important for capsule biosynthesis, modification, transport and immunologic reactivity, *N. meningitidis* can be divided into 13 different serogroups, with serogroups A, B, C, W, X and Y being most commonly found in cases with invasive

meningococcal disease (Tzeng & Stephens, 2000; Rosenstein *et al.*, 2001; Spinosa *et al.*, 2007). Several vaccines have already been developed against these polysaccharide capsules; however, *N. meningitidis* is capable of switching serogroups via phase variation or genetic exchange (Rosenstein *et al.*, 2001; Vogel & Claus, 2011). Interestingly, this capsulation as a virulence factor is not seen in commensal *Neisseria* spp. or *N. gonorrhoeae*.

N. gonorrhoeae is an obligate human pathogen that infects the mucosal cells of the reproductive tract, causing the sexually transmitted infection (STI), gonorrhoea. *N. gonorrhoeae* infection rates are relatively high, with an estimated 106 million people being newly infected each year according to the World Health Organisation (WHO) (World Health Organization, 2024), making it the second most common STI globally, after chlamydia. Due to the nature of the reproductive tract, infections differ between females and males, with symptoms often presenting as urethritis or epididymitis in men, and cervicitis or vaginal pruritus in women (Miller, 2006; Haese *et al.*, 2021). Furthermore, *N. gonorrhoeae* infections in women can often ascend up the reproductive tract, causing disseminated upper genital tract infections like pelvic inflammatory disease that presents as severe pain (present in 10-20% of female infections), infertility or even ectopic pregnancy (Morse & Knapp, 1992; Miller, 2006). Despite this, *N. gonorrhoeae* infections in females most often produce little or no cytokine production, and hence effects minimal inflammatory response, leading to a considerable proportion of infections in females being asymptomatic (~40-60%) (Hedges *et al.*, 1998; van Duynhoven, 1999). Conversely, infections in men are more cell-invasive, leading to a robust inflammatory response, culminating in symptomatic infections (~85-99%) (Steichen *et al.*, 2008; Falsetta *et al.*, 2011). *N. gonorrhoeae* may also colonise the oropharyngeal tract and the rectum, although these infections are commonly asymptomatic with low prevalence (Chan *et al.*, 2016).

Currently, no vaccines have been successfully developed against *N. gonorrhoeae* due to its highly panmictic nature and lack of capsulation (Haese *et al.*, 2021). This means that the current line of defence against *N. gonorrhoeae* relies on antibiotics. However, *N. gonorrhoeae* has quickly become resistant to almost all antibiotic therapies that have been used against it, making treatment increasingly difficult (Unemo & Shafer, 2011, 2014). The rapid emergence of both multi drug-resistant

(MDR) and eXtensive drug-resistant (XDR) strains of *N. gonorrhoeae* has led to the declaration of the bacterium as a high-priority pathogen by the WHO, stressing the importance of further research into new ways to tackle this bacterium (Lee *et al.*, 2017).

2.3.4.2 Commensal *Neisseria* spp.

Despite the extensive attention being given to the two pathogenic *Neisseria* spp., a majority of the human-colonising members of the genus are commensal or non-pathogenic. The majority of them share a similar habitat in the oro- or nasopharyngeal region of the human host (highlighted in Table 2.1) and make up a very small percentage of the human microbiome (Zaura *et al.*, 2009; Kahler, 2021). Some may be associated with disease in immunocompromised individuals; however, virulence is low, and these are normally identified and presented as case studies (Liu *et al.*, 2015; Walsh *et al.*, 2023). Due to the lack of serious symptoms, less research has been undertaken on these species; however, they are likely important for maintaining healthy interactions with other beneficial microbes. Furthermore, they may also be important in supressing pathogenic *Neisseria.* For example, randomised controlled trials with human volunteers showed that individuals inoculated with *Neisseria lactamica* had lower meningococcal carriage rates, likely because *N. lactamica* prevented *N. meningitidis* from colonising the nasopharyngeal tract by competing for nutrients and space, subsequently limiting their colony sizes and motilities (Li *et al.*, 2006; Deasy *et al.*, 2015; Custodio *et al.*, 2020). This effect may not only be due to physical suppression however, as a clinical trial using a vaccine based on the outer membrane vesicles of *N. lactamica* showed induction of a weak, but broad immune response against *N. meningitidis* (Gorringe *et al.*, 2009). *Neisseria mucosa* has also been reported to produce a secondary metabolite that inhibits *N. gonorrhoeae* growth *in vitro* (Aho *et al.*, 2020), although other results in literature are conflicting (Abdellati *et al.*, 2022).

Table 2.1 Characterised commensal *Neisseria* spp. that colonise human mucosal surfaces.

Species	**Habitat**	**Characteristics and potential infections**
Neisseria cinerea (Knapp *et al.*, 1984)	Oropharynx Genitourinary tract	Reports of neonatal conjunctivitis obtained from the mother (Dolter *et al.*, 1998; Fiorito *et al.*, 2019).
Neisseria elongata (Bøvre & Holten, 1970)	Nasopharynx and oropharynx	Rod-shaped. Associated with endocarditis and infected lung bulla (Youssef *et al.*, 2019; Moriya *et al.*, 2022).
Neisseria lactamica (Hollis *et al.*, 1969)	Nasopharynx	Same habitat and similar traits as *N. meningitidis*; potentially protective against *N. meningitidis*. Common in children and neonates, but infection rates decrease with age.
Neisseria mucosa (Veron *et al.*, 1959) (incl. previously classified *Neisseria sicca* (Bennett *et al.*, 2012))	Nasopharynx and oropharynx	May be opportunistic in immunocompromised patients (i.e endocarditis) (Sommerstein *et al.*, 2013; Altdorfer *et al.*, 2021; Ren *et al.*, 2023). Is the most commonly reported invasive commensal *Neisseria* spp. (Walsh *et al.*, 2023).
Neisseria polysaccharea (Riou & Guibourdenche, 1987)	Nasopharynx and oropharynx	Able to produce extracellular polysaccharides from sucrose. No reports of disease (Walsh *et al.*, 2023).

Neisseria subflava (Benson *et al.*, 1928) (incl. previously classified *Neisseria perflava* and *Neisseria flavescens* (Bennett *et al.*, 2012))	Upper respiratory tract and genitourinary tract	May be opportunistic in immunocompromised patients (i.e. endocarditis, meningitis, septicaemia) but these are rare (Chong *et al.*, 1975; Pollack *et al.*, 1984; Sinave & Ratzan, 1987).

In addition to this, there is evidence that commensal *Neisseria* species can inhibit pathogenic *Neisseria* growth via DNA uptake, due to the different methylation patterns on its DNA. Using a mouse model of lower genital tract infections, Kim and colleagues demonstrated that *N. gonorrhoeae* is able to take up pieces of DNA released by commensal *N. elongata*. Uptake of this DNA with a foreign methylation pattern resulted in chromosomal damage and cell death of *N. gonorrhoeae*, which led to its clearance from the mouse vagina (Kim *et al.*, 2019). This phenomenon was also shown to occur with *N. meningitidis* in a liquid-assay, and is now a patented strategy, clearly showing the importance of these lesser known *Neisseria* spp. that infect humans (Kim *et al.*, 2019).

2.3.5 Biofilm formation of *Neisseria* spp.

A common feature across all *Neisseria* species is their ability to form biofilms; these are communities of bacteria that are held together in aggregates by a self-produced ECM. Implicated in ~ 80% of all chronic bacterial infections in humans, including on medical devices, biofilms often form in stressful environments where they aid in the survival and persistence of the bacteria (Stickler, 2008; Rather *et al.*, 2021). There are four major steps involved in biofilm formation (Figure 2.9). In the first step, contact is made between the bacteria and the surface of interest, resulting in a monolayer of cells on the surface (O'Toole *et al.*, 2000; Watnick & Kolter, 2000; Sauer *et al.*, 2022). This attachment is reversible and the bacteria are able to return to their planktonic state if disturbed by external factors like shear forces (Chandki *et al.*, 2011). In the second step, establishment of a monolayer is followed by the production of an ECM, making the attachment irreversible, and allowing for the formation of multi-layered microcolonies. In the third step, the bacteria grow and divide into mature three-dimensional biofilms (O'Toole *et al.*, 2000; Watnick &

Kolter, 2000; Chandki *et al.*, 2011). This is followed by the final stage, where cells disperse from the biofilm, releasing individual bacteria into the environment (Watnick & Kolter, 2000; Sauer *et al.*, 2022).

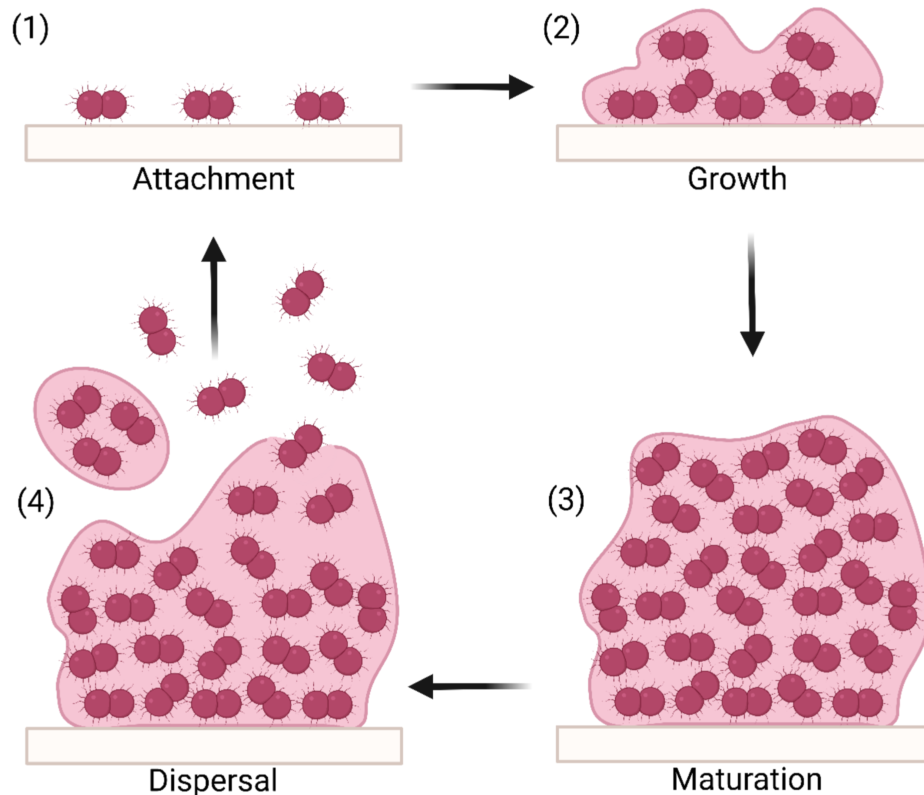

Figure 2.9 Steps in general bacterial biofilm formation. (1) Formation of a monolayer on the surface of interest. (2) Cell division and production of an extracellular matrix. (3) Maturation into a three-dimensional structure. (4) Dispersal and detachment of individual cells. Created in https://BioRender.com.

Biofilms offer a myriad of benefits to the bacteria they enclose. By increasing the spatial proximity of individual bacteria within the microcolony, biofilms are effective at facilitating communication and substrate exchange between neighbouring cells (Jefferson, 2004). This includes the possible transfer and exchange of new antibiotic resistance genes, further facilitating its spread throughout the population (Cvitkovitch *et al.*, 2003; Aliane & Meliani, 2021). In fact, *N. gonorrhoeae* exhibits high rates of gene transfer during early and permeable biofilms as opposed to older and denser biofilms (Kouzel *et al.*, 2015). Biofilms also create a physical barrier between the cells and their environment which shields cells at the centre of the biofilm from environmental stressors (i.e. oxidative stress, dehydration, shear forces, UV radiation), host stressors such as the immune response, and external agents like antimicrobials (Jefferson, 2004; Colvin *et al.*,

2011; Aliane & Meliani, 2021). For example, *N. meningitidis* inside a biofilm has been shown to be more resistant to penicillin than its planktonic counterparts, potentially due to the physical barrier and depth that the antibiotic has to traverse to reach the centre of the biofilm (Lappann *et al.*, 2006). Furthermore, aggregation in *N. gonorrhoeae* has also been linked to the upregulation of genes involved in antibiotic tolerance and the subsequent observed tolerance to ciprofloxacin (Wielert *et al.*, 2023; Kraus-Römer *et al.*, 2025). This physical barrier also translates to the creation of separate heterogenous physiochemical microenvironments within the biofilm with unique gradients of conditions (i.e. substrates, nutrients, pH) that make it more difficult for the host to specifically target due to the different resultant phenotypes, behaviours and metabolic responses of the bacteria (Steichen *et al.*, 2008; Neil & Apicella, 2009; Aliane & Meliani, 2021). Finally, biofilm formation aids in the physical attachment of pathogenic bacteria to their target host cells, which increases their chances of infection and invasion, and hence contributes to their pathogenicity and virulence (Vestby *et al.*, 2020).

2.3.5.1 Role of biofilms in *Neisseria* pathogenesis

The importance of biofilm formation in pathogenic *Neisseria* was initially demonstrated when some clinical strains of *N. meningitidis* were shown to form biofilms *in vitro* (~30% of carriage isolates and 12.5% of invasive disease isolates) which allowed them to be resistant against penicillin (Lappann *et al.*, 2006). The biofilm-forming abilities of *N. meningitidis in vivo* were then demonstrated in clinical samples of removed tonsillar tissue using immunolabelling, where microcolonies were formed just below the epithelial mucosal surfaces (Sim *et al.*, 2000). Initially, only unencapsulated strains of *N. meningitidis* were shown to form biofilms; however, some capsulated strains isolated from patient samples have since been shown to generate biofilms on airway epithelial cells *in vitro* (Yi *et al.*, 2004; Lappann *et al.*, 2006; Neil *et al.*, 2009; Lappann & Vogel, 2010). The finding that unencapsulated strains of *N. meningitidis* were more likely to form biofilms was further supported by transcriptomic analysis, which showed a downregulation of capsule synthesis genes during biofilm formation (O'Dwyer *et al.*, 2009). This is likely due to the strong repulsion between hydrophilic abiotic substrates and the hydrophobic cellular capsular surfaces which would not be conducive for initial attachment and biofilm formation in capsulated strains (O'Dwyer *et al.*, 2009). This is interesting, as capsulated *N. meningitidis* strains are typically more invasive and

often lead to disease; however, it was also shown that some meningococcal strains that do express capsule synthesis genes would later lose their capsulation via phase variation, potentially due to their ability to better associate and attach to surfaces without the hydrophobic capsular layer (Weber *et al.*, 2006). Hence, modulation of capsule expression and subsequent biofilm formation in *N. meningitidis* is reliant on environmental cues to ensure efficient attachment and colonisation before invasion into the host cells.

Similarly, biofilm formation by *N. gonorrhoeae* has also been demonstrated in both flow chambers and on primary urethral epithelial and cervical cells, where it can persist for up to eight days without substantial damage to the tissue (Greiner *et al.*, 2005). These biofilms were also observed in cervical biopsy samples from patients, highlighting the importance of biofilms in *N. gonorrhoeae* pathogenesis in women (Steichen *et al.*, 2008). *N. gonorrhoeae* has also been shown to colonise intrauterine contraceptive devices where it exists as a minor part of a mixed biofilm (~2%) with other organisms like *Staphylococcus aureus* and *Candida albicans* (Pruthi *et al.*, 2003). Interestingly, there is a lack of evidence for *N. gonorrhoeae* biofilm formation in male patients, which, as a majority of male infections are symptomatic, may indicate a role for biofilm formation to evade the host immune response during cases of gonococcal infections in women (Falsetta *et al.*, 2011). In fact, transcriptomic and proteomic profiling of gonococcal biofilms showed an upregulation of genes important for stress response and anaerobic respiration such as the nitric oxide reductase, *norB*, and the cytochrome c peroxidase, *ccp*, especially by cells in the biofilm base layers (Falsetta *et al.*, 2009; Phillips *et al.*, 2012; Kraus-Römer *et al.*, 2025). Disruption of these genes in *N. gonorrhoeae* negatively affected biofilm biomass and thickness, suggesting that anaerobic respiration occurs in *N. gonorrhoeae* biofilms (Falsetta *et al.*, 2009).

More specifically, during these oxygen-limiting conditions, *N. gonorrhoeae* uses partial denitrification to reduce nitrite to nitrous oxide, a pathway of which has also been demonstrated to occur in *N. meningitidis* (Rock & Moir, 2005; Falsetta *et al.*, 2009). Through this nitrite reduction in biofilms, *N. gonorrhoeae* is capable of breaking down any host-produced nitrous products, which subsequently dampens the host inflammatory and immune responses and results in asymptomatic infections that are extremely common in female patients (Falsetta *et al.*, 2009). The

formation of biofilms by *N. gonorrhoeae* as a means to survive against oxidative and disulfide stressors is further supported by the inability of mutants deficient in some redox-related genes such as the oxidative stress regulatory protein, *oxyR*, and the glutathione reductase, *gor*, to form biofilms (Seib *et al.*, 2007; Potter *et al.*, 2009; Kraus-Römer *et al.*, 2025).

2.3.5.2 Role of biofilms in commensal *Neisseria* survival and colonisation

Despite our increased understanding of the role of biofilms in pathogenic *Neisseria* spp., less is known about their prevalence and function in commensal *Neisseria*. Due to their similarities both in genes and habitat however (Wolff & Stern, 1995; Marri *et al.*, 2010; Baerentsen *et al.*, 2022), it is likely that commensal *Neisseria* are able to form biofilms in a similar way to their pathogenic counterparts. In fact, commensal and pathogenic *Neisseria* often form mixed biofilms together. For example, *N. lactamica* and *N. meningitidis* that coexist in the nasopharyngeal tract have been shown to interact together to form mixed-species biofilms (Pérez-Ortega *et al.*, 2017). Other evidence for biofilm formation in commensal *Neisseria* includes the formation of microcolonies of *N. cinerea* on human epithelial cells (Custodio *et al.*, 2020), as well as the formation of biofilms by *N. subflava* with a unique dispersal pattern that points in the direction of higher temperatures on petri dishes (Kaplan & Fine, 2002). Rather than having a primary role in invasion and infection however, it is more likely that these commensal *Neisseria* biofilms aid in the colonisation and survival against other competitors in their specific niche (e.g. physical competition between *N. meningitidis* and *N. lactamica*) and would thus be heavily influenced by their surrounding microbiota.

2.3.5.3 Structure and composition of *Neisseria* biofilms

The ECM makes up a large component of bacterial biofilms (~75-90%) compared to the cells themselves (~10-25%) (Rather *et al.*, 2021). In most bacteria, self-produced exopolysaccharide (EPS) is the primary ECM constituent (~50-90% of the biofilm) where they aid in cell-cell and cell-surface adhesion, as well as the protection of cells (Flemming *et al.*, 2000; Limoli *et al.*, 2015). The classic biofilm model organism, *P. aeruginosa* for example, produces three different types of EPS; Psl, Pel and alginate, the latter of which is often implicated in cystic fibrosis infections (Govan & Deretic, 1996; Friedman & Kolter, 2004).

Neisseria by contrast lack the genes necessary for EPS production (Greiner *et al.*, 2005). Instead, neisserial biofilms rely on outer membrane vesicles that pinch off the outer membrane which shape the matrix and stabilise the biofilm (Greiner *et al.*, 2005; Steichen *et al.*, 2008). Mutation of the *msbB* gene that leads to decreased membrane blebbing reduces the ability of *N. gonorrhoeae* to form biofilms in flow cells (Steichen *et al.*, 2008). The membranous biofilm matrix of *N. gonorrhoeae* also contains multiple water channels and pores that facilitate nutrient and substrate exchange (Greiner *et al.*, 2005). On the other hand, the structure of biofilms formed by *N. meningitidis* are very strain-dependent with some exhibiting very well-defined connection channels, while others seem to show no evidence of any defined connections (Arenas & Tommassen, 2017).

The most notable feature of all neisserial biofilms however, is the large quantity of exDNA present in the ECM which comprises significantly more than the 1-2% found in most other bacterial species.

2.3.6 Extracellular DNA as a component of biofilms

2.3.6.1 Extracellular DNA in other bacterial biofilms

Initially the exDNA observed in many bacterial biofilms was considered to be an artefact of processes like cell lysis, and it was not until the demonstration that early biofilms in *P. aeruginosa* were inhibited by the addition of a general nuclease, DNaseI, that the concept of an essential role for exDNA in biofilms gained traction (Whitchurch *et al.*, 2002; Flemming *et al.*, 2007; Sarkar, 2020). Further development of this notion – which included characterisations of the structure of mature *P. aeruginosa* biofilms (Klausen *et al.*, 2003; Allesen-Holm *et al.*, 2006) and a wider understanding of how patient cells contribute to sputum viscosity – gave rise to an aerosolised formulation of recombinant human DNaseI, which is now administered as a therapeutic Pulmozyme® (dornase alfa) for cystic fibrosis patients (Bakker & Tiddens, 2007; Konstan & Ratjen, 2012; Terlizzi *et al.*, 2022). The results obtained from the *P. aeruginosa* studies then prompted wider consideration for the role of exDNA in early biofilm formation of other bacteria including *Campylobacter jejuni*, *Haemophilus influenzae*, *Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus mutans*, with many reports demonstrating that DNase could either disperse early biofilms or prevent new

biofilms from forming (Table 2.2), suggesting an important role of exDNA during the initial steps of bacterial biofilm formation (Tang *et al.*, 2013; Schlafer *et al.*, 2017). The evidence for exDNA in the ECM is not restricted to singular-species biofilms however, as it has also been identified in the vicinity of cells that form mixed natural oral biofilms in dental plaques (Rostami *et al.*, 2017).

Table 2.2 Examples of other studies looking into the importance of extracellular DNA (exDNA) in the extracellular matrix of different bacterial biofilms. **(*) denotes bacteria with a genomically-encoded copy of the Lig E enzyme, which as described below, appears to act on exDNA in the biofilm.**

Organism	Medical implications of biofilm formation	Main findings/observations
Gram-negative bacteria		
Acinobacter baumanii	Pneumonia and catheter infections	- Addition of exDNA led to biofilm augmentation of up to 224.64%, while addition of DNaseI dispersed preformed biofilms by 59.41% (Sahu *et al.*, 2012)
Burkholderia pseudomallei	Melioidosis	- Positive correlation found between biofilm biomass and the amount of exDNA present. DNaseI significantly decreased attachment during early biofilm development (0-24 h-old), but not in mature biofilms (45 h-old) (Pakkulnan *et al.*, 2019). - Constant living:dead cell ratios throughout growth suggest exDNA likely sourced from living cells (Pakkulnan *et al.*, 2019).
Campylobacter jejuni *	Gastroenteritis	- Biofilms sensitive to DNaseI (Svensson *et al.*, 2009). - ExDNA-mediated biofilm formation induced in response to aerobic and starvation stress, which allowed the bacterium to persist for twice as long as that of planktonic cells (Feng *et al.*, 2016).

		- ExDNA closely associated with bacterial lysis (Feng *et al.*, 2018).
Haemophilus influenzae *	Otitis media, pneumonia	- DNaseI inhibited biofilm formation after 6 h, which was not observed when the biofilms were similarly treated with Proteinase K (Izano *et al.*, 2009)
Vibrio cholerae *	Cholera	- DNase disrupted 24 h-old biofilms, but not 72 h-old biofilms (Seper *et al.*, 2011).
Gram-positive bacteria		
Enterococcus faecalis	Endocarditis, enteritis	- DNaseI decreased adhesion of cells to dentin and increased biofilm susceptibility to chlorhexidine (Li *et al.*, 2012)
Mycobacterium avium hominissuis	Lung/respiratory tract infection	- ExDNA important for high biofilm-producing strains. DNaseI reduced the biomass of biofilms as it formed, as well as that of established biofilms on abiotic surfaces and on human pharyngeal epithelial cells (Rose *et al.*, 2015) - DNase-treated biofilms more susceptible to moxifloxacin and clarithromycin (Rose *et al.*, 2015) - Source of exDNA likely from cell lysis as random amplified polymorphic DNA (RAPD) analysis indicated likely genomic origins (Rose *et al.*, 2015)

Staphylococcus aureus	Forms on medical devices and chronic wound infections	- ExDNA is the most common component in most strains (Sugimoto *et al.*, 2018) and is important for intercellular adhesion polymer-independent biofilms (Izano *et al.*, 2008) - DNaseI inhibited new biofilm formation and detached preformed biofilms, which increased their sensitivity to detergents (Izano *et al.*, 2008) - ExDNA likely from lysed cells that release genomic DNA (Rice *et al.*, 2007)
Staphylococcus epidermidis	Forms on medical devices	- DNase affected early biofilms (6 h-old), but not older biofilms (12 h-old) (Qin *et al.*, 2007)
Streptococcus mutans	Dental caries and endocarditis	- ExDNA production in 5- and 24-h biofilm increased by >3 and >1.6-fold respectively compared to planktonic cells, and addition of DNaseI significantly reduced biofilm formation (Liao *et al.*, 2014) - ExDNA produced via lysis-independent membrane vesicles (Liao *et al.*, 2014)

Despite this, the organisation of exDNA in the ECM may differ, with some groups describing them as a 'fuzzy cloud' that accumulates around cells in a biofilm (Tang *et al.*, 2013). In *P. aeruginosa*, the exDNA arranges itself in a grid-like structure on the surface (Allesen-Holm *et al.*, 2006), while that of the non-typeable *H. influenzae* organises itself within a heavily interwoven mesh-like arrangement, which was later observed to form a cruciform-like structure with the arms of four double-stranded DNA joined together (Jurcisek & Bakaletz, 2007; Devaraj *et al.*, 2019). Localisation of exDNA onto filamentous structures seem to be a common organisational pattern, as observed in *E. faecalis* (Barnes *et al.*, 2012) and *S. epidermidis* (Zatorska *et al.*, 2017), as well as the nanofibre-like structures in *Streptococcus mutans* (Liao *et al.*, 2014). The clear organisational pattern of exDNA highlights its critical role in maintaining the structural integrity of the ECM in bacterial biofilms, potentially including those formed by *Neisseria* spp.

2.3.6.2 Extracellular DNA in pathogenic *Neisseria* biofilms

ExDNA has also been shown to be important in early biofilm formation for pathogenic clonal complexes of *N. meningitidis*, although less so for low prevalence or commensal strains (Lappann *et al.*, 2010). More specifically, meningococcal exDNA interacts with specific DNA binding proteins such as the heparin-binding protein, NhbA, and the α-peptide of the IgA protease located on the cell surface; these protein-DNA interactions are critical for maintaining *N. meningitidis* biofilms as protein-free DNA extracts were unable to restore *N. meningitidis* biofilms to the same extent in the laboratory (Lappann *et al.*, 2010; Arenas *et al.*, 2013; Arenas & Tommassen, 2017). In fact, it is hypothesised that exDNA may play a dual-role in the ECM at different stages of meningococcal biofilm formation; the first being the support of initial attachment of the bacteria to the substratum during early biofilm stages when the levels of exDNA are still relatively low, and the second being the maintenance of mechanical integrity during late stages when the levels of exDNA are higher (Lappann *et al.*, 2010).

As mentioned earlier, the importance of exDNA for *N. meningitidis* biofilm formation differs between strains; this difference has given rise to the 'settler/spreader' biofilm model where exDNA-independent clonal complexes, or 'spreaders', form unstable, fragile biofilms that are more sensitive to shear forces, but have higher transmission rates between different hosts due to their ease of

detachment (Lappann *et al.*, 2010; Pérez-Ortega *et al.*, 2017). Conversely, exDNA-dependent clonal complexes, or 'settlers', form stronger, more robust biofilms that are quickly established and allow for more stable interactions and long-term colonisations of the human host to occur (Lappann *et al.*, 2010). The combination of both 'settler' and 'spreader' phenotypes in a population allow *N. meningitidis* to effectively colonise and spread among different hosts over long periods of time, promoting its virulence and pathogenicity (Lappann & Vogel, 2010).

Similar to the experiments with *P. aeruginosa*, DNase-inhibition of *N. gonorrhoeae* biofilm formation was crucial in establishing exDNA as a major component of gonococcal biofilms (Falsetta *et al.*, 2011; Steichen *et al.*, 2011). In fact, single-stranded DNA is important for initial biofilm formation in *N. gonorrhoeae*, while double-stranded DNA makes up the majority of exDNA in mature gonococcal biofilms (Zweig *et al.*, 2014). Furthermore, work by the Maier group showed that the length of exDNA governs its mobility within gonococcal colonies and hence its subsequent retention and incorporation into the ECM (Bender *et al.*, 2022). This, coupled with the lack of EPS-encoding genes greatly support a major role of exDNA in *N. gonorrhoeae* biofilms.

2.3.6.3 Extracellular DNA in commensal *Neisseria* biofilms

Considering the scarcity of reports on biofilm formation of commensal *Neisseria* spp., it is not surprising that little is known about the presence of exDNA in their ECM. Nevertheless, as both pathogenic and commensal species occupy similar habitats and share high genetic similarities (including the lack of genes necessary to produce EPS) we predict that exDNA plays an equivalent role in commensal *Neisseria* biofilms as well (Wolff & Stern, 1995; Marri *et al.*, 2010; Baerentsen *et al.*, 2022). Recently, Shaikh and colleagues have shown that the synthetic drug molecule Ebselen is capable of dispersing *N. mucosa* biofilms by degrading pieces of exDNA and attenuating their quorum sensing-induced urease and protease activities against host proteins (Shaikh *et al.*, 2022). Considering the mixed-species biofilms formed between *N. meningitidis* and *N. lactamica*, we predict that the composition of their biofilms are likely very similar. More specifically, due to their spatial proximity, they are likely to share the same ECM as they release exDNA and other materials into the same extracellular environment in the same location, benefiting both species equally. However, to our knowledge, this has not been

verified yet. We are hopeful that this gap will be filled soon as more evidence of the importance of commensal *Neisseria* spp. comes to light.

2.3.7 Potential functions of extracellular DNA in *Neisseria* biofilms

2.3.7.1 Adhesion, structure, stability and antimicrobial protection

One of the ways that exDNA plays a critical role in the establishment of neisserial biofilms is by bridging and overcoming the weak repulsive interactions that exist between the bacteria and the surfaces of interest. More specifically, attachment of cells to a surface involves both attractive long-range Liftshitz-van der Waal interactions and weak repulsive interactions that arise from the mutual net negative charges that exist on most cellular and abiotic surfaces (Okshevsky & Meyer, 2015; Maier, 2021). Despite this, exDNA is able to facilitate closer range interactions by adsorbing to and extending out of cellular surfaces in long loop structures (Figure 2.10). These structures are then able to interact with any acidic (electron-accepting) or basic (electron-donating) groups via close-range acid-base interactions (Lappann *et al.*, 2010; Das *et al.*, 2011a; Das *et al.*, 2011b; Okshevsky & Meyer, 2015). Not surprisingly, the strengths and distances of the initial long-range repulsive forces determine the potentiality of these short-range acid-base interactions that occur between neighbouring *Neisseria* cells. Hence, the higher the molecular weight of exDNA that extends out, the more likely it is able to penetrate the repulsive barrier and allow for positive interactions to occur between neighbouring cells and surfaces (Figure 2.10) (Regina *et al.*, 2014; Okshevsky & Meyer, 2015). This initial bacterial attachment forms the monolayer that the *Neisseria* biofilm grows from.

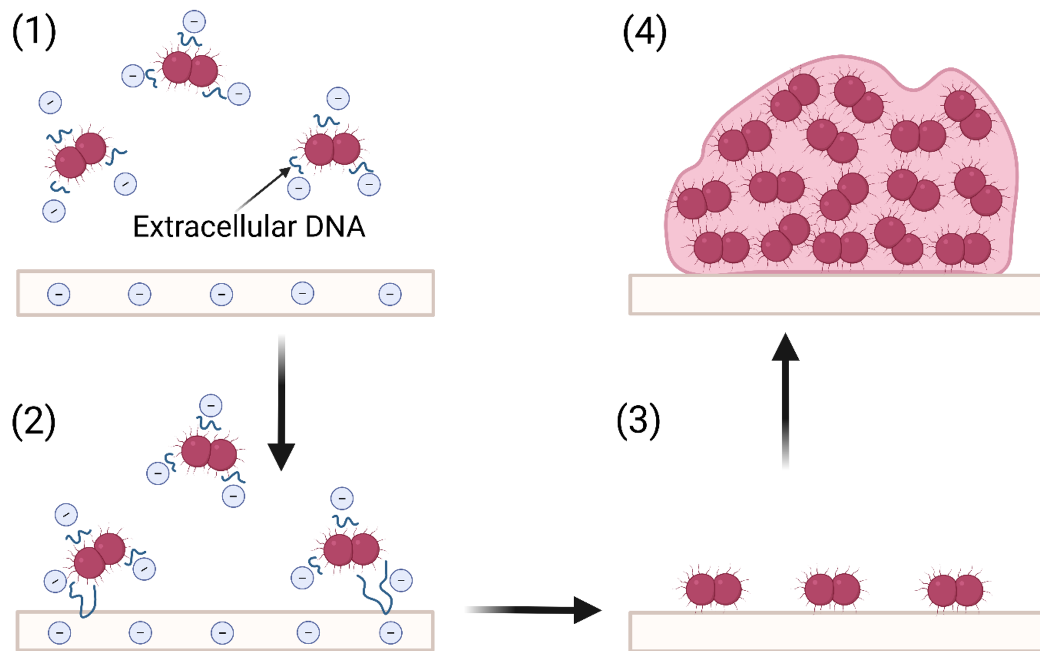

Figure 2.10 Role of extracellular DNA in bridging repulsive forces during biofilm formation initiation in *Neisseria*. (1) Weak repulsive forces exist between the bacteria and surface of interest. (2) High molecular weight extracellular DNA extends out and bridges the repulsive forces, facilitating close-range interactions between the bacteria and surface. (3) Reversible formation of a monolayer of cells on the surface of interest. (4) Cell replication and extracellular matrix production with extracellular DNA (not shown) leads to the formation of mature, multilayered three-dimensional biofilms. Created in https://BioRender.com.

Perhaps the best recognised role of exDNA in biofilms however, is its role in maintaining the structure and stability of the biofilm matrix. After the formation of the monolayer on surfaces, the division of *Neisseria* cells is accompanied by different interactions between neighbouring cells that allow them to be maintained as three-dimensional microcolonies. Here, the negative exDNA likely acts as an adhesive net by interacting electrostatically with any positively-charged surface-exposed matrix proteins of neighbouring cells (Dengler *et al.*, 2015; Arenas & Tommassen, 2017; Serrage *et al.*, 2021). Interestingly, exDNA also contributes to the organisation and motility of cells in other bacterial biofilms like *P. aeruginosa* by organising furrows or pathways for coordinated bacterial migration to occur (Gloag *et al.*, 2013). However, no direct link between *Neisseria* mobility in biofilms and exDNA has been elucidated as of yet.

It has been well established that biofilms physically hinder diffusion of antimicrobial agents into the centre of bacterial colonies. However, the negatively-charged exDNA may also contribute to antimicrobial resistance itself by chelating

positively-charged antibiotics and antimicrobial peptides produced by the host immune system, or by binding critical cations and acidifying the ECM, which in turn induces the expression of different genes that offer protection against these agents (Mulcahy *et al.*, 2008; Johnson *et al.*, 2013; Wilton *et al.*, 2016). Although this chelation has not been directly proven in *Neisseria* spp. yet, this resistance has been observed in other bacteria that form exDNA-dependent biofilms like non-typable *Haemophilus influenzae* against human β-defensin-3, ampicillin and ciprofloxacin (Jones *et al.*, 2013; Cavaliere *et al.*, 2014), as well as the restoration of aminoglycoside resistance by the addition of exogenous DNA in *P. aeruginosa* (Mulcahy *et al.*, 2008; Chiang *et al.*, 2013).

2.3.7.2 A pool of DNA for nutrients, DNA repair and recombination into the genome

The large quantity of exDNA in *Neisseria* biofilms provides a pool of available DNA that may be used for other processes that include the three main theories for the role of competence in bacteria; 'DNA for food', 'DNA for genome repair' and 'DNA for evolution'. DNA represents a significant source of phosphate, nitrogen and carbon, and recycling exDNA decreases the energy requirements for *de novo* nucleotide synthesis under stressful conditions (Redfield, 2001; Jakubovics & Burgess, 2015). Although not yet observed in *Neisseria* spp., the recent discovery that exDNA is produced during biofilm establishment in *Bacillus subtilis* before its global degradation in a spatiotemporally-coordinated pulse has led to the conclusion that exDNA acts as a 'metabolic reservoir' or 'food' that can be used when nutrient availability decreases in later stages (Lander *et al.*, 2024). Alternatively, it is possible that competent *Neisseria* may incorporate these exDNA for genomic DNA repair processes. This theory is supported by the observation that only exDNA with a specific DNA uptake sequence is internalised by *Neisseria* spp., guaranteeing that only closely-related DNA suitable for homologous recombination and repair is internalised (Seitz & Blokesch, 2013). Finally, exDNA in *Neisseria* biofilms offers potential for acquisition of novel genes from neighbouring cells including new antibiotic resistance genes that may increase the overall fitness and survival of the colony (Veening & Blokesch, 2017). Considering the natural competence of *Neisseria* at all stages of growth, the use of exDNA for repair and horizontal gene acquisition is highly likely.

Recent studies using fluorescently-labelled DNA suggest an interplay between genetic transformation and biofilm formation in *N. gonorrhoeae* based on the length of exDNA present (Bender *et al.*, 2022). Here, small fragments of DNA of around 300 bp are able to diffuse freely through *N. gonorrhoeae* colonies and will likely be taken up by the cells, whereas those exceeding 3000 bp face hindered diffusion and are more likely to be slowly incorporated into the ECM of biofilms (Kouzel *et al.*, 2015; Bender *et al.*, 2022). This is supported by the observation that DNA transformation and acquisition of multi-drug resistance in *N. gonorrhoeae* is highly efficient during early biofilm formation (<24 h), which is where exDNA is predicted to contribute to *Neisseria* biofilm formation the most, but was strongly reduced after 24 h, independent of biofilm density (Kouzel *et al.*, 2015). This feature is not specific to *Neisseria* however, as higher transformation or conjugation rates have also been observed in *Escherichia coli* biofilms compared to planktonic cells (Hausner & Wuertz, 1999), in mixed biofilms from dental plaque (Roberts *et al.*, 1999), and in *S. mutans* upon activation of proteins involved in competence (Li *et al.*, 2002; Petersen *et al.*, 2004; Nagasawa *et al.*, 2020). Taken together, these observations indicate that biofilms are highly important at facilitating horizontal gene transfer in general (Okshevsky & Meyer, 2015).

2.3.8 Sources of extracellular DNA in *Neisseria* biofilms

2.3.8.1 Cell lysis and release of genomic DNA

Perhaps the most widely-recognised source of exDNA in *Neisseria* biofilms is genomic DNA released through autolysis (Figure 2.11) (Hebeler & Young, 1975; Elmros *et al.*, 1976; Rouphael & Stephens, 2012). In *N. gonorrhoeae*, autolysis is associated with a prolonged cell division process and is most common when growth stops due to a depletion of energy or environmental stressors such as low pH and temperature which negatively affects mechanical stability (Hebeler & Young, 1975; Elmros *et al.*, 1976). On the other hand, *N. meningitidis* undergoes autolysis to a lesser extent which predominantly occurs in pathogenic meningococcal strains and may be more tuned for endotoxin release and other virulence phenotypes (Elmros *et al.*, 1976; Lappann *et al.*, 2010; Rouphael & Stephens, 2012). These pathogenic *N. meningitidis* clonal complexes utilise a cytoplasmic N-acetylmuramyl-L-alanine-amidase, AmiC, and a lytic transglycosylase, LtgA, to hydrolyse the cell wall for exDNA release during early biofilm formation, as well as the autolytic

activity of the outer membrane phospholipase A to release exDNA that work against mechanical forces in mature biofilms (Lappann *et al.*, 2010; Lappann & Vogel, 2010; Sigurlásdóttir *et al.*, 2019).

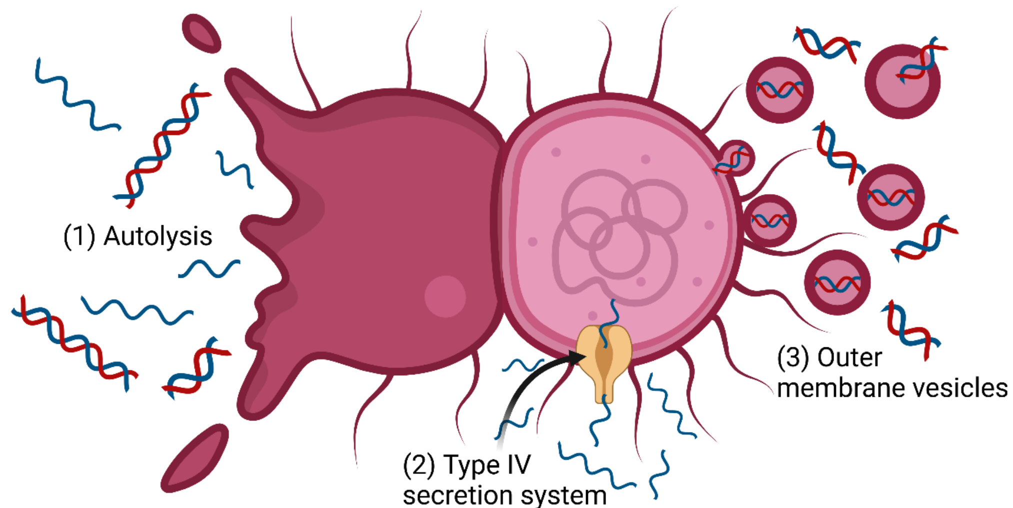

Figure 2.11 Potential sources of self-derived extracellular DNA in *Neisseria* biofilms. (1) Autolysis releasing genomic DNA. (2) Secretion of single-stranded DNA via the Type IV secretion system. (3) Release of double-stranded DNA via outer membrane vesicles. Created in https://BioRender.com.

On the other hand, commensal *Neisseria* exhibit autolysis to a lesser extent, where it likely contributes more to the recycling of nutrients for the benefit of their survival. While very early work on *Neisseria pharyngis* (now reclassified as *N. sicca/N. mutans*) did not detect autolysis (Elmros *et al.*, 1976), more recent findings hint at the ability of commensal *Neisseria* to undergo similar autolytic processes to pathogenic species. For example, all commensal *Neisseria* have orthologs of AmiC and LtgA implicated in meningococcal autolysis (Garcia & Dillard, 2006; Kohler *et al.*, 2007; Rouphael & Stephens, 2012). Similarly, the discovery that *N. elongata* is capable of killing both *N. meningitidis* and *N. gonorrhoeae* in a DNA-dependent manner based on DNA uptake and DNA methylation meant that the release mechanism of the 'killer' DNA is likely autolysis based on its genomic composition (Kim *et al.*, 2019). Regardless, autolysis seems to be a method by which all *Neisseria* spp. can use to release exDNA, which positively contributes to the maintenance of their biofilms.

2.3.8.2 Active DNA secretion

Another method for DNA release into the ECM is active secretion, which may have evolved as a source of exDNA release that does not trigger the host immune system to the same extent as autolysis (Salgado-Pabón *et al.*, 2007). The best characterised example of this can be observed in *N. gonorrhoeae*, which secretes single-stranded chromosomal DNA directly into the extracellular space using its Type IV secretion system (T4SS) (Figure 2.11) (Dillard & Seifert, 2001; Hamilton *et al.*, 2005). This complex is encoded on a 57 kb gonococcal genetic island (GGI) and was the first genetic locus responsible for DNA secretion to be characterised (Dillard & Seifert, 2001; Hamilton *et al.*, 2005). The single-stranded DNA released via the T4SS complex is heavily methylated and has its 5' end blocked, meaning that only its 3' end is susceptible to degradation by single-stranded DNA-specific nucleases (Hamilton *et al.*, 2005; Salgado-Pabón *et al.*, 2007). Although shown to be important during early biofilm formation, this single-stranded DNA is not retained in mature gonococcal biofilms (Hamilton *et al.*, 2005; Salgado-Pabón *et al.*, 2007; Zweig *et al.*, 2014). Two proteins have been identified to be important for *N. gonorrhoeae* T4SS exDNA release; the lytic transglycosylase, AtlA, which is likely involved in hydrolysing the peptidoglycan layer without inducing lysis, and the partitioning protein, ParB, which likely guides the DNA-to-be-secreted to the T4SS complex, both of which are also encoded on the GGI (Dillard & Seifert, 2001; Kohler *et al.*, 2007; Ramsey *et al.*, 2011).

The GGI encoding the T4SS is integrated into the chromosomal replication terminus of the gonococcal chromosome, with half of it encoding genes related to the T4SS machinery, while the other half encode hypothetical proteins, some of which are DNA-binding or DNA-processing related (Callaghan *et al.*, 2017). Interestingly only ~ 80% of *N. gonorrhoeae* clinical isolates carry the GGI (Callaghan *et al.*, 2017). A notable exception is FA1090, a commonly used *N. gonorrhoeae* strain in the laboratory, which lacks this GGI but is still capable of forming biofilms (Greiner *et al.*, 2005; Zola *et al.*, 2010). It would thus be interesting to study the ratio of single- and double-stranded DNA in GGI-lacking gonococci such as FA1090 to elucidate how these strains release or obtain single-stranded DNA that is typically used to initiate biofilm formation, or whether double-stranded DNA acts as a substitute instead. Perhaps this active secretion has evolved to compensate for the low amount of cell lysis that occurs during early

growth and biofilm formation and hence is not required at later stages due to the high frequency of autolysis, or it may be unnecessary in strains that undergo high rates of autolysis at all stages of growth (Zweig *et al.*, 2014). It would also be interesting to compare the thickness and stability of the biofilms formed between these GGI-containing and GGI-lacking strains of *N. gonorrhoeae* to detect any differences in the formation rates or biofilm morphologies, or the resultant pathogenicities of both types of gonococci and their correlation to asymptomatic gonorrhoea infections.

In contrast, only ~18 % of *N. meningitidis* clinical isolates carry the GGI and where this is present, genes encoding the T4SS are disrupted by insertions and deletions which likely renders it non-functional and unlikely to be the source of exDNA (Woodhams *et al.*, 2012). Although the GGI has also been found in the genome of the commensal *Neisseria bacilliformis*, none of the 15 commensal *Neisseria* spp. (including *N. cinerea*, *N. lactamica* and *N. mucosa*), encode the *atlA* or *parB* genes needed for single-stranded DNA release (Pachulec *et al.*, 2014). Therefore it appears that the wide-spread single-stranded DNA secretion is specific to *N. gonorrhoeae* as an adaptation to its niche in the reproductive tract, and not a common feature of other *Neisseria* spp. (Dillard & Seifert, 2001).

2.3.8.3 Outer membrane vesicles

Extracellular DNA in *Neisseria* spp. may also be exported via outer membrane vesicles, a process closely associated with active secretion, albeit less tightly regulated (Figure 2.11) (Dorward & Garon, 1990). These outer membrane vesicles or 'outer membrane blebs' were found to harbour large quantities of DNA and DNA-binding proteins in *N. gonorrhoeae* biofilms (Dorward & Garon, 1989; Dorward *et al.*, 1989). Outer membrane vesicles are also abundant in *N. meningitidis* and other commensal *Neisseria* like *N. cinerea*, suggesting a universal mode of exDNA transport across the *Neisseria* genus. (Lappann *et al.*, 2013b; Piliou *et al.*, 2023). This mode of transport has been observed in a variety of other bacteria as well including *A. baumannii*, *H. pylori* and *P. aeruginosa*, the latter of which seems to package its vesicles during the exponential phase of growth (Renelli *et al.*, 2004; Sahu *et al.*, 2012; Grande *et al.*, 2015). Investigations into the nature of DNA associated with these vesicles in other bacteria show strong similarity to genomic DNA with high scattering around the ECM and arrangement in a structured network

(Liao *et al.*, 2014; Xu *et al.*, 2022). The packaging of DNA into these vesicles is proposed to occur either as a spontaneous side-effect or consequence of cell lysis, or as a result of cell division (Marinacci *et al.*, 2023). Questions into the type of DNA transported in these outer membrane vesicles and the stage of growth they are activated are still outstanding for members of the genus *Neisseria*.

2.3.8.4 Other potential sources or interplay of extracellular DNA release

There are perhaps many other ways for exDNA to be released into the ECM that are unexplored. Considering the pathogenic nature of both *N. meningitidis* and *N. gonorrhoeae*, it is possible that some of the exDNA in their biofilm matrix originates from the human host. In particular, neutrophil extracellular traps (NETs) released by neutrophils as a defence mechanism consist of fibres or strings of DNA or chromatin, as well as proteins that are capable of binding and engulfing the targeted pathogens (Brinkmann *et al.*, 2004). It is possible that the release of DNA from these NETs may also contribute to *Neisseria* biofilm exDNA, as has been implicated in other infections like *P. aeruginosa*-related cystic fibrosis (Lethem *et al.*, 1990; Vitkov *et al.*, 2009; Chiang *et al.*, 2013). Although lacking evidence in publications, we predict that this is also a likely source of exDNA for *Neisseria* biofilms, especially as both *N. meningitidis* and *N. gonorrhoeae* are able to induce the formation of and interact with NETs via nucleases that degrade them and potentially release their DNA into the environment (Lappann *et al.*, 2013a; Juneau *et al.*, 2015).

Furthermore, multiple mechanisms of exDNA release may occur in a single *Neisseria* species at a given time, which may depend on the environmental conditions or the metabolic activity of the subpopulation. For example, cells inside a neisserial colony that are metabolically inactive may undergo cell lysis, whereas those at the surface may display the more structured outer membrane vesicle-mediated release (Sarkar, 2020). Alternatively, there may be an interplay between quorum sensing-dependant and quorum sensing-independent mechanisms of exDNA release, with the quorum sensing-independent mechanism being responsible for maintaining a basal level of DNA in the extracellular milieu (Das *et al.*, 2013). Many factors would obviously be at play, and it is clear that this is an important area of research to pinpoint the exact sources of exDNA that contribute to *Neisseria* biofilm formation during specific conditions of growth.

2.3.9 Proteins important for extracellular DNA-mediated biofilms in *Neisseria* spp.

2.3.9.1 Type IV pilus

The Type IV pilus (T4P) is a surface-exposed structure that exhibits dynamic twitching mobility which aids with the motility, adhesion, competence, microcolony formation, protein secretion and virulence of many bacteria (Strom & Lory, 1993; Craig & Li, 2008). In *Neisseria*, this retractable T4P is involved in DNA uptake and provides a channel for DNA to cross the cell membrane (Muschiol *et al.*, 2015). In addition, the T4P aids in biofilm formation by facilitating cell-to-surface interactions which are essential for biofilm initiation, as well as enabling cell-cell interactions which promote cellular aggregation and microcolony formation (Pohlschroder & Esquivel, 2015). This T4P-reliant cell-cell contact may either be explained by a cell attachment model, where the T4P binds with surface proteins of other cells to form a web that prevents individual cells from moving independently, or a pilus binding model, where individual T4P from different cells interact directly with each other to form bundles that inherently tie them together (Kirn *et al.*, 2000; Piepenbrink & Sundberg, 2016; Maier, 2021; Ellison *et al.*, 2022). In both models, the T4P interactions control the rate, timing, number and location of cell-cell interactions that occur and are conducive to the eventual formation of multicellular three-dimensional biofilms.

Post-translational modifications of the T4P in *N. gonorrhoeae* via phase and antigenic variation may also affect the rupture forces necessary to interrupt cell-cell interactions (Oldewurtel *et al.*, 2015; Zöllner *et al.*, 2017; Maier, 2021). This in turn triggers bacterial cell sorting or spatial reorganisation into clusters in early biofilms, as cells tend to move in the direction of the highest rupture force where they can be pulled the strongest (Oldewurtel *et al.*, 2015; Maier, 2021; Ronish *et al.*, 2022). The differential attraction, pulling, contact and retracting active forces generated by the heterogenous gene expression or modification of the T4P are responsible for generating a mechanical force which spatially sorts the *Neisseria* cells, leading to the defined architecture of the mixed microcolony (Bonazzi *et al.*, 2018; Pönisch *et al.*, 2018; dos Santos Souza *et al.*, 2020; Maier, 2021). In fact, non-piliated *N. gonorrhoeae* with a low rupture force tend to move toward the expanding front or the boundary of an expanding colony, where it would have a stronger competitive advantage (i.e. optimal space and nutrient availability), as well as a higher chance

of dispersal to a new biofilm attachment site (Oldewurtel *et al.*, 2015; Zöllner *et al.*, 2017; Maier, 2021). Over time, the cells in a specific region of the biofilm will start to interact with neighbouring cells with differential pulling and subsequent sorting depending on their surface structures, and hence move in a direction where the rupture force is the highest (Oldewurtel *et al.*, 2015).

Similarly, microcolony formation in *N. meningitidis* has been shown to be reliant on the T4P (PilX)-mediated twitching motility, which if disrupted, leads to flat, unstructured biofilms that are less viscous and more solid-like, and unable to colonise vascular networks as efficiently (Winther-Larsen *et al.*, 2001; Yi *et al.*, 2004; Lappann *et al.*, 2006; Lappann & Vogel, 2010). It is of note however, that the role of T4P in meningococcal biofilms is not directly related to its organisation, and that those that lack this twitching motion are still able to form biofilms. Rather, it is the microcolony formation and bacterial aggregation and hence formation of a three-dimensional architecture that will be impacted (Yi *et al.*, 2004; Lappann *et al.*, 2006). Furthermore, it has been elegantly demonstrated by manipulation of the mean number of exposed pili, that different levels of piliation are required for different processes in *N. meningitidis*, with DNA uptake only requiring ~20% piliation (i.e. ~1 pilus per cell), other cellular processes like endothelial plasma cell membrane reorganisation requiring >70% piliation (i.e. 3-5 pili per cell), and aggregation and adhesion for biofilm formation requiring >40% piliation (i.e. 2 pili per cell) (Imhaus & Duménil, 2014), stressing the importance of the T4P for the structure and organisation of *Neisseria* biofilms.

2.3.9.2 Extracellular thermonucleases

Another group of proteins that have been implicated in many bacterial biofilms are extracellular thermonucleases, which are enzymes that degrade DNA and may in turn modulate the structure of these exDNA-dependent biofilms. These nucleases have been identified in a variety of bacteria including *S. aureus*, *V. cholerae* and *B. subtilis* where its disruption often leads to the formation of thicker biofilms with higher levels of matrix-associated higher molecular weight exDNA, as well as larger cell aggregates with lower rates of detachment (Tang *et al.*, 2008; Lauderdale *et al.*, 2009; Mann *et al.*, 2009; Kiedrowski *et al.*, 2011; Seper *et al.*, 2011; Tang *et al.*, 2011; Kaplan & Horswill, 2024; Lander *et al.*, 2024). These extracellular nucleases appear to possess a dual-role in remodelling the three-dimensional

structure of biofilms via exDNA manipulation, as well as the dispersal and detachment of mature biofilms, leading to the availability of exDNA for other processes like nutrient recycling.

A similar nuclease homologous to the secreted thermonuclease from *S. aureus* has been identified and implicated in the development of *N. gonorrhoeae* biofilms (Steichen *et al.*, 2011). Deletion of this thermonuclease, Nuc, led to the formation of significantly thicker and larger gonococcal biofilm biomass, which was directly attributed to an increased level of exDNA via microscopy (Steichen *et al.*, 2011). It is suggested that this thermonuclease may be working on exDNA in the periplasm before its release into the ECM as a method to control DNA release, rather than to degrade exDNA already present in the ECM itself (Steichen *et al.*, 2011). Apart from directly digesting and interacting with exDNA, this Nuc thermonuclease is also capable of degrading NETs in *N. gonorrhoeae*, which then leads to NET DNA release into the ECM, potentially balancing its negative implications on biofilm formation via exDNA digestion (Juneau *et al.*, 2015). Although little is known about the presence of external nucleases in other *Neisseria* spp., preliminary analysis by our group shows that a similar nuclease is present in the genome of *N. meningitidis* and many commensal *Neisseria* like *N. lactamica* and *N. cinerea*, highlighting its potential role in biofilm formation across the *Neisseria* genus.

2.3.9.3 Other DNA-interacting proteins

The observation that crude chromosomal DNA, but not purified protein-free DNA, can promote biofilm formation in *N. meningitidis* and other bacteria strongly suggests that other DNA-interacting proteins also play essential roles in biofilm formation (Harmsen *et al.*, 2010; Lappann *et al.*, 2010). For example, in *N. meningitidis*, the DNA-binding lipoprotein antigen, NhbA, and the IgA protease appear to be involved in exDNA-mediated biofilm formation (Arenas *et al.*, 2013). These proteins are expressed on the surface of cells and are cleaved by the autotransporter protease, NalP, which releases positively-charged peptides into the matrix that limits exDNA-binding and exDNA-dependent biofilms from forming (Arenas *et al.*, 2013). Furthermore, the autotransporter A protein, AutA, is capable of both self-interaction and binding to DNA which promotes subsequent aggregation in *N. meningitidis* (Arenas *et al.*, 2015). However, it is worth noting that this gene is disrupted in ~75% of *N. meningitidis* strains, and that the reliance

on exDNA binding for meningococcal auto-aggregation is strain-dependent (Arenas *et al.*, 2015).

In addition to this, our group has recently shown that the disruption of the ATP-dependent DNA ligase Lig E in *N. gonorrhoeae* reduces biofilm formation, providing further evidence that DNA, the substrate for DNA ligases, is important for biofilm formation (Pan *et al.*, 2024). Lig E is also found in many other bacteria where exDNA seems to play a central role in biofilm formation including *C. jejuni*, *H. influenzae*, *N. meningitidis* and *V. cholerae* (Pan *et al.*, 2021). Considering the presence of an extracellular localisation signal peptide on the N-terminus of Lig E, we hypothesise that this ligase works to enhance the amount of high molecular weight exDNA, potentially counteracting the effects of the thermonuclease. As exDNA is also predicted to be situated on the lattice of membrane proteins in the outer membrane blebs of *N. gonorrhoeae* (Dorward & Garon, 1989; Phillips *et al.*, 2012), we further postulate that Lig E may be working on exDNA in the periplasm or in the membranous blebs to remodel it and ensure the integrity of the biofilm. In fact, we believe that the ligase Lig E works in favour of the aforementioned concentration gradient of exDNA in the interplay between genetic transformation and biofilm formation in *N. gonorrhoeae* (Kouzel *et al.*, 2015; Bender *et al.*, 2022), increasing the lengths of fragmented pieces of exDNA, which would be more likely to be incorporated into the biofilm.

In addition to this, a number of other DNA-interacting proteins that have a role in biofilm formation in other bacteria have been identified (Table 2.3). We postulate that it is worth investigating and searching for homologues or proteins with similar functions in different species of *Neisseria* to gain a better understanding of other exDNA-protein interactions that promote *Neisseria* biofilm formation.

Table 2.3 List of some examples of DNA-interacting proteins identified from other bacteria that have been implicated in biofilm formation.

Protein	Bacteria and reference	Summary
β-toxin enzyme	*Staphylococcus aureus* (Huseby *et al.*, 2010)	- Forms covalent self-cross-links by binding exDNA and forms insoluble nucleoprotein complexes via its "DNA biofilm ligase activity" - Resultant conformation change increases resistance to detergents and allows structuring of exDNA for stable biofilm matrix formation
Amyloids	*Staphylococcus aureus* (Schwartz & Boles, 2013; Schwartz *et al.*, 2016)	- Protein aggregates that arise due to protein misfolding, implicated in biofilm formation - Made up of small phenol soluble modulin peptides that are cationic and capable of interacting with exDNA, leading to their polymerisation, and acquisition of a protease-resistant biofilm matrix
DNABII family of DNA-binding proteins; integration host factor (IHF) and the histone-like protein (HU)	*Escherichia coli* (Goodman *et al.*, 2011) *Haemophilus influenzae* (Brandstetter *et al.*, 2013; Jurcisek *et al.*, 2017)	- Ubiquitous among all eubacteria, disruption rapidly disrupts biofilm matrix - Stabilises DNA by binding at the vertices of crossed exDNA strands in a Holiday junction-like configuration - Bends DNA upon binding, with higher binding affinities with bent DNA over unbent DNA, stabilising the three-dimensional structure of the biofilm (Goodman & Bakaletz, 2022) - Option for targeting to disrupt biofilms in cystic fibrosis patients (Gustave *et al.*, 2013)

GAPDH and enolase	*Staphylococcus aureus* (Foulston *et al.*, 2014; Dengler *et al.*, 2015)	- Predicted to be released via autolysis during the stationary phase - When pH is low, they acquire a net positive charge, allowing them to interact strongly with the negatively-charged DNA and protecting them from matrix-degrading enzymes - 'Moonlighting' effect in biofilms
Lipoproteins i.e SaeP	*Staphylococcus aureus* (Kavanaugh *et al.*, 2019)	- Leads to increased retention of high molecular weight exDNA on cellular surfaces and subsequent enhancement of exDNA-dependent biofilms, as well as reduced nuclease production - Believed to stabilise the negatively charged DNA via their positive charges in the cytoplasm before export to allow for favourable electrostatic interaction to occur with negatively charged surfaces - Deletion has minimal impacts on biofilm formation itself, but increases biofilm porosities

2.3.10 Therapeutic implications of extracellular DNA in *Neisseria* biofilms

It is now clear that many human pathogens form DNA-rich biofilms as a way to survive and invade the human host. This makes exDNA an appealing target to disperse these biofilms via DNase enzymes that have compatible temperature optima to bacterial growth conditions (Lauková *et al.*, 2020). This approach has been successful as the aforementioned Pulmozyme® (dornase alfa) treatment for cystic fibrosis patients and may be applicable to other bacterial pathogens and associated diseases (Fuchs *et al.*, 1994; Konstan & Ratjen, 2012; Fisher *et al.*, 2021; Terlizzi *et al.*, 2022). The use of DNase to disperse young *Neisseria* biofilms, in conjunction with antibiotics, may be an alternative therapeutic strategy against pathogenic *Neisseria* that are becoming increasingly resistant to new antibiotic therapies. However, DNase-dependent therapies come with a few limitations including their relatively short half-life (~3-4 h) and potential inhibition by proteases *in vivo* or action against the host DNA (Stamm *et al.*, 2024).

Alternatively, it may be possible to target other proteins or matrix components essential to the neisserial biofilm, or the DNA secretion process itself (i.e. the T4SS secretion system). Much attention has been placed on targeting the exDNA-binding integration host factor (IHF) (Table 2.3) in other bacteria, which often leads to the collapse of the biofilm and the release of individual bacteria that are more susceptible to antimicrobial agents (Goodman *et al.*, 2011; Novotny *et al.*, 2013; Brockson *et al.*, 2014). In contrast to the efficacy of DNase only in early biofilms, anti-DNABII disruption was shown to be effective for mature biofilms that were up to two weeks old (Brockson *et al.*, 2014; Goodman & Bakaletz, 2022), with limited off-target effects in humans (Rogers *et al.*, 2022). Phase 2a trials of the monoclonal antibody CMTX-101 against members of the DNABII family began in late 2024 for cystic fibrosis patients, with results expected in 2025 (Rogers *et al.*, 2022). It has to be considered however, that these proteins are a key component of normal human gut microbiota biofilms that may also be affected if used as a drug therapy, and hence may affect commensal *Neisseria* if used as a treatment against their pathogenic counterparts.

Further difficulties arise in choosing the delivery mechanisms of these potential therapeutics against *Neisseria* biofilms. As highlighted by Jakubovics & Burgess (2015), various aspects need to be considered including: a) their safety in humans, b) the compatibility and stability of the enzyme with the delivery system components, c) the ability of the delivery system to reach the biofilm of interest, d) the ability of the enzyme to be activated when in contact with the biofilm, and e) the overall stability of the delivery mechanism. Although the delivery of Pulmozyme® (dornase alfa) via aerosols provides a direct route to the lungs and hence clearance of the biofilms, this is not applicable for other infections like *N. gonorrhoeae* which colonises the reproductive tract, or other infections in hard to reach areas like *N. meningitis* past the blood-brain barrier. Current delivery options of DNaseI include surface coatings (Swartjes *et al.*, 2013), intravenous infusions of DNase1-coated melanin-like nanospheres (Park *et al.*, 2020), as well as neutrophil-mediated delivery to sites of inflammation which would have reduced immune response (Chu *et al.*, 2015). Cost is another issue as mammalian DNase requires glycosylation after translation and hence require mammalian cells for production (Okshevsky *et al.*, 2015). A cheaper option may be the use of bacterial nucleases that can be expressed in prokaryotes, although care has to be taken to minimise eliciting an immune response in the human host against a foreign protein (Okshevsky *et al.*, 2015).

The targeting of DNA in biofilms may have important implications in many areas where biofilms play an important role including treatment of dental plaques, prevention of biofilms on medical devices or even in environmental applications. It is tempting to say that these techniques are easily applicable to *Neisseria* spp. However, variability amongst different bacterial biofilms and exDNA release creates complications and the need to tailor the strategies to *Neisseria* specifically (Peterson *et al.*, 2013; Okshevsky *et al.*, 2015). Further investigations into commensal *Neisseria* biofilm and their reliance on exDNA would also open more pathways on targeting pathogenic *Neisseria* by potentially utilising commensal *Neisseria* exDNA as a therapeutic strategy against *N. gonorrhoeae* and *N. meningitidis* instead.

2.3.11 Conclusions

The discovery of the importance of exDNA in *N. meningitidis* and *N. gonorrhoeae* that arise from a variety of sources including cell lysis and active secretion have opened up new avenues for targeting these persistent pathogens that are becoming increasingly resistant. In particular, it offers a potential pathway to target the persistence of *N. meningitidis* ('settler/spreader' phenotype) and on stopping the spread of asymptomatic *N. gonorrhoeae* infections. However, it is clear that there is a big gap in the knowledge of how commensal *Neisseria* biofilms work. Considering the localisation of these commensal *Neisseria* in the same habitat, it is possible that there is an interplay between their biofilms and that of their pathogenic counterparts, which may be utilised to keep the pathogenic *Neisseria* at bay. More research into this area is necessary to bridge the gaps in research that often exist between pathogenic and commensal *Neisseria* to gain a better understanding of the inner workings of the genus as a whole. This may eventually lead to alternative cheaper pathways that can be utilised against increasingly resistant pathogenic *Neisseria* in the future.

2.3.12 References

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3 Chapter Three

Characterising the Phenotype and Structure of Ngo-Lig E: A Potential Role for the ATP-Dependent DNA Ligase Lig E of *Neisseria gonorrhoeae* in Biofilm Formation

3.1 Preface

Despite being the first b-ADL to be characterised, relatively little is known about the characteristics and function of Lig E. To our knowledge, until the publication of the manuscript below, no *in vivo* characterisation had been reported on this enzyme. In this chapter, I provide the first example of *in vivo* characterisation of Lig E from *N. gonorrhoeae*, termed Ngo-Lig E. In particular, I focus on the importance of Ngo-Lig E for the survival, behaviour and pathogenesis of *N. gonorrhoeae* including its growth, biofilm formation, DNA uptake, DNA-damaging stress response, and host cell adhesion and infection. Furthermore, as stated in Chapter Two, although two Lig E structures from different organisms have been solved, none has been determined from human pathogens which computer modelling predicts include disulfide bonds (Pan *et al.*, 2021). In this chapter, I present the first solved structure of Lig E from a human pathogen, *N. gonorrhoeae*. I also performed a variety of activity assays using the recombinant Ngo-Lig E protein that provided insight into the mechanisms of Lig E ligation.

The work in this chapter is presented as a manuscript that has been published in the peer-reviewed journal, *BMC Microbiology*. This manuscript describes the characterisation of *ngo-lig E in vivo*, as well as the structure and activity of recombinant Ngo-Lig E via X-ray crystallography, and addresses **Objectives One** and **Two** (see Chapter One). Although the published title ‘A role for the ATP-dependent DNA ligase Lig E of *Neisseria gonorrhoeae* in biofilm formation’ implies that the paper focuses on the potential role of Ngo-Lig E in biofilm formation specifically, I note that this was based on a crude crystal violet assay performed on the *ngo-lig E* mutants as part of its general phenotypic

characterisation and was not the focal point of this paper. Rather, this formed the basis for further experiments presented in Chapter Four. Hence the title of this chapter has been refined to better encapsulate the experiments conducted. Supplementary material associated with this work can be found in Appendix A:

Pan, J., Singh, A., Hanning, K., Hicks, J., & Williamson, A. (2024). A role for the ATP-dependent DNA ligase Lig E of *Neisseria gonorrhoeae* in biofilm formation. *BMC Microbiology, 24*(1), 29.

Any changes made to the paper presented here are highlighted in blue (i.e. changes in nomenclature to ensure consistency between chapters).

3.1.1 Author contributions and acknowledgements

As first author, I led the experimental work including construction and characterisation of the *N. gonorrhoeae* mutants and recombinant Ngo-Lig E, as well as crystallisation and solving of the Ngo-Lig E structure. Adele Williamson assisted with the crystallisation and solving of the protein structure, as well as writing and editing of the manuscript. Avi Singh helped carry out DNA extraction and sequencing of *N. gonorrhoeae* mutants while I was away at Cardiff University, while Kyrin Hanning analysed the sequencing data. The study and experimental design of the work was conceptualised by me, my co-supervisor Joanna Hicks, and my Chief supervisor Adele Williamson. The co-authorship form for this manuscript can be found in Appendix D.

This research was undertaken in part using the macromolecular crystallography 2 MX2 beamline at the Australian Synchrotron, part of Australia's Nuclear Science and Technology Organisation (ANSTO), and made use of the Australian Cancer Research Foundation (ACRF) detector.

A role for the ATP-dependent DNA ligase Lig E of *Neisseria gonorrhoeae* in biofilm formation

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3.2 Abstract

Background

The ATP-dependent DNA ligase, Lig E, is present as an accessory DNA ligase in numerous proteobacterial genomes, including many disease-causing species. Here we have constructed a genomic *lig E* knock-out in the obligate human pathogen *Neisseria gonorrhoeae* and characterised its growth and infection phenotype.

Results

Results demonstrate that *N. gonorrhoeae* Lig E (Ngo-Lig E) is a non-essential protein, and its deletion does not cause defects in replication or survival of DNA-damaging stressors. Knock-out strains were partially defective in biofilm formation on an artificial surface as well as adhesion to epithelial cells. In addition to *in vivo* characterisation, we have recombinantly expressed and assayed Ngo-Lig E and determined the crystal structure of the enzyme-adenylate engaged with a DNA substrate in an open non-catalytic conformation.

Conclusions

These findings, coupled with the predicted extracellular/periplasmic location of Lig E indicates a role in extracellular DNA joining as well as providing insight into the binding dynamics of these minimal DNA ligases.

3.3 Introduction

The DNA ligase Lig E is the most recently delineated form of the diverse bacterial ATP-dependent DNA ligases (b-ADLs) which are found in the genomes of many bacterial species in addition to their replicative NAD^+-dependent DNA ligase. The b-ADLs characterised to date are auxiliary enzymes which join DNA breaks as part of stationary phase DNA-repair pathways. These structurally diverse enzymes are

categorised by both the composition of their appending domains, some of which have autonomous catalytic functions, and their operonic arrangement with other pathway enzymes. The best-studied b-ADLs, Lig C and Lig D, function in stationary-phase base excision repair and non-homologous end joining respectively and are found adjacent to other genes that carry out earlier nucleolytic, polymerase or DNA end-binding steps (Shuman & Glickman, 2007; Zhu & Shuman, 2010; Płociński *et al.*, 2017). Likewise, the co-localisation of the Lig B class of b-ADLs with a conserved Lhr-helicase and a pair of nucleases implies a role in genomic DNA repair (Ejaz & Shuman, 2018; Ejaz *et al.*, 2019).

In contrast to these b-ADLs, Lig E does not exhibit syntenic conservation with any repair-associated genes and its biological function remains undefined (Williamson *et al.*, 2016; Pan *et al.*, 2021). Lig E is distinguished by having a minimal structure lacking typical globular DNA binding domains or loop regions. Despite this, *in vitro* characterisations of recombinant Lig E enzymes from a range of bacteria have demonstrated that they are fully-functional ATP-dependent ligases with preferential activity on singly-nicked DNA and some, albeit weaker, activity on cohesive ends (Williamson & Pedersen, 2014; Williamson *et al.*, 2014; Williamson *et al.*, 2018; Berg *et al.*, 2019). Structures of Lig E bound to adenylated nicked DNA, or as the enzyme-adenylate without DNA, show that it engages this substrate through highly conserved basic residues in the oligonucleotide binding (OB) domain and the inter-domain linker (Williamson *et al.*, 2014; Williamson *et al.*, 2018). The most intriguing structural aspect of Lig E however, is the N-terminal leader sequence which is predicted to direct its localisation to the periplasm. Removal of this sequence increases both the stability and activity of the enzyme, and both Lig E structures, as well as most *in vitro* characterisations, have been undertaken on the mature leader-less form (Williamson & Pedersen, 2014).

Examination of the phylogenetic distribution of Lig E indicates that it is widespread among, but restricted to proteobacteria, and is typically the only b-ADL found in a particular species' genome (Williamson *et al.*, 2016). Notably, Lig E has been annotated in the genomes of several naturally competent, biofilm-forming pathogenic bacteria, several of which have acquired antibiotic resistance traits and present potential multidrug-resistance threats (Pan *et al.*, 2021). An example is the obligate human pathogen *Neisseria gonorrhoeae* which colonises and infects

mucosal cells of the male and female reproductive tracts and is the causative agent of the sexually transmitted infection (STI), gonorrhoea. One of the most concerning features of *N. gonorrhoeae* is its ability to gain antibiotic resistance due to its natural competence. Its propensity to take up conspecific DNA is enhanced 20–100-fold by the presence of a 10 bp DNA uptake sequence (DUS) which is common in the gonococcal genome and allows the bacterium to differentiate species-specific DNA from other DNA in the environment (Unemo & Shafer, 2014; Cehovin & Lewis, 2017). Gonorrhoea infection may cause urethritis in men, cervicitis or pelvic inflammatory disease in women and neonatal conjunctivitis if contracted during birth; however, a high proportion of infections are asymptomatic (approximately 50% in females) leading to its undetected spread and delayed treatment (Unemo *et al.*, 2019; Martín-Sánchez *et al.*, 2020). *N. gonorrhoeae* readily forms a biofilm on epithelial cells during infection which likely contributes to this ability to evade the host immune system through the help of a heterogeneous physical barrier or the potential subsequent induction of oxidative stress defence mechanisms, and additionally can modulate the spread of antibiotic resistance by horizontal gene transfer (Greiner *et al.*, 2005; Steichen *et al.*, 2008; Falsetta *et al.*, 2011; Kouzel *et al.*, 2015). Extracellular DNA (exDNA) comprises a large fraction of gonococcal biofilms and is produced by active DNA secretion as well as the contribution of genomic DNA from lysed gonococcal cells (Steichen *et al.*, 2011; Phillips *et al.*, 2012; Zweig *et al.*, 2014).

Despite extensive *in vitro* characterisation of Lig E from a range of pathogenic and environmental bacteria, the biological function of Lig E remains unknown. However, the probable extracellular location of Lig E, coupled with its presence in the genomes of bacteria known to be competent for exDNA uptake and which can form exDNA-rich biofilms suggest the involvement of this DNA ligase in one or both of these processes. Here we report the first *in vivo* study of the function of Lig E via generation of a knock-out in *N. gonorrhoeae.* Although no impact was observed on planktonic growth rates, the deletion mutant was partially defective in biofilm formation and adhesion to epithelial cells, as well as exhibiting aberrant growth rates in the presence of DNA-damaging antibiotics. In addition, we have characterised recombinantly produced *N. gonorrhoeae* Lig E (Ngo-Lig E) *in vitro* and determined the structure of the enzyme-adenylate in an open DNA-engaged conformation.

3.4 Methods

3.4.1 *N. gonorrhoeae* strains and cultivation

All *N. gonorrhoeae* used in this study were generated from the MS11 strain (ATCC: BAA-1833™). Gonococci were grown at 37 °C and 5% CO_2 either on gonococcal base (GCB) agar (Difco™) or in gonococcal base liquid (GCBL) media (15 g/L proteose peptone #3, 4.0 g/L, K_2HPO_4, 1.0 g/L, KH_2PO_4, 1.0 g/L NaCl), both supplemented with 1% *Kellogg's* supplement (22.22 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM $Fe(NO_3)_3$) (Dillard, 2011).

3.4.2 *N. gonorrhoeae* mutant construction

To generate different variants, constructs were designed to introduce insertions and/or deletions into the MS11 genome (GenBank: CP003909.1) by homologous recombination of flanking sequences. Briefly, the *Δngo-lig E^{kan}* mutant contained a disruption of the *ngo-lig E* gene (NGFG_RS11310, discontinued locus tag: NGFG_01849) via a kanamycin resistance cassette, while the *ngo-lig E-his^{kan}* mutant had a 6-histidine (His)-tag at the C-terminus of the intact *ngo-lig E* gene as well as an additional kanamycin resistance cassette behind the gene. The *P_{opaB}-ngo-lig E-his^{kan}* mutant was generated at an intergenic site between the previously labelled open reading frames NGFG_RS14495 and NGFG_RS13185. This site has since been updated as a new locus tag encompassing the 7318 base pair (bp) region (NGFG_RS15145) as an uncharacterised 'phage protein'. Here, a codon-optimised *ngo-lig E* gene was used to avoid aberrant recombination with the native *ngo-lig E* copy while still encoding the same amino acid sequence. This was inserted behind the constitutive *opaB* promoter (P_{opaB}) and included a 6-His-tag at the C-terminus and a kanamycin resistance cassette for selection. All DNA constructs were ordered as gene fragments or clonal genes (from Integrated DNA Technologies (IDT) or Twist Biosciences).

Strains were generated via spot transformation (Dillard, 2011; Callaghan & Dillard, 2019). Briefly, colonies determined to be piliated by observation under a dissecting microscope were streaked through 10 ng spots of the DNA constructs on GCB agar (Dillard, 2011). After a 24 h incubation, colonies growing at the spotted locations were re-streaked onto GCB plates with kanamycin (50 µg/mL) for selection. These mutants were verified via polymerase chain reaction (PCR) and sequencing

analyses using primers detailed in Table A. 1. To ensure only the desired changes were introduced, the *Δngo-lig E*^{kan} and wild-type (*wt*) genomes were re-sequenced using Oxford Nanopore technology (Oxford Nanopore technologies, Oxford, UK). Genomic DNA was extracted using the Thermo Scientific GeneJet Genomic DNA Purification kit (ThermoFisher Scientific) and libraries were prepared using the Native Barcoding Kit 24 V14 (SQK-NBD114.24, Oxford Nanopore technologies, Oxford, UK) following the manufacturer's protocol. Sequencing was conducted on the Oxford Nanopore MinION using the R10.4.1 flow cell compatible with Kit 14 chemistry, and basecalling and multiplexing were carried out using Dorado V0.40 (https://github.com/nanoporetech/dorado). Resulting reads were assembled via a long-read consensus approach using Tricycler (Wick *et al.*, 2021) and compared in the Geneious Prime 2021.1.1 software (http://www.geneious.com/) via the Mauve plugin (Wick *et al.*, 2021). Sequencing data have been deposited with the identifier PRJNA1051170 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1051170/).

3.4.3 Statistical methods for phenotypes and assays

Statistical analyses of phenotypic characterisation experiments and enzyme assays were performed using the GraphPad Prism 9.4.0 software (https://www.graphpad.com/). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to compare the different measurements and p values < 0.05 were deemed statistically significant.

3.4.4 Growth experiments in liquid culture

Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. Suspensions with an OD_{600} of 0.05 were prepared and aliquoted into 12-well plates (1 mL per well, 3 replicates each), where each 12-well plate represented one time point. Gonococcal cells were harvested at 1.5 h intervals by scraping the cells from the bottom of the wells and vortexing vigorously for 2 min. Growth was monitored by measuring the OD_{600} of the cell resuspensions before serially diluting and plating onto GCB agar. The number of colonies on the agar plates were counted after 48 h to obtain colony forming units (CFUs).

3.4.5 H_2O_2 oxidative stress assay

Oxidative stress assays were carried out as described previously (Cahoon *et al.*, 2011). Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. Suspensions with an OD_{600} of 0.05 were prepared and aliquoted into separate wells in 12-well plates. After 9 h of growth, the gonococci were subjected to 0, 2, 5, 10, 25 or 50 mM hydrogen peroxide (H_2O_2) treatment for 20 min. Cells were then scraped from the bottom of the wells, pelleted, and washed with 300 μL GCBL to remove excess H_2O_2, then resuspended in 1 mL GCBL before being serially diluted and plated onto GCB agar. CFU readings were obtained by counting the number of colonies formed after 48 h.

3.4.6 UV survival assay

Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. Suspensions with an OD_{600} suspension of 0.6 were prepared and serially diluted before plating onto GCB agar. The agar plates were subjected to ultraviolet (UV) irradiation at 80 J for 0, 5, 7.5 and 10 min using a BLX-254 crosslinker (Stohl & Seifert, 2001). The plates were then incubated for 48 h before counting to obtain CFU readings.

3.4.7 Nalidixic acid treatment assay

Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. Suspensions with an OD_{600} of 0.6 were prepared and serially diluted before plating onto GCB agar with 1.25 mg/L nalidixic acid (Nal-acid) (Stohl & Seifert, 2001). The plates were incubated for 48 h before counting to obtain CFU readings.

3.4.8 Cell infection and adhesion assays

The ME-180 (HTB-33^{TM}) endocervical cell line was used for the host association assays and was maintained in McCoy's 5 A media (Gibco) supplemented with 10% foetal bovine serum (FBS). Based on previously described work (Hockenberry Alyson *et al.*, 2016), ME-180 cells were seeded in 12-well plates 48 h prior to use to achieve 80–100% confluency on the day of the experiment. Piliated, Opa negative (Opa^{-}) gonococci from a 24 h streak were lawned for 16 h before

resuspension in GCBL media. The OD_{600} of the resuspensions were measured and back-calculated to give units of CFU/mL. The suspensions were then used to infect the ME-180 cells at a multiplicity of infection (MOI) of 25 in McCoy's 5 A with 10% FBS for 6 h. All experiments were done in triplicate.

For planktonic measurements, the supernatants were aspirated and the wells were washed three times with 1 mL GCBL. The pooled supernatant and washes were vortexed (2 min), serially diluted and plated onto GCB agar. To measure adhered cells, the remaining cell monolayers were subjected to 0.5% saponin treatment (1 mL in GCBL) for 20 min, then scrapped and vortexed vigorously for 2 min before serial dilution and plating onto GCB agar. The number of colonies on the agar were counted after 48 h to obtain CFU readings for planktonic and adhered cells respectively. Gonococcal adherence and planktonic growth were calculated as the proportions of total CFUs and expressed as percentages.

For invasion measurements, the media was aspirated before treatment of infected ME-180 cells with 50 µg/mL gentamicin for 1 h. The wells were washed three times with 1 mL GCBL before being subjected to 0.5% saponin (1 mL in GCBL) for 20 min. The cells were then scraped and vortexed vigorously for 2 min before serial dilution and plating onto GCB agar. The number of colonies on the agar were counted after 48 h to obtain CFU readings for cells that had invaded the cervical cell monolayer. The extent of invasion was calculated as a proportion of the total number of cells.

3.4.9 Biofilm microtiter assays

Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. Suspensions with an OD_{600} of 0.05 were prepared and aliquoted into separate wells in 96-well plates (100 µL per plate, 8 replicates each). After 24 h, the wells were washed three times with sterile water before staining with 125 µL 0.8% crystal violet for 15 min. The wells were then washed four times with sterile water before air-drying overnight. The dye was resolubilised in 125 uL 30% acetic acid and the solubilised crystal violet solutions were transferred to a new 96-well plate. The extent of biofilm formed was determined by the absorbance at 560 nm as previously described (O'Toole, 2011).

3.4.10 RNA extraction and RT-qPCR

RNA was isolated from *wt* gonococci and the three variant strains under planktonic and biofilm conditions. Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. Suspensions with an OD_{600} of 0.05 were prepared and aliquoted into separate wells in 12-well plates. After 24 h, the cells were either harvested without scraping (planktonic fraction) or harvested with scraping of the wells (biofilm fraction). Total RNA was isolated using the Direct-zol RNA Miniprep Kit (Zymo Research). RNA concentration and quality were measured using the DeNovix DS-11 spectrophotometer and the Denovix RNA quantification assay kit. Reverse transcription was performed on 18 ng/µL RNA (162 ng total) to obtain complementary DNA (cDNA) using the SuperScriptIII First-Strand Synthesis System (Invitrogen). Reverse transcription-quantitative PCR (RT-qPCR) was performed on a Mic qPCR cycler using the Hot Fire Pol DNA polymerase kit (Solis Biodyne) with specific probes and primers for the *ngo-lig E* gene and the 16S ribosomal RNA (rRNA) housekeeping gene as listed in Table A. 2. Relative quantification of gene transcription was performed using the comparative Ct method (Schmittgen & Livak, 2008) after normalising to the 16s rRNA gene.

3.4.11 Subcellular fractionation

Subcellular fractionation was performed on gonococcal cells to separate cytoplasmic, cell-membrane, periplasmic and extracellular proteins (Ramsey *et al.*, 2014). Piliated gonococci from a 24 h streak were lawned for 16 h before resuspension in GCBL media. 30 mL cultures with an OD_{600} of 0.05 were prepared with GCBL media and cultivated overnight before harvesting by centrifugation (5000 xg, 15 min) to separate the pellet and supernatant. Extracellular proteins were recovered from the supernatant by precipitation with 20% trichloroacetic acid, incubation for 1 h on ice, and collection by centrifugation at 20,000 xg. The resultant pellet was washed with ice-cold 90% acetone three times before air drying and resuspension in 10 mM Tris (pH 8.0). The periplasmic fraction was isolated from the pelleted cells by addition of 1 mL of buffer 1 (0.2 M Tris (pH 8.0), 0.1 M EDTA, 20% sucrose) before incubation on ice (20 min) and centrifugation (20,000 xg 15 min, 4 °C). The pellet was resuspended in 1 mL buffer B (10 mM Tris, 5 mM $MgSO_4$, 0.2% SDS, 1% Triton X100) before incubation on ice (20 min) and

centrifugation (20,000 xg, 15 min, 4 °C). The resultant supernatant was the periplasmic portion. To isolate cytoplasmic fraction proteins, the remaining pellet was treated with 1 mL Bug Buster (Sigma-Aldrich) and agitated for 20 min before centrifugation (10,000 rpm, 10 min, 4 °C). The supernatant was recentrifuged at maximum speed (1 h) and the resultant supernatant was isolated as the cytoplasmic portion. To isolate the membrane fraction isolation, the remaining pellet was resuspended in 0.01 M Tris (pH 8), spun, and the resultant pellet was isolated as the membrane portion.

3.4.12 His-tagged protein detection

To enrich for His-tagged proteins, each subcellular fraction was incubated with pre-washed Ni Sepharose High Performance nickel resin beads (Cytiva) for 15 min. After this time, the beads were sedimented by centrifugation, washed twice with lysis buffer (50 mM Tris pH 8.0, 750 mM NaCl, 1 mM $MgCl_2$, 5% glycerol) and electrophoresed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Western blotting was performed with nitrocellulose membranes. After protein transfer, membranes were blocked for 1 h with 5% milk in Tris buffered saline-Tween 20 (TBS-T). The membrane was probed with 1:500 anti-His-tag mouse monoclonal (HIS.H8), sc57598 igG2b antibody (Santa Cruz Biotechnology; 10 µg/mL) overnight, and 1:1000 goat anti-mouse polyclonal IgG antibody conjugated to horseradish peroxidase ab97023 (Abcam; 1 mg/mL) for 1 h. The membranes were incubated with the SuperSignal^{TM} West Femto Maximum Sensitivity Substrate for 5 min before imaging using the iBright Imaging System (Invitrogen).

3.4.13 Recombinant expression of Ngo-Lig E

The position of the Ngo-Lig E N-terminal leader sequence was predicted using SignalP 5.0 (Petersen *et al.*, 2011). Pre-cloned constructs encoding mature native- and C-terminally His-tagged Ngo-Lig E in the pDONR221 plasmid were synthesised from Twist BioScience with codon optimisation for *E. coli*. Constructs were sub-cloned into the pDEST17 and pHMGWA vectors using the Gateway system and recombinant Ngo-Lig E was expressed and purified from BL21(DE3)pLysS at 15 °C as described for other Lig E proteins previously (Williamson & Pedersen, 2014).

Briefly, native mature Ngo-Lig E expressed from pDEST17 with an N-terminal His-tag was purified with a primary immobilised metal affinity chromatography (IMAC) step on a 5 mL His trap HP column with buffer A (50 mM Tris pH 8, 750 mM NaCl, 10 mM imidazole, 5% glycerol) and eluted with buffer B (50 mM Tris pH 8, 750 mM NaCl, 500 mM imidazole, 5% glycerol). After exchange into TEV protease cleavage buffer C (50 mM Tris pH 8, 100 mM NaCl, 5% glycerol, 1 mM DTT), the N-terminal His-tag was cleaved overnight with TEV protease (0.1 mg/ml) and the de-tagged protein was recovered by a reverse-IMAC step. A final size-exclusion chromatography (SEC) was carried out using a Hi Load 16/600 Superdex 75 column. Native mature Ngo-Lig E and C-terminally tagged mature Ngo-Lig E expressed with N-terminal His-maltose binding protein (MBP) tags were purified in the same way, but an additional chromatographic step was included after size exclusion to separate any residual His-MBP tag that had carried over after cleavage. Pooled Ngo-Lig E/Ngo-Lig E-His were loaded onto an MBPTrap HP column in MBP binding buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) and eluted using a linear gradient of MBP elution buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM maltose). All proteins were evaluated as being purified to homogeneity by the appearance of a single band on SDS-PAGE gels.

3.4.14 DNA ligation assays

Gel-based endpoint assays were used to measure ligation activity as described previously (Sharma *et al.*, 2020; Pan *et al.*, 2021). Standard assay conditions included 80 nM of fluorescently-labelled nicked substrate, 1.0 mM ATP, 10 mM $MgCl_2$, 10 mM DTT, 50 mM NaCl and 50 mM Tris pH8.0. Ngo-Lig E or Ngo-Lig E-His (0.1 µM) were incubated at 25 °C for 30 min before quenching with 95% formamide stop buffer. Products were electrophoresed on 20% urea-PAGE gels and fluorescence was detected using the iBright imaging system (Invitrogen) and quantified using ImageJ (Schneider *et al.*, 2012). The assay was repeated with variations in the pH (Tris buffer for pH 7.1-9.0; MES buffer for pH 5.5–6.2) and amount of ATP used in the reaction buffer, as well as different combinations of substrate oligonucleotides to generate different ligatable DNA breaks (Table A. 3 and Table A. 4). Incubation conditions for the different DNA substrates were 25 °C, 30 min for single nick, overhang and mismatched substrates, and 15 °C overnight for blunt-ended and gapped substrates.

3.4.15 Crystallisation and structure determination of the Ngo-Lig E–DNA complex

Double-stranded nicked DNA for co-crystallisation was assembled as described previously (Williamson *et al.*, 2018; Pan *et al.*, 2021) using high-performance liquid chromatography (HPLC)-purified oligos purchased from IDT with the sequences CAC TAT CGG AA (5'-PO_4 strand); ATT GCG ACC (3'-OH strand) and TTC CGA TAG TGG GGT CGC AAT (complementary strand). His-tagged Ngo-Lig E (478.7 µM) was incubated with a 1.2 molar excess of the nicked duplex DNA and additional 5 mM ethylenediaminetetraacetic acid (EDTA) for 1 h on ice prior to commencing crystallisation screening. Crystals with a plate morphology were grown by hanging drop diffusion at 18 °C in 0.5 M potassium thiocyanate and 0.1 M Bis Tris Propane pH 8.0, and were mounted in cryoloops and directly flash frozen in liquid nitrogen for data collection. Diffraction data to 2.44 Å was measured at the Australian Synchrotron MX2 beamline (Aragão *et al.*, 2018) and integrated, scaled and merged using the XDS and Aimless programmes (Kabsch, 2010; Winn *et al.*, 2011). A model of Ngo-Lig E was built using AlphaFold via the CoLab server (Jumper *et al.*, 2021) and processed using the Process Predicted Model utility in the Phenix suite (Adams *et al.*, 2010). The processed NTase and OB domains were used as search models for molecular replacement in Phaser-MR (McCoy *et al.*, 2007) together with iteratively-truncated portions of double-stranded DNA from the Ame-Lig co-crystal (6GDR). The initial model was improved by iterative rounds of refinement using Phenix.refine (Afonine *et al.*, 2012) and manual rebuilding in COOT (Emsley *et al.*, 2010). Data collection and statistics are listed in Table 3.1 and the structure was deposited to the Protein Data Bank (PDB) with the identifier 8U6X.

Table 3.1 Data collection and refinement statistics. Statistics for the highest-resolution shell are shown in parentheses.

	Ngo-Lig E (8U6X)
Wavelength	0.9537
Resolution range	43.67 – 2.44 (2.527 – 2.44)
Space group	P 21 21 2
Unit cell	39.398 167.68 51.159 90 90 90

Total reflections	115,570 (11,213)
Unique reflections	13,286 (1269)
Multiplicity	8.7 (8.8)
Completeness (%)	99.86 (99.61)
Mean I/sigma(I)	11.88 (1.64)
Wilson B-factor	53.04
R-merge	0.1076 (1.08)
R-meas	0.1144 (1.146)
R-pim	0.03774 (0.3757)
CC1/2	0.997 (0.694)
CC*	0.999 (0.905)
Reflections used in refinement	13,273 (1266)
Reflections used for R-free	1329 (127)
R-work	0.2301 (0.3423)
R-free	0.2809 (0.3688)
CC(work)	0.937 (0.764)
CC(free)	0.891 (0.577)
Number of non-hydrogen atoms	2295
macromolecules	2222
ligands	43
solvent	42
Protein residues	247
RMS(bonds)	0.003
RMS(angles)	0.51

Ramachandran favored (%)	95.92
Ramachandran allowed (%)	4.08
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	2.96
Clashscore	3.21
Average B-factor	61.64
macromolecules	58.78
ligands	66.12
solvent	44.75

3.5 Results

3.5.1 Ngo-Lig E is not essential for gonococcal growth and survival

To evaluate the importance of Ngo-Lig E for *N. gonorrhoeae* viability, physiology and stress survival, we constructed a knock-out of the *ngo-lig E* gene (Δ*ngo-lig E*^{kan}) by interruption of the *ngo-lig E* open reading frame with a kanamycin resistance cassette (*kan*^{R}) which removed a stretch of 795 nucleotides from the centre of the gene (Figure 3.1A). A second construct was generated which appended a 6-His-tag to the C-terminus of native Ngo-Lig E and inserted a *kan*^{R} cassette behind the *ngo-lig E* gene (*ngo-lig E-his*^{kan}). The purpose of this was to provide an immunogenic handle on natively-produced Ngo-Lig E, and this construct additionally served as a control for the knock-out with both strains containing equivalent *kan*^{R} insertions in their genomes. A third construct (P_{opaB}-*ngo-lig E-his*^{kan}) generated a constitutive high-expression genotype, by inserting a second copy of His-tagged *ngo-lig E* under the control of the strong constitutive P_{opaB} promoter at a separate location in the genome (NGFG_RS15145).

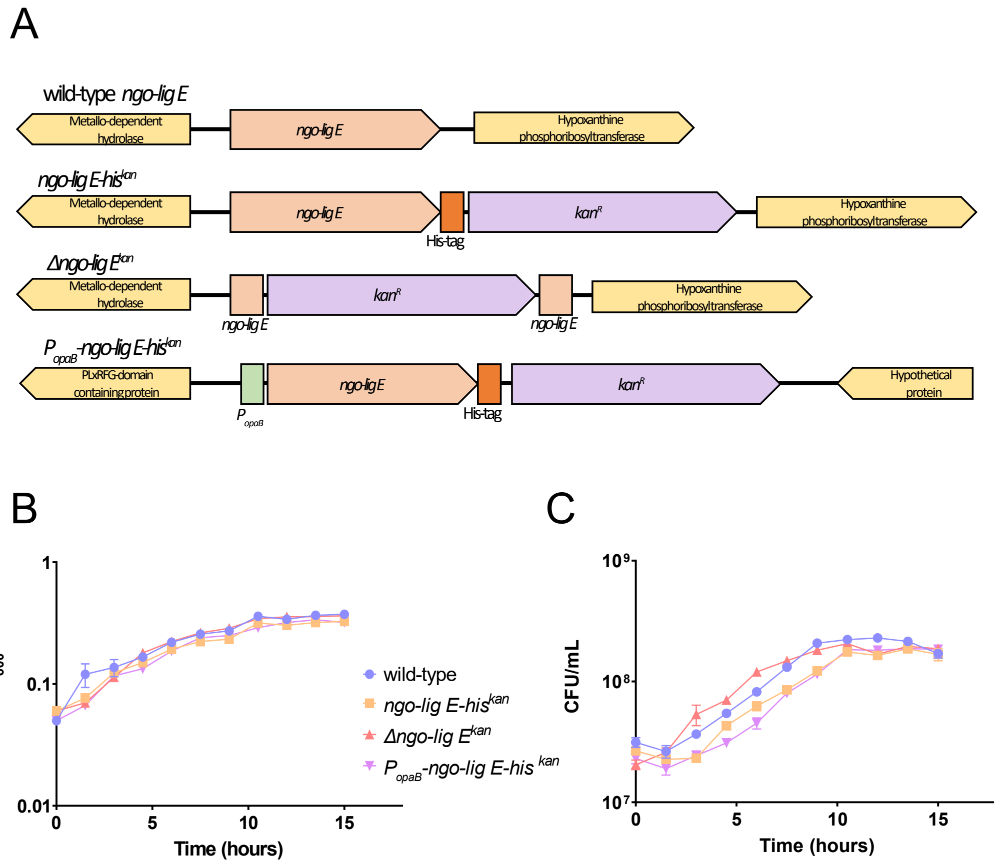

Figure 3.1 Growth of *Neisseria gonorrhoeae* variants. (A) Schematic of the genetic constructs for His-tagged *ngo-lig E* (*ngo-lig E-his*^{kan}), the *ngo-lig E* deletion mutant (*Δngo-lig E*^{kan}) and constitutively upregulated *ngo-lig E* (P_{opaB}*-ngo-lig E-his*^{kan}). (B) Growth curve of *N. gonorrhoeae* variants monitored by OD_{600}. (C) Number of viable *N. gonorrhoeae* cells monitored by colony forming unit (CFU) counts for each culture. Points are the mean of triplicate measurements and error bars represent the standard error of the mean.

The resulting clones were sequenced to confirm the correct genotypes. qPCR indicated a 89-fold upregulation of *ngo-lig E* transcripts from the P_{opaB}*-ngo-lig E-his*^{kan} strain and confirmed that *ngo-lig E* gene expression was eliminated from the *Δngo-lig E*^{kan} strain (Table A. 5 and Figure 3.2A). Growth in liquid culture measured by both OD_{600} and CFU counts showed no significant differences in growth between *wt N. gonorrhoeae*, *Δngo-lig E*^{kan}, *ngo-lig E-his*^{kan} and P_{opaB}*-ngo-lig E-his*^{kan} which suggests that *ngo-lig E* is neither an essential gene, nor is its overexpression deleterious to cell survival in planktonic culture (Figure 3.1B and C). Attempts were made to visualise Ngo-Lig E-His expression by Western blot against the 6-His-tag in both *ngo-lig E-his*^{kan} and P_{opaB}*-ngo-lig E-his*^{kan} during different growth stages; however, despite evidence of gene expression by qPCR, neither strain showed detectable immunologic signal by this method (Figure A. 2).

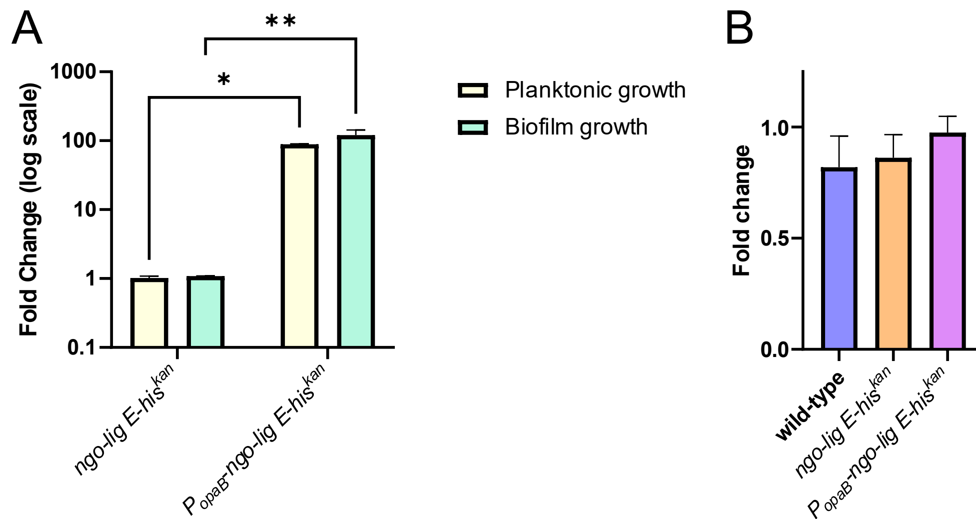

Figure 3.2 Comparisons of *ngo-lig E* gene expression with normalisation to the 16S rRNA gene. (A) Fold changes of *ngo-lig E* expression for *ngo-lig E-his*^{kan} and *P*$_{opaB}$*-ngo-lig E-his*^{kan} compared to *wt Neisseria gonorrhoeae* under either planktonic or biofilm conditions. (B) Fold changes of *ngo-lig E* expression for *wt*, *ngo-lig E-his*^{kan}, and *P*$_{opaB}$*-ngo-lig E-his*^{kan} under biofilm conditions compared to planktonic conditions. Points are the mean of triplicate measurements and error bars represent the standard error of the mean. Significance values are given as * $p \leq 0.05$; ** $p \leq 0.01$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

3.5.2 Response to oxidative and DNA-damaging stressors

Given the role of DNA ligases in genomic DNA repair and replication, we asked whether Ngo-Lig E plays a role in surviving DNA damage or oxidative stress in *N. gonorrhoeae* by subjecting the *Δngo-lig E*^{kan}, *ngo-lig E-his*^{kan}, *P*$_{opaB}$*-ngo-lig E-his*^{kan} and *wt* strains to genotoxic stressors. No significant differences in survival were seen between either the Ngo-Lig E deficient *Δngo-lig E*^{kan} strain, or the *P*$_{opaB}$*-ngo-lig E-his*^{kan} overexpressor when exposed to increasing concentrations of H_2O_2 or UV dosages (Figure 3.3A and B). As H_2O_2 and UV are both expected to damage chromosomal DNA, the absence of higher mortality in either of these strains suggests that Ngo-Lig E does not play a significant role in repair of chromosomal DNA damage. A significant increase in survival was observed for the *Δngo-lig E*^{kan} mutant when treated with nalidixic acid, with almost twice as many cells surviving at a concentration of 1.25 mg/L relative to the *wt* (Figure 3.3C). Nalidixic acid induces double-stranded breaks in chromosomal DNA, suggesting that although Ngo-Lig E may not be involved directly in intracellular DNA repair processes, it is still able to interact with chromosomal DNA.

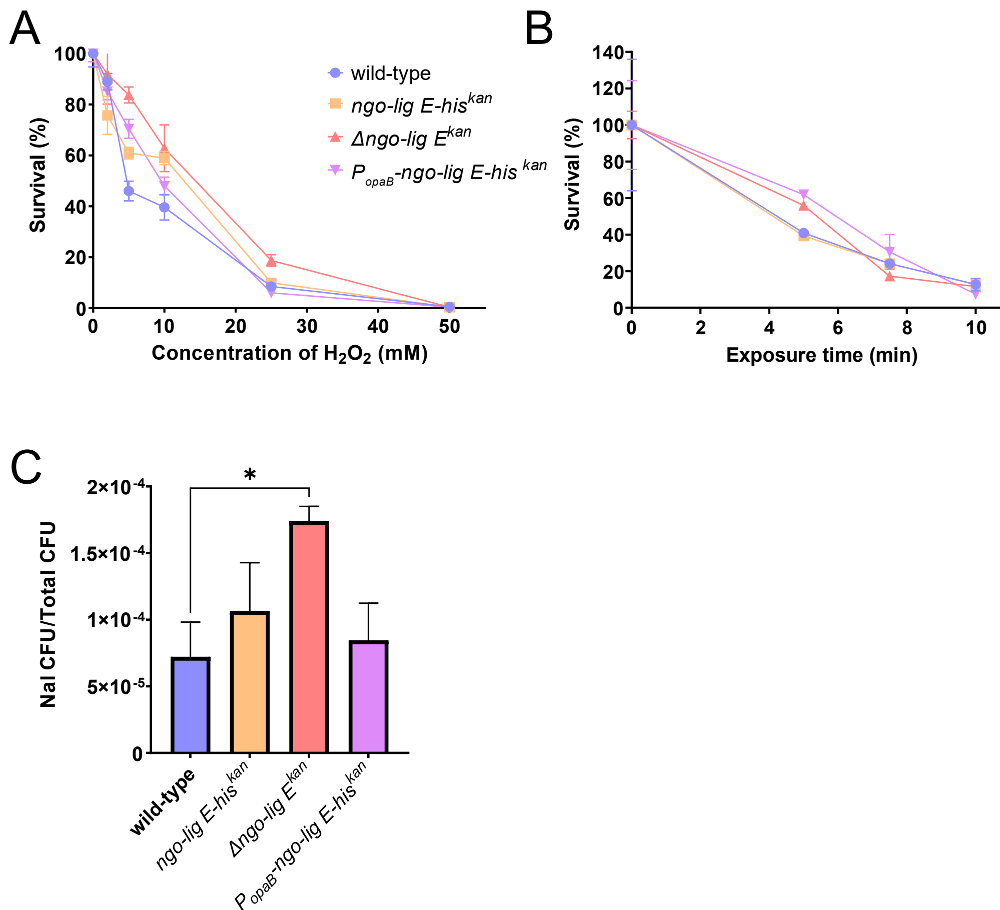

Figure 3.3 Damage and oxidative stress assays with *Neisseria gonorrhoeae* strains. (A) Survival after treatment with hydrogen peroxide. (B) Survival after irradiation with UV light. (C) Survival after treatment with nalidixic acid. Points are the mean of triplicate measurements and error bars represent the standard error of the mean. Significance values are given as * $p \leq 0.05$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

3.5.3 Ngo-Lig E deletion impacts biofilm formation and cell adhesion

Due to its predicted periplasmic location, we considered whether Ngo-Lig E could influence biofilm formation. Crystal violet quantification of strains cultivated for 24 h in 96-well plates indicated that deletion of *ngo-lig E* decreased the extent of biofilm produced by *Δngo-lig E*^{kan}; however, upregulating the expression of Ngo-Lig E did not increase biofilm production above that of the wild-type (Figure 3.4A). qPCR indicated that transcription of *ngo-lig E* was not upregulated in *wt* during biofilm formation relative to liquid culture which suggests that although deletion of Ngo-Lig E diminishes biofilms, it is not necessary to upregulate its expression to produce biofilms (Figure 3.2B).

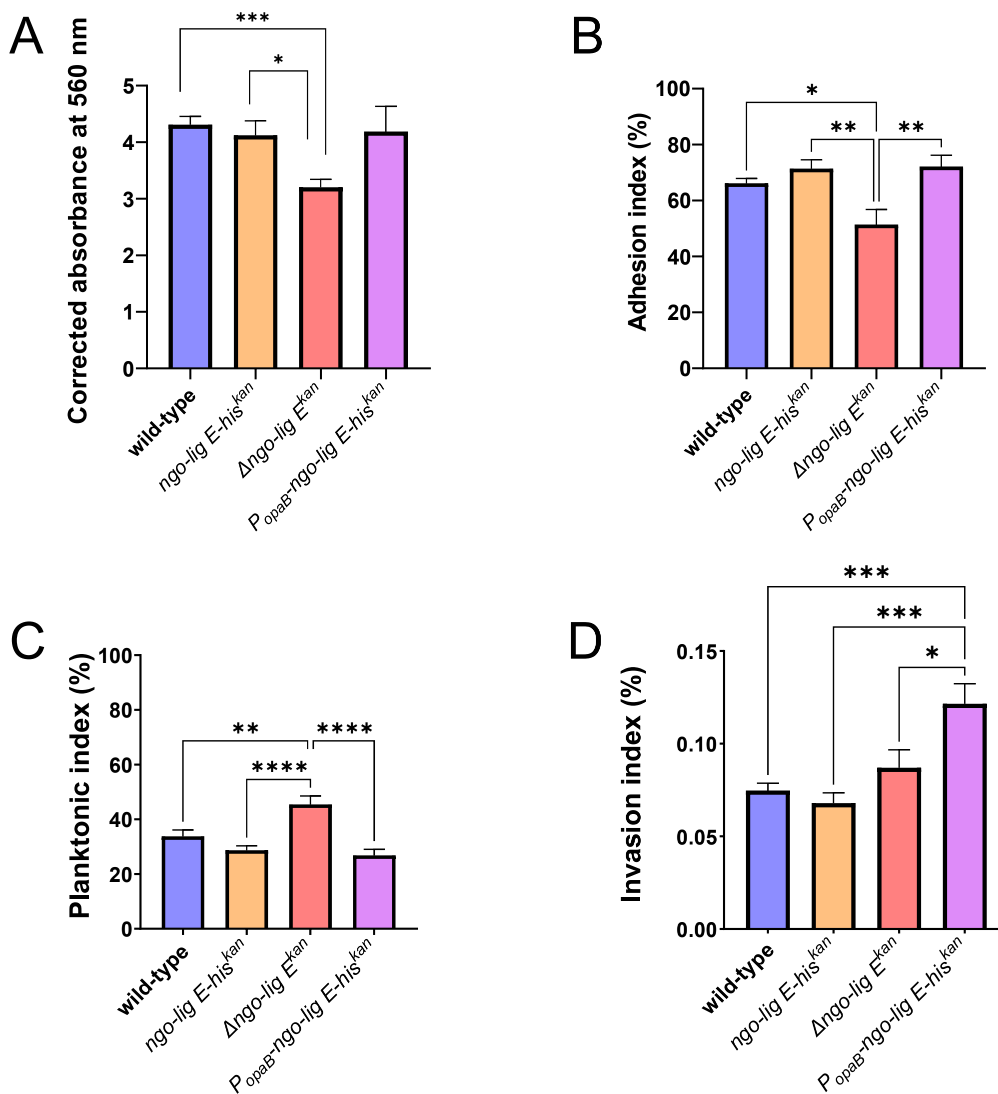

Figure 3.4 Characterisation of *Neisseria gonorrhoeae* biofilm formation and interactions with human cells. **(A) Biofilm formation in liquid culture by *N. gonorrhoeae* strains. (B) Proportion of *ngo-lig E* mutants adhering to epithelial cells. (C) Proportion of *ngo-lig E* mutants remaining in the non-adhered (planktonic) state when co-cultured with ME-180 epithelial cells. (D) Proportion of *ngo-lig E* mutants invading ME-180 epithelial cells relative to the total *N. gonorrhoeae* cells (planktonic and adhered). Points are the mean of triplicate measurements and error bars represent the standard error of the mean. Significance values are given as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.**

Given this result, we further asked whether the absence of Ngo-Lig E could influence the adhesion behaviour or infectivity of *N. gonorrhoeae* to human epithelial cells. Comparison of adhesion on endocervical cells indicates that *Δngo-lig E*^{kan} is impaired in its ability to attach to the eukaryotic cell surface, with a significantly greater portion of *ngo-lig E* knock-out cells remaining in the planktonic fraction (Figure 3.4B and C); however, increased production of Ngo-Lig E did not enhance cell adhesion. Conversely, the decreased adhesion of the Ngo-

Lig E-deficient strain did not translate into defects in infectivity, however, the strain over-expressing Ngo-Lig E had increased rates of cell invasion (Figure 3.4D). There were no significant differences in the behaviour of the His-tagged *ngo-lig E-his*^{kan} strain compared to the *wt* for cell infection, adhesion or biofilm formation.

To ensure only the *ngo-lig E* gene was disrupted and no unintended changes had occurred during mutant construction, both the *Δngo-lig E*^{kan} and *wt* genomes were re-sequenced. Alignment of the consensus sequences confirmed the presence of the desired *ngo-lig E* interruption (Figure A. 1) and that there were no significant differences between the two genomes outside of this region. Particular attention was given to the 64 phase variable genes which have been implicated in phenotypic differences between *N. gonorrhoeae* strains (Jordan *et al.*, 2005). All were found to be more than 99% identical between our deletion and *wt* strains, and none had evidence of recombination, rearrangements or other significant insertion and deletions.

3.5.4 ATP-dependent nick-sealing activity and structure of Ngo-Lig E

To compare the substrate specificity of Ngo-Lig E to that of other previously-characterised species, and to examine whether the His-tag impacts activity, mature Ngo-Lig E without the predicted N-terminal periplasmic leader sequence was recombinantly expressed and purified. *In vitro* assays with 5’ 6-carboxyfluorescein (FAM)-labelled substrates demonstrated that Ngo-Lig E has highest activity on singly-nicked DNA. It is also able to join double-stranded breaks with 4 bp cohesive overhangs and has detectable activity on substrates with a mismatched base pair at the 3’-OH end of the break (Figure 3.5A and B). Maximal activity was observed at pH 7.1 and while significant joining was seen at higher pH, little to no activity was observed below this maximum (Figure 3.5C). Inclusion of a C-terminal His-tag had no deleterious effect on Ngo-Lig E activity with nicked DNA substrates, and actually appeared to enhance ligation efficiently (Figure A. 3).

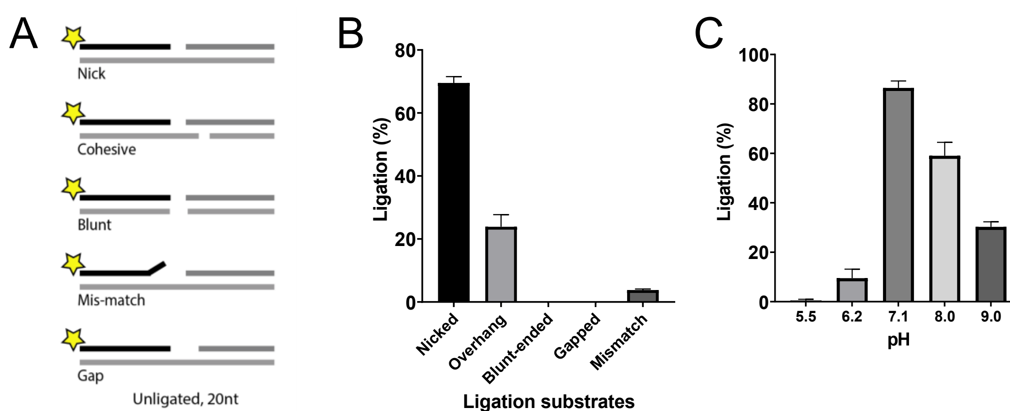

Figure 3.5 Activity of recombinant Ngo-Lig E. **(A) Schematic of FAM-labelled DNA substrates with ligatable breaks. (B) Specific activity of Ngo-Lig E on different double-stranded breaks. (C) pH dependence of Ngo-Lig E specific activity on singly-nicked DNA. Values represent the percentage of total substrate ligated quantified from band intensities and are the mean of three replicates. Error bars represent the standard error of the mean.**

To enable structural comparison with other Lig E proteins, Ngo-Lig E was crystalised in the presence of a 21 bp piece of DNA with a centrally placed phosphorylated nick. The resulting structure showed Ngo-Lig E in an open conformation bound to the DNA via interactions with its OB domain only (Figure 3.6A). Covalent adenylation at lysine (Lys) 22 is present in the NTase domain, indicating that we have captured a pre-step 2 state before complete encirclement of the DNA, which would position the nick in the active site (Figure 3.6B). Analysis of the Ngo-Lig E structure confirms the presence of the disulphide bond between cysteine (Cys) 176 and Cys 197 in the OB domain which was previously predicted by computational modelling (Figure 3.6C and Figure A. 5A) (Pan *et al.*, 2021). Examination of the linker region reveals a network of polar and electrostatic interactions that stabilise the open conformation of the domains including Lys 176 and serine (Ser) 170 from the linker with glutamic acid (Glu) 141 from the NTase domain; tyrosine (Tyr) 172, Tyr 179 and His 201 from the OB domain with Lys 165, Glu 8 and Lys 10 of the NTase domain; and glutamine (Gln) 171 of the linker with arginine (Arg) 230 of the OB domain (Figure 3.6D).

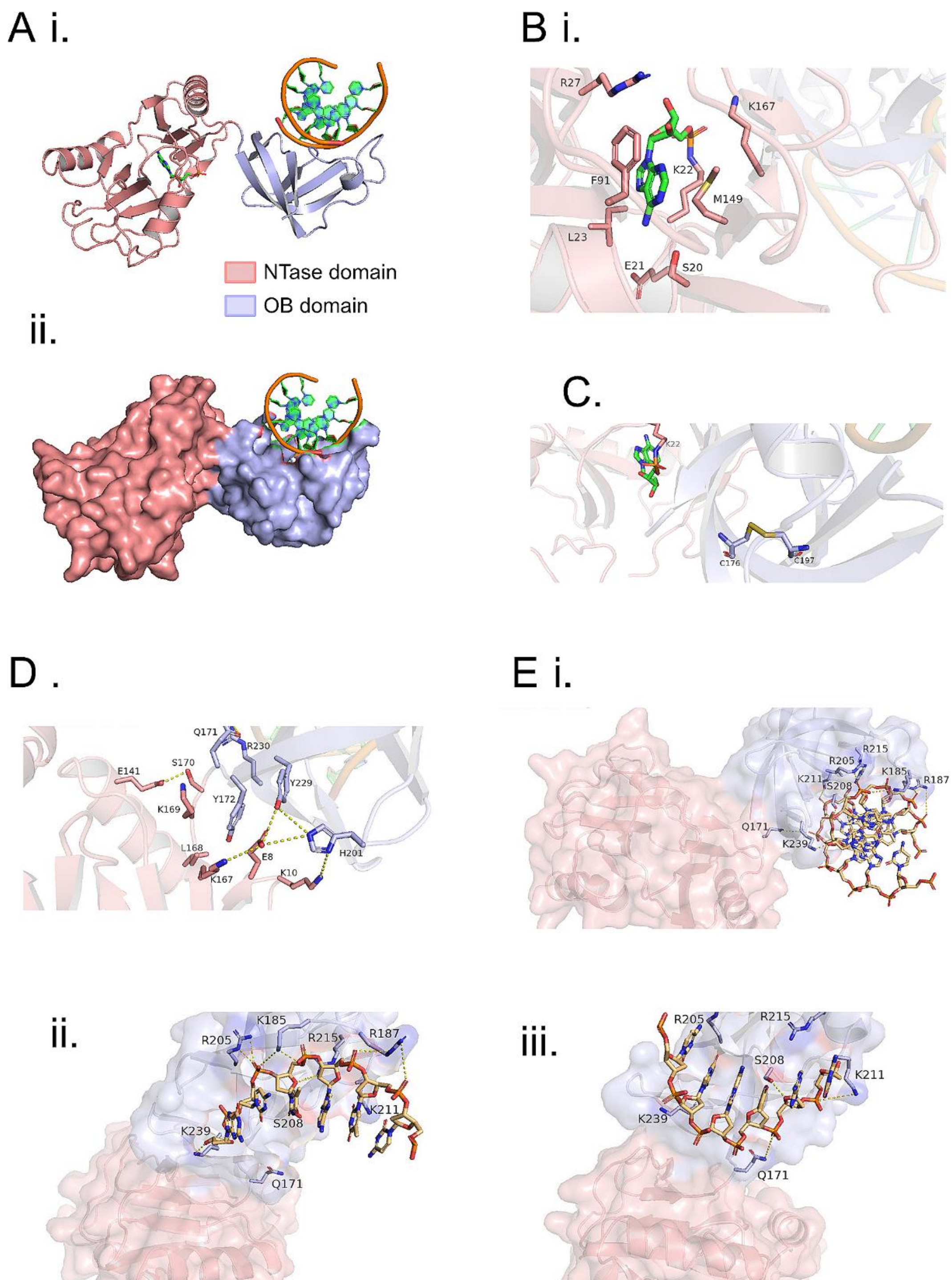

Figure 3.6 Structure of Ngo-Lig E. (A) Overall structure of DNA-engaged Ngo-Lig E coloured by domain shown as a (i) cartoon or (ii) as a surface. (B) View of the NTase domain active site including the adenylated catalytic lysine (K) residue. (C) Position of the disulphide bond in the oligonucleotide-binding domain. (D) Inter-domain interactions surrounding the linker. Electrostatic and polar interactions less than 5Å are indicated by dashed yellow lines. (E) (i) Interaction of Ngo-Lig E with the DNA duplex, (ii) specific interactions between conserved residues and the 'complement' strand of the duplex, (iii) specific interactions between residues of the OB domain and the equivalent of the 'nicked' strand where the DNA break is located in other DNA-bound ligase structures.

Despite the use of a 21-mer DNA fragment in the crystallisation condition, only 6 bps were visible in the final structure. Examination of crystal packing (Figure A. 4) shows that the DNA forms a continuous filament throughout the crystal with protein subunits arranged along it. We suspect that in the absence of the specific interactions imparted by nick-binding from the NTase domain, the Ngo-Lig E subunits have assembled non-specifically along the DNA filament; thus the 6 bp fragment in our structure represents a sample of the entire 21-mer piece. Key interactions with the DNA primarily involve basic residues from the OB domain and linker including Lys 239, Arg 205, Lys 185, Arg 215, Lys 211 and Arg 187. These residues contact the backbone phosphates of the 'complement' strand which is base-paired opposite the nicked strand in other ligase-DNA structures (Figure 3.6E ii.). There are fewer interactions with the 'nick' strand with the only contacts being Lys 239, Gln 171 and Ser 208 (Figure 3.6E iii.). The electron density is continuous in this region of the phosphodiester backbone which is consistent with the ligase engaging an unbroken section of the DNA in a non-specific manner (Figure A. 5 B). The nucleobases that are modelled in the structure represent the 6-mer combination giving the lowest R-free value during refinement and are in a region of the original 21-mer substrate before the nick; however, the density between base pairs is more symmetrically-distributed than would be expected for a well-ordered purine-pyrimidine pair, again supporting our suggestion that we have sampled an average of the 21-mer sequence in the present structure (Figure A. 1 C).

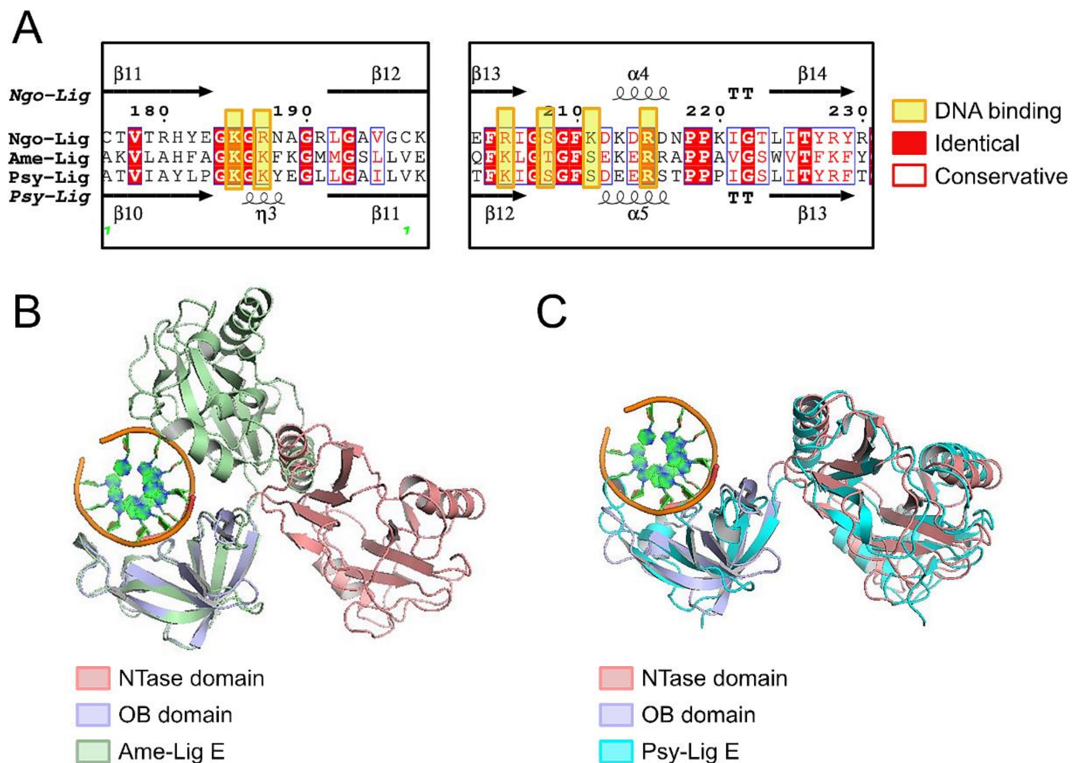

Figure 3.7 Comparisons of Ngo-Lig E to other solved structures of Lig E. **(A) Sequence alignment of other Lig E proteins that have been structurally characterised from *Alteromonas mediterranea* (Ame-Lig E) and *Psychromonas* sp. SP041 (Psy-Lig E). (B) Ngo-Lig E and Ame-Lig E structures superimposed by alignment of the OB domains. (C) Ngo-Lig E and Psy-Lig E structures superimposed by alignment of the OB domains. DNA shown is from the Ngo-Lig E structure.**

Comparison of the Ngo-Lig E structure with Lig E from *Alteromonas mediterranea* (Ame-Lig E) in a DNA-bound closed conformation shows the same conserved OB-domain residues are involved in DNA binding, despite the differences in overall conformation (Figure 3.7A and B). Meanwhile superposition of Ngo-Lig E with Lig E from *Psychromonas* sp. SP041 (Psy-Lig E) which was crystalised in the open-state shows that the domains of both proteins are in an identical configuration, despite the absence of DNA in the latter (Figure 3.7C).

3.6 Discussion

Despite extensive *in vitro* characterisation of Lig E from a range of pathogenic and environmental Gram-negative bacteria (Cheng & Shuman, 1997; Magnet & Blanchard, 2004; Williamson & Pedersen, 2014; Williamson *et al.*, 2014; Williamson *et al.*, 2016; Williamson *et al.*, 2018; Berg *et al.*, 2019) and the availability of structures of Lig E in both DNA-bound and DNA-free states (Williamson *et al.*, 2014; Williamson *et al.*, 2018) little is known about its

biological function. Here we have examined the effect of knocking out *lig E* in *N. gonorrhoeae*, or *ngo-lig E*, as well as the impact of inserting a second copy of *ngo-lig E* under the control of the strong constitutive P_{opaB} promoter. Our results demonstrate that neither deletion nor upregulation of *ngo-lig E* impacts *N. gonorrhoeae* survival in liquid culture, or its planktonic growth rate. The dispensability of Ngo-Lig E under these conditions is counter to earlier studies which ascribed the inability to generate a viable deletion in *Haemophilus influenzae* to *lig E* being an essential gene (Cheng & Shuman, 1997). Although the reason for this discrepancy is not clear, all bacterial genomes identified as possessing *lig E* also include the canonical NAD^+-dependent *lig A* DNA ligase responsible for chromosomal replication and most highly-conserved DNA repair pathways, arguing for a non-essential function for *lig E* (Williamson *et al.*, 2016).

Similarly, Ngo-Lig E does not appear to mitigate the effects of oxidative stress and UV irradiation, both of which are anticipated to damage chromosomal DNA. Hydrogen peroxide treatment is a physiologically-relevant stressor as reactive oxygen species are produced during the host inflammatory response and by commensal *Neisseria* in the urogenital tract (Seib *et al.*, 2006). Although UV exposure is not a natural source of damage in the urogenital mucous membranes that *N. gonorrhoeae* inhabits, this serves as a convenient proxy for generic DNA damage. The lack of its participation in DNA-damage survival is consistent with the predicted periplasmic location of Ngo-Lig E based on its N-terminal leader sequence, indicating that its primary substrate is not breaks or damages in chromosomal DNA. The enhanced survival of the *ngo lig E* knock-out to treatment with nalidixic acid relative to the wild-type is somewhat counterintuitive; however, an equivalent but even starker example of this has been reported with knock-outs of the *phrB* deoxyribodipyrimidine photolyase ortholog which modulates DNA supercoiling (Cahoon *et al.*, 2011). There, the striking 7,000-fold increased survival of nalidixic acid treatment was ascribed to mis-regulation of DNA topology by functional PhrB in the presence of DNA breaks caused by the treatment. In the case of the *ngo-lig E* deletion, we propose that improper recruitment of functional Ngo-Lig E to nalidixic acid-induced DNA breaks may interfere with the resolution of this damage by canonical repair mechanisms. Previous *in vitro* studies have demonstrated that Lig E has a high affinity for nicked double-stranded DNA and we anticipate that Ngo-Lig E will bind tightly to the doubly-nicked chromosomal

DNA caused by nalidixic acid-poisoning of the gyrase B protein (Williamson *et al.*, 2018). The difference in the effect of nalidixic acid compared with UV and H_2O_2 may be due to their modes of action. UV and H_2O_2 typically impact the nitrogenous bases by oxidation or generation of dimerised photoproducts, but do not directly cause breaks in the phosphodiester backbone and therefore would not provide high-affinity binding sites for Lig E prior to initiation of repair pathways (Cadet & Wagner, 2013).

Appending a 6-His-tag to the C-terminus of native Ngo-Lig E in the *N. gonorrhoeae* chromosome had essentially no impact on its growth or survival characteristics, suggesting that neither the His-tag nor the kanamycin selection cassette were deleterious to the strains. The slightly elevated levels of nick-sealing activity in the recombinant His-tagged enzyme relative to its untagged counterpart may be due to the increased positive charge in the C-terminal OB domain enhancing the enzyme's interaction with the DNA substrate, or from minor differences in the purification protocol. It is not entirely clear why the His-tagged Ngo-Lig E expressed under P_{opaB} was unable to be detected immunologically in gonococcal cells despite being transcribed at elevated levels as demonstrated by qPCR. Other groups have cited similar difficulties in detection of extracellular/periplasmic gonococcal proteins using immunological and enzymatic methods (Steichen *et al.*, 2011), which they ascribed potentially being due to dispersion of the enzyme into the culture supernatant. It is possible that our His-tagged Ngo-Lig E is likewise diluted to undetectable levels in the culture supernatant.

On the basis of Ngo-Lig E's predicted periplasmic/extracellular location, we investigated its impact on biofilm formation. ExDNA is an important structural component of gonococcal biofilms and is derived from the *N. gonorrhoeae* chromosome, either secreted through the T4SS system or released by autolysis (Greiner *et al.*, 2005; Falsetta *et al.*, 2011). The role of the extracellular thermonuclease, Nuc, is well established in modulating the properties of gonococcal biofilms, with deletion of this gene resulting in thicker and higher biofilm biomass as well as the inability to escape chromatin-rich neutrophil extracellular traps (NETs) (Steichen *et al.*, 2011; Juneau *et al.*, 2015). Our results, which indicate that the *ngo-lig E* knock-out produces less biofilm, suggest that Ngo-Lig E acts in opposition to Nuc, potentially linking double-stranded segments of

DNA. Although Ngo-Lig E is incapable of joining blunt-end double-stranded breaks, it demonstrated robust cohesive end joining as well as some activity on mismatched breaks, indicating that it could potentially link regions of microhomology. In addition to decreased biofilm production, our results indicate that the *ngo-lig E* knock-out is less effective at adhering to epithelial cells, although it is not impaired in cell invasion. The cell adhesion process of *N. gonorrhoeae* involves microcolony attachment to the host cell surface via the Type IV pilus, followed by interactions between *N. gonorrhoeae* opacity (Opa) proteins, epithelial receptors and other surface molecules (Winther-Larsen *et al.*, 2001; Higashi *et al.*, 2007; LeVan *et al.*, 2012; Quillin & Seifert, 2018; Walker *et al.*, 2023). Although the rationale for Ngo-Lig E impacting cell adhesion is not clear, this could also be related to defects in biofilm formation as biofilm is suggested to play a role in cell-surface colonisation (Greiner *et al.*, 2005; Falsetta *et al.*, 2011).

An emerging area of research in *N. gonorrhoeae* is the impact of biofilm architecture on dissemination of antibiotic resistance genes. In particular, the age and density of biofilm modulates the horizontal transfer of genes with more rapid spread observed in early biofilm and decreased dispersal in mature biofilms (Kouzel *et al.*, 2015). Biofilms are also considered to act as potential exDNA reservoirs as the mobility of DNA fragments through this matrix is hindered by increasing DNA length and the presence of DUS, leading to accumulation of *N. gonorrhoeae*-specific exDNA (Bender *et al.*, 2022). In light of this, the potential of Ngo-Lig E to modify the mobility of transformable exDNA by modulating biofilm properties, or potentially by acting on the transformation substrate itself by increasing its length or rejoining nicks resulting from nuclease or oxidative damage, is of considerable interest. One of the earliest studies of recombinant Lig E from *Neisseria meningitidis* suggested a possible function in competence for Lig E based on its periplasmic localisation signal and it has been noted that the majority of Lig E-possessing bacteria are known to be competent in natural transformation and/or encode essential competence genes in their genomes (Magnet & Blanchard, 2004; Williamson *et al.*, 2016). Immunological detection in the present study via an introduced His-tag was insufficient to define the extracellular or periplasmic distribution, however it is feasible that double-stranded DNA could serve as a substrate for periplasmically-localised Ngo-Lig E prior to import across the plasma membrane.

Finally, our DNA-engaged open-conformation structure of Ngo-Lig E reveals the specific interactions made between the OB domain and the DNA backbone, which are independent of sequence or phosphodiester-backbone continuity. Ngo-Lig E, like other members of this group of DNA ligases, lacks any additional DNA binding domains or 'latch' regions such as those found in *Chlorella* virus-type ligases which allow the enzyme to encircle the DNA duplex. Instead, Lig E ligases rely on the well-structured positively-charged surface of the OB domain for affinity. The conformation of the Ngo-Lig E enzyme-adenylate is consistent with the scanning mechanism previously proposed for locating breaks in the duplex (Bauer *et al.*, 2017). Here, non-specific interactions rapidly interrogate the substrate, and upon encountering a discontinuity in the duplex, the ligase-adenylate re-orients its core domains about the linker region to position the active site for subsequent catalysis. The Ngo-Lig E structure suggests that in these minimal ligases, the OB domain is responsible for localising the enzyme to DNA and conformational scanning, prior to productive binding.

3.7 Conclusions

Lig E is widely distributed among Beta-, Epsilon- and Gammaproteobacteria including some of the most prevalent human and agricultural pathogens, several of which are considered high-priority due to their emerging multi-antibiotic resistance. Our demonstration of a physiological role for Lig E in *N. gonorrhoeae* biofilm formation and cell adhesion recommends this enzyme for further study to understand its impact on virulence and pathogenicity, as well as potential roles among commensal and environmental proteobacteria. Future directions will include more detailed phenotypic studies of the impact of *ngo-lig E* deletion on biofilm architecture and its interplay with other biofilm-modulating enzymes and processes. There is also the outstanding issue of the specific extracellular location of Ngo-Lig E, as well as exploration of a potential role in the uptake and transformation of exDNA which we anticipate will provide a more extensive picture of the biological function of this enigmatic DNA ligase protein.

3.8 Data availability

Sequencing data have been deposited with the identifier PRJNA1051170. Structure coordinates for the Ngo-Lig E protein-DNA complex have been deposited to the PDB with the identifier 8U6X.

3.9 Supplementary

Supplementary tables and figures supporting this manuscript can be found in Appendix A.

3.10 References

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4 Chapter Four

Influence of the ATP-Dependent DNA Ligase, Lig E, on *Neisseria gonorrhoeae* Microcolony and Biofilm Formation

4.1 Preface

In the manuscript presented in Chapter Three, I reported on phenotypic characterisations of *N. gonorrhoeae* strains where *ngo-lig E* was deleted, which led to the discovery of Ngo-Lig E's importance in biofilm formation, a feature that is often implicated in disease caused by many human pathogens. However, this phenotype was determined via a relatively crude end-point crystal violet assay. In Chapter Four, I further explore the likely involvement of Ngo-Lig E in biofilm formation via confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). This involved growing the bacteria under constant shear forces and fresh media in CDC Biofilm Reactors® in the laboratory of our collaborators at the Cardiff University School of Dentistry, which was followed by visualisation via CLSM at the Cardiff University School of Biosciences. Infection of reconstituted three-dimensional human vaginal epithelial cells with the gonococcal variants was also conducted to determine if any defects in biofilm formation would affect the infectivity and pathogenicity of *N. gonorrhoeae*.

The research in this chapter supports my prior findings and is presented as a manuscript that has been submitted in a peer-reviewed journal (*Biofilm*, manuscript number: BIOFLM-D-25-00038) and is awaiting review. This manuscript explores the potential role of Ngo-Lig E in biofilm formation in *N. gonorrhoeae* and hence addresses **Objective Three** (see Chapter One). A preprint of this manuscript has also been deposited on *bioRxiv* (**doi:** https://doi.org/10.1101/2025.02.17.638724). Supplementary material associated with this work can be found in Appendix B:

Pan, J., Albarrak, A., Hicks, J., Williams, D., & Williamson, A. (2025). Influence of the ATP-dependent DNA ligase, Lig E, on *Neisseria gonorrhoeae* microcolony and biofilm formation. (*Submitted*).

4.1.1 Author contributions and acknowledgements

As first author, I led all the experimental work including the construction, growth, biofilm formation in CDC Biofilm Reactors®, rHVE infection and confocal visualisation of the *N. gonorrhoeae* mutants. Abdullah Albarrak provided expertise and guidance and assisted with setting up the CDC Biofilm Reactors®, rHVE infections, as well as confocal imaging of the coupons and tissue slides. The study and experimental design of the work was conceptualised by me, our collaborator from Cardiff University David Williams, my co-supervisor Joanna Hicks, and my Chief supervisor Adele Williamson, who all provided guidance with the research. The co-authorship form for this manuscript can be found in Appendix D.

Biofilm research and confocal imaging in this manuscript was undertaken at the School of Dentistry and School of Biosciences at Cardiff University.

Influence of the ATP-dependent DNA ligase, Lig E, on *Neisseria gonorrhoeae* microcolony and biofilm formation

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4.2 Abstract

Background

Neisseria gonorrhoeae, the causative agent for the sexually transmitted infection, gonorrhoea, is known to form biofilms rich in extracellular DNA on human cervical cells. Biofilm formation is conducive to increased antimicrobial resistance and evasion of the host immune system, potentially causing asymptomatic infections. Using plate-based assays, we have previously shown that the disruption of a potential extracellular DNA ligase, Lig E, in *N. gonorrhoeae* (Ngo-Lig E) impacts biofilm formation. In this research, we further explored this phenotype using confocal and scanning electron microscopy to directly visualise the morphology of microcolonies and biofilm formation. Biofilm growth on artificial surfaces and on three-dimensional human vaginal epithelial tissue was evaluated for strains where *ngo-lig E* was either disrupted or overexpressed.

Results

Results demonstrated that Ngo-Lig E is important for the formation of robust, compact *N. gonorrhoeae* microcolonies, as well as extensive biofilms on artificial surfaces. The *ngo-lig E* deletion strain also had the highest tendency to be retained on the surface of epithelial tissues, with decreased invasion and damage to the host cell layers.

Conclusions

These findings support a role for Ngo-Lig E to be secreted from *N. gonorrhoeae* cells for the purpose of inter-cell adhesion and biofilm formation. We suggest that

Ngo-Lig E strengthens the extracellular matrix and hence microcolony and biofilm formation of *N. gonorrhoeae* by ligation of extracellular DNA.

4.3 Introduction

Neisseria gonorrhoeae is a Gram-negative diplococcus bacterium responsible for the sexually transmitted infection (STI), gonorrhoea. With the World Health Organization estimating 106 million new cases each year, gonorrhoea is the second most common STI in the world (Unemo & Shafer, 2014; World Health Organization, 2024). Infections occur in the mucosal epithelial cells of the urogenital tract, causing inflammation that presents as pain with urination in men (urethritis) and abnormal bleeding and pain in women (cervicitis) (Edwards & Apicella, 2004; Tapsall, 2005). Additionally, infections in women may spread to the upper reproductive tract, causing pelvic inflammatory disease, as well as ectopic pregnancy or infertility (McCormack, 1981). If left untreated, infections in pregnant women may also lead to neonatal conjunctivitis and blindness in the newborn (McCormack, 1981; Dillard, 2011). In females, a high proportion of *N. gonorrhoeae* infections are asymptomatic (≥50%), allowing the bacterium to spread undetected in the community (McCormack, 1981; Unemo & Shafer, 2014). This trait is often attributed to the bacterium's ability to readily form biofilms, which aid in oxidative stress survival, attachment to surfaces and evasion from the host immune system (Greiner *et al.*, 2005; Falsetta *et al.*, 2009).

Interestingly, *N. gonorrhoeae* lacks the genes necessary to produce exopolysaccharides that contribute to biofilm architecture in other bacterial species (Greiner *et al.*, 2005). Instead, *N. gonorrhoeae* utilises extracellular DNA (exDNA) as its major matrix component which provides structural integrity to the biofilm (Steichen *et al.*, 2011). This exDNA may originate from the frequent autolysis that occurs in *N. gonorrhoeae*, or from active secretion of DNA via the type IV secretion system (T4SS) (Morse & Bartenstein, 1974; Hebeler & Young, 1975; Elmros *et al.*, 1976; Zola *et al.*, 2010; Zweig *et al.*, 2014). In addition, the membranous extensions or blebs of the outer membrane that are extruded during gonococcal biofilm formation often harbour DNA (Dorward *et al.*, 1989; Greiner *et al.*, 2005; Steichen *et al.*, 2008). The abundance of exDNA in the biofilm has the potential to act as a pool for gene exchange and acquisition of new antibiotic resistance genes (Kouzel

et al., 2015); however, the extent of DNA diffusion through established gonococcal biofilm is modulated by its maturity and density, which may limit horizontal gene transfer by this mechanism (Kouzel *et al.*, 2015; Bender *et al.*, 2022).

The DNA component of gonococcal biofilms is enzymatically remodelled by a secreted thermonuclease, Nuc, that degrades exDNA in the biofilm matrix, as well neutrophil extracellular traps (NETs), the latter aiding in bacterial escape from NET killing (Steichen *et al.*, 2011; Juneau *et al.*, 2015). However in addition to this nuclease, *N. gonorrhoeae* also encodes a minimal ATP-dependent DNA ligase, Lig E, which like Nuc, possesses an N-terminal signal peptide that is predicted to direct its extracellular secretion. Removal of this signal sequence has been shown to increase both stability and activity of recombinantly-expressed Lig E, which promotes the view that it is the cleaved isoform that represents the biologically-relevant mature protein. Lig E is encoded in the genomes of many Gram-negative bacteria without any syntenic organisation or consistent co-localisation with other genes (Williamson *et al.*, 2016; Pan *et al.*, 2021). This, together with the presence of the N-terminal signal sequence, suggests a function of Lig E other than chromosomal DNA repair and that it may act on exDNA (Magnet & Blanchard, 2004; Williamson *et al.*, 2014). Consistent with this is the fact that Lig E is found in many biofilm-forming and competent proteobacteria like *N. gonorrhoeae* (Williamson *et al.*, 2016). Recently, we reported that deletion of *lig E* from *N. gonorrhoeae* (*ngo-lig E*) negatively impacted the extent of biofilm formation when measured via an indirect crystal violet assay, as well as impacting *N. gonorrhoeae* adhesion to host human cervical cells (Pan *et al.*, 2024) (Chapter Three).

In the present study, we further explore this phenotype, visualising the biofilms formed by *ngo-lig E* deletion and overexpressing strains of *N. gonorrhoeae* using microscopy. We provide the first reported use of the Centre for Disease Control (CDC) Biofilm Reactor® (BioSurface Technologies) to generate constant shear forces during growth of *N. gonorrhoeae* biofilms in conjunction with confocal laser scanning microscopy (CLSM). We also used commercially available three-dimensional (3-D) reconstructed human vaginal epithelium (rHVE) (SkinEthic Laboratories) in lieu of traditional two-dimensional (2-D) cell lines for host-cell assays. Such reconstructed epithelial models account for different cell morphologies, tissue architecture and differentiation that occur during normal

microbial infection *in vivo* (Malfa *et al.*, 2023). Use of these approaches demonstrated the potential importance of Ngo-Lig E on *N. gonorrhoeae* microcolony and biofilm formation, and allowed us to quantify the damage conducted by the bacterium onto human tissue.

4.4 Methods

4.4.1 *Neisseria gonorrhoeae* manipulation

All *N. gonorrhoeae* used in this study were of the MS11 strain (GenBank: CP003909.1). Gonococci were grown at 37°C with 5% CO_2 either on gonococcal base (GCB) agar (Difco™) or in gonococcal base liquid (GCBL) (15 g/L Bacto™ Protease Peptone No. 3, 4 g/L K_2HPO_4, 1g/L KH_2PO_4, 1 g/L NaCl), both supplemented with 1% *Kellogg's* supplement (22.22 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM $Fe(NO_3)_3$) (Dillard, 2011). Liquid growth was supplemented with sodium bicarbonate (0.042%), while solid growth was maintained in a 5% CO_2 atmosphere. Piliation status was determined by morphology under a dissecting microscope at the start of each experiment.

The *Δnuc*^{kan} mutant (Table 4.1) was generated via spot transformation in the same manner as the previously-described mutants used in this study (Dillard, 2011; Pan *et al.*, 2024). Briefly, the transforming DNA construct was ordered as a gene fragment from Twist Biosciences with flanking sequences surrounding the *nuc* site to facilitate homologous recombination. Piliated, Opa negative (Opa-) colonies were streaked through 10 ng spots of the DNA construct. Mutants were selected on GCB agar with 50 µg/mL kanamycin before verification via PCR and sequencing.

The generation of other strains used in this study (Table 4.1) has been reported previously (Pan *et al.*, 2024). As described, the *Δngo-lig E*^{kan} and *wt* genomes were previously re-sequenced to confirm that there were no significant differences between the two genomes apart from the disruption of *ngo-lig E* (Pan *et al.*, 2024).

Table 4.1 List of *Neisseria gonorrhoeae* MS11 mutants used in this study (GenBank: CP003909.1).

Mutant	Insertion	Purpose	Source
Δngo-lig E^{kan}	Kanamycin resistance cassette disrupting the *ngo-lig E* gene (NGFG_RS11310).	*ngo-lig E* knock-out mutant.	(Pan *et al.*, 2024)
ngo-lig E-his^{kan}	6-His-tag and a kanamycin resistance cassette inserted at the C-terminus of *ngo-lig E*.	Control for the insertion of the kanamycin resistance cassette in the *Δngo-Lig E*^{kan} mutant, with the His-tag serving as an epitope tag.	(Pan *et al.*, 2024)
P_{opaB}-*ngo-lig E-his*^{kan}	Codon-optimised *ngo-lig E* gene under the constitutive *opaB* promoter inserted in a neutral site in the genome (NGFG_RS15145, annotated as a phage protein).	*ngo-lig E* under a strong constitutive promoter in a neutral site in the genome to observe the effects of overexpression of Ngo-Lig E.	(Pan *et al.*, 2024)
Δnuc^{kan}	Kanamycin resistance cassette interrupting the *nuc* (thermonuclease) gene, NGFG_RS05400, as outlined by Steichen *et al.*, 2011.	Positive control for increased biofilm formation in *N. gonorrhoeae* for scanning electron microscopy.	This work, based on a previous publication (Steichen *et al.*, 2011)

4.4.2 Biofilm formation in CDC Biofilm Reactors®

To facilitate imaging via confocal microscopy, *wt N. gonorrhoeae* and the *ngo-lig E* mutants (Table 4.1) were each transformed with the pEG2 cryptic plasmid (Christodoulides *et al.*, 2000) which was kindly gifted to us by the Radcliff laboratory (University of Auckland). The pEG2 plasmid contains an *sfGFP* gene under a *porA* promoter and was introduced to the MS11 variants via spot transformation with selection via erythromycin (10 µg/mL). Successful transformants were verified by fluorescence of the sfGFP under blue light, and the plasmids were continuously maintained in *N. gonorrhoeae* by addition of erythromycin to the culture media.

The pEG2 transformants of the *N. gonorrhoeae* variants were streaked and cultured for 24 h on GCB plates with erythromycin. Piliated bacteria were then lawned and cultured for 16 h on chocolate agar before resuspension in GCBL. For each mutant, a 1 mL suspension of an OD_{600} of 0.05 was used to inoculate media for growth in CBR 90 Standard CDC Biofilm Reactors® (Biosurface Technologies) which were assembled as per the manufacturer's protocol (Figure 4.1). Briefly, polycarbonate coupons (diameter: 12.7 mm; thickness: 3.8 mm) were fitted into vertical polypropylene rods in the biofilm reactor. The 1 mL inoculum was introduced via the inlet port into the vessel containing GCBL with erythromycin and sodium bicarbonate (333 mL). Batch growth with stirring was performed for 6 h before continuous flow with sterile GCBL at 0.6 mL per min for 16-17 h. The coupons were then extracted from the rods and rinsed twice in water before CLSM.

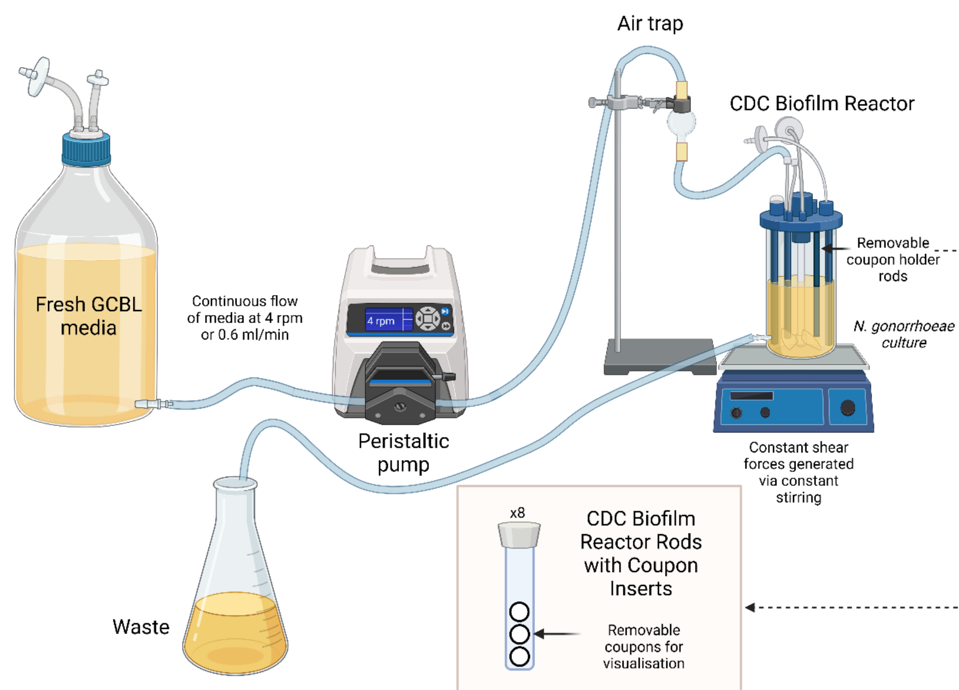


Figure 4.1. Schematic of the set up used to generate *Neisseria gonorrhoeae* biofilms using CBR 90 Standard CDC Biofilm Reactors® (Biosurface Technologies). Created in https://BioRender.com.

4.4.3 Infection of reconstructed human vaginal epithelium (rHVE)

SkinEthic™ HVE tissue (0.5 cm^2, age day 5; HVE/S/5) were ordered from Episkin (Lyon, France). These are vulval epidermoid carcinoma A431 cells seeded onto a polycarbonate filter in inserts and maintained at the air-liquid interface (Figure 4.2). Upon receipt, cells were equilibrated with the SkinEthic™ Maintenance Medium (Episkin (Lyon, France)) for 4 h in 12-well plates in a humidified chamber (37°C, 5% CO_2), before replacement with fresh media (1 mL). *N. gonorrhoeae* pEG2 cultures prepared in the same medium (100 µL) were inoculated onto the tissue at 1x10^8 CFU per cm^2 and left for 16-17 h in a humidified chamber (37°C, 5% CO_2). The supernatant under each tissue insert was recovered and used for lactase dehydrogenase (LDH) activity assays, while the cell inserts were washed twice with phosphate-buffered saline (PBS). The tissue and their membranes were then isolated from the inserts for microscopic imaging.

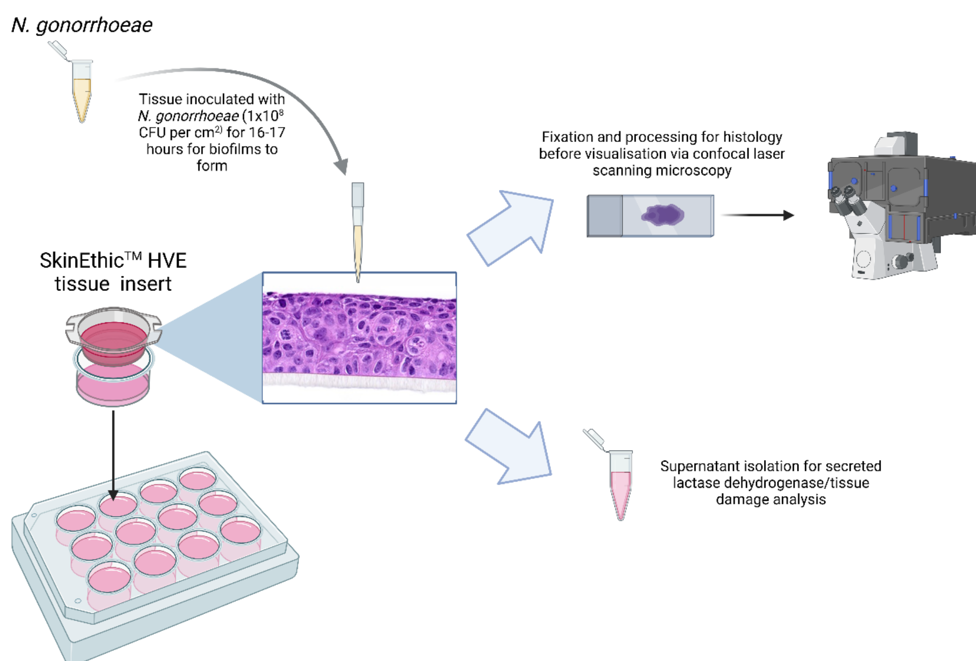


Figure 4.2. Schematic of the infection work-flow with the SkinEthic™ HVE tissue model obtained from Episkin (Lyon, France). Created in https://BioRender.com. Image of HVE cells obtained from https://www.episkin.com/HVE-Vaginal-Epithelium.

4.4.4 Histological techniques

The isolated cells and membranes were individually wrapped in Surgipath® Bio-Wraps™ (Leica Biosystems) and placed in cassettes before soaking in Reagecon Formal Buffered Saline for 3 h. Dehydration was performed using the LeicaASP300S Fully Enclosed Tissue Processor (90% v/v ethanol (1 h), 95% v/v ethanol (1 h), 100% v/v ethanol (4x1 h), xylene (3x1 h)). The cassettes were embedded in Surgipath® Formula 'R' paraffin wax (Leica Biosystems) using the Leica EG1150 Modular Tissue Embedding Center. Transverse sectioning was performed at the BioImaging Hub at the School of Biosciences at Cardiff University to obtain 20 µm sections on microscope slides. Wax was removed with xylene (5 min) before washes with 70% v/v ethanol (5 min) and 100% v/v ethanol (5 min), followed by rehydration in water (5 min). VECTASHIELD® Antifade Mounting Medium with DAPI (H-1200-10, Vector Laboratories) (20 µL) was added onto the sections before visualisation using CLSM.

4.4.5 Confocal laser scanning microscopy (CLSM)

CLSM was conducted at the Cardiff University Bioimaging Hub Core Facility (RRID:SCR_022556). Biofilms grown on polycarbonate coupons were visualised using the Zeiss Cell Discoverer 7 microscope at x40 magnification using the suGFP channel (laser excitation (exc) wavelength: 480 nm, laser emission wavelength (em): 505 nm) to image the sfGFP-expressing gonococcal cells. Infected rHVE tissues were visualised using the Zeiss LSM 880 with Airyscan microscope at x63 magnification. rHVE nuclei were visualised using the DAPI channel (exc: 405 nm, em: 449 nm) and sfGFP-expressing *N. gonorrhoeae* were visualised using the GFP channel (exc: 488 nm, em: 519 nm). Five random fields-of-view (z-stacks) were obtained for each slide/coupon.

Images were analysed using COMSTAT 2.1 (Heydorn *et al.*, 2000; Vorregaard, 2008) in an OME-TIFF format (Otsu thresholding). Quantified parameters included biovolume/biomass (volume over area, $\mu m^3/\mu m^2$), average thickness (biomass) (height distribution of biomass-containing columns, µm), average thickness (entire area) (height distribution of the biofilm for the entire observed area including empty columns, µm), maximum thickness (highest point of the biofilm ignoring empty voxels, µm), surface to biovolume ratio of the biofilms (total surface facing the void over biovolume, $\mu m^2/\mu m^3$) and dimensionless roughness coefficient, R_a^* (variability in biofilm height; $R_a^* = \frac{1}{N}\sum_i^N \frac{|L_{fi} - \overline{L_f}|}{\overline{L_f}}$ where N = number of measurements, L_{fi} = i'th individual thickness measurement and $\overline{L_f}$ = average thickness) (Nowicki, 1985; Murga *et al.*, 1995; Heydorn *et al.*, 2000; Vorregaard, 2008).

To ensure consistency in acquisition of parameters, all confocal images using the Zeiss LSM 880 with Airyscan microscope were acquired at the same zoom setting (zoom setting 3), which in the infection experiments, focused on the biofilm layer at the surface of the rHVE tissue. As described below, differences in tissue thickness were observed after inoculation with different strains, meaning that the lower membrane was not visible in some fields-of-view. To account for this difference, the depth of *N. gonorrhoeae* invasion was normalised relative to the depth of remaining tissue in the same field-of view.

4.4.6 Scanning electron microscopy (SEM)

N. gonorrhoeae variants were lawned on GCB agar for 16 h before resuspension in GCBL. The bacteria were seeded into 12-well plates with a starting OD_{600} of 0.05 and onto 0.2 µM pore size filter papers. After 9 h (exponential phase, based on growth experiment from Pan *et al*., 2024) the medium was removed and the bacteria were fixed overnight in 2.5% glutaraldehyde. The bacteria were washed (x4) in 0.1 M sodium cacodylate and distilled water before successive dehydration with 50% ethanol (1 h), 75% ethanol (1 h), 95% ethanol and four rinses with 100% ethanol. The critical point drying process and coating with platinum (5 nm) was performed by the Electron Microscope Facility at the University of Waikato. Images were obtained via the Hitachi SU8230 microscope (3 kV acceleration). Three fields-of-view were taken for each sample. The area of microcolonies formed (μm^2) was quantified via ImageJ (Schneider *et al*., 2012).

4.4.7 Lactase dehydrogenase (LDH) activity assay

LDH quantification was performed on the isolated rHVE supernatant after *N. gonorrhoeae* infection using the CyQUANT™ LDH Cytotoxicity Assay kit as per the manufacturer's instructions. Absorbances at 490 and 680 nm were measured and the background absorbance at 680 nm was subtracted from that at 490 nm.

4.4.8 Statistical methods

Statistical analyses were preformed using the GraphPad Prism 9.4.0 software (https://www.graphpad.com/). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to compare the different measurements and p values < 0.05 were deemed statistically significant.

4.5 Results

4.5.1 Adhesion and biofilm formation of *Neisseria gonorrhoeae* on polycarbonate surfaces is dependent on Ngo-Lig E

To determine if Ngo-Lig E impacted the ability of *N. gonorrhoeae* to adhere to and to form biofilms on abiotic surfaces, their growth on polycarbonate coupons after 16-17 h cultivation in CDC Biofilm Reactors® under constant shear forces was studied. Confocal images of the coupons (Figure 4.3 and Figure B. 1) indicated that

N. gonorrhoeae had relatively low adherence to the polycarbonate surfaces compared to other organisms that we have studied using a similar set-up, such as *Candida albicans* and *Enterococcus faecalis* (Nassar *et al.*, 2023).

Despite this, CLSM images of the coupons after growth showed a clear decrease in the ability of the *Δngo-lig E*^{kan} mutant to attach to surfaces and to form extensive or continuous biofilms compared to *wt N. gonorrhoeae* (Figure 4.3A and Figure B. 1). Conversely, biofilms formed when *ngo-lig E* was overexpressed (P_{opaB}-*ngo-lig E-his*^{kan}) were more extensive across the surface, while *wt* and *ngo-lig E-his*^{kan} seemed to extend similarly to each other. Quantification via COMSTAT analysis showed no significant differences in the total biomass among the different gonococcal variants (volume per area, Figure 4.3B). However, there was a significant reduction in both the thickness of the entire area of growth (indicative of spatial size of the biofilm across the entire area, (Figure 4.3C)) and the thickness of the biomass (thickness distribution of only biomass-containing columns, Figure 4.3D) when *ngo-lig E* was disrupted compared to *wt* and *ngo-lig E-his*^{kan}. Although the overexpressing P_{opaB}-*ngo-lig E-his*^{kan} mutant had a significantly higher average biomass thickness compared to the other three variants (Figure 4.3D), its average thickness or spatial spread over the entire area was similar to that of *wt N. gonorrhoeae* (Figure 4.3C), while also displaying higher overall maximum biofilm thickness (highest point of the biofilm, Figure 4.3E) and lower surface:biovolume ratio (ratio of total surface facing the void over biovolume, Figure 4.3F) than the other variants. Furthermore, the dimensionless roughness coefficient indicated slightly higher roughness or variability in the height of the biofilms formed by the P_{opaB}-*ngo-lig E-his*^{kan} mutant compared to the other *N. gonorrhoeae* variants (Figure 4.3G).

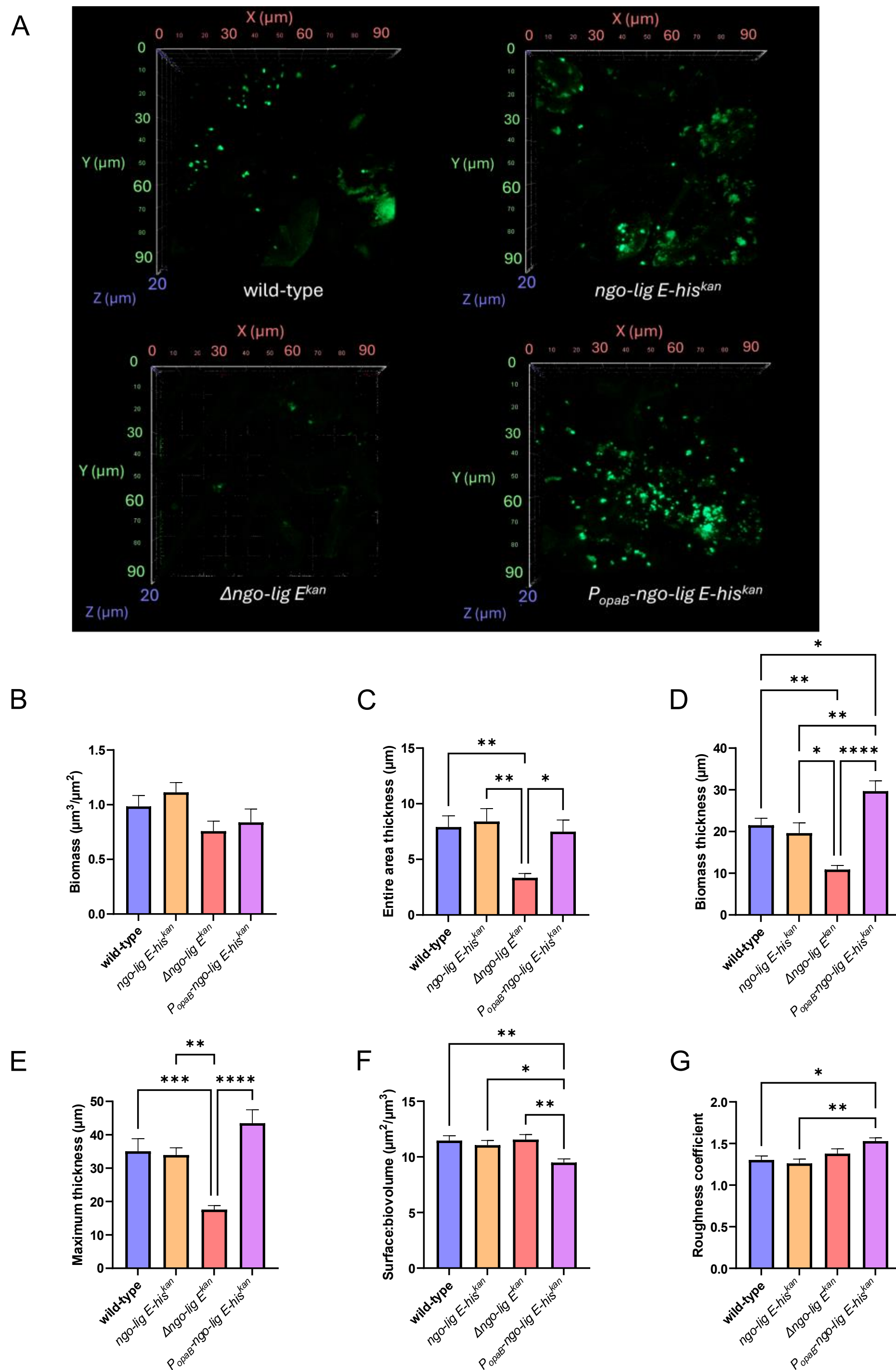


Figure 4.3. Biofilm formation and adhesion of *Neisseria gonorrhoeae* pEG2 (expressing sfGFP) on polycarbonate coupons in CDC Biofilm Reactors®. (A) Representative CLSM z-stack images (x40 objective magnification, em: 480 nm, exc: 505 nm). Five fields-of-view were imaged for each of the three biological replicates. Additional supporting images can be found in Figure B. 1. (B) Biomass/biovolume. (C) Surface to biovolume ratio. (D) Dimensionless roughness coefficient. (E) Average thickness (entire area). (F) Average thickness (biomass). (G) Maximum thickness. Parameters were calculated using the COMSTAT 2.1 software (Heydorn *et al.*, 2000; Vorregaard, 2008). Points in the bar graphs are the mean of values from five fields-of-view of three biological replicates and error bars represent the standard error of the mean. Significance values are given as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

4.5.2 Ngo-Lig E increases the damage and migration of *Neisseria gonorrhoeae* into reconstructed human vaginal epithelium (rHVE) tissue

To further explore the impact of this sparse biofilm phenotype of the *N. gonorrhoeae ngo-lig E* deletion strain on pathogenicity and virulence, we investigated its ability to form biofilms on a 3-D SkinEthic^{TM} HVE tissue model. The intended experiments involved allowing the *N. gonorrhoeae* pEG2 cells to establish biofilms on the polycarbonate coupons in CDC Biofilm Reactors® before placing these in direct contact with the rHVE tissues. However, as extensive biofilms were not formed on the coupons, we inoculated cultures of the *N. gonorrhoeae* pEG2 strains directly onto the rHVE cells to allow them to form stable biofilms on a more biologically relevant surface. Confocal imaging of these infections (Figure 4.4A and Figure B. 2) showed increased depths of invasion of *wt N. gonorrhoeae*, the His-tagged mutant (*ngo-lig E-his^{kan}*) and the overexpressor (P_{opaB}-*ngo-lig E-his^{kan}*) into the tissue model, while *Δngo-lig E^{kan}* remained on the upper surface of the tissue. Furthermore, tissues infected with the *Δngo-lig E^{kan}* mutant appeared more intact after inoculation, while those infected by the other *N. gonorrhoeae* variants appeared more damaged or were perforated. The final z-stack images obtained for quantification via COMSTAT were focused and zoomed onto the surface of the rHVE cells where *N. gonorrhoeae* was predicted to form biofilms (zoom setting 3). This was an optimal setting for *wt N. gonorrhoeae*, the His-tagged mutant (*ngo-lig E-his^{kan}*) and the overexpressor (P_{opaB}-*ngo-lig E-his^{kan}*) as it showed the extent of the damage caused by the bacteria to the host cells which decreased tissue thickness and hence the bottom membrane was visible for most. However, as a majority of the *Δngo-lig E^{kan}*-infected tissue were more intact, the membranes were only visible on a lower zoom setting (Figure B. 3).

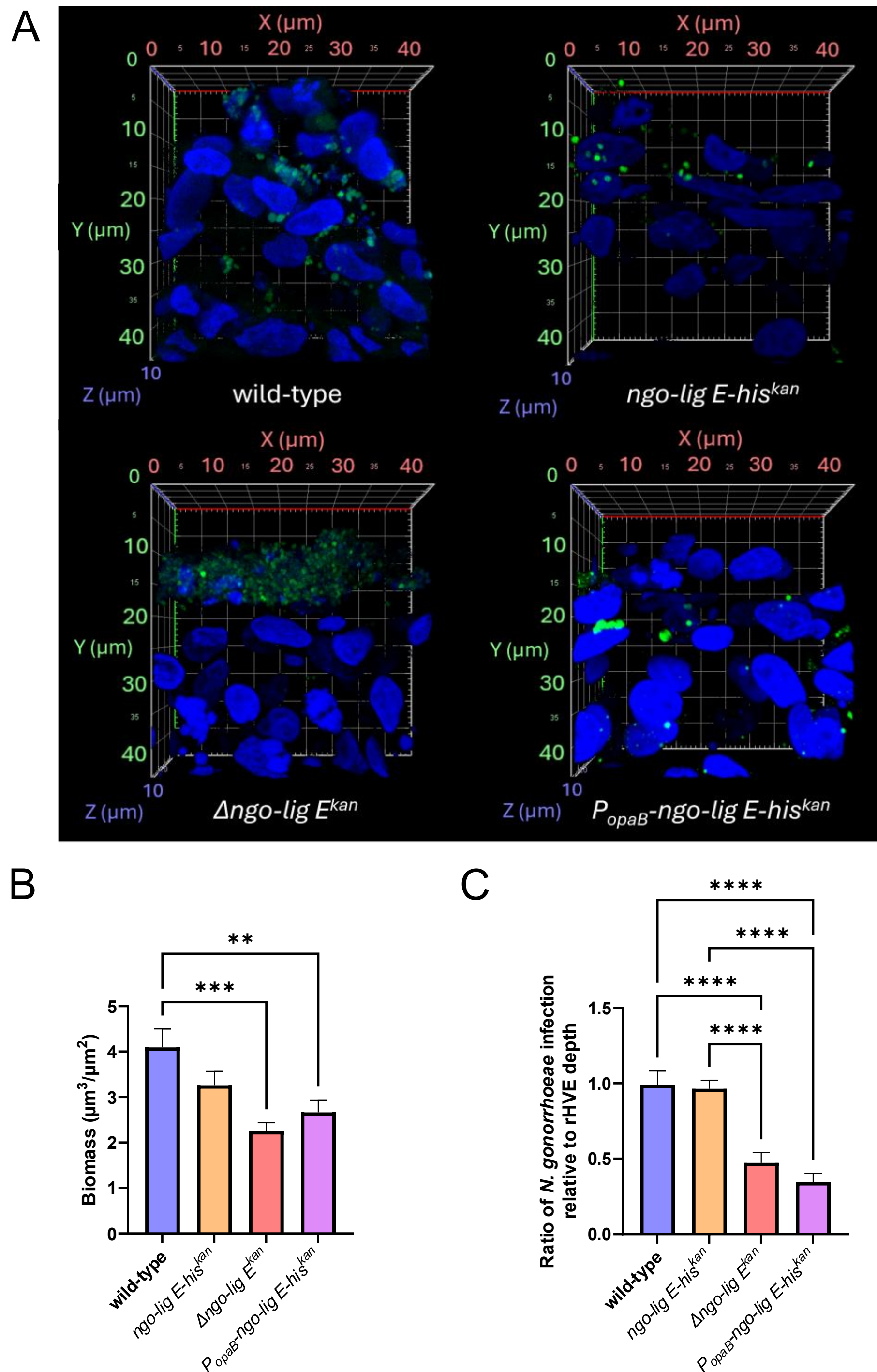


Figure 4.4. Infection and invasion of SkinEthic™ HVE tissue by *Neisseria gonorrhoeae*. (A) CLSM z-stack images (x63 objective magnification, zoom setting 3) of the *N. gonorrhoeae*-infected HVE cells (20 μm) with *N. gonorrhoeae* pEG2 (expressing sfGFP) shown in green (exc: 488 nm, em: 519 nm) and the nuclei of the HVE cells in blue (exc: 405 nm, em: 449 nm). Additional supporting images can be found in Figure B. 2. (B) Biomass/biovolume of *N. gonorrhoeae* growth (green channel) quantified using the COMSTAT 2.1 software (Heydorn *et al.*, 2000; Vorregaard, 2008). (C) Quantification of the depth of *N. gonorrhoeae* infection on the y-axis (green channel) relative to the depth of remaining HVE cells on the y-axis (blue) in the same field-of-view. Points in the bar graphs are the mean of values from five fields-of-view of three biological replicates, with two 20 μm sections each (30 field-of-views in total). Error bars represent the standard error of the mean. Significance values are given as ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

COMSTAT quantification of the biofilms formed by *N. gonorrhoeae* on the rHVE cells showed a slightly lower biomass for *Δngo-lig E*^{kan} and the overexpressor *P*$_{opaB}$-*ngo-lig E-his*^{kan} compared to *wt* on the host tissue cells (Figure 4.4B). However, this did not take into account the extent of tissue damage induced by the bacteria. To measure this, the range of *N. gonorrhoeae* infection (highest to lowest point on the y-axis), relative to the height of remaining HVE in that particular field-of-view was calculated (Figure 4.4C). Results showed that both *Δngo-lig E*^{kan} and *P*$_{opaB}$-*ngo-lig E-his*^{kan} had significantly lower invasion rates than *wt* and *ngo-lig E-his*^{kan}, the latter two strains having similar depth ratios.

To further quantify the extent of epithelial cell damage, the amount of LDH released in the supernatant after biofilm establishment was measured as a proxy for epithelial membrane disruption. LDH levels were significantly lower for *Δngo-lig E*^{kan}-infected rHVE cells relative to infection with the *wt* strain (Figure 4.5), mirroring the trend observed when the invasion depth was calculated from the CLSM images (Figure 4.4C). Interestingly, although the amount of LDH released by cells infected with the overexpressor *P*$_{opaB}$-*ngo-lig E-his*^{kan} was also significantly lower than that of *wt*, this decrease was not as large as that caused by the *Δngo-lig E*^{kan} mutant.

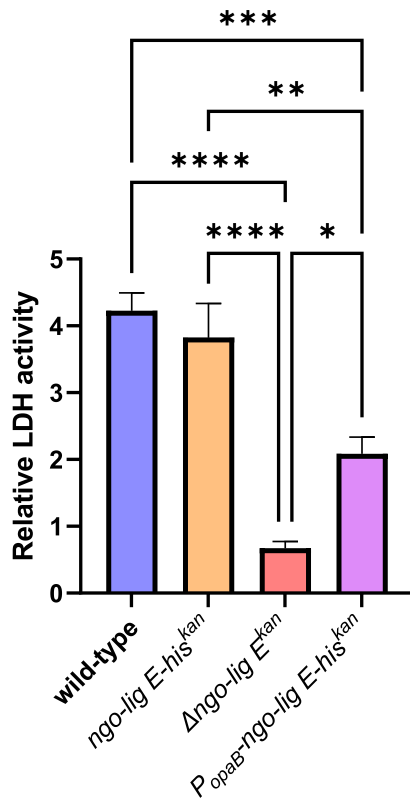


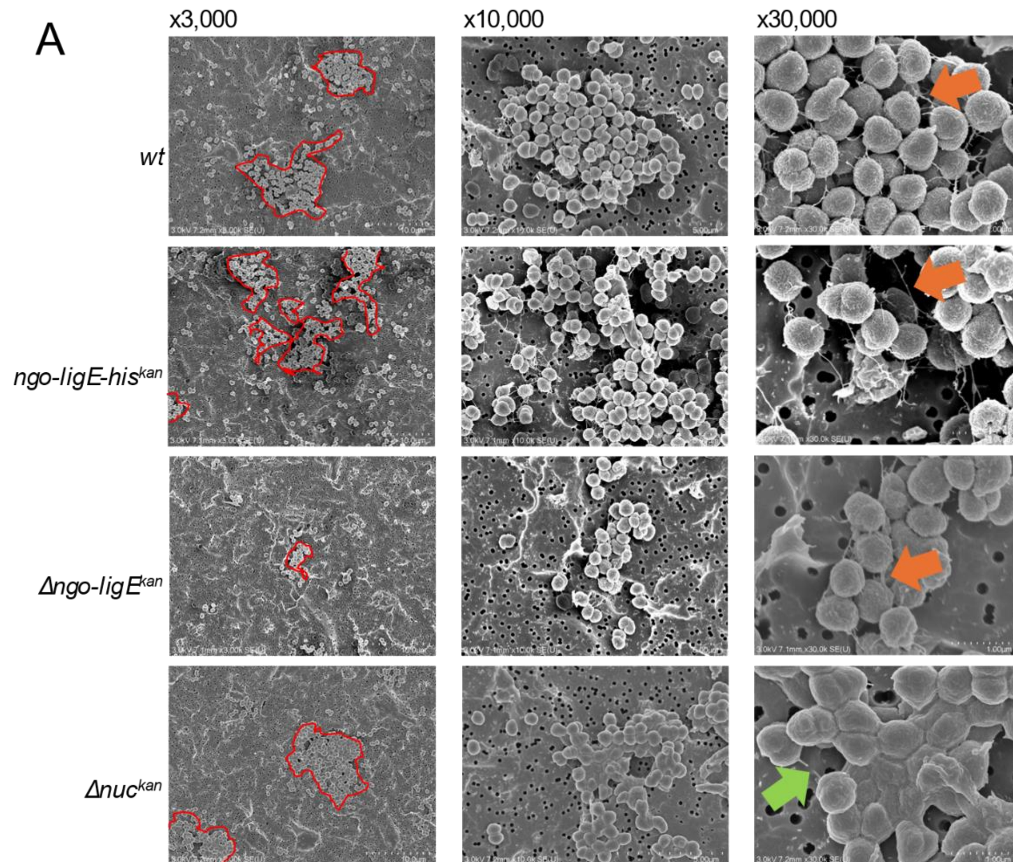
Figure 4.5. Quantification of lactase dehydrogenase (LDH) release in the supernatant after *Neisseria gonorrhoeae* infection of rHVE tissue. Relative LDH activity was obtained via absorbance at 490 nm relative to the absorbance of blank media and corrected for by the background absorbance at 680 nm. Points are the mean of values from three technical replicates of each biological replicate and error bars represent the standard error of the mean. Significance values are given as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$ Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

4.5.3 Ngo-Lig E is important for microcolony formation in *Neisseria gonorrhoeae*

To determine if the observed differences in biofilm morphology were attributable to altered microcolony formation, SEM microscopy on the *N. gonorrhoeae* strains during the exponential phase of growth was performed. These images show that *Δngo-lig E*^{kan} formed markedly fewer and smaller microcolonies compared to *wt N. gonorrhoeae* (Figure 4.6A and Figure B. 4), and that these covered significantly lower surface areas (Figure 4.6B). Furthermore, the microcolonies formed by *ngo-lig E*^{kan} were slightly more dispersed and 'loose' compared to the cohesive microcolonies formed by the *wt* strain. Meanwhile the microcolonies of the His-tagged control (*ngo-lig E-his*^{kan}) covered a similar total surface area to *wt* (Figure 4.6B). To evaluate the impact of increased exDNA on microcolony formation, microcolonies formed by the *nuc* deletion strain (*Δnuc*^{kan}) were imaged. Deletion

of *nuc* had previously been demonstrated to increase biofilm formation in *N. gonorrhoeae* (Steichen *et al.*, 2011), and here, we show that the microcolonies formed by this mutant were similar in size to that formed by *wt*, albeit slightly denser and more compact (Figure 4.6A). Furthermore, the *Δnuc*^{kan} cells also adhered to one another more closely in what appears to be a thicker extracellular matrix (ECM).

Images at a higher magnification (x30,000) showed piliation filaments that seemed to tether individual bacteria to each other in the microcolonies formed by both the *wt* and the His-tagged control (*ngo-lig E-his*^{kan}) (Figure 4.6A, indicated via orange arrows). Although also present in *Δngo-lig E*^{kan}, these piliation filaments were not as extensive when *ngo-lig E* was disrupted. Interestingly, the extensions observed projecting between cells in the *Δnuc*^{kan} mutant were thicker than the pili filaments observed in the other variants (Figure 4.6A, indicated by the green arrow), which could be due to a covering of ECM.



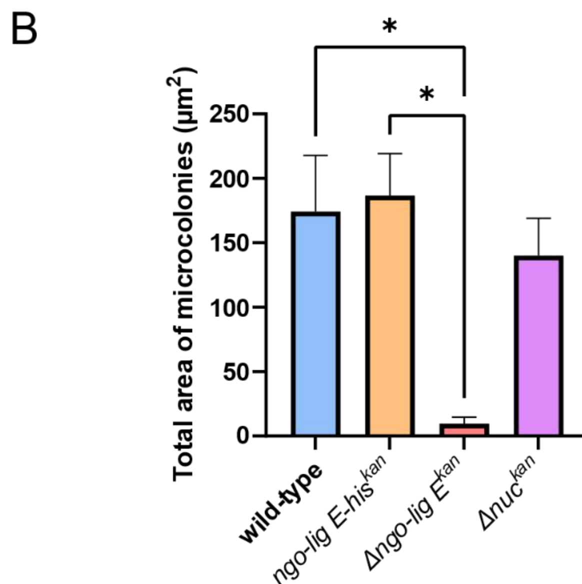


Figure 4.6. ***Neisseria gonorrhoeae*** **microcolony formation. (A) Representative SEM images of *N. gonorrhoeae* microcolonies formed on 0.2 µm pore size filter papers during the exponential phase of growth (9 h). The approximate outline of microcolonies in the field-of-view are shown in red and were annotated manually after image collection. Orange arrows indicate pili filaments, while the green arrow points at a similar extended filamentous structure. Additional supporting fields-of-view can be found in Figure B. 4. (B) Quantification of the average total area covered by the microcolonies in each field-of-view via ImageJ (Schneider *et al.*, 2012). Points are the mean of values of the total surface area covered in each field-of view and error bars represent the standard error of the mean. Significance values are given as * $p \leq 0.05$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.**

4.6 Discussion

4.6.1 Ngo-Lig E and its potential role in biofilm formation in *Neisseria gonorrhoeae*

In a previous study, we showed via an end-point crystal violet assay that the disruption of the DNA ligase Ngo-Lig E negatively affected biofilm formation of *N. gonorrhoeae* (Pan *et al.*, 2024). Here, we have expanded on this finding by culturing the same mutants under constant shear forces and in continuous media flow in CDC Biofilm Reactors®. The morphologies of the resulting biofilms were assessed and demonstrated a marked reduction in colonisation and biofilm formation of *N. gonorrhoeae* on artificial surfaces when *ngo-lig E* was disrupted compared to *wt* and the *ngo-lig E-his*^{kan} mutant. The latter strain served as a control for the insertion of the kanamycin resistance marker which was used to construct the deletion and overexpressing strains, and indicated that the resistance cassette was not responsible for the observed phenotype. Conversely, overexpression of *ngo-lig E* (P_{opaB}*-lig E-his*^{kan}) formed denser and thicker biofilms which were more compact relative to *wt*. We previously demonstrated a 90-fold upregulation of *ngo-lig E* expression in this strain, indicating that Ngo-Lig E activity contributed positively to biofilm formation (Pan *et al.*, 2024, Chapter Three). Our previous work (Pan *et al.*, 2024) also revealed no differences in growth rates between any of the *ngo-lig E* variants and *wt N. gonorrhoeae,* and since the inocula were normalised before each experiment, the observed differences in biofilm formation were not due to differences in initial cell density or growth rates.

Consistent with the phenotype on artificial surfaces, the *ngo-lig E* deletion also caused defects in *N. gonorrhoeae* attachment to human cells and invasion into human tissue. Previous results from our group showed decreased adhesion of the *Δngo-lig E*^{kan} mutant onto a monolayer of ME-180 human cervical cells (Pan *et al.*, 2024). Here, we also showed the reduced capabilities of the same mutant to invade and migrate into reconstituted 3-D HVE tissue, which in turn equates to less cellular damage by the deletion mutant relative to *wt N. gonorrhoeae*. Instead, *Δngo-lig E*^{kan} accumulated on the upper surfaces of the tissue, which appeared healthier and less perforated than the *wt*-infected sections. This reduced invasion depth has important implications on the extent of *N. gonorrhoeae* infection in the human host as *N. gonorrhoeae* invasion and transcytosis into tissues may lead to disseminated

infections after crossing the subepithelial space (Edwards & Apicella, 2004). Although the overexpressor P_{opaB}-*ngo-lig E-his*^{kan} appeared to follow this trend as well, we note that most of the bacteria were closer to the membrane, and we speculate that this could be indicative of deeper invasion past the membrane into the supernatant. We predict that due to the decreased ability of *Δngo-lig E*^{kan} to attach to the surfaces of cells, this strain is less able to invade human cells and cause damage. However, as we were only able to obtain end-point images after 16-17 h of infection, we did not capture the step-by-step process of internalisation and transcytosis of the other mutants into the host cells as it occurred (e.g. via time-lapse microscopy).

Our observations of the consequences of *ngo-lig E* deletion on biofilm morphology are consistent with our hypothesis that this ligase acts on exDNA to form high molecular weight substrates that better contribute to initial biofilm formation by overcoming any repulsive forces between the ECM and the surface (Maier, 2021). Unlike many other bacterial biofilms, exDNA is likely the primary structural biopolymer of gonococcal biofilms, with dsDNA being a critical component of mature *N. gonorrhoeae* biofilms (Zweig *et al.*, 2014; Kouzel *et al.*, 2015). Work by Bender *et al*. has demonstrated that for *N. gonorrhoeae* larger pieces of DNA (>3000 bp) are more likely to have a high concentration gradient outside the colony and integrate into the biofilm (Bender *et al.*, 2022).

We hypothesise that extracellular Ngo-Lig E is important for repairing breaks in free exDNA fragments, increasing their lengths and integrity, allowing them to be retained outside the colonies and therefore contribute to the pool of exDNA that stabilises the extracellular biofilm matrix of *N. gonorrhoeae*. This action would counteract the activity of the extracellular Nuc, which remodels gonococcal biofilms through its cleavage and degradation of exDNA (Steichen *et al.*, 2011) and suggests that some level of interplay or regulatory control between these two opposing activities is likely. Here, we show that the *nuc* deletion strain formed microcolonies of similar sizes to *wt N. gonorrhoeae*; however, these microcolonies appeared more globular, with filamentous extensions that seemed to indicate thicker ECM. It is possible that this observed morphology was due to the inability of the *nuc* deletion to regulate exDNA content, and suggests that in the *wt* strain, Nuc works in conjunction with Ngo-Lig E to manipulate exDNA, and thus optimises

and modulates the architecture of the biofilm. We also question if the increase in exDNA length and integrity performed by Ngo-Lig E allows the exDNA to interact with other DNA binding proteins, which may assemble to create a more stable framework for the biofilm to build on.

Here, we have shown that Ngo-Lig E affects biofilm formation, potentially via its activity on exDNA, which in turn affects the adhesion of *N. gonorrhoeae* to human cells and their subsequent invasion and damage. Based on these observations, we consider extracellular Ngo-Lig E to be important for the pathogenicity and virulence of *N. gonorrhoeae*, making it an appealing target for future drug design against this incredibly resistant bacterium. Despite this, many questions about Ngo-Lig E remain, including its specific cellular location, its regulation, and its direct consequences for the exDNA fraction. The presence of an N-terminal signal peptide indicates that Ngo-Lig E is transported to the periplasmic space, however, it may be further transported to the extracellular milieu via membranous blebs that contribute to the ECM of *N. gonorrhoeae* biofilms (Dorward & Garon, 1989; Dorward *et al.*, 1989; Steichen *et al.*, 2008). Additionally, it remains unknown if Ngo-Lig E is required for biofilm maintenance, or if it is more important during early biofilm formation when exDNA is most critical (Zweig *et al.*, 2014). Answering this specific question would require quantifying the amount and arrangement of exDNA in the ECM when *ngo-lig E* is disrupted (i.e via exDNA staining); this was attempted in the present study, however we encountered difficulties in its visualisation after growth in the Biofilm Reactors, potentially due to the constant stirring that may damage the exDNA. A final area of interest is how Ngo-Lig E activity, and especially a potential influence on DNA size, may affect gene transfer which is more frequent in early biofilms (Kouzel *et al.*, 2015). This would involve studying biofilm formation by the different gonococcal mutants at different timepoints.

4.6.2 New tools and methods for understanding *Neisseria gonorrhoeae* biofilm and pathogenicity

Previously, our group had used a crystal violet assay to demonstrate the importance of Ngo-Lig E on biofilm formation, which although rapid and convenient, was an indirect method that assessed static biofilms (Pan *et al.*, 2024). Other more

advanced techniques that have been used to study *N. gonorrhoeae* biofilms include the growth of the bacterium on glass or patterned silicone coverslips (the latter to study the effects of surface topography) in continuous flow chambers or cells, or even static growth on glass dishes (Greiner *et al.*, 2005; Falsetta *et al.*, 2009; Falsetta *et al.*, 2011; Steichen *et al.*, 2011; Kwiatek *et al.*, 2014; Kouzel *et al.*, 2015; Oldewurtel *et al.*, 2015; Płaczkiewicz *et al.*, 2019). While such methods have greatly increased our understanding of gonococcal biofilm formation, they often require bespoke laboratory equipment and can be subjected to technical complications such as bubble formation in the flow channels that disrupt cellular adhesion.

Here, we report the first use of a commercially-available CDC Biofilm Reactor® to study *N. gonorrhoeae* biofilms, involving the continuous flow of fresh media controlled via a periplasmic pump into a growth chamber filled with retrievable coupons that the bacterium can adhere to, while maintaining a constant shear force across the surface (Kocot *et al.*, 2021). Adhesion of *N. gonorrhoeae* to polycarbonate coupons was not as extensive as we had anticipated, however we attribute this to the polycarbonate material used. This was readily available in our laboratory, but has not been widely used for *N. gonorrhoeae* biofilm studies, with glass being the preferred substrate for *N. gonorrhoeae* surface adhesion for other reports (Greiner *et al.*, 2005; Falsetta *et al.*, 2009; Falsetta *et al.*, 2011; Steichen *et al.*, 2011; Kwiatek *et al.*, 2014; Płaczkiewicz *et al.*, 2019). In addition, the constant stirring of the bacterial culture may have damaged exDNA, which since it is a major constituent of *N. gonorrhoeae* biofilms, would also affect the adhesion and biofilm formation in this setting. Despite this, we believe that with further optimisations, CDC Biofilm Reactors® offer great promise for further studies of biofilm formation in *N. gonorrhoeae*, especially if a glass surface and slower shear forces are used.

We also showed the importance of a 3-D model for studying bacterial-host interactions, which allowed us to examine the effects of Ngo-Lig E on *N. gonorrhoeae* migration into host tissues, a phenotype not observable in a cell monolayer. Common techniques employed by other groups involve the use of a primary cell line or biopsy samples which can be directly coated onto glass coverslips for easy microscopy (Greiner *et al.*, 2005; Płaczkiewicz *et al.*, 2019). The search for appropriate 3-D models for *N. gonorrhoeae* study is of increasing

interest, with one particular group developing a model for this purpose using porcine small intestinal submucosa as a scaffold (Heydarian *et al.*, 2019). Until models like this are readily available however, the SkinEthic HVE^{TM} tissue model used here from Episkin (Lyon, France) provides a good substitute as it is easy to obtain, reproducible, and provides a more biologically-relevant model with different tissue cell types and morphologies.

4.7 Conclusions

The DNA ligase, Lig E, is present in many bacteria like *N. gonorrhoeae* that form exDNA-dependent biofilms. Here we show that Lig E from *N. gonorrhoeae* (Ngo-Lig E) influences the formation of gonococcal biofilms and microcolonies on artificial surfaces, as well as the invasion into and damage of 3-D reconstituted HVE tissue. We propose that Ngo-Lig E may be acting on fragmented exDNA in the extracellular space of *N. gonorrhoeae*, which is conducive for microcolony formation and proto-biofilm interactions to occur. Future directions include studying the role of Ngo-Lig E at different stages of gonococcal biofilm formation, as well as investigations into the potential interplay between Ngo-Lig E and Nuc on exDNA-mediated biofilm remodelling. Regardless, the results presented in this report highlight the importance of Ngo-Lig E on the virulence and pathogenicity of *N. gonorrhoeae*. We predict that this may open up new avenues and pathways for targeting not only *N. gonorrhoeae*, but also other human pathogens that express this minimal ligase, potentially finding a way to target extensive biofilm formation in many clinical settings.

4.8 Acknowledgements

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4.9 Supplementary

Supplementary tables and figures supporting this manuscript can be found in Appendix B.

4.10 References

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5 Chapter Five

DNA Uptake During Natural Competence? A Proposed Location and Role of the ATP-Dependent DNA Ligase, Lig E, in *Neisseria gonorrhoeae*

5.1 Preface

Perhaps the most interesting characteristic of Lig E is the presence of a signal peptide on its N-terminus that indicates likely export outside the cell. Despite experiments with recombinant protein indicating that the leaderless Lig E is the mature form, there is to date, no direct evidence for extracellular localisation or the exact destination of Lig E (i.e periplasm, membrane or extracellular environment). I have previously attempted to track constitutively expressing Ngo-Lig E via immunoblotting, although this was met with difficulties due to poor detection (Chapter Three). In the current chapter, I used a different approach to track the localisation of a superfolder green fluorescent protein (sfGFP) fused to the signal peptide sequence of Ngo-Lig E via fluorescence and immunoblotting. From this, I obtained preliminary results indicating Ngo-Lig E's likely localisation in the periplasmic and extracellular space of *N. gonorrhoeae*, where it may perform its biological function on pieces of exDNA. Work presented in Chapter Three and Chapter Four show that this localisation may be related to its role in biofilm formation. However, as noted in the introduction, Lig E-expressing bacteria also encode competence-related genes, which has led me to hypothesise that Ngo-Lig E may also play a role in the natural transformation of DNA.

In this chapter, I explore the potential role of Ngo-Lig E in DNA uptake of both nicked and intact DNA in *N. gonorrhoeae*, an activity that may aid in its rapid acquisition of antibiotic resistance genes and any other genes that would promote its survival and persistence in the human host. This was conducted using a fluorescent reporter construct generated by Golden Gate assembly. Cloning and

characterisation of two fluorescent reporter constructs were conducted by a Master's student, Avi Singh, under my supervision and guidance.

The research in this chapter is presented as a manuscript that is in preparation for submission. This manuscript explores both the likely cellular localisation of Ngo-Lig E, as well as its potential role in *N. gonorrhoeae* DNA uptake and hence addresses **Objectives Three** and **Four** (see Chapter One). I acknowledge that other experiments still need to be conducted before this manuscript is ready for submission, which I have outlined in Chapter Six; these experiments are currently in progress. I also note that there are some discrepancies in the methodology of this manuscript compared to those presented in Chapter Three and Chapter Four including the use of OD_{550} vs OD_{600} to measure *N. gonorrhoeae* optical density, or the use of different mutants for different experiments (e.g. *Δngo-lig* E^{kan} vs *Δngo-lig* E^{erm}). While conducting the results presented in this chapter, our laboratory had changed several methodologies for *N. gonorrhoeae* manipulation to ones that were more optimised, under the guidance of our collaborators Maria Rendón, Katherine Rhodes and Magdalene So from The University of Arizona. Furthermore, construction of DNA uptake constructs involved the use of a kanamycin resistance cassette, and hence a different antibiotic resistance gene (erythromycin resistance) had to be used for the *N. gonorrhoeae* knock-out strain for these experiments.

Supplementary material associated with this work can be found in Appendix C:

Pan, J., Singh, A., Hicks, J., & Williamson, A. (2025). DNA uptake during natural competence? A proposed location and role of the ATP-dependent DNA ligase, Lig E in *Neisseria gonorrhoeae*. (*In preparation for submission*).

5.1.1 Author contributions

As first author, I led the experimental work including the design and cloning of constructs, DNA uptake assays, RNA isolation and sequencing analysis, as well as *N. gonorrhoeae* subcellular fractionation and immunoblotting. Avi Singh optimised the Golden Gate cloning protocol and construction of two constructs under my supervision and assistance. The study and experimental design of the work was conceptualised by me, my co-supervisor Joanna Hicks, and my Chief supervisor Adele Williamson, who also provided guidance with the research and support with

data analysis. The co-authorship form for this manuscript can be found in Appendix D.

DNA uptake during natural competence? A proposed location and role of the ATP-dependent DNA ligase, Lig E, in *Neisseria gonorrhoeae*

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5.2 Abstract

Background

The DNA ligase Lig E is a periplasmic-targeted ATP-dependent isoform found in many Gram-negative bacteria including *Neisseria gonorrhoeae*, where it has been shown to have a role in biofilm formation. Interestingly, many Lig E-possessing bacteria are also naturally competent, suggesting a second possible function in DNA uptake. Here, we investigate this potential dual-role of Lig E from *N. gonorrhoeae* (Ngo-Lig E) by measuring the ability of Lig E mutant strains to be transformed by damaged DNA encoding fluorescent and antibiotic reporters.

Results

We demonstrated that Ngo-Lig E is important for the uptake of nicked double-stranded DNA for genomic integration in *N. gonorrhoeae*, and that this function is dependent on the provision of extracellular ATP. We also investigated the *in vivo* location of Ngo-Lig E by fusion of its N-terminal sequence elements with a fluorescent reporter designed via Golden Gate cloning. Results from this show that the mature Ngo-Lig E is located in both the periplasm and the extracellular milieu of *N. gonorrhoeae*.

Conclusions

These findings highlight the potential locations and roles of Ngo-Lig E in both natural competence and biofilm formation in the periplasm and extracellular space of *N. gonorrhoeae*, emphasising its likely importance in its survival and persistence and recommending it as a potential drug target against this incredibly antibiotic-resistant bacterium.

5.3 Introduction

Ligases are enzymes that seal breaks in the phosphodiester backbones of DNA and RNA during repair, replication and recombination (Zimmerman *et al.*, 1967). These enzymes are made up of two core domains, an adenylation (AD) or NTase domain that binds an adenylate donor, either ATP or NAD^+, and an oligonucleotide-binding (OB) domain that engages the nucleic acid substrate (Subramanya *et al.*, 1996; Doherty & Wigley, 1999). All bacteria express an NAD^+-dependent DNA ligase that joins Okazaki fragments during DNA replication and most repair processes. However, some also encode additional bacterial ATP-dependent ligases (b-ADLs) that vary in structure and function (Subramanya *et al.*, 1996; Weller *et al.*, 2002; Gong *et al.*, 2004; Williamson & Pedersen, 2014; Płociński *et al.*, 2017). This includes Lig E, a minimal-structure b-ADL with only the two core domains, which displays autonomous DNA-joining activity that does not require the participation of other subunits (Subramanya *et al.*, 1996; Odell *et al.*, 2000; Williamson & Pedersen, 2014; Williamson *et al.*, 2014; Williamson *et al.*, 2016; Pan *et al.*, 2021; Pan *et al.*, 2024). The majority of other accessory b-ADLs have been demonstrated to be involved in stationary phase DNA damage responses, often forming complexes with operonic or co-localised gene products to effect repair (Lewis & Resnick, 2000; Zhu & Shuman, 2007; Płociński *et al.*, 2017; Williamson & Leiros, 2020).

However, the biological function of Lig E has remained something of an enigma. Furthermore, Lig E, which is found only in Gram-negative proteobacteria, seems to form a distinct clade from other DNA-repair b-ADLs and it lacks the syntenic organisation observed with the other b-ADLs (Pergolizzi *et al.*, 2016; Williamson *et al.*, 2016; Pan *et al.*, 2021). Most intriguingly, it has a 20-40 amino acid-long periplasmic localisation signal peptide on its N-terminus, which when deleted *in vitro*, increases its activity and stability (Magnet & Blanchard, 2004; Williamson *et al.*, 2014). This potential extracellular export of a DNA ligase is unexpected considering the cytosolic location of bacterial genomic DNA. Based on its predicted cellular location, we postulate that Lig E is working on extracellular DNA (exDNA) in the periplasmic space of Lig E-expressing bacteria. Further supporting this is the presence of Lig E in organisms that express a homologue of the competence factor, ComEA, suggesting that they are able to take up pieces of exDNA as part of the

DNA transformation process. This includes the obligate human pathogen, *Neisseria gonorrhoeae* (Chen & Gotschlich, 2001).

N. gonorrhoeae is a biofilm-forming obligate human pathogen responsible for the second most common sexually transmitted infection (STI) in the world, gonorrhoea (Unemo & Shafer, 2014; World Health Organization, 2024). *N. gonorrhoeae* is naturally competent and is able to take up pieces of exDNA from its environment at every stage of its growth using its type IV pili (T4P), provided that they include a specific DNA uptake sequence (DUS) (Biswas *et al.*, 1977; Hamilton & Dillard, 2006). The high frequency of natural transformation in *N. gonorrhoeae* creates high structural and antigenic diversity, leading to a panmictic population, and has allowed it to become resistant to almost all classes of antibiotics that have been used for its disease treatment (Hamilton & Dillard, 2006). The precise molecular mechanisms involved in the DNA uptake process in *N. gonorrhoeae* are still being studied, however, it is known that recognition of the DUS on the cellular surface triggers the movement of DNA across the cell membrane in its double-stranded form into a DNase-resistant state with potential protection against nucleases via the periplasmic competence factor, ComE (Biswas & Sparling, 1981; Facius *et al.*, 1996; Chen & Gotschlich, 2001; Aas *et al.*, 2002; Hamilton & Dillard, 2006). Some of the double-stranded DNA (dsDNA) is then believed to be converted to single-stranded DNA (ssDNA) in the periplasm, while plasmid DNA is often subjected to restriction (Biswas *et al.*, 1986; Stein *et al.*, 1988; Chaussee & Hill, 1998; Chen & Dubnau, 2004; Hamilton & Dillard, 2006). Considering the oxidative nature of the human reproductive environment, especially during infections, it is likely that these pieces of exDNA derived from the extracellular environment are fragmented or otherwise damaged, and that a system is in place to ensure the integrity of the DNA before integration into the genome of *N. gonorrhoeae*, which we hypothesise may involve Lig E.

Previous studies have implicated Lig E in *N. gonorrhoeae* biofilm formation and attachment to host cells, with ablation of the *lig E* gene causing a decrease in both the extent and volume of biofilm formed on artificial surfaces and reconstructed human epithelial sections, while compromising its ability to adhere to and invade reconstructed tissue (Pan *et al.*, 2024; Pan *et al.*, 2025) (Chapter Four). However, as noted previously, there is a high correlation between the presence of Lig E and

demonstrated natural competence of the bacteria, as well as the presence of known competence genes in their genomes (Williamson *et al.*, 2016). For example, many well-studied species that have been model organisms for DNA uptake systems encode Lig E, including important human and agricultural pathogens where natural competence presents an antimicrobial resistance risk. We hypothesise that Lig E may have a role outside the cytosol, where it acts on pieces of exDNA. In the present work, we test the impact of Lig E deletion on DNA transformation in *N. gonorrhoeae* with both intact and nuclease-restricted DNA using a newly-established *N. gonorrhoeae* reporter system, which demonstrated its important role in this process. We also probe the localisation of Lig E from *N. gonorrhoeae* (hereby referred to as Ngo-Lig E) using a superfolder GFP (sfGFP) reporter protein under the periplasmic leader sequence of Ngo-Lig E *in vivo*, showing the potential translocation of the protein into the periplasmic and extracellular space of *N. gonorrhoeae*.

5.4 Methods

5.4.1 *Neisseria gonorrhoeae* manipulation and mutant construction

All *N. gonorrhoeae* used in this study were of the MS11 strain. Gonococci were grown at 37°C with 5% CO_2 either on gonococcal base (GCB) agar (Difco^{TM}) or in gonococcal base liquid (GCBL) (15 g/L Bacto^{TM} Protease Peptone No. 3, 4 g/L K_2HPO_4, 1g/L KH_2PO_4, 1 g/L NaCl), both supplemented with 1% *Kellogg's* supplement (22.22 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM $Fe(NO_3)_3$) (Dillard, 2011). Piliation status was determined via morphology under a dissecting microscope at the beginning of each experiment.

To generate different variants, constructs were designed to introduce insertions and/or deletions into the *N. gonorrhoeae* MS11 genome (GenBank: CP003909.1) by homologous recombination of flanking sequences as described previously (Dillard, 2011). Briefly, the *Δngo-lig E^{erm}* mutant contained a disruption of the *ngo-lig E* gene (NGFG_01849) by an erythromycin resistance cassette. A *P_{opaB}-ngo-lig E-his^{kan}* mutant was generated previously (Pan *et al.*, 2024) at a neutral site in the genome (NGFG_RS15145) with a codon-optimised *ngo-lig E* gene inserted behind the constitutive *opaB* promoter (*P_{opaB}*), as well as both a 6-His-tag and a kanamycin

resistance cassette at the C-terminus. The same insertion was generated in *Δngo-lig E*^{erm} giving a *ngo-lig E* overexpressing complement (*Δngo-lig E*^{erm}, *P*$_{opaB}$*-ngo-lig E-his*^{kan}). All DNA constructs were ordered as gene fragments or clonal genes (from Integrated DNA Technologies (IDT) or Twist Biosciences).

Strains were generated via spot transformation (Dillard, 2011; Callaghan & Dillard, 2019). Briefly, piliated, Opa negative (Opa-) colonies were streaked through 10 ng spots of the DNA constructs. Mutants were selected for via selection on GCB agar with the antibiotic of interest (50 µg/mL kanamycin, 10 µg/mL erythromycin) before verification via PCR and sequencing.

5.4.2 Extracellular DNA uptake assay

5.4.2.1 Rifampicin resistance gene (*rpoB*) construct

A rifampicin resistant *N. gonorrhoeae* MS11 strain with a mutation in the *rpoB* gene (NGFG_RS10255) (Schaeffer *et al.*, 2022) was kindly gifted to us by the So laboratory (University of Arizona). This strain contained a C>T mutation at position 1657 of the gene, changing residue 553 from a histidine to a tyrosine. The genomic DNA of the strain was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific™) according to the manufacturer's protocol before amplification of the *rpoB* gene via PCR using the primers listed in Table C. 1.

5.4.2.2 Construction of a fluorescent reporter gene toolbox

The Geneious Prime 2021.1.1 software (http://www.geneious.com/) was used to analyse the *N. gonorrhoeae* MS11 genome (GenBank: CP003909.1) to select three neutral sites for integration of fluorescent reporters for DNA uptake. Criteria for selection were that adjacent genes did not have an essential function and that coding regions would not be disrupted. In addition, consideration was placed to ensure no disturbance of any gene promoters, terminators or operonic groups, including the presence of any favourable DUS sequences or restriction sites for gene insertion, and avoiding large differences in GC content, as well as any repeat regions that may affect homologous recombination. Eventually, candidate 'Site 1' was used in our assays based on the relative transformation efficiencies of a kanamycin resistance cassette into each site.

The different insertion parts of the toolbox were designed individually (Table 5.1) and were ordered as gene blocks, while the vector backbone containing 500 bp 5' and 3' flanks homologous to the insertion region of interest in Site 1 was ordered as a plasmid, all from Twist Biosciences. Each part was designed with recognition sites for the TypeIIS restriction enzyme, BsaI, to create specific overhangs to ensure the correct order for insertion into the vector backbone during cloning (promoter-reporter gene-antibiotic resistance gene). Constructs were assembled from individual parts using Golden Gate cloning (Engler *et al.*, 2009) to create different combinations of complete fluorescent reporter plasmids for expression. Briefly, desired parts were combined in one reaction at 18°C overnight at a 3:1 molar ratio of each insert:backbone, in combination with BsaI-HFv2 (30 units) and T4 DNA ligase (1000 units). The reactions were terminated at 60°C for 5 minutes before transformation into chemically competent DH5α *E. coli* cells. Positive colonies were selected for via the loss of the *laczα* gene in the vector backbone which presented as white colonies (instead of blue) in the presence of X-gal. The plasmids of interest were isolated using E.Z.NA.® Endo-Free Plasmid DNA Midi Kit for later integration into *N. gonorrhoeae* via spot transformation.

Table 5.1 Different inserts used to construct fluorescent reporter genes via Golden Gate cloning. Individual sequences are provided in Hyperlink C. 1.

Part	Feature
Promoter	
P_{pilE}	Constitutive *pilE* promoter
P_{opaB}	Constitutive *opaB* promoter
Reporter genes	
sfGFP	Superfolder GFP (excitation wavelength (exc): 488 nm, emission wavelength (em): 510 nm)
mCherry	mCherry (exc: 587 nm, em: 610 nm)
Antibiotic resistance gene	
kan^R	Kanamycin resistance cassette for selection

The fluorescence intensities of the generated constructs in both *E. coli* and *N. gonorrhoeae* were determined via microscopy and spectroscopy to determine their

suitability as reporters for DNA uptake assays. Briefly, the fluorescence of resuspended lawns (5 µL on microscope slides) were visualised using the Zeiss Axiostar *plus* transmitted light microscope via the blue (exc: 450-490 nm, dichroic (beam splitter): 510 nm, long pass filter (em): 515 nm) and green (exc: 546/12, dichroic (beam splitter): 580 nm, long pass filter (em): 590 nm) light filter sets (wavelengths) at x40 magnification. Prepared lawns were also serially diluted and the fluorescence intensities were quantified using the SpectraMax® M4 Microplate Reader (exc: 485 nm, em: 525 nm for sfGFP fluorescence; exc: 587 nm, em: 610 nm for mCherry fluorescence).

5.4.2.3 Transformation assay for the uptake of extracellular DNA

N. gonorrhoeae liquid transformation assays were performed as outlined by Dillard, 2011. Briefly, piliated gonococci from a 24 h streak were lawned for 7.5 h. Cultures with a starting OD_{550} of 0.07 were prepared from the lawn and were left shaking overnight with sodium bicarbonate (0.042%) (30°C, 50 rpm). 5 mL cultures with a starting OD_{550} of 0.3 were then generated and left shaking for 1.5 h (37°C, 250 rpm), before adjusting the OD_{550} to 0.6.

The respective DNA constructs of interest (4000 ng) were warmed in 200 µL of GCBL media supplemented with $MgSO_4$ (5 mM) in 12-well plates for 15 min at 37°C. Prepared gonococci that had settled for 5 min (40 µL from the bottom of the inoculum, OD_{550} 0.6) were added to the DNA mix and incubated for 30 min at 37°C. GCBL (2 mL) was added to the transformation mix before incubation at 37°C for 2 h. The gonococci were then scrapped from the wells and serially diluted before plating onto GCB agar with the desired antibiotics (50 µg/mL kanamycin or 40 µg/mL rifampicin). The number of colonies on the agar plates were counted after 48 h to obtain colony forming unit (CFU) measurements. Colonies transformed with the *sfGFP* construct were verified via fluorescence under blue light. Transformation efficiencies were calculated as the CFU of transformants (on kanamycin plates) over total CFU (non-selected).

To introduce nuclease damage to the exDNA, fluorescent reporter constructs were treated with restriction enzymes. This included double-stranded breaks (DSBs) in the form of overhangs (NcoI) and single-stranded breaks (SSBs) in the form of nicks (Nb.BtsI) (New England Biolabs®). Briefly, 10,000 ng of DNA was digested

according to the manufacturer's instructions for each 50 uL reaction. NcoI digestion was performed at 37°C for 2 h, followed by inactivation at 65°C for 10 min, while Nb.BtsI digestion was performed at 37°C for 1 h, followed by inactivation at 80°C for 20 min. The restriction digest mixtures were used directly in the transformation assays without further purification.

For experiments to test the effect of exogenous ATP on DNA uptake, 1 mM ATP was added along with the DNA of interest. For transformations under oxidative stress, transformations were conducted as above with gonococci that had been exposed to H_2O_2 (25 mM) for 20 min. Untreated controls were included in parallel for each treatment condition/experiment for direct comparison.

5.4.2.4 Evaluation of piliation status by immunoblotting against PilE

To ensure piliation status did not differ between strains, the levels of PilE expression were quantified via immunoblotting after each DNA uptake assay. The remaining cultures from the DNA uptake assays were isolated and freeze-thawed five times to lyse the cells and were later run on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Western blotting was performed with nitrocellulose membranes. After protein transfer, membranes were blocked for an hour with 5% milk in Tris buffered saline-Tween 20 (TBS-T). The membrane was probed overnight with a primary antibody against the EYLLN motif on the conserved N-terminus domain of the T4P major pilin, PilE (SM-1, 1:1000) (Virji *et al.*, 1989), which was kindly gifted to us by the So Laboratory (University of Arizona). This was used in combination with a goat anti-mouse polyclonal IgG antibody conjugated to horseradish peroxidase ab97023 (Abcam, 1:5000) for 1 h. The membranes were incubated with the SuperSignal™ West Femto Maximum Sensitivity Substrate for 5 min before imaging using the iBright Imaging System (Invitrogen).

5.4.2.5 RNA isolation and sequencing

Remaining scrapped cells from the DNA uptake assays were pelleted and snap-frozen after resuspension in 800 µL TRI Reagent (Zymo Research) to preserve RNA. Total RNA was isolated from two separate assays using the Direct-zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions, while RNA integrity and concentration were determined using the DeNovix RNA Assay

kit and the Agilent High Sensitivity RNA ScreenTape on the Agilent TapeStation 4150. Sequence library preparation using the TruSeq Stranded Total RNA Library Prep Gold kit and whole transcriptome sequencing using the Illumina NovaSeqX system was performed by Macrogen Oceania with 151 bp paired-end reads. Macrogen Oceania also performed data processing via Trimmomatic (version 0.38) (Bolger *et al.*, 2014), genome alignment to the reference genome (ASM15685v2, NCBI RefSeq assembly: GCF_000156855.2) via Bowtie (version 1.12) (Langmead *et al.*, 2009) and gene expression quantification via HTSeq version 0.10.0 (Anders *et al.*, 2015). The DESeq2 package (Love *et al.*, 2014) was used to perform differential gene expression in R version 4.2.2 (R Core Team, 2022) with the parameters adjusted *p*-value (*padj*) ≤ 0.05 and log2 fold change ≥ 1 being deemed as differentially expressed, which was later expanded to *padj* ≤ 0.05 and log2 fold change ≥ 0.5 to identify genes with more subtle expression differences. Functional annotation of *N. gonorrhoeae* MS11 is poorly annotated and hence gene ontology (GO) annotations were obtained from the National Center for Biotechnology Information (NCBI) database of already characterised proteins, or of identical proteins in the database.

5.4.3 Location tracking

5.4.3.1 Location tracking construct generation

Golden Gate cloning was used to generate two DNA constructs for location tracking as outlined above. The first construct (Location Construct 1, Figure C. 1) included the same destination vector backbone mentioned earlier, the strong P_{pilE} promoter and the kanamycin resistance cassette with a modified *sfGFP* gene which had the signal peptide of *ngo-lig E* and 11 linker amino acids attached to its N-terminus (MIKKIIGGIIPIFTAVFIPASAGAADLMLAQEYKG), as well as a 6-His-tag on its C-terminus (Figure C. 1). Attempts were also made to generate a recombination construct where the full-length Ngo-Lig E was fused to the sfGFP reporter (Location Construct 2, Figure C. 1); however, difficulties were encountered with generating the full plasmid in *E. coli*.

N. gonorrhoeae mutants were generated with Location Construct 1 using the spot transformation protocol outlined above.

5.4.3.2 Subcellular fractionation and sfGFP-His protein detection

Piliated gonococci transformed with Location Construct 1 were lawned on agar and left for 16 h. The lawns were resuspended in GCBL and left to grow with sodium bicarbonate (0.042%) for 2 h (37°C, 250 rpm). Subcellular fractionation was performed as outlined previously (Pan *et al.*, 2024) to isolate the extracellular, periplasmic, cytoplasmic and membranous fractions. The location of the sfGFP-His protein was tracked in the fractions via fluorescence under blue light. To enrich for His-tagged proteins, an equal amount of each fraction (140 µg) was incubated with Ni Sepharose High Performance nickel resin beads (Cytiva) for 15 min. The volumes loaded onto the beads were normalised to the total protein concentration. The beads were centrifuged and washed twice with lysis buffer (50 mM Tris pH 8, 750 mM NaCl, 1 mM $MgCl_2$, 5% glycerol) before running them on 12% SDS-PAGE gels to separate the proteins by size.

Western blotting was performed on the samples obtained from subcellular fractionation as outlined above using an anti His-tag mouse monoclonal (HIS.H8): sc57598 igG2b antibody (Santa Cruz Biotechnology; 1:500) and a goat anti-mouse polyclonal IgG antibody conjugated to horseradish peroxidase ab97023 (Abcam, 1:1000). The membranes were incubated with the SuperSignal West Femto Maximum Sensitivity Substrate for 5 min before imaging using the iBright Imaging System (Invitrogen).

5.4.4 Statistical methods

Statistical analyses were preformed using the GraphPad Prism 9.4.0 software (https://www.graphpad.com/). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to compare the different measurements and p values < 0.05 were deemed statistically significant.

5.5 Results

5.5.1 Homologous recombination involving a single nucleotide change in *Neisseria gonorrhoeae* is dependent on Ngo-Lig E

Given the evidence of a likely periplasmic location of Ngo-Lig E, we investigated the potential function of Ngo-Lig E on gonococcal transformation with exDNA. To

test this, we performed DNA uptake assays with a mutated *rpoB* gene segment that had a single nucleotide mutation, changing residue 553 from a histidine to a tyrosine. This gene encodes the β-subunit of the RNA polymerase which is the target of rifampicin, while the mutation prevents proper binding of rifampicin to the RNA polymerase causing resistance (Schaeffer *et al.*, 2022). As *N. gonorrhoeae* already has a native *rpoB* gene, the mutated gene would offer a selective advantage when rifampicin is present. Results (Figure 5.1) show a significant decrease in the ability of the bacterium to take up and integrate the provided *rpoB* gene block via homologous recombination when *ngo-lig E* was disrupted compared to *wt*. Interestingly, this phenotype did not seem to be restored by the complement with a constitutively expressing *ngo-lig E* ($\Delta ngo\text{-}lig\ E^{erm}$, $P_{opaB}\text{-}ngo\text{-}lig\ E\text{-}his^{kan}$). The constitutively expressing *ngo-lig E* control without the native *ngo-lig E* disrupted ($P_{opaB}\text{-}ngo\text{-}lig\ E\text{-}his^{kan}$) also displayed similar low transformation efficiencies to the complement.

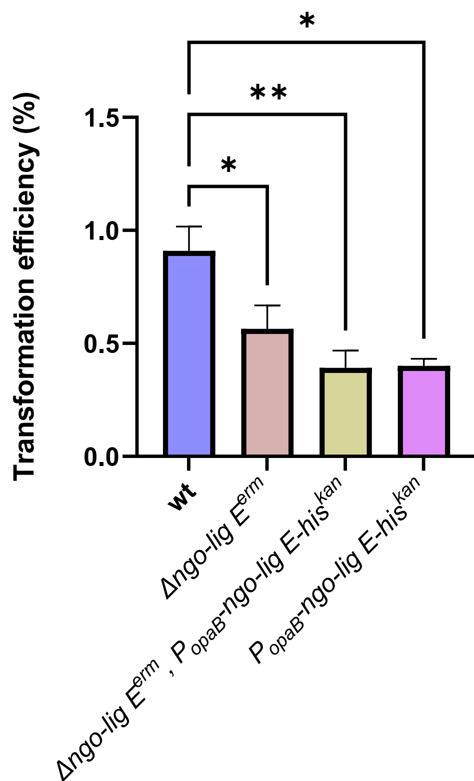
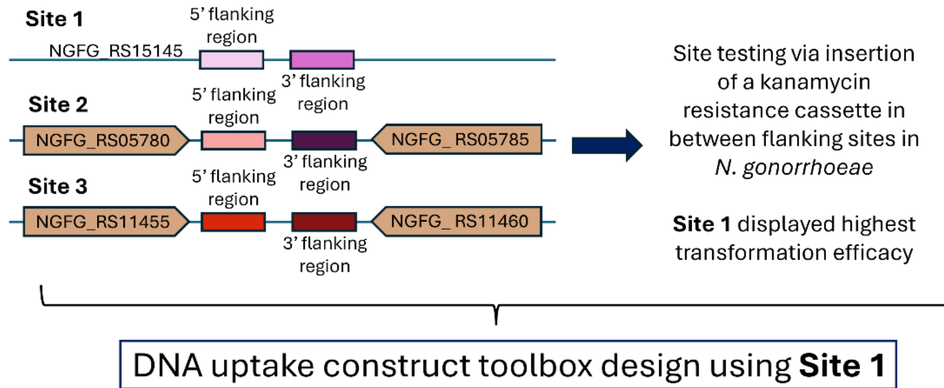


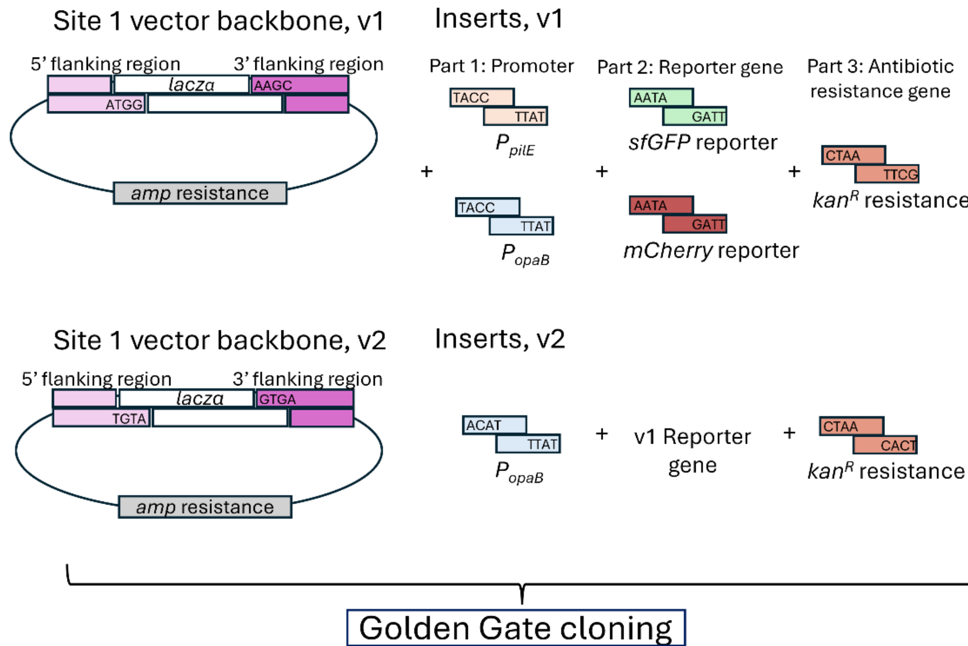
Figure 5.1 Transformation efficiencies of *Neisseria gonorrhoeae* with an *rpoB* gene block with a single nucleotide substitution (C>T), where mutants were selected for via rifampicin resistance. Transformation efficiencies were calculated as a percentage over total colony forming units. Points are the mean values of triplicates while error bars represent the standard error of the mean. Significance values are given as *$p \leq 0.05$; ** $p \leq 0.01$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

N. gonorrhoeae regularly undergoes antigenic and phase variations that affect its piliation status, which in turn could affect transformation efficiency. To ensure that the differences in transformation efficiencies observed were not due to any differences in PilE expression (the major pilin subunit), the levels of PilE expression were quantified via immunoblotting after each DNA uptake assay, which showed equal amounts of PilE expression for the different *N. gonorrhoeae* mutants of interest (Figure C. 2). This was also confirmed visually via microscopy (Figure C. 3).

5.5.2 Development of fluorescent reports to measure DNA uptake

As *N. gonorrhoeae* already has a native *rpoB* gene, transformation with the mutated *rpoB* construct would only require homologous recombination of a very small insert to incorporate the single nucleotide change that confers antibiotic resistance. We were also interested in determining if Ngo-Lig E is similarly involved in the acquisition of novel genes not present in the gonococcal genome. To determine this, we developed a DNA toolkit that would allow us to vary the lengths of DNA constructs and investigate the uptake of multiple genes that could be subjected to damage. This involves having multiple promoters, reporters (with DUS) and antibiotic resistance gene parts that can be assembled in different combinations via Type IIS restriction or Golden Gate cloning (Engler *et al.*, 2009) (Figure 5.2).





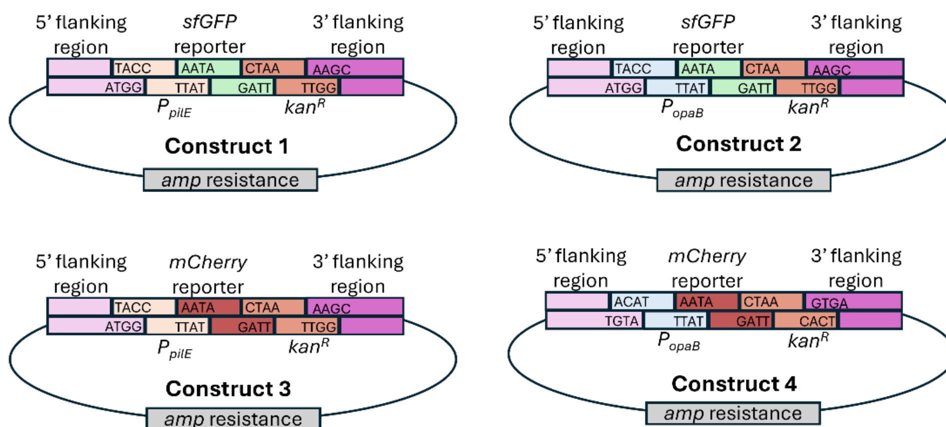


Figure 5.2 Workflow of the development of fluorescent reporter constructs to measure extracellular DNA repair via Golden Gate assembly. Constructs 1-3 were successfully cloned with version 1 (v1) parts, while construct 4 was created with version 2 (v2) parts. The individual parts were designed with specific recognition sites for the restriction enzyme BsaI. Genbank sequences of the individual parts and the final constructs can be found in Appendix C (Hyperlink C. 1).

The vector backbone contained the 5’ and 3’ 500 bp flanking regions homologous to the site of insertion at a neutral site in the genome (NGFG_RS15145, previously labelled as an intergenic region, now as ‘phage protein’). Neutral sites for the DNA uptake assay were chosen based on the criteria outlined in the Methods section and with the intention of utilising the site for gene complementation in the future. Based on this, constructs for three candidate sites were selected (Site 1: NGFG_RS15145; Site 2: between NGFG_RS05780 and NGFG_RS05785; Site 3: between NGFG_RS11455 and NGFG_RS11460). Transformation trials of these sites with a kanamycin resistance gene cassette in between 5’ and 3’ 500 bp flanking regions of each location showed greater transformation rates with the NGFG_RS15145 region (data not included), which was the final site chosen for the DNA uptake assays (Site 1). We note that attempts were also made to utilise other published complementation neutral sites (i.e. *trpB-iga* and *aspC-lctP*) (Ramsey *et al.*, 2012). However, we experienced difficulties in reliably generating mutants in these locations. Furthermore, we were worried about the implications of interrupting the genome close to an essential *N. gonorrhoeae* gene (*iga*) when we had already compromised the genome by disrupting *ngo-lig E* (Remmele *et al.*, 2014).

The *opaB* and *pilE* promoters were chosen as they are strong constitutive promoters and would drive the expression of both the reporter and antibiotic resistance genes (Fyfe *et al.*, 1995; Belland *et al.*, 1997; Ramsey *et al.*, 2012; Bender *et al.*, 2022). All four reporter constructs were generated successfully and were confirmed via Sanger sequencing. To enable future compatibility with other toolbox-development initiatives in our team (Flemming *et al.*, in preparation) and in an attempt to improve assembly efficiencies, a second-generation (v2) backbone was designed which differed to the first version (v1) only in the 4 bp overhangs used to insert the 5’ end of the promoter block and the 3’ end of the resistance block into the backbone. V1 inserts were used to generate Constructs 1-3 (P_{pilE}-*sfGFP-kan*^{R}, P_{opaB}-*sfGFP*-kan^R and P_{pilE}-*mCherry-kan*^{R}), while v2 inserts were used to create the fourth construct (P_{opaB}-*mCherry-kan*^{R}) which was unsuccessful with v1.

All four constructs showed expression of the reporter genes in *E. coli* via fluorescence (Figure 5.3A) with visibly stronger expression of both sfGFP and mCherry under P_{pilE} *compared* to P_{opaB}. Transformation of the constructs into *N. gonorrhoeae* MS11 also showed stronger expression and fluorescence of sfGFP

under P_{pilE} than P_{opaB} (Figure 5.3B and C), as well as some fluorescence and expression of mCherry under P_{pilE} (Figure 5.3B and D). No fluorescence was detectable from mCherry under P_{opaB} (Figure 5.3B). Based on the strength of expression, we concluded that DNA Construct 1 (P_{pilE}-*sfGFP-kan*^{R}) was ideal as a reporter for DNA uptake of novel genes. In principle, this construct would allow us to measure both unselected transformations via sfGFP fluorescence (uptake of gene cassette in the absence of antibiotic selection) as well as selected transformations via kanamycin resistance (after plating with kanamycin). However, due to the generally low transformation efficiencies of new genes into *N. gonorrhoeae*, we found that the *wt* background was so high in the absence of antibiotic selection that transformants were not detectable unless selection via kanamycin was included at the same time.

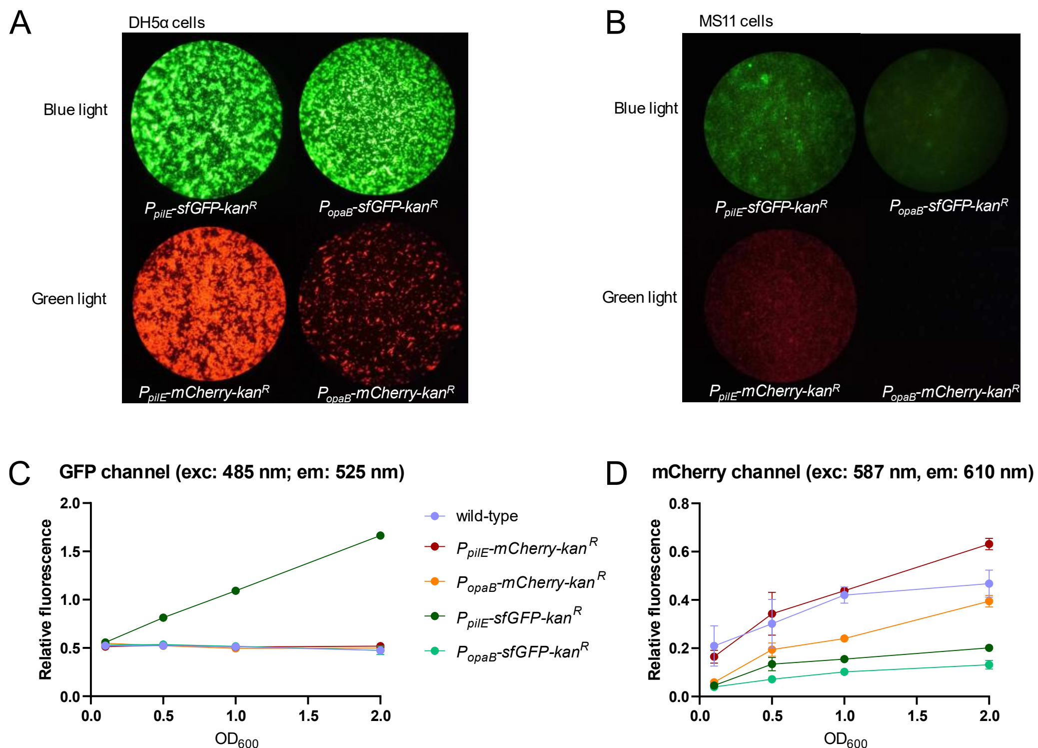


Figure 5.3 Expression of fluorescent DNA uptake reporter constructs generated via Golden Gate cloning. Expression in (A) *Escherichia coli* DH5α and (B) *Neisseria gonorrhoeae* MS11 cells visualised via microscopy (x40 objective magnification). Exc: 485 nm, em: 525 nm for sfGFP fluorescence under blue light; exc: 587 nm, em: 610 nm for mCherry fluorescence under green light. Quantified fluorescence of the *N. gonorrhoeae* mutants measured via spectroscopy using the (C) GFP channel (exc: 485 nm; em: 525 nm) and (D) mCherry channel (exc: 587 nm; em: 610 nm). Points are the mean values of triplicate measurements with error bars representing the standard error of the mean.

Ngo-Lig E is important for the repair and uptake of novel extracellular DNA in *Neisseria gonorrhoeae*

Using Construct 1 from Figure 5.2 (P_{pilE}-*sfGFP-kan*^{R}), we investigated whether Ngo-Lig E aids with the uptake and integration of novel genes into the genome of *N. gonorrhoeae* by providing the construct to the cells in an undigested circular form. Results show a significant decrease in the ability of the *Δngo-lig E*^{erm} mutant to take up the new piece of uncut circular DNA and integrate it into its genome compared to *wt N. gonorrhoeae* (Figure 5.4). We also investigated whether Ngo-Lig E has a role in the repair of digested exDNA before integration into the genome by supplying the same construct after restriction enzyme treatment to generate either single-stranded nicks or double-stranded overhangs. Results of these transformations show that *Δngo-lig E*^{erm} is impaired in its ability to take up and integrate DNA with both kinds of damage relative to *wt N. gonorrhoeae* (Figure 5.4) which echoes the results with intact DNA. For both *wt* and *Δngo-lig E*^{erm}, transformations were significantly higher for the nicked DNA than the overhang substrate. Notably, *Δngo-lig E*^{erm} was only ~60-70% as efficient at taking up the nicked DNA compared to its uptake of intact DNA, providing some evidence of repair of the breaks by this enzyme (Figure 5.4), although *wt* was almost 100% as efficient at taking up nicked DNA as it was with intact DNA. On the other hand, transformation efficiencies with the overhang damage were considerably lower for all strains, supporting our previous results where recombinant Ngo-Lig E preferred a nicked substrate (Pan *et al*., 2024) (Chapter Three). Specifically, the *wt* strain was only 1% as efficient at taking up constructs with a DSB compared to intact DNA, while *ngo-lig E*^{erm} was only ~ 0.5% as efficient in incorporating the same damaged construct compared to its intact counterpart (Figure 5.4, Table C. 2), although differences between the two variants were not statistically significant.

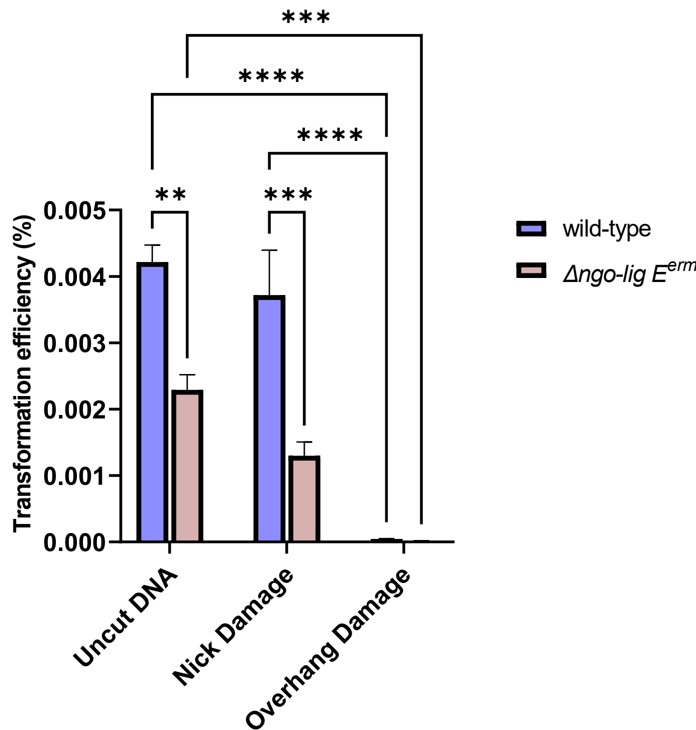


Figure 5.4 Transformation efficiencies of *Neisseria gonorrhoeae* with uncut and damaged (nick and overhang) P_{pilE}-*sfGFP-kan*^{R} constructs. Successful transformants were selected for via fluorescence and kanamycin resistance. Transformation efficiencies were calculated as a percentage over total colony forming units and average values can be found in Table C. 2 (for reference for the low overhang damage values). Assays were conducted in triplicates and error bars represent the standard error of the mean with significance values given as ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

To determine whether Ngo-Lig E is utilising exogenous ATP as a cofactor during exDNA joining, we repeated the DNA transformation experiments with the addition of 1 mM ATP alongside the exDNA. Results (Figure 5.5A) showed no significant changes in the uptake efficiencies of uncut DNA for either *wt N. gonorrhoeae* or *Δngo-lig E*^{erm} with ATP supplementation. However, addition of ATP significantly increased the transformation efficiencies of the nicked DNA for *wt N. gonorrhoeae* to almost double the frequency without supplementation (Figure 5.5B), while the transformation rates were not improved for the *Δngo-lig E*^{erm} mutant.

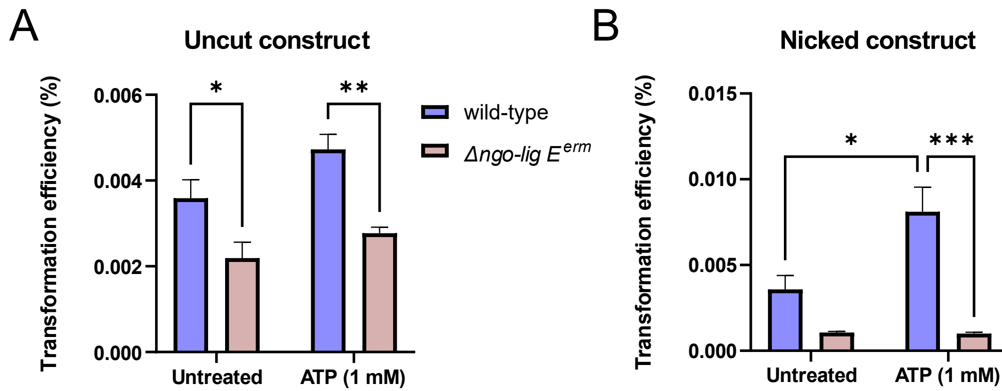


Figure 5.5 Effect of ATP supplementation (1 mM) on the transformation efficiencies of *Neisseria gonorrhoeae* with the P_{pilE}-*sfGFP-kan*^{R} construct. (A) Uncut construct control. (B) Nicked construct. Transformation efficiencies were calculated as a percentage of total CFU. Successful transformants were detected via fluorescence and kanamycin resistance. Assays were conducted in triplicates. Error bars represent the standard error of the mean with significance values given as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

5.5.3 Ngo-Lig E-related DNA uptake in *Neisseria gonorrhoeae* is not linked to oxidative stressors in the environment

To test whether oxidative stress stimulated Ngo-Lig E-mediated transformations, the uptake of intact and nicked P_{pilE}-*sfGFP-kan*^{R} was measured in the presence of H_2O_2. No effect of H_2O_2 on transformation efficiencies was observed for either *wt* or *Δngo-lig E*^{erm} with uncut (Figure 5.6A) or nicked DNA (Figure 5.6B), suggesting that any Ngo-Lig E-related DNA uptake is not a response of oxidative stress. Results from this experiment did not reveal major changes in gonococcal numbers immediately after H_2O_2 treatment, or after the 2 h recovery period following transformation (data not included). Immunoblotting (Figure C. 2) indicated that PilE was similarly expressed between the two variants.

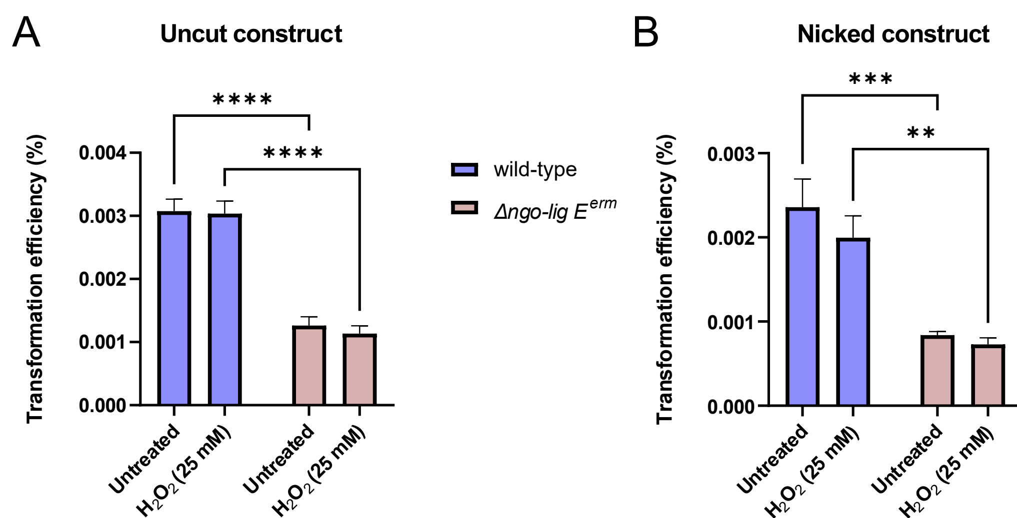


Figure 5.6 Effect of H_2O_2 (25 mM) on the transformation efficiencies of *Neisseria gonorrhoeae* with the *P_{pilE}-sfGFP-kan^R* construct. (A) Uncut construct control. (B) Nicked construct. Successful transformants were selected for via fluorescence and kanamycin resistance. Assays were conducted in triplicates. Error bars represent the standard error of the mean with significance values given as ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Comparisons which showed no significant difference ($p > 0.05$) are not indicated.

5.5.4 Differential gene expression is observed in *N. gonorrhoeae* when *ngo-lig E* is disrupted

RNA sequencing analysis showed no differentially expressed genes between *wt N. gonorrhoeae* during periods of DNA uptake under both normal conditions and hydrogen peroxide treatment, or during DNA uptake of either uncut or damaged exDNA. However, three genes were differentially expressed between *wt* and *Δngo-lig E^{erm}* (*padj* ≤ 0.05 and log2 fold change ≥ 1) (Table 5.2). Specifically there was undetectable expression (~90-fold decrease) of the *ngo-lig E* gene (NGFG_RS11310), an approximately 35-fold decrease in the expression of a hypothetical pilin gene (NGFG_RS11475), and an approximately 3-fold increase in the expression of a pilin gene (NGFG_RS14890) in *Δngo-lig E^{erm}* compared to *wt*. Manual reannotation of these genes by comparison to identical protein sequences from other strains indicated that the downregulated NGFG_RS11475 in *ngo-lig E^{erm}* is *pilEuss2*, which is a unique silent copy of *pilE* just downstream of *pilE* (Rotman *et al.*, 2016). Meanwhile, NGFG_RS14890 is located within a *pilS* locus. Despite these differentially expressed pilin genes, immunoblotting (Figure C. 2) and visual confirmation under a dissecting microscope (Figure C. 3) indicated that both variants were similarly piliated and that this should not affect the results obtained during the DNA uptake assays.

Table 5.2 Differentially expressed genes (padj ≤ 0.05, log2 fold change ≥ 1) between *Δngo-lig E^{erm}* and *wt Neisseria gonorrhoeae* MS11 (ASM15685v2, NCBI RefSeq assembly: GCF_000156855.2). Values with a log2 fold change ≥ 0.5 are shown in Table C. 3.

Locus tag	Gene annotation	Log2 Fold change	*padj*
NGFG_RS11310	DNA ligase	-5.73845	4.26e-10
NGFG_RS11475	Hypothetical/Pilin	-4.72958	8.46e-5
NGFG_RS14890	Pilin	1.529562	0.005084

We also evaluated the data with more permissible cutoff parameters (*padj* ≤ 0.05 and log2 fold change ≥ 0.5) to identify genes with more subtle expression changes. This identified seven additional genes that were all 1.5-2x fold more expressed in *Δngo-lig E^{erm}* compared to *wt* (Table C. 3). These genes included products involved in energy production and conversion, inorganic ion transport, amino acid transport and metabolism and replication, recombination and repair; however, in all cases the log2 fold change values of these genes were relatively low.

5.5.5 The signal peptide of Ngo-Lig E directs export of proteins to both the periplasmic and extracellular space of *N. gonorrhoeae*

Based on the presence of a predicted signal peptide on its N-terminus, it is likely that Ngo-Lig E is trafficked outside the cytosolic space. To identify the likely destination of mature Lig E, we constructed a fusion between sfGFP and the N-terminal sequence of Ngo-Lig E (Location Construct 1, Figure C. 4). This comprised the predicted periplasmic-targeted Ngo-Lig E leader peptide and 11 residues of the unstructured section after the predicted cleavage site, which were joined directly to the N-terminus of the sfGFP. In addition, a His-tag was included at the C-terminus of the sfGFP to allow for enrichment via nickel affinity. This construct was placed under the control of the P_{pilE} promoter and inserted into the neutral site locus (Site 1, Figure 5.2). The resulting construct displayed visible fluorescence in both *E. coli* and *N. gonorrhoeae*, allowing us to track the localisation of sfGFP in different sub-cellular fractions of *N. gonorrhoeae.*

Strong fluorescence was detected at approximately 28 kDa on SDS-PAGE in the crude periplasmic fraction, which was close to the predicted size of the cleaved protein (27.6 kDa) (Figure 5.7A). This was further enhanced after enrichment via nickel pull-down against the C-terminal His-tag (Figure 5.7A). No other fluorescent band corresponding to the uncleaved protein product was detected (estimated 32.3 kDa). Furthermore, Ngo-Lig E is predicted to be transported via the Sec pathway (Figure C. 4), which typically occurs in an unfolded and hence its inactive state before cleavage of the signal peptide.

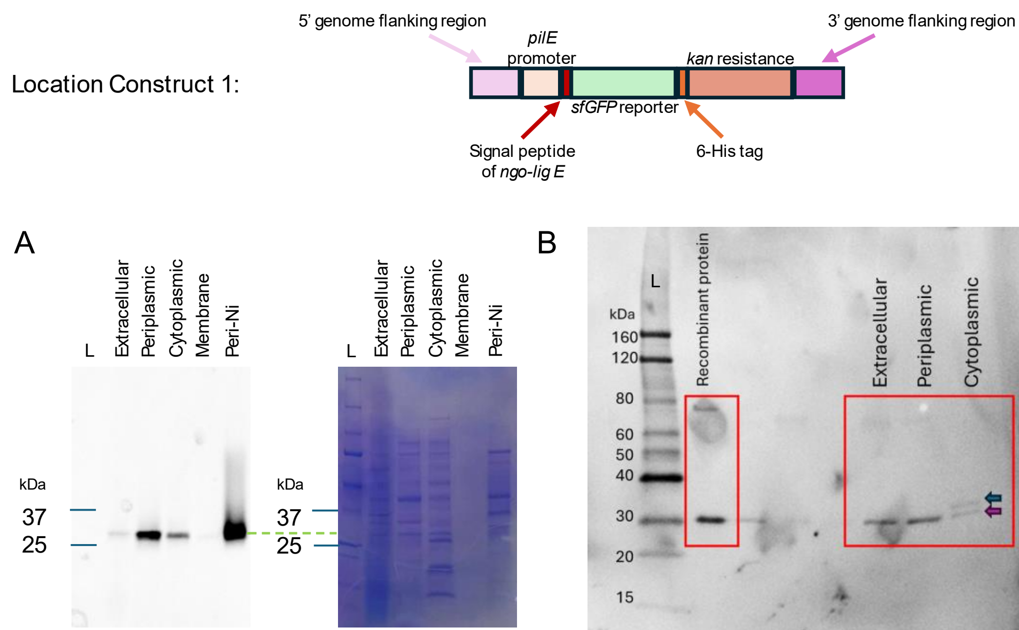


Figure 5.7 Tracking of sfGFP under the signal peptide of Ngo-Lig E (schematic of construct used shown in the top panel) (A) 12% SDS-PAGE gel of the unconcentrated subcellular fractions directly after extraction of the signal-peptide-sfGFP *Neisseria gonorrhoeae* variant, visualised via fluorescence using the green fluorescent protein fluorescence channel (exc: 455-485 nm, em: 508-557 nm) on the iBright Imaging System (left) and via Coomassie staining of the same gel (right). The sizes of the bands visualised via fluorescence were estimated by marking the gel (denoted by the green line) and aligning the images after Coomassie staining. 'Peri-Ni' = the periplasmic fraction after a nickel-affinity enrichment via the His-tag. Note, protein concentrations were not normalised before loading (same volume of each fraction loaded). (B) Western blot against the His-tag of the subcellular fractions obtained from the signal peptide-sfGFP-His mutant in *N. gonorrhoeae* after enrichment by nickel pull-down. The sizes of the purified recombinant protein control mix (Ngo-Lig E-His) are 29.2 kDa and 73.9 kDa for the MBP-tagged version. The blue arrow shows the predicted uncleaved leader-sfGFP protein (32.3 kDa) while the pink arrow shows the predicted cleaved sfGFP product (27.6 kDa). 'L' in both figures refers to the Protein Precision Plus^{TM} All Blue Prestained Protein Standards (Bio-Rad) ladder used.

To further support these results, we performed a Western blot against the C-terminal His-tag of protein after a nickel pull-down (Figure 5.7B). Interestingly, only an

approximately 28 kDa band was detected in the periplasmic and extracellular space, with a stronger signal in the periplasmic space. Two bands were detectable in the cytoplasmic fraction, the smaller approximately 28 kDa band, and a slightly larger band (>30 kDa), the latter of which was predicted to be the uncleaved protein (estimated: 32.3 kDa).

5.6 Discussion

5.6.1 Potential role of Ngo-Lig E on DNA uptake during natural transformation in *Neisseria gonorrhoeae*

In this work, we have further investigated the biological function of the minimal ligase, Ngo-Lig E, which is an accessory DNA ligase encoded in the *N. gonorrhoeae* genome in addition to its replicative NAD^+-dependent ligase, Lig A. We have previously demonstrated a role for Lig E from *N. gonorrhoeae* (Ngo-Lig E) in gonococcal biofilm formation (Pan *et al.*, 2024) (Chapter Four). However, as many Lig E-expressing bacteria like *N. gonorrhoeae* also encode competence-related genes, they are likely able to take up pieces of exDNA which are stored in the periplasmic space during translocation (Gangel *et al.*, 2014), providing a potential substrate for Ngo-Lig E activity. Here, we first demonstrated that Ngo-Lig E is important for transformation with DNA containing single nucleotide mutations in a gene that provides a survival advantage (i.e. rifampicin resistance) and that uptake decreases when *ngo-lig E* is deleted.

It has to be noted however, that overexpression, as well as deletion of *ngo-lig E*, had a negative effect on DNA uptake, indicated by the decreased ability of the overexpressing variant (P_{opaB}-*ngo-lig E-his*) to be transformed by the rifampicin resistance-encoding DNA. This likely explains why complementation of the *ngo-lig E* deletion did not restore DNA uptake to *wt* levels. Previously, we have shown that constitutively expressing *ngo-lig E* does not affect *N. gonorrhoeae* growth and stress response, although it significantly enhances invasion into human cells and biofilm formation (Pan *et al.*, 2024; Pan *et al.*, 2025) (Chapter Three). We predict that the overexpression of Ngo-Lig E in this work may come at an energy cost to the bacterium, especially considering its ATP requirements, causing it to lower its DNA uptake or repair frequencies to compensate for this overproduction. It is also possible that *ngo-lig E* overexpression interferes with the recombination process

itself, as it was previously shown that deletion of the gene increased survival of DNA-damaging antibiotics which was ascribed to the tendency of mistrafficked Ngo-Lig E to bind tightly to nicked DNA intracellularly (Pan *et al.*, 2024).

We then asked if Ngo-Lig E is important for the uptake of larger DNA fragments. Initially, this was attempted by incorporating a novel *β-lactamase* gene which would provide resistance to ampicillin (data not shown). However, despite being able to express the β-lactamase after introducing it into the MS11 chromosome and showing it provided the appropriate resistance, this construct gave too few selectable transformants when used in the DNA uptake assays to be suitable as a reporter construct. To combat this, we developed a multi-part toolbox based on Golden Gate assembly that allowed us to create a range of novel fluorescent reporter genes under different promoters for incorporation into the gonococcal genome. Results from this show that Ngo-Lig E is also important for the uptake of 'intact' novel genes in *N. gonorrhoeae*. Transformation efficiencies with the novel fluorescent construct were lower than that with the mutated *rpoB* gene block, the latter of which displayed an almost 80-fold higher efficiency. This is likely attributable to the incorporation of two large novel genes (*sfGFP* and kan^R) relative to the singe nucleotide change in the *rpoB* gene.

Next, we demonstrated that Ngo-Lig E is important, albeit not essential, for the uptake of damaged dsDNA with single-stranded breaks in *N. gonorrhoeae.* Prior experiments with recombinant Ngo-Lig E showed its preference to ligate singly-nicked dsDNA (SSBs) with ~ 35% more efficiency than overhang substrates (DSBs) (Pan *et al.*, 2024). We thus created both damages on the P_{pilE}-*sfGFP*-kan^R construct via restriction enzyme digestion, with the nicks and overhangs occurring in either the *sfGFP* gene or the kanamycin resistance cassette. Both variants displayed considerably lower efficiencies with the overhang damage; however, *wt* was still twice as efficient at incorporating the construct compared to when *ngo-lig E* was disrupted, strongly suggesting that the ATP-dependent Ngo-Lig E is responsible for sealing damages in exDNA in *N. gonorrhoeae.* We then showed that the addition of external ATP significantly enhances transformation of *wt N. gonorrhoeae* by nicked DNA to levels that actually exceeded those of the non-digested construct; meanwhile, transformation of nicked DNA by the *ngo-lig E* deletion strain remained low both with and without supplementary ATP. This result provides

extremely strong evidence that Ngo-Lig E is participating in exDNA ligation of nicked constructs, and is utilising the supplied extracellular ATP to do so. In light of this, it interesting to note that there was also lower transformation with the uncut construct when *ngo-lig E* was disrupted, both in the presence and absence of additional ATP, and that supplementation of ATP enhanced the uptake of uncut substrates in *wt N. gonorrhoeae*. We postulate that the provided 'intact' substrate may have been damaged or sheared before uptake, either during the preparation of the plasmid construct in the laboratory, or by any external nucleases in the culture. Furthermore, the P_{pilE}*-sfGFP-kan*^{R} construct used for the assay was in a circular state, which would be subjected to restriction as it enters the cell (Biswas *et al.*, 1977; Sox *et al.*, 1979; Stein *et al.*, 1988). This explains the very similar transformation efficiencies of both nicked and intact DNA for *wt N. gonorrhoeae*.

Considering the oxidative nature of the reproductive tract and the constant presence of oxidative stressors from neutrophils which may damage exDNA, we also predicted that Ngo-Lig E may aid in DNA repair and uptake during periods of oxidative stress (Seib *et al.*, 2006; Stohl & Seifert, 2006). Previous data from our group showed no significant differences between the survival of *wt N. gonorrhoeae* and a *Δngo-lig E* mutant when exposed to H_2O_2 (Pan *et al.*, 2024). However, other groups have demonstrated that DNA repair is enhanced during periods of oxidative stress in *N. gonorrhoeae*, which is common due to the constant threat from neutrophil extracellular traps that induce oxidative stress (Stohl & Seifert, 2006). These oxidative stressors would also damage exDNA outside the cells. Thus, we predicted that Ngo-Lig E-dependent exDNA repair may be enhanced in these conditions due to the increased DNA damage that necessitates increased genomic repair. To test this hypothesis, we repeated the transformation assay with the P_{pilE}*-sfGFP-kan*^{R} construct after treatment of the bacterium with 25 mM H_2O_2. However, we showed that this Ngo-Lig E-dependant DNA uptake and repair is not influenced by oxidative stress, with no significant differences in *ngo-lig E* expression during DNA uptake after a 2 h recovery period when exposed to H_2O_2.

RNA sequencing showed no significant differences in gene expression in *wt N. gonorrhoeae* during DNA uptake during conditions of stress, as well as during uptake of nicked vs uncut DNA. However, there was markedly decreased expression of a silent *pilE* copy when *ngo-lig E* was disrupted. Despite this, regular

immunoblotting of PilE and verification of morphology via microscopy before each experiment showed no visible changes in piliation status for any cells, and we are henceforth confident that this differential expression did not affect the DNA uptake phenotype observed.

5.6.2 Potential cellular location of Ngo-Lig E in *Neisseria gonorrhoeae*

Previously, we have hypothesised that Lig E is being transported into the periplasmic space of Gram-negative bacteria based on the predicted signal peptide on its N-terminus, as well as the potential for disulphide bond in its OB domain which would form in the oxidising environment of the periplasm (Pan *et al.*, 2024). Lig E also displays higher ligation activity when its signal peptide is absent, suggesting that this is its active form (Magnet & Blanchard, 2004; Williamson & Pedersen, 2014). However, to date, the sub- or extracellular location of Lig E has not been confirmed *in vivo*. Previous attempts by our group to visually track a Ngo-Lig E-GFP fusion in its native location in *N. gonorrhoeae* were unsuccessful, potentially due to the relatively weak expression of Ngo-Lig E from its native promoter (data not published). We have also found it difficult to track the location of a C-terminally His-tagged Ngo-Lig E under the constitutive *opaB* promoter via the His-tag in a neutral genomic location (Pan *et al.*, 2024) (Chapter Three). Other groups have experienced similar difficulties with the extracellular thermonuclease, Nuc, which also possesses an N-terminal signal peptide in *N. gonorrhoeae*, highlighting the difficulties in tracking potentially periplasmic gonococcal proteins (Steichen *et al.*, 2011).

To overcome these setbacks, we attempted to generate a construct expressing Ngo-Lig E-sfGFP under the constitutive P_{pilE} promoter (Location Construct 2, Figure C. 1); however, this was also unsuccessful with no transformants being obtained in *E. coli* after multiple attempts at Golden Gate cloning, indicating that Ngo-Lig E may be slightly toxic in *E. coli*. Therefore, as an alternate strategy, we instead generated a fusion construct of sfGFP-His with the first 35 amino acids of Ngo-Lig E (including its signal peptide) on its N-terminus (Location Construct 1, Figure C. 1) which we have cloned into Site 1 in the *N. gonorrhoeae* chromosome. This enabled dual-tracking of the protein's location visually via fluorescence and

immunologically via detection of the His-tag. sfGFP was specifically chosen as it is able to fold properly without mixed disulfide-linked oligomers and hence still fluoresces in oxidative environments like the periplasm (Dinh & Bernhardt, 2011).

Our signal peptide-sfGFP fusion showed that the signal peptide of Ngo-Lig E directs the transport of proteins to both the periplasmic and extracellular space of *N. gonorrhoeae*, with some protein also being retained in the cytoplasm. The presence of a slightly higher band only in the cytoplasmic fraction strongly indicates that the signal peptide is likely cleaved off during export, and that the mature protein is directed across the inner membrane into the extracellular/periplasmic space of *N. gonorrhoeae*. Distinction between the two locations is difficult however, as the subcellular fractionation procedure can be prone to cross-contamination of proteins between fractions. To overcome this, our group is currently working on tracking the exportation of Ngo-Lig E directly via super resolution microscopy. Furthermore, nanobodies specific to Ngo-Lig E are currently being developed in our laboratory which would have superior specificity to the target protein than the anti-His antibodies used. This will be used for future Ngo-Lig E tracking experiments.

5.6.3 A proposed model for Lig E in periplasmic repair of damaged DNA and biofilm formation

Consistent with our experimental findings, we propose that Ngo-Lig E is transported to the periplasmic space where it has a role in natural transformation and repair of nicked DNA in *N. gonorrhoeae*. Although the general steps of gonococcal natural transformation have been outlined and are believed to be similar to those of other proteobacteria such as *Vibrio cholera* (Hamilton & Dillard, 2006), much is still unknown about the exact mechanisms involved, and hence it is difficult to place where Ngo-Lig E may fit in this process. It has been postulated however, that after linear dsDNA crosses the cell membrane into the periplasmic space of Gram-negative bacteria, it is separated into single strands to prevent endonuclease restriction, with one strand crossing into the cytoplasm for homologous recombination, while the other is degraded into nucleotides in the periplasm (Chaussee & Hill, 1998; Chen & Dubnau, 2004). Similarly, small circular DNA (<11.5 kb) is often non-specifically cleaved or processed into its linear double-

stranded form as it crosses the outer membrane without undergoing further restriction via endonucleases in the periplasm (Biswas *et al.*, 1977; Sox *et al.*, 1979; Stein *et al.*, 1988). Although *N. gonorrhoeae* takes up both linear and circular DNA with the same efficiency, it displays higher transformation efficiencies with circular DNA, which is believed to be integrated into the genome after re-circularisation (Biswas *et al.*, 1986). Therefore, rather than being critical for the translocation of DNA into the cell or its genomic integration, we believe that Ngo-Lig E enhances the rate and frequency of DNA transformation in *N. gonorrhoeae* by augmenting the amount of available intact DNA for recombination, especially when it offers a selective advantage (i.e. antibiotic/rifampicin resistance). We propose two major routes for this:

1. Sealing nicks or breaks in linear dsDNA obtained from the environment (e.g. damaged extracellularly due to the highly oxidative human environment), increasing the likelihood that either single strand of the duplex are available as templates for homologous recombination in the cytoplasm, and hence enhancing transformation rates, or-

2. Sealing nicks or breaks in circular dsDNA that occurs as it crosses the outer membrane, either restoring its circular topology in the periplasm or repairing the nicks that may have randomly occurred in protein-encoding genes

In addition to a role in the periplasm, we believe that Ngo-Lig E is also transported to the extracellular space, potentially via outer membrane blebs, which are common in *N. gonorrhoeae* biofilms (Dorward *et al.*, 1989; Greiner *et al.*, 2005; Steichen *et al.*, 2008). As the blebs pinch off the membrane, the periplasmic contents are enveloped, carrying DNA, lipids and proteins which may also include periplasmic Ngo-Lig E. These all contribute to the biofilm matrix, which we predict is where extracellular Ngo-Lig E works on maintaining biofilm stability or initiating biofilm formation (Pan *et al.*, 2025). The interplay between biofilm formation and DNA transformation in *N. gonorrhoeae* has been studied by other researchers, with DNA transformation being most efficient during early biofilm formation (Kouzel *et al.*, 2015). We propose that as larger pieces of DNA tend to be retained outside gonococcal colonies and be incorporated in biofilms, the ‘leaky’ Ngo-Lig E in the

extracellular milieu may be working on repairing breaks in exDNA for biofilm initiation, whereas periplasmic Ngo-Lig E may work on repairing breaks in DNA to enhance homologous recombination efficiency (Bender *et al.*, 2022). A fascinating next-step in this research would be to further probe the interplay between these two processes and the role of Ngo-Lig E in each.

5.7 Conclusions and future work

The elucidation of the potential location of Lig E in *N. gonorrhoeae* (Ngo-Lig E) has allowed us to discern its potential function in relation to its cellular location. Here we show that Ngo-Lig E is likely exported to the periplasm of *N. gonorrhoeae* where it may be important for sealing nicks in linear dsDNA destined for homologous recombination, enhancing the probability of successful integration into the genome, as well as sealing nicks in circular DNA as it enters the periplasm. This has important implications for its ability to take up long contiguous pieces of DNA, and hence its rapid acquisition of novel antibiotic resistance genes. We also propose that Ngo-Lig E is transported to the extracellular space of *N. gonorrhoeae* via membranous blebs, which may work on fragmented exDNA that contribute to their retention and incorporation into biofilms.

One outstanding question that remains is the source of the ATP cofactor used by Lig E enzymes for ligation. We predict that the Ngo-Lig E, which is transported outside the cytoplasm, is utilising ATP in the extracellular environment for enhanced ligation and repair, such as that provided during the supplementation experiment. Given the evidence of growth phase-regulated extracellular ATP in some Gram-negative bacteria and the possibility of *N. gonorrhoeae* accessing host ATP as it invades human cells, it is possible that Ngo-Lig E is activated with external ATP outside the cytoplasm when the gonococcus is in its native environment (Mempin *et al.*, 2013; Spari & Beldi, 2020). However, we cannot rule out that the increased activity with the addition of ATP may be due to the uptake of ATP into the cells rather than being used in the periplasmic space. Our group is currently developing a method to quantify the amount of ATP in the periplasmic space of *N. gonorrhoeae*, both during its normal growth and during the uptake of nicked DNA. We also acknowledge the lack of a complement for the assays conducted with the P_{pilE}-*sfGFP-kan*^{R} construct due to difficulties working with

other antibiotics in *N. gonorrhoeae*; however, this is also currently in development with our strains. We likewise note the differential expression in the two silent pilin genes in the *Δngo-lig E*^{erm} mutant. *N. gonorrhoeae* is capable of undergoing pilin antigenic variation via non-reciprocal recombination from transcriptionally silent *pilS* copies onto *pilE*, generating bacteria with different piliation status, the process of which is dependent on the guanine quadruplex (G4) motif upstream of *pilE* (Hill & Davies, 2009; Wachter *et al.*, 2015; Wielert *et al.*, 2023). However, DNA uptake and natural transformation in *N. gonorrhoeae* is dependent on the T4P machinery (Hamilton & Dillard, 2006). Hence, we are also working on creating 'locked pilus' variants of all our mutants where the G4 motif is disrupted, based on work that has been conducted by Maier and colleagues (Zöllner *et al.*, 2017; Bender *et al.*, 2022). By preventing this pilus antigenic variation from occurring, we can ensure that the differentially expressed silent pilin genes do not affect the results observed with our transformation assays.

Although further experiments will need to be conducted to investigate the interplay between the two proposed roles and locations of Ngo-Lig E, the results presented in this report highlight the importance of Ngo-Lig E on the virulence and pathogenicity of *N. gonorrhoeae*. We anticipate that this will open up new avenues and pathways for targeting not only *N. gonorrhoeae*, but other human pathogens that express this ligase as well, avoiding a potential future plagued by endlessly resistant 'superbugs'.

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5.9 Supplementary

Supplementary tables and figures supporting this manuscript can be found in Appendix C.

5.10 References

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6 Chapter Six

Conclusions and Future Perspectives

6.1 Thesis summary

The presence of the accessory ATP-dependent ligase, Ligase E (Lig E), in some Gram-negative proteobacteria is a fascinating phenomenon considering its non-operonic organisation, and the lack of common gene synteny between different Lig E-expressing bacteria. Despite being the first b-ADL characterised (Cheng & Shuman, 1997), little has been elucidated about the biological role of this very minimal enzyme. Why then, would some bacteria expend the energy to express an enzyme that also requires a high-energy cofactor (ATP) if it does not benefit them? Perhaps the most intriguing feature about this enzyme however, is the presence of a 20-40 amino acid N-terminal signal peptide that indicates likely transportation of the protein outside the cell, which is opposed to the cytosolic location of genomic DNA. And so, several major questions are outstanding in regards to this localisation tag. Does this feature point to a likely external source of DNA that requires ligation and repair? And if so, how would this exDNA benefit the bacterium that expresses this ligase? In fact, the narrow subset of bacteria that express Lig E includes bacteria that inhabit aqueous marine environments, as well as human pathogens that express a homologue of the competence gene, ComEA, which may hint at the need to take up pieces of exDNA to their advantage (Williamson *et al.*, 2016). One of the Lig E-expressing human pathogens of note is *N. gonorrhoeae*, the causative agent for gonorrhoea, which has a reputation of effectively acquiring new antibiotic resistance genes at a fast rate due to its unregulated natural competence, making it incredibly difficult to treat. In this context, our group hypothesised that perhaps Lig E is working on repairing fragmented exDNA in the periplasm, enhancing their integrity and likelihood that they would be successfully integrated into the bacterial genome during homologous recombination.

Therein comes my doctoral thesis, with the primary aim of characterising Lig E from *N. gonorrhoeae*, or Ngo-Lig E, to elucidate its phenotype, structure, location and function. To do so, I have created several *ngo-lig E* mutants in *N. gonorrhoeae* and studied their phenotypes in terms of their growth rates and behaviour. Results

from this were presented as a published manuscript in Chapter Three and indicate that Ngo-Lig E is not essential for growth in *N. gonorrhoeae*, and has no significant impact on *N. gonorrhoeae* UV and H_2O_2 DNA damage response. Interestingly, I also showed that when Ngo-Lig E was absent, *N. gonorrhoeae* displayed some defects in the formation of biofilms, as well as adhesion onto a monolayer of ME-180 cervical epithelial cells. Although this was not part of the original hypothesis, it led me to wonder if this phenotype may be related to its potential function: Is Ngo-Lig E important for biofilm formation in *N. gonorrhoeae* as well? And if so, is this related to its original hypothesised role in DNA transformation?

This observation from the phenotypic study shaped my approach in investigating the potential function of Ngo-Lig E. Instead of a linear approach, I investigated its potential role from two angles that each involved exDNA repair; a biofilm approach presented as a manuscript in Chapter Four, as well as a DNA transformation approach discussed in Chapter Five. Through a collaboration with the Williams laboratory at the School of Dentistry at Cardiff University, I observed that when subjected to constant shear forces, *N. gonorrhoeae* struggled to form microcolonies and stable biofilms on artificial surfaces. Unlike many other bacterial biofilms, exDNA is likely the primary substrate of gonococcal biofilms, with dsDNA being a critical component of late biofilms in *N. gonorrhoeae* (Zweig *et al.*, 2014; Kouzel *et al.*, 2015). In fact, work by other research teams (Bender *et al.*, 2022) showed that higher molecular weight DNA (>3000 bp) are more likely to have a large concentration gradient outside the colony and integrate into the biofilms of *N. gonorrhoeae*. Hence, I hypothesised that Ngo-Lig E may be working on repairing breaks in fragmented free-floating exDNA in early biofilms to ligate and create larger substrates that are more likely to be retained on the outside of colonies and be incorporated into the biofilm ECM. There, the larger DNA would extend further out and work better at bridging repulsive long-range van der Waals forces to enable short-range acid-base cell-surface interactions that are necessary for *N. gonorrhoeae* to form a monolayer on surfaces, and hence result in subsequent microcolony and biofilm formation (Das *et al.*, 2013; Regina *et al.*, 2014; Okshevsky & Meyer, 2015; Okshevsky *et al.*, 2015). Not only is this biofilm formation important for its survival in the human host (i.e. antibiotic resistance or protection against the host immune system), it also aids with its infection and pathogenicity. More specifically, I showed that when Ngo-Lig E was absent, *N.*

gonorrhoeae struggled to attach to the host epithelial tissue, potentially due to the low biofilm attachment, which subsequently decreased its likelihood of cellular invasion and damage and hence subsequent inflammatory response in the human host. From this perspective, it seems as though Ngo-Lig E indirectly affects the extent of *N. gonorrhoeae* host cell damage and the severity of gonorrhoea-related symptoms in humans via its role in early biofilm formation. This predicted function of Ngo-Lig E is in contrast to the potentially extracellular Nuc, which remodels gonococcal biofilms through its cleavage and degradation of exDNA (Steichen *et al.*, 2011). I thus speculate that Ngo-Lig E may be working in conjunction with Nuc to optimise and modulate the architecture of biofilms in *N. gonorrhoeae* via exDNA manipulation. I also postulate that the increase in exDNA length and integrity performed by Ngo-Lig E allows the exDNA to interact with other DNA binding proteins, which arrange themselves to create a more stable framework for the biofilm to build on.

As highlighted in the review in Chapter Two however, the exDNA present in *N. gonorrhoeae* biofilms have other possible functions, including to serve as a pool of DNA for DNA transformation. As Lig E-expressing bacteria express a ComEA homologue that indicates likely natural competence, I also looked at the potential role of Ngo-Lig E in the repair and uptake of exDNA in *N. gonorrhoeae*. In this context, I consistently observed lower transformation efficiencies with intact and nicked plasmid DNA when Ngo-Lig E was absent, the constructs of which were designed using the Golden Gate assembly toolbox. Although the plasmid DNA was uncut in this context, it would have been subjected to restriction after crossing the outer membrane of *N. gonorrhoeae* (Biswas *et al.*, 1986; Stein *et al.*, 1988). It may be that there is a system in place that decides which single-strand gets transported across the inner membrane for recombination, and which gets degraded for other processes, in which case, the damaged strand would be more likely to be targeted by nucleases for degradation to increase the likelihood of successful integration. If so, I predict that any repair, and hence stabilisation of damage in the double-stranded substrate before separation into single strands by a ligase would increase the likelihood that both single strands would cross the inner membrane, increasing the chances of successful homologous recombination. Furthermore, I also reported an increase in the transformation efficiencies of nicked DNA when exogenous ATP was added. These findings presented in Chapter Five strongly indicate that Ngo-

Lig E is likely involved in the repair of nicked DNA in the periplasm before the formation of single-strands and its transportation across the inner membrane for genomic integration. Thus, Ngo-Lig E may be important in the rapid transfer of genes between bacteria that increase their fitness and survival and potentially the rapid acquisition of antibiotic resistance in the community.

Both hypothesised functions of Ngo-Lig E listed above relies on the transportation of the ligase out of the cytosolic space, which is consistent with the presence of the signal peptide on its N-terminus, the removal of which enhances its stability (Williamson & Pedersen, 2014). Attempts to track the transportation of constitutively expressed Ngo-Lig E under an *opaB* promoter were met with difficulties, potentially due to low expression in the cell under normal conditions. Through various optimisations and expression trials, I have identified a stronger constitutive *pilE* promoter that allowed us to track the transportation of sfGFP in *N. gonorrhoeae* under the direction of the N-terminal signal peptide of Ngo-Lig E, highlighted in the manuscript in Chapter Five. From this, I found expression of the cleaved sfGFP protein in both extracellular and periplasmic fractions that supports the likely transportation of Ngo-Lig E into these locations. Thus, I put forward the following hypothesis for the dual-role and location of Ngo-Lig E (Figure 6.1): Perhaps, Ngo-Lig E is transported to the periplasmic space where it repairs nicks in DNA fated for genomic integration in *N. gonorrhoeae*. However, outer membrane blebs or vesicles that also contribute to the biofilms of *N. gonorrhoeae* would pinch off parts of the periplasmic material as they form. I believe that this may include periplasmic Ngo-Lig E, thus transporting and incorporating it into the ECM where it aids in the formation of high molecular weight DNA substrates that form a strong framework and bridging substrate for initial biofilm formation for *N. gonorrhoeae*.

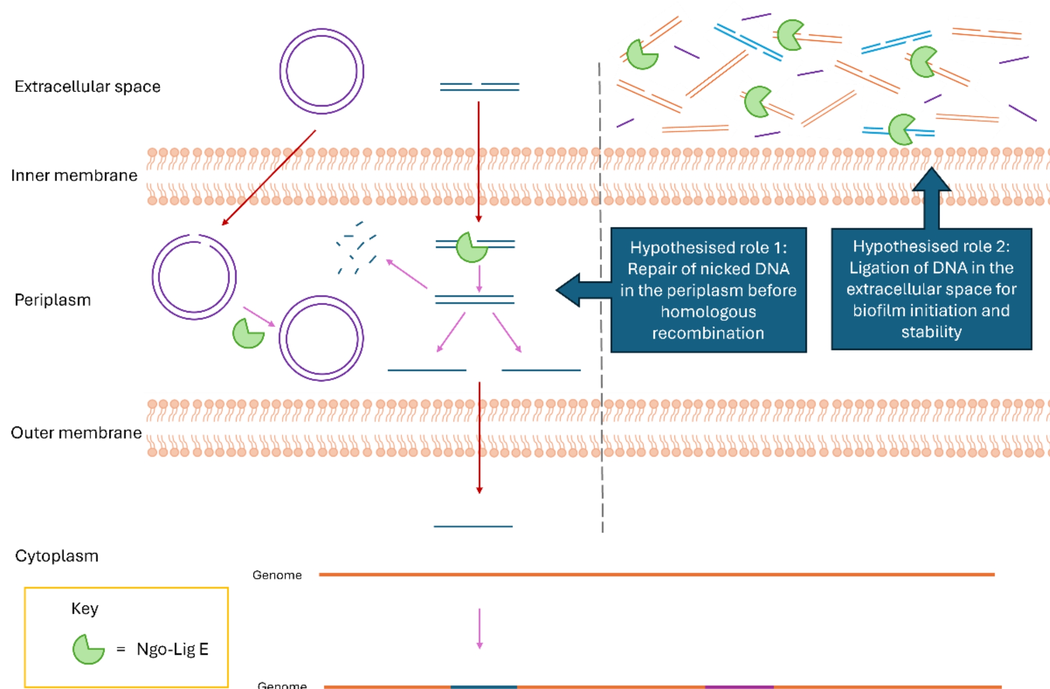

Figure 6.1 Proposed dual role and location of the ATP-dependent ligase, Lig E, in *Neisseria gonorrhoeae* (Ngo-Lig E) involving the repair of nicked extracellular DNA in the periplasm, contributing to DNA uptake (hypothesised role 1) and the repair of extracellular DNA for biofilm initiation and stability (hypothesised role 2).

In addition to this, I have also determined the structure of recombinant Ngo-Lig E in an open confirmation with the OB domain engaging its substrate DNA via its well-structured positively-charged surface, which I have presented in the published manuscript in Chapter Three. This structure demonstrated that complete encirclement is unnecessary for ligation to occur since Ngo-Lig E has no additional domains. Furthermore, I also showed evidence of a disulphide bond in the OB domain which I previously predicted via computational analysis, potentially supporting its likely maturation in the oxidative periplasmic space as well (Pan *et al.*, 2021). This structure serves as the first solved structure of Lig E from a human pathogen and has been deposited in the Protein Data Bank (8U6X). Data from this structure is currently being used in our laboratory to design conformational inhibitors against Ngo-Lig E and other b-ADLs.

Thus, I have identified a potential dual-role and location of Ngo-Lig E in DNA uptake and biofilm formation in the periplasmic and extracellular space in *N. gonorrhoeae*, which in turn highly influences its pathogenicity and virulence. The manuscripts presented in this thesis are crucial in filling the gaps about this enzyme that had been left unanswered for so long. From not knowing anything about its *in*

vivo characteristics, we now have significant information on the phenotype, potential cellular location as well as the potential functions of Lig E. Structural studies from this thesis have also helped us better understand how this enzyme works and allow us to make informed predictions on how it interacts with its substrates. It is clear, that despite its unassuming appearance, there is more to this small enzyme that one would expect. In fact, it could potentially be key to tackling the ever-persistent and ever-resistant *N. gonorrhoeae* that we have been searching for all along.

6.2 Future directions

6.2.1 Elucidating the functional context of Ngo-Lig E, or its interactions with other proteins

The discovery that Ngo-Lig E may have a role in biofilm formation in *N. gonorrhoeae* is greatly reminiscent of the previously mentioned endogenous nuclease, Nuc, which also possesses an N-terminal signal peptide and controls the incorporation of DNA in gonococcal biofilms, although in an opposite manner to Ngo-Lig E (Steichen *et al.*, 2011). It would thus be interesting to determine if there is an interplay between the two likely exported proteins in manipulating the length of exDNA that is incorporated into gonococcal biofilms during different stages of growth or different conditions of stress. This would in turn affect the subsequent biofilm biomass or thickness. More specifically, Ngo-Lig E may be working at creating higher molecular weight exDNA substrates that strengthen and stabilise the biofilm while the nuclease may work antagonistically to refine the amount of incorporated exDNA for biofilm remodelling.

Both *in vivo* and *in vitro* work have been planned to study this potential interaction. As stated in Chapter Four, I have generated separate *Δnuc*^{kan} and a *Δngo-lig E*^{erm} mutants in *N. gonorrhoeae* which exhibit different microcolony-formation behaviours, with that of *Δngo-lig E*^{erm} being more dispersed while that of *Δnuc*^{kan} were more globular. In addition to this, I have generated a double *Δnuc*^{kan} and *Δngo-lig E*^{erm} mutant in *N. gonorrhoeae* which I have visualised via SEM (Figure 6.2). These SEM images show similar microcolony architecture of the double mutant to that formed by the *Δnuc*^{kan} mutant, potentially indicating that Ngo-Lig E is not as substantial at remodelling gonococcal biofilms as the nuclease. Other *in vivo*

experiments need to be performed to further investigate this potential interaction including a comparison of growth rates, biofilm formation on artificial surfaces as well as the ability of the mutants to infect and adhere to epithelial cells.

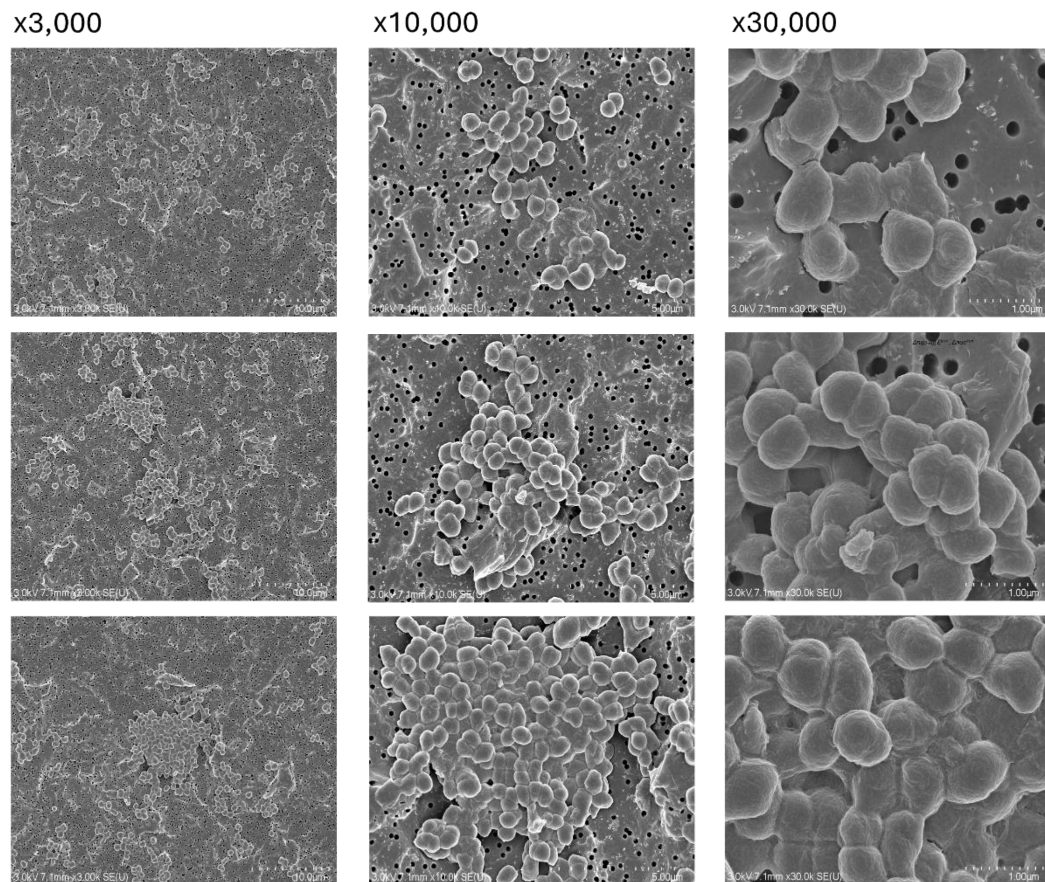

Figure 6.2 SEM images of microcolonies formed by a *Δnuc*^{kan} and *Δngo-lig E*^{erm} double mutant in *Neisseria gonorrhoeae* on 0.2 µm pore size filter papers during the exponential phase of growth (9 h).

In addition to this, I plan to recombinantly co-express both Nuc and Ngo-Lig E to study their interactions *in vitro* to gain a better insight into any co-regulation and complex formation that may occur between the two proteins. Furthermore, preliminary analysis via AlphaFold 3 (Abramson *et al.*, 2024) show potential interactions between Ngo-Lig E and several copies of the competence factor, ComE, that may interact with the substrate DNA at the same time (Figure 6.3). Characterising the potential interactions between these two proteins in *N. gonorrhoeae* may offer further insight into the potential role of Ngo-Lig E in competence and DNA uptake. It is also possible to perform a pull-down assay using affinity chromatography or coimmunoprecipitation to determine what other

proteins Ngo-Lig E interacts with, and hence gain a better picture of its functional context and regulation.

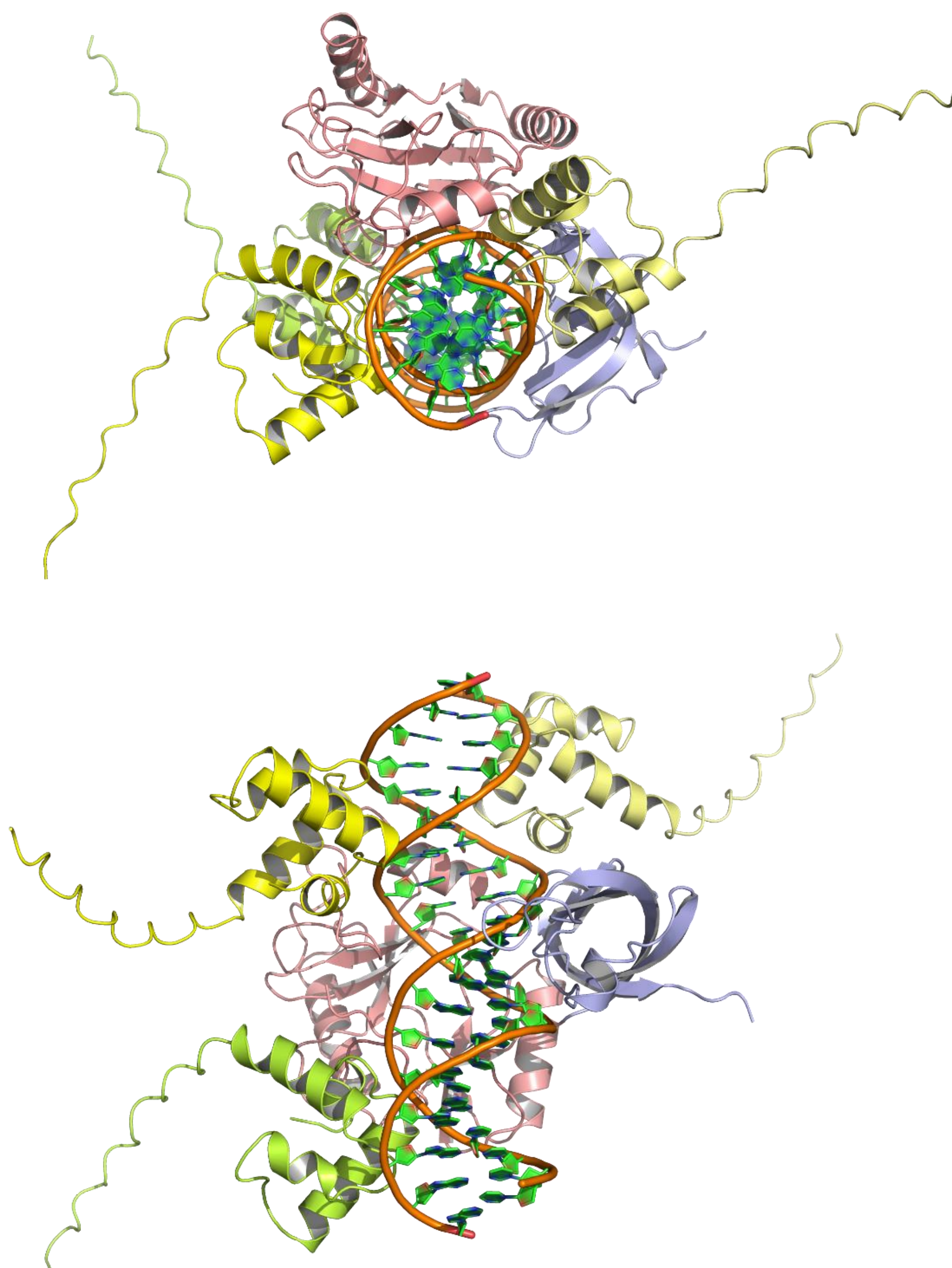

Figure 6.3 Top-down view (top) and side view (bottom) of an AlphaFold 3 (Abramson et al., 2024) prediction of Ngo-Lig E (AD domain in pink, OB domain in purple) and three copies of ComE (three shades of yellow) from *Neisseria gonorrhoeae* with substrate DNA. The sequences of Ngo-Lig E and ComE were entered without their predicted signal peptide determined via SignalP 6.0 (Teufel et al., 2022). The DNA sequences entered were the sequences used for the crystallisation of Ngo-Lig E in Chapter Three.

6.2.2 Gaining further insight into the DNA substrates of Ngo-Lig E

As established in this thesis, exDNA is the likely substrate of Ngo-Lig E. I am thus interested in further exploring the exact type of substrate Ngo-Lig E interacts with, which would allow us to infer the exact role of this ligase during biofilm formation and DNA uptake. Due to time constraints, I was unable to optimise the visualisation of *N. gonorrhoeae* exDNA during biofilm formation via confocal microscopy. Future work in this area includes staining the exDNA in these biofilms with propidium iodide to quantify any differences in the amount of exDNA incorporated in the ECM when Ngo-Lig E is absent. This can be followed up by exDNA quantification of *N. gonorrhoeae* at different stages of growth. Activity assays reported in Chapter Three and the solved structure of Ngo-Lig E highly suggest that dsDNA is the primary substrate of Ngo-Lig E. However, it may be worth investigating if Ngo-Lig E works on ssDNA as well, especially considering the predicted separation of dsDNA into single-strands in the periplasm before it transverses the inner membrane, and the important role of ssDNA during initial biofilm formation in *N. gonorrhoeae* (Zweig et al., 2014). Furthermore, I hypothesise that the interplay of exDNA length and its likely fate in a gonococcal colony, which was established by the Maeir laboratory, may be modulated by Ngo-Lig E (Bender *et al.*, 2022). I plan to investigate this hypothesis by supplying fluorescent exogenous DNA of differing lengths to the *N. gonorrhoeae* mutants and comparing the rates of both DNA uptake and biofilm formation during this assay, similar to previous work by other groups (Bender *et al.*, 2022).

6.2.3 Confirming the localisation of Ngo-Lig E

Despite the promising results in Chapter Five on the potential export of Ngo-Lig E into the periplasmic space of *N. gonorrhoeae*, one has to note that this was based on the tracking of sfGFP under the signal peptide of Ngo-Lig E in a neutral site of the genome, rather than the tracking of the native protein itself. I am hoping to confirm this localisation by tracking the transportation of Ngo-Lig E itself from its native site. Our group is currently in the process of engineering nanobodies with high specificity to Ngo-Lig E that I can use for immunoblotting after subcellular fractionation of *wt N. gonorrhoeae* rather than relying on commercial anti-His antibodies that may exhibit broad off-target activities. Furthermore, I am proposing

to visually track the protein transportation via single-molecule super-resolution microscopy at our collaborator's laboratory at the La Trobe University in Australia. This could be done via the anti-His-tag in the *ngo-lig E-his*^{kan} and P_{opaB}-*ngo-lig E-his*^{kan} mutants, followed by microscopy. Other future work in this area include investigations into the transportation mechanisms of Ngo-Lig E and its potential extracellular transport via outer membrane blebs. Quantification of Ngo-Lig E in the extracellular and periplasmic space in a *N. gonorrhoeae* mutant that exhibits reduced membrane blebbing (via interfering with the MsbB aceyltransferase involved in LOS biosynthesis) compared to *wt* could offer insight into the mechanics of Ngo-Lig E's localisation in the cell (Steichen *et al.*, 2008). Results from these experiments will be added onto the manuscript presented in Chapter Five to provide a more well-rounded understanding.

6.2.4 Creation of locked pilus models of the mutants, as well as true complements in *N. gonorrhoeae*

RNA sequencing data from the manuscript in Chapter Five shows a downregulation in the expression of a unique silent copy of *pilE* when *ngo-lig E* was disrupted. This in turn may affect any piliation antigenic variation that may occur in the mutant. As piliation status and the T4P is important for DNA uptake and competence, it is important that I ensure that this differential expression did not affect the phenotype observed. Hence, I plan on creating a 'locked pilus' mutant of all our *N. gonorrhoeae* variants by interfering with the G4 motif which is important for RecA-dependent pilin antigenic variation. This is based on work that has been conducted by the Maier group (Zöllner *et al.*, 2017; Bender *et al.*, 2022) who has kindly gifted us with the genomic DNA of their *wt* mutant, which I will use to create our mutants. After doing so, I will repeat the experiments highlighted in Chapter Five to ensure that this differential expression had no effects on the lower transformation efficiencies observed by the *Δngo-lig E*^{erm} mutant.

Furthermore, I am currently working on creating a true complement of *Δngo-lig E*^{erm}. Rather than inserting a copy of *ngo-lig E* in a neutral site of the genome of *Δngo-lig E*^{erm}, I plan on replacing the erythromycin resistance cassette with a copy of *ngo-lig E* in its native genomic environment. Repeating the DNA uptake assays with *wt, Δngo-lig E*^{erm} and this complement will allow me to be confident that the

observed phenotype was due to the absence of *ngo-lig E* and not any other factors related to the spot transformation process. These results will also be included in the manuscript in Chapter Five to support the data presented.

6.2.5 Understanding Lig E as a whole

This doctoral thesis serves as the first example of *in vivo* characterisations of Lig E from any bacteria, as well as the first solved structure of Lig E from a human pathogen. Despite this, there may be some differences between Lig E from different organisms depending on the environment they inhabit. For example, computer modelling of Lig E from *C. jejuni* predicted two disulphide bonds in its OB domain, while those from the aqueous *A. mediterranea* or *Psychromonas* sp. have none (Williamson & Pedersen, 2014; Williamson *et al.*, 2014; Pan *et al.*, 2021). Characterisations of Lig E from other organisms will offer us insight into any unique functions of Lig E pertaining to the different bacterial environments, and corroborate the results presented above. In fact, our laboratory has recently obtained access to *Vibrio natriegens*, a fast-growing Lig E-expressing marine bacteria that would allow us to manipulate and study Lig E at a faster rate. It is my strong belief, that by expanding the plethora of characterised Lig E from different bacteria, we will gain a better understanding of the purpose and role of this small but significant enzyme.

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Appendix A

Supplementary Material for Chapter Three

Table A. 1 Primers for mutant confirmation and sequencing.

Primer name	**Sequence (5’ to 3’)**	**Purpose**
Primer_1_KO_FD	CGG GGA GAA TTT CGT AAC GT	*ngo-lig E* forward primer
Primer_2_Rev	CAC TTT TTG CAG TGC GGC A	*ngo-lig E* reverse primer
Primer_4_His_FD	CAC CAC CAC CAC CAC CAC T	*ngo-lig E* forward primer
Primer_seq_Lig-E_Fd	CAA ATC AGG GTG CAG TTT TGG CGG AA	*ngo-lig E* forward sequencing primer
Primer_seq_Lig-E_Rev	GCC CCG CCC ATC ACG GGC AGG AGC A	*ngo-lig E* reverse sequencing primer
NS10_Construct_Fd	GTC CGC AAT GCG CCG AAT	Neutral site forward primer
NS10_Construct_Rev	GCC TGT CCG CGT CTG AAA	Neutral site reverse primer
NS10_External_Fd	GTA ACG GTT TTC AAT GCC	Neutral site forward sequencing primer
NS10_External_Rev	GAT ATG CGC GGA CAT TAT	Neutral site reverse sequencing primer

Table A. 2 qPCR primers and probes.

Oligonucleotide name	Sequence (5' to 3')	Purpose
Lig E primer Fd	CGT ATT GGG ACG GAA AGC A	*ngo-lig E* gene forward primer
Lig E primer Rev	AAT CTG CTC GAA CTG ACC AC	*ngo-lig E* gene reverse primer
Lig E probe	CAA AGG CTT TAC CGC GCA GTT TCC	*ngo-lig E* gene probe
16s primer Fd	CTG GGA TAA CAC TGA CGT TCA T	*16S rRNA* gene forward primer
16s primer Rev	GCA ATC AAG TTG CCC AAC AG	*16S rRNA* gene reverse primer
16s probe	AGT CCA CGC CCT AAA CGA TGT CAA	*16S rRNA* gene probe

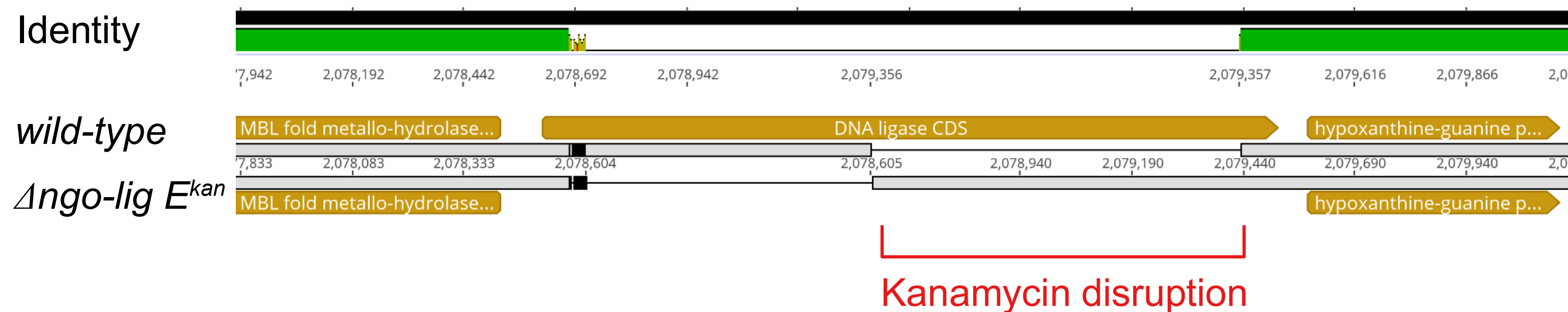


Figure A. 1 Mauve alignment of *Δngo-lig E^{kan}* and wild-type *Neisseria gonorrhoeae* highlighting the disruption of *ngo-lig E* (annotated as 'DNA ligase').

Table A. 3 Oligonucleotide sequences for generating ligatable double-stranded DNA substrates. Combinations used for different ligatable breaks are given in Table A. 4.

Oligonucleotide	Composition
L1	5'-(6-carboxyfluorescein) AGG CCA TGG CTG ATA TCG CA-3'
L2	5'-(phosphate) TAG GCA TTC GAG CTC CGT CG-3'
L3	5'- CGA CGG AGC TCG AAT GCC TAT GCG ATA TCG GCC ATG GCC T -3'
L6	5'-CGA CGG AGC TCG AAT GCC TA-3'
L7	5'-(phosphate) TGC GAT ATC AGC CAT GGC CT-3'
L8	5'-(phosphate) ATA TCA GCC ATG GCC T-3'
L9	5'-CGA CGG AGC TCG AAT GCC TAT GCG-3'
L10	5'-CGA CGG AGC TCG AAT GCC TAC GCG ATA TCA GCC ATG GCC T-3'
L11	5'-CGA CGG AGC TCG AAT GCC TAG TGC GAT ATC AGC CAT GGC CT-3'

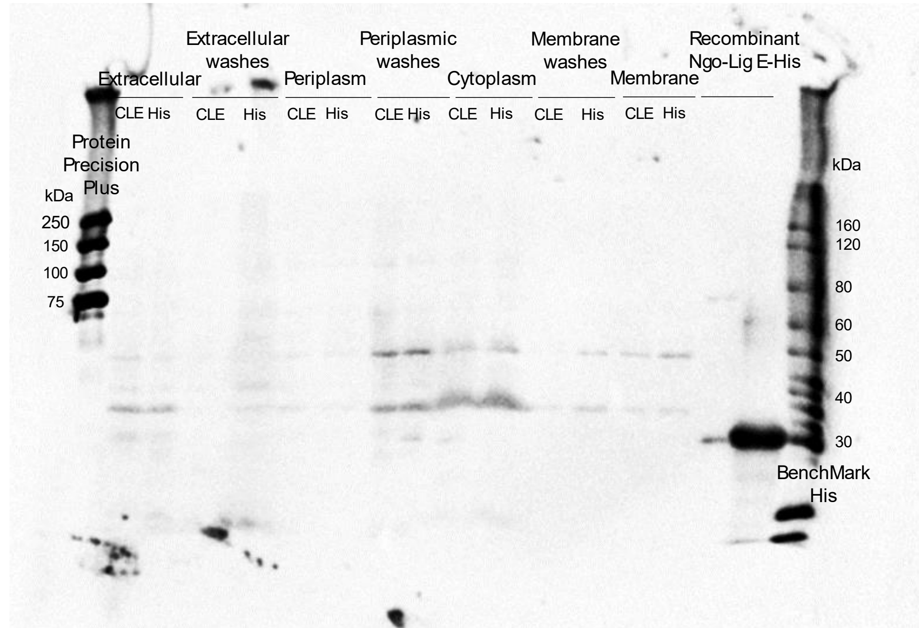
Table A. 4 Oligonucleotide combinations used to generate different double-stranded ligatable substrates. Sequences are given in Table A. 3

Substrates	Ligatable strand	Complementary strand
Single nick	L1, L2	L3
Blunt ended	L1, L2	L6, L7
Overhang	L1, L2	L8, L9
Mismatch	L1, L2	L10
Gapped	L1, L2	L11

Table A. 5 Table of gene expression measured by qPCR and expressed as fold changes between mutants in biofilm and planktonic cultures. Data were collected in duplicate, and errors represent the standard error of the mean.

	Planktonic	Biofilm
Δngo-lig E^{kan}	ND	ND
ngo-lig E-his^{kan}	1.022 (± 0.061)	1.081 (± 0.008)
P_{opaB}-*ngo-lig E-his*^{kan}	88.62 (± 1.282)	120.423 (± 22.244)

A



B

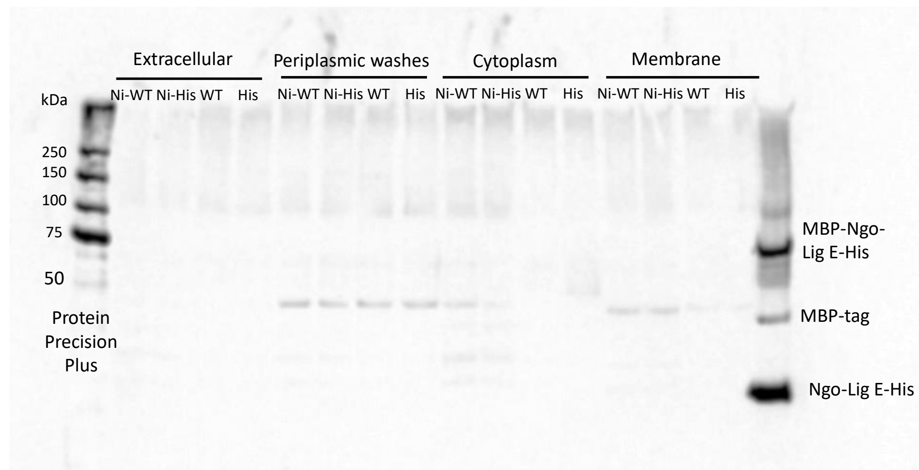


Figure A. 2 Western blots of different cellular fractions of *Neisseria gonorrhoeae* MS11 using an anti-His antibody. (A) Comparison of the different fractions from the His-tagged constitutively upregulated Ngo-Lig E mutant (*P_{opaB}-ngo-lig E-his^{kan}*; 'CLE') and the His-tagged Ngo-Lig E mutant (*ngo-lig E-his^{kan}*; 'His') mutants. (B) Comparisons of fractions from wild-type *N. gonorrhoeae* ('WT') and *ngo-lig E-his^{kan}* ('His') as well as the respective nickel pull-downs of each fraction ('Ni-WT' and 'Ni-His' respectively). Ladders are indicated in kilodalton (kDa). Sizes of the recombinant protein controls were 32 kDa for recombinant Ngo-Lig E-His, 44 kDa for the MBP tag and 76 kDa for MBP-Ngo-Lig E-His.

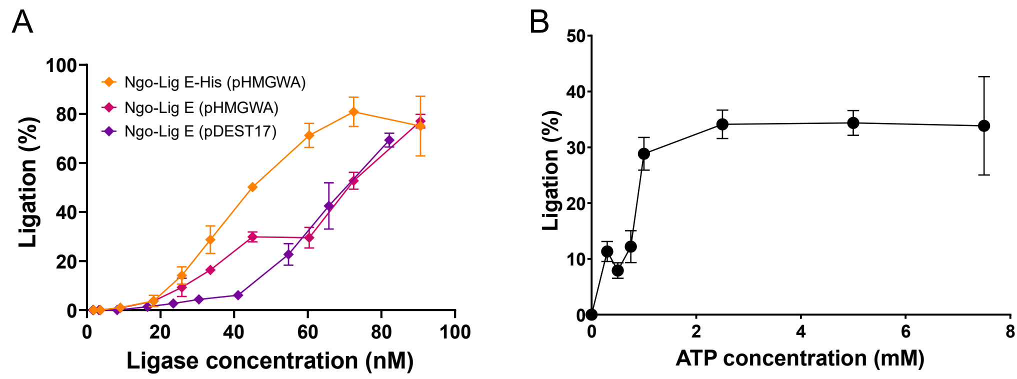


Figure A. 3 (A) Ligase activity on singly-nicked DNA substrates for various recombinantly-produced Ngo-Lig E constructs including mature Ngo-Lig E purified from an N-terminal His-tagged fusion (pDEST17) with the tag removed ('Ngo-Lig E (pDEST17)') and Ngo-Lig E purified from an N-terminal MBP-fusion (pHMGWA) with and without the tag removed ('Ngo-Lig E (pHMGWA)' and 'Ngo-Lig E-His (pHMGWA)'). (B) ATP optimum of Ngo-Lig E ligation. Measurements were made in triplicate. Error bars represent the standard error of the mean.

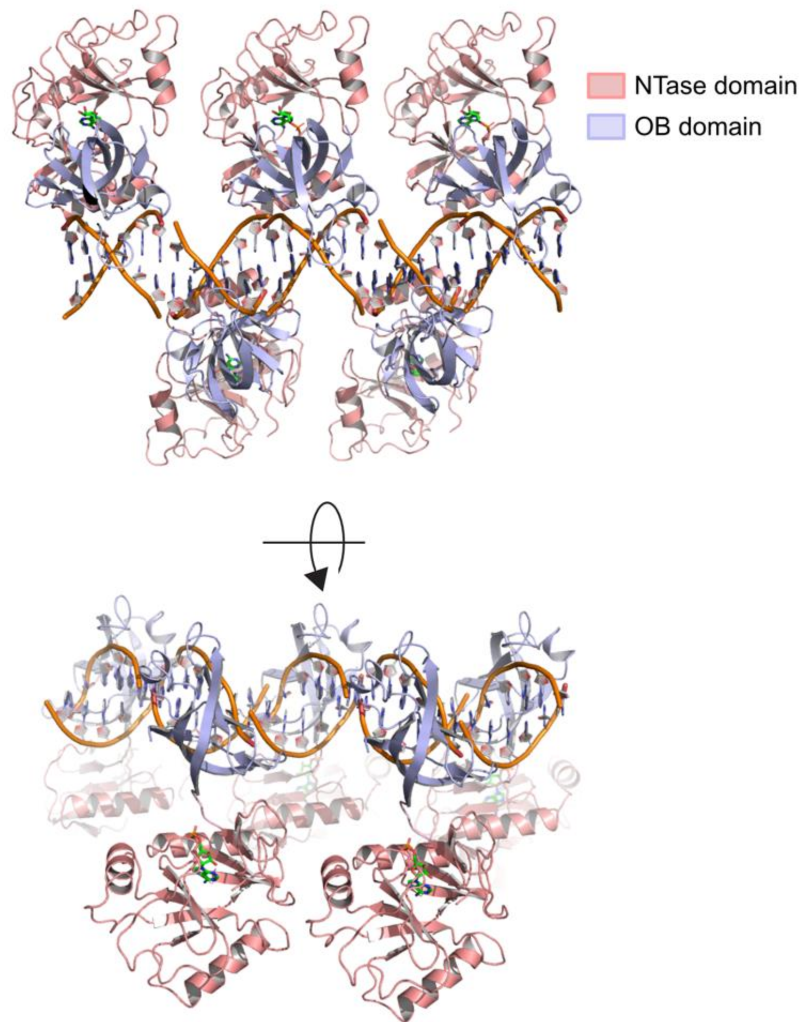


Figure A. 4 Symmetry-related molecules in the Ngo-Lig E crystal. Symmetry-related mates within 4 Å are shown generating the continuous DNA filament.

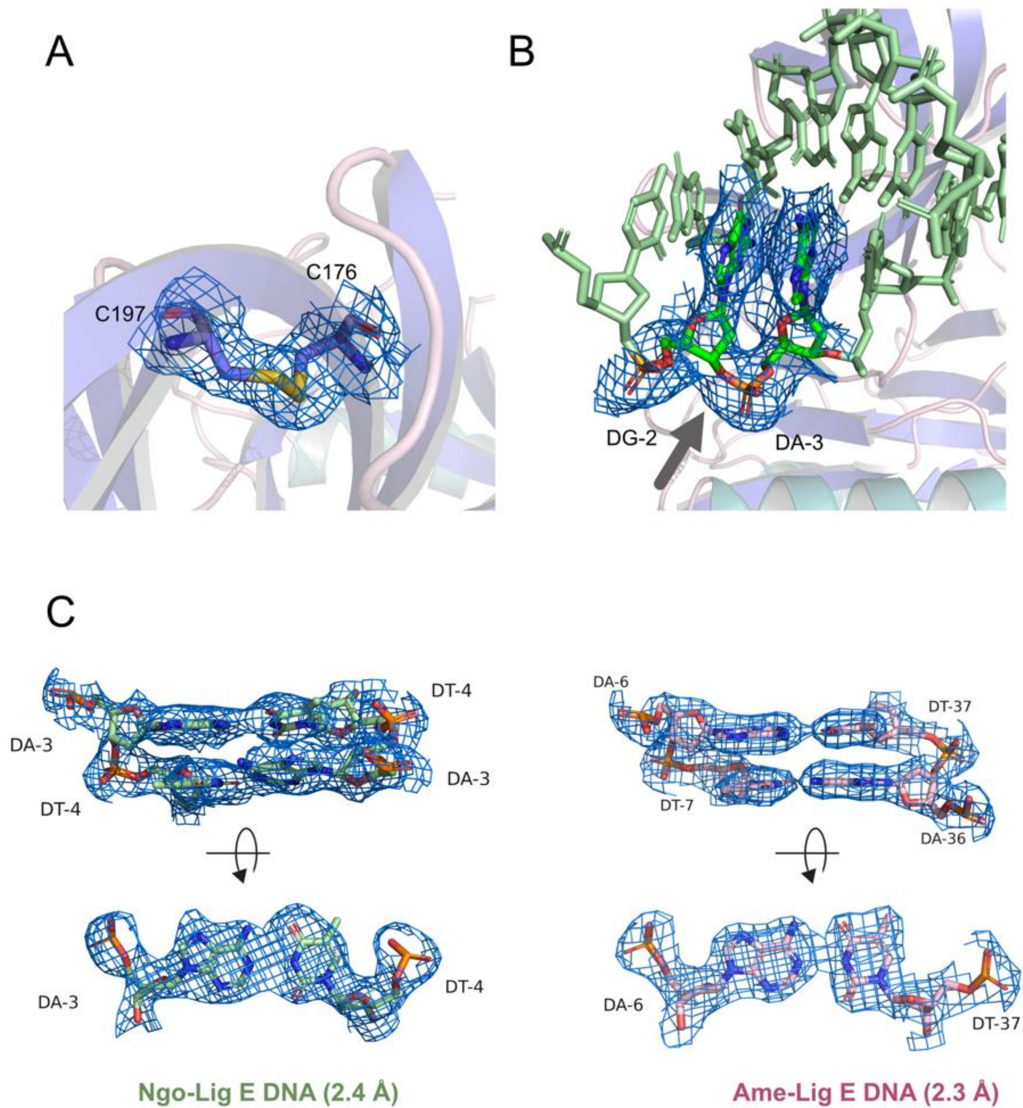


Figure A. 5 Observed electron density map (2Fo-Fc) surrounding key features of the Ngo-Lig E structure including: (A) The disulphide bond, displayed at the 1.0 Å level. (B) The DNA strand that is equivalent to the nick in other DNA ligase-DNA structures, showing continuous density in the present structure. Map is displayed at the 0.5 Å level. (C) Comparisons of density surrounding centrally-placed base-pairs in the Ngo-Lig E structure and the equivalent base-pair in the Ame-Lig E structure (6GDR).

Appendix B

Supplementary Material for Chapter Four

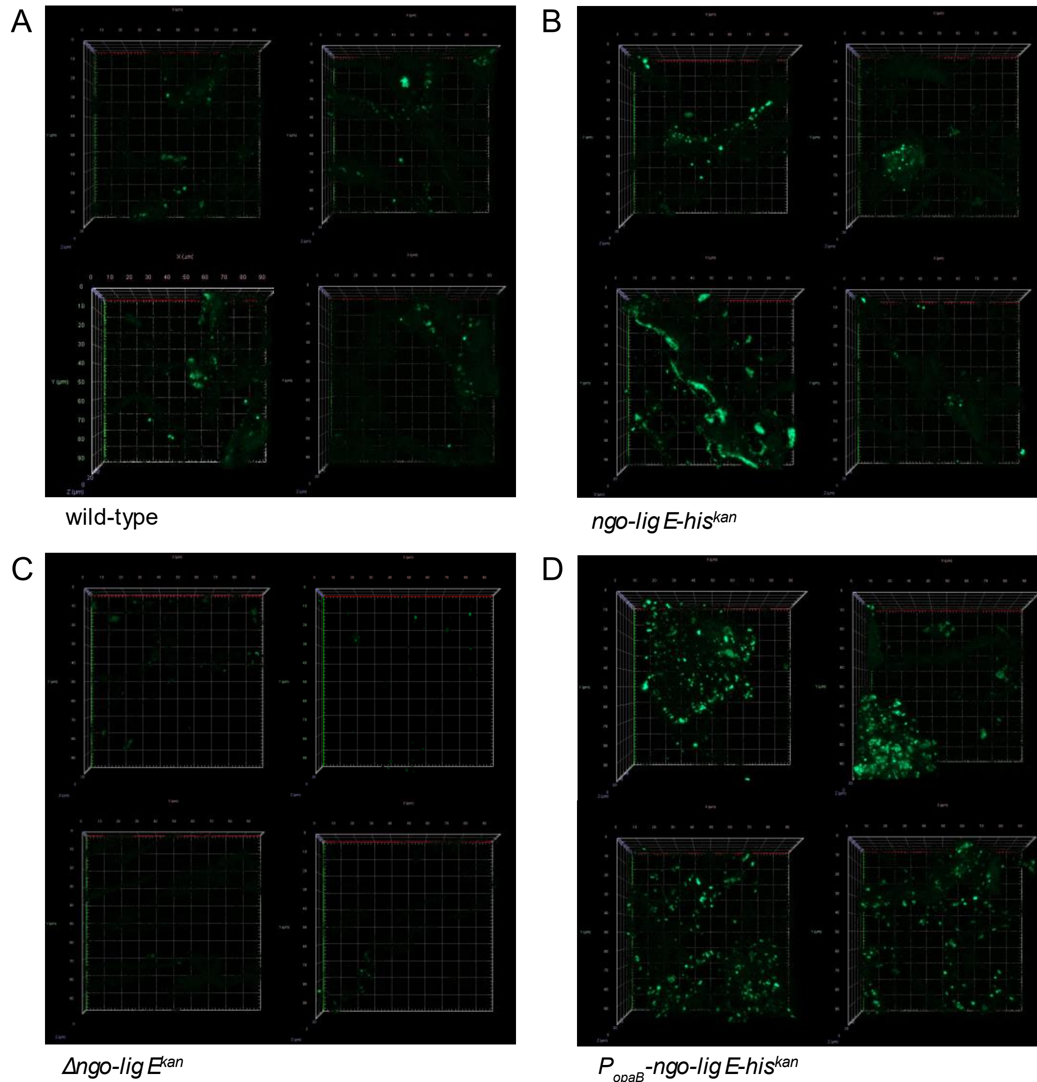


Figure B. 1 Additional CLSM z-stack images of representative fields-of-view of *N. gonorrhoeae* pEG2 (expressing sfGFP) biofilm formation and adhesion onto polycarbonate coupons in CDC Biofilm Reactors® (x40 objective magnification, em: 480 nm, exc: 505 nm). (A) Wild-type *N. gonorrhoeae* (B) *ngo-lig E-his^{kan}* (C) *Δngo-lig E^{kan}* (D) *P_{opaB}-ngo-lig E-his^{kan}*.

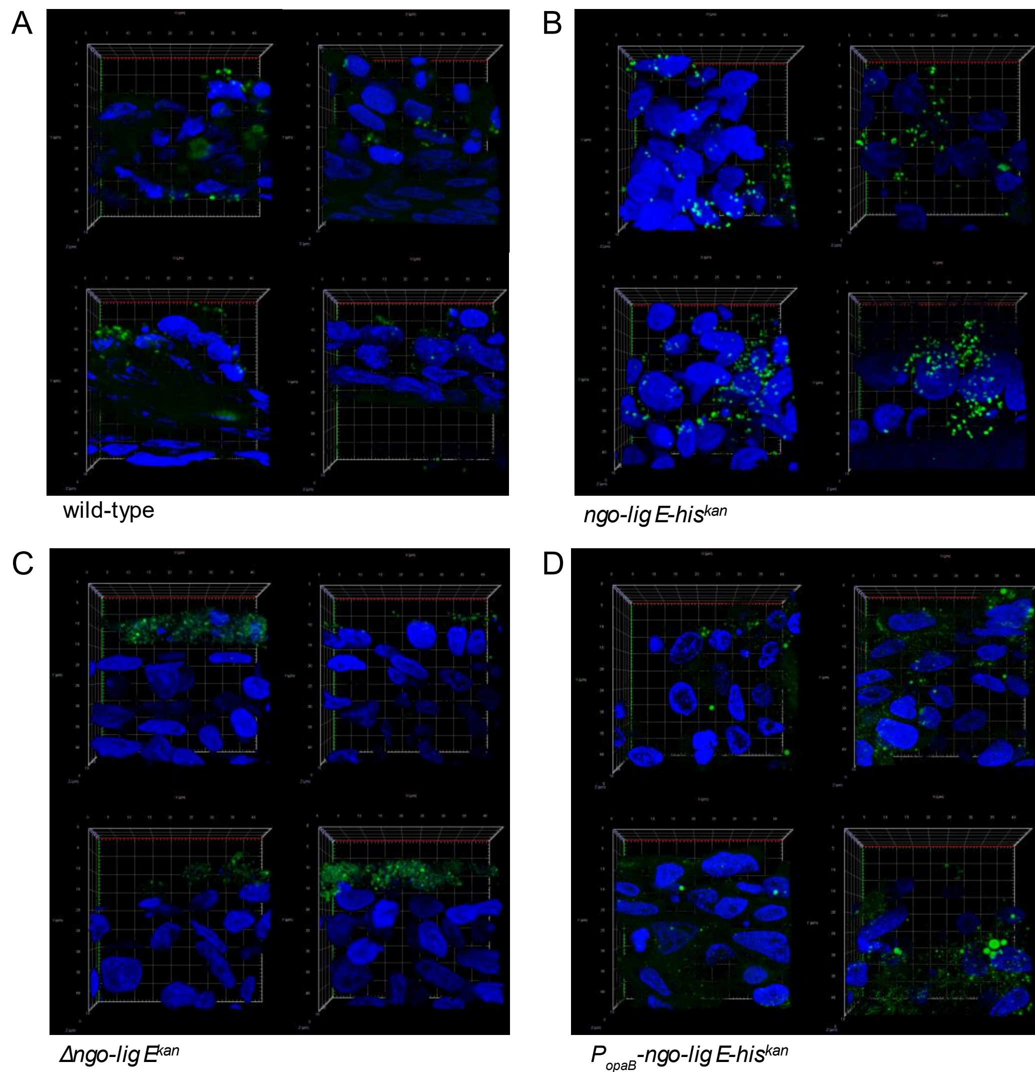


Figure B. 2 Additional CLSM z-stack images (x63 objective magnification, zoom setting 3) of representative fields-of-view of the infection and invasion of SkinEthic™ HVE cells by *Neissiera gonorrhoeae*. (A) Wild-type *N. gonorrhoeae* (B) *ngo-lig E-his*^{kan} (C) *Δngo-lig E*^{kan} (D) P_{opaB}-*ngo-lig E-his*^{kan}. *N. gonorrhoeae* pEG2 (expressing sfGFP) is shown in green (exc: 488 nm, em: 519 nm) and the nuclei of the rHVE cells in blue (exc: 405 nm, em: 449 nm).

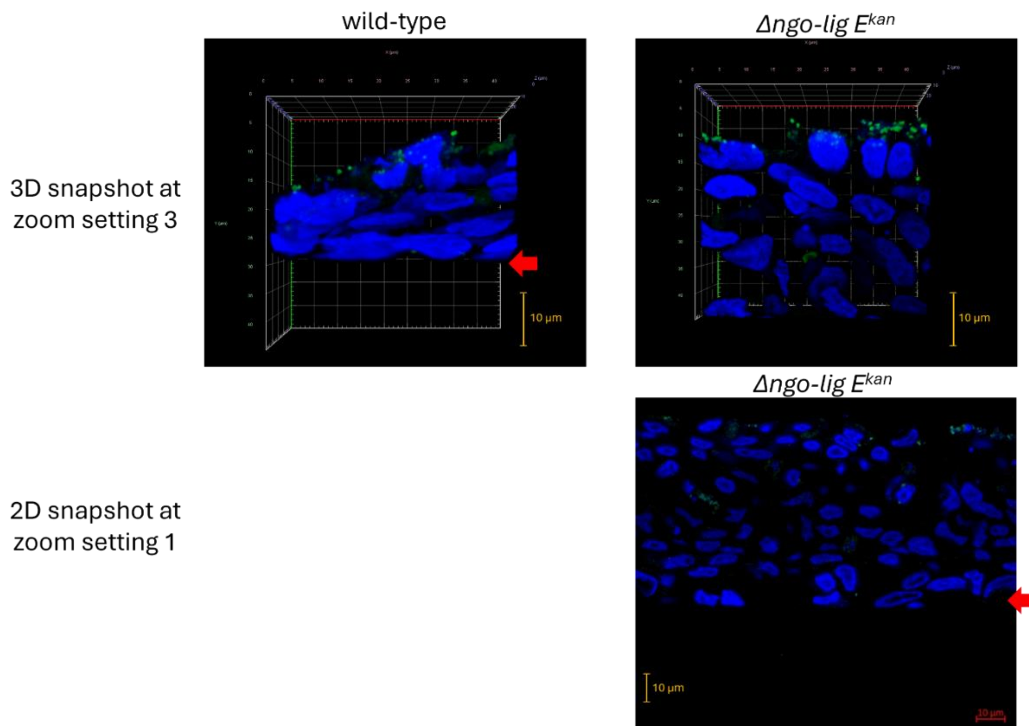


Figure B. 3 CLSM images (x63 magnification) of SkinEthic™ HVE cells (blue, exc: 405 nm, em: 449 nm) infected with *Neisseria gonorrhoeae* pEG2 (green, exc: 488 nm, em: 519 nm) showing the extent of tissue damage. The top row shows the three-dimensional snapshot of wild-type and *Δngo-lig E^kan^ N. gonorrhoeae*-infected tissue at zoom setting 3 (final zoom setting used), showing the depth and length of the remaining intact tissue, with the tissue membrane visible for *wt* at that setting (red arrow). The bottom row shows a two-dimensional snapshot of the same *Δngo-Lig E^kan^*-infected tissue slide at zoom setting 1 which finally shows the membrane of the tissue (red arrow).

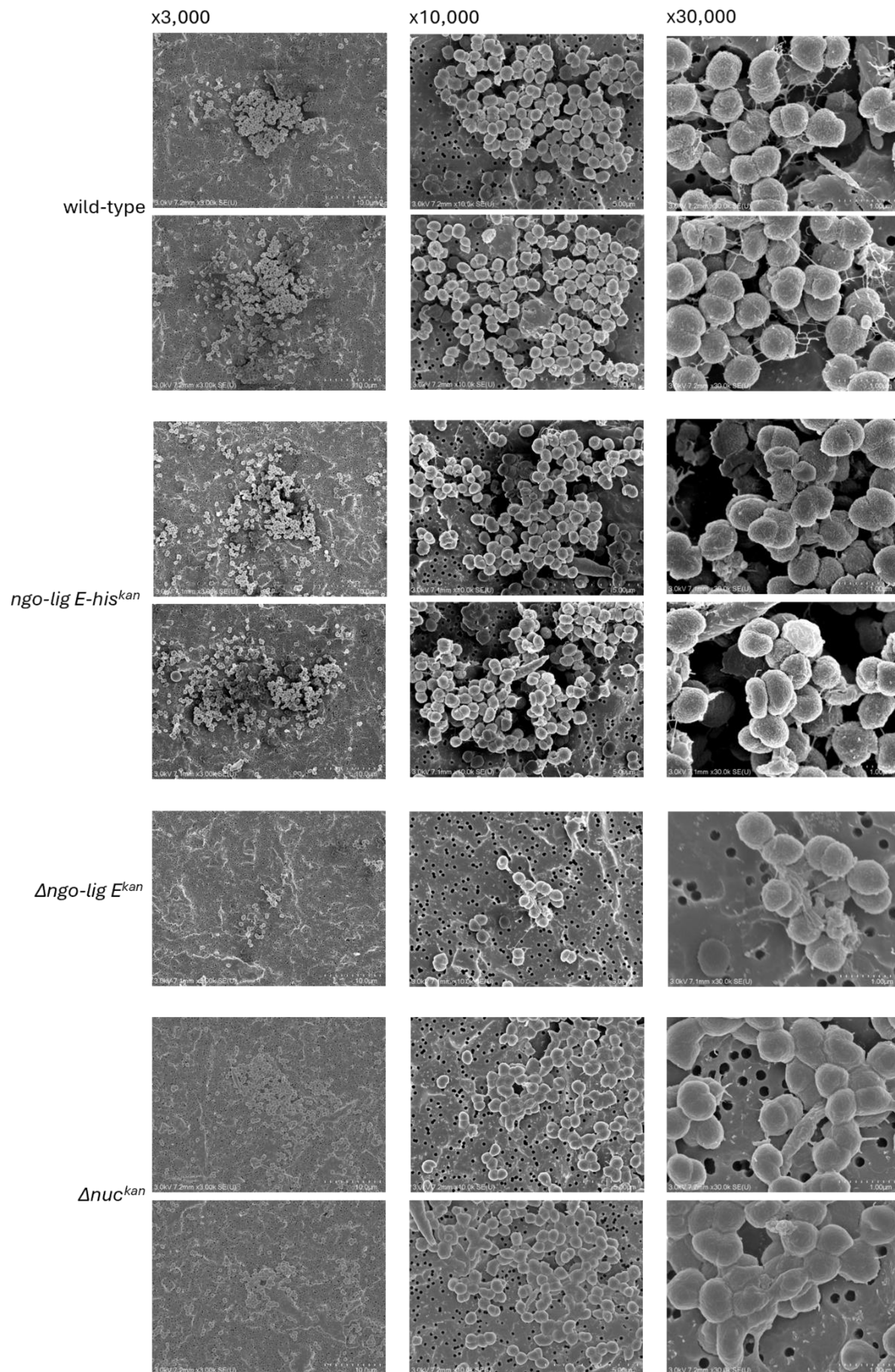


Figure B. 4 Additional supporting SEM images of *Neisseria gonorrhoeae* microcolonies formed on 0.2 µm pore size filter paper during the exponential phase of growth (9 h). Note, only one other field-of-view is shown for *Δngo-lig E^{kan}* due to the lack of microcolonies formed by this mutant.

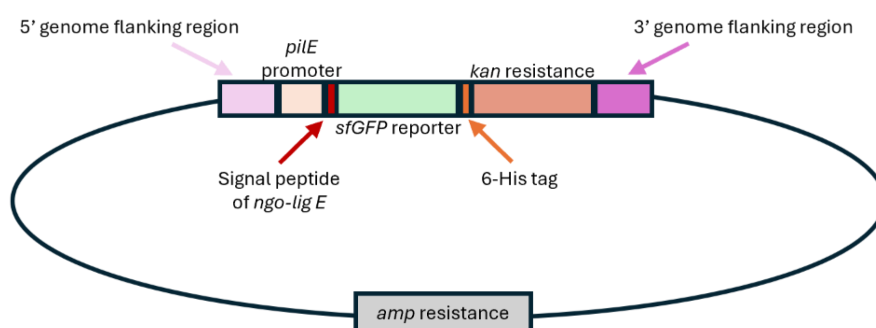
Appendix C

Supplementary Material for Chapter Five

Table C. 1 Primers used for *rpoB* gene amplification.

Name	Sequence (5’-3’)
rpoB_Fd	CAG ATT GAT GCG TAC CG
rpoB_Rev	GGC GGA TTT TCG ATA TGG

Location Construct 1



Location Construct 2

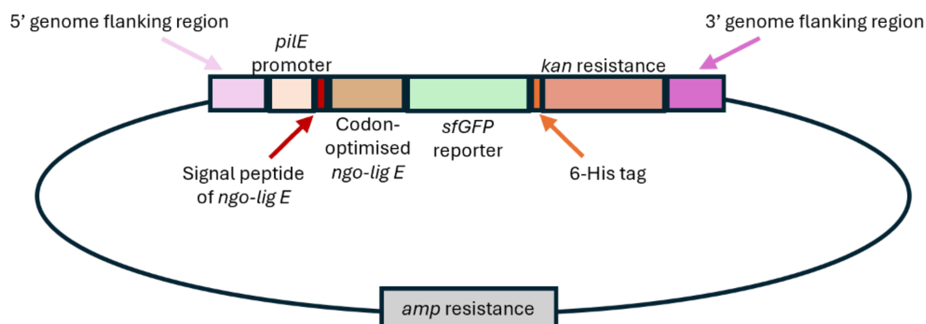


Figure C. 1 Schematic of the location constructs designed for tracking the cellular localisation of the signal peptide of Ngo-Lig E, generated via Golden Gate cloning. Location Construct 1 (top) included a strong *pilE* promoter (P_{pilE}) and the kanamycin resistance cassette with a modified *sfGFP* gene which had the signal peptide of *ngo-lig E* attached to its N-terminus and a 6-His-tag on its C-terminus. Location Construct 2 (bottom) included the native *ngo-lig E* in front of the *sfGFP* gene. The 5’ and 3’ flanks were homologous to 500 bp regions within a neutral site in the genome (NGFG_RS15145).

Table C. 2 Mean and standard error values of *Neisseria gonorrhoeae* transformation efficiencies with the P_{pilE}-*sfGFP-kan*^{R} construct plotted in Figure 5.4. Transformation efficiencies were calculated as a percentage over total CFU.

	wild-type	***Δngo-lig E*^{erm}**
Uncut DNA	0.0042152 ± 0.0002563	0.0022900 ± 0.0002295
Nick damage	0.0000428 ± 0.0000073	0.0000139 ± 0.0000040
Overhang damage	0.0037182 ± 0.0006764	0.0012966 ± 0.0002098

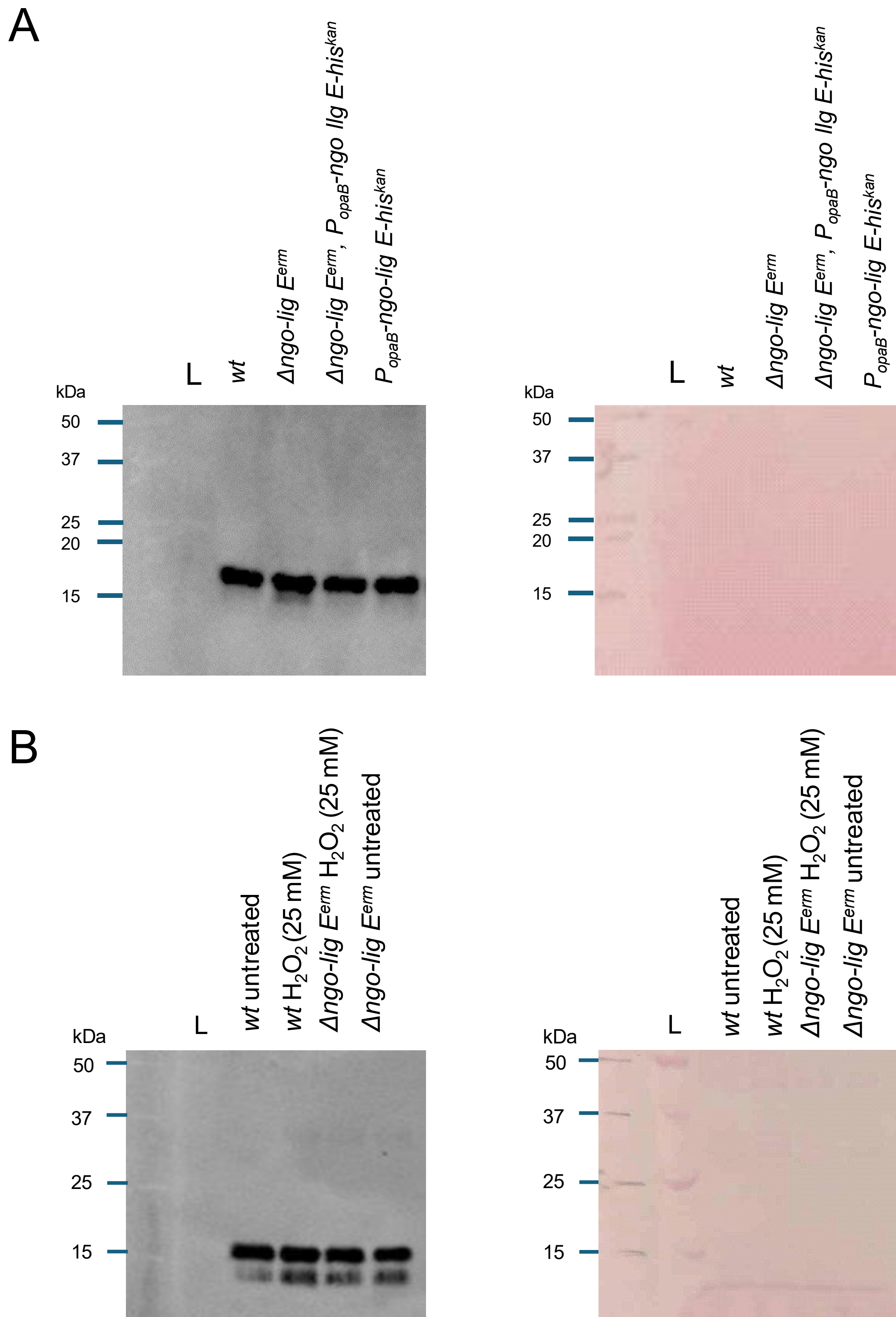


Figure C. 2 Western blot (left) of *Neisseria gonorrhoeae* cultures from DNA uptake assays against the EYLLN motif on the conserved N-terminus domain of PilE, with the respective Ponceau S stained membrane on the right. (A) *N. gonorrhoeae* cultures used for assays with the *rpoB* gene block. (B) *N. gonorrhoeae* cultures used for P_{pilE}-*sfGFP-kan*^{R} H_2O_2 transformation assays. Equal amounts of protein were loaded for each well per gel/blot. The predicted size of PilE in *N. gonorrhoeae* MS11 is ~18kDa. The sizes of the ladder (L, Protein Precision Plus^{TM} All Blue Prestained Protein Standards (Bio-Rad)) were marked on the membrane after Ponceau staining to determine the sizes of the bands after probing and visualisation.

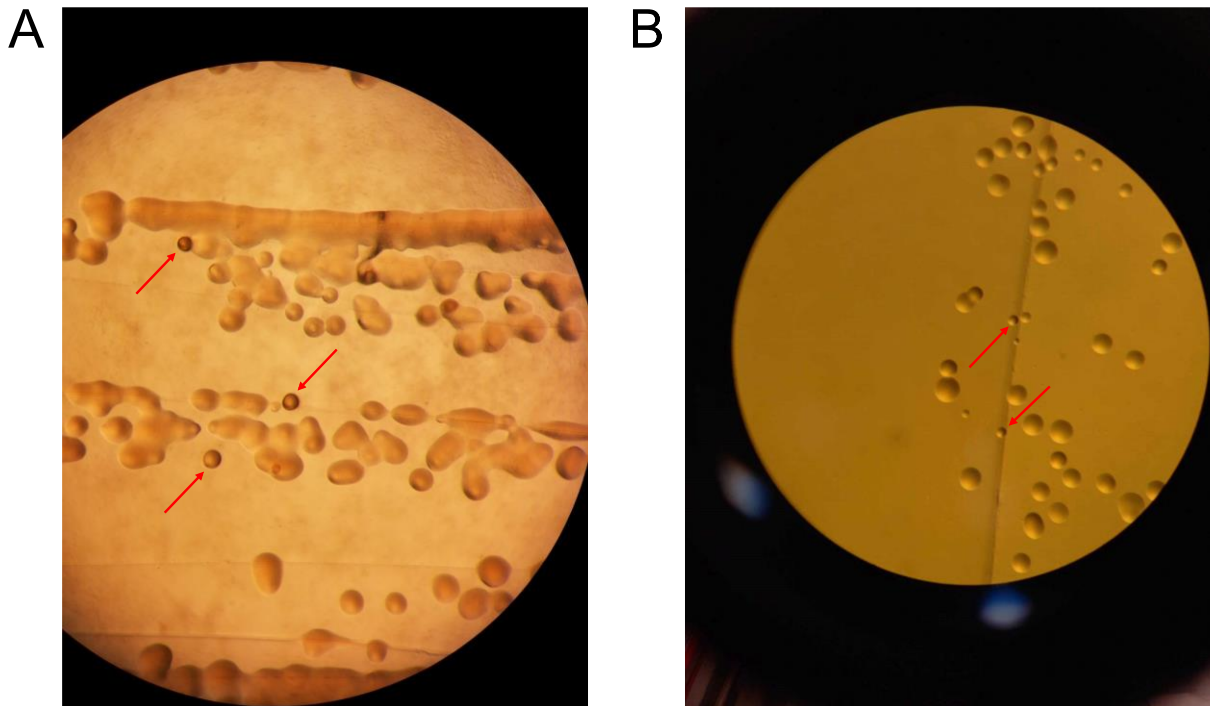


Figure C. 3 Morphology of the piliation status of (A) wild-type and (B) *Δngo-lig E^{erm} Neisseria gonorrhoeae* MS11 under a dissecting microscope. Piliated gonococci (red arrow) were determined morphologically via the presence of a dark ring around the colony. The wild-type image was taken by PhD students, Emma Walker and Stacy van Niekerk when visiting the So laboratory at the University of Arizona while learning new *N. gonorrhoeae* manipulation techniques.

Hyperlink C. 1 Genbank files of individual inserts used to generate the DNA reporter constructs via Golden Gate assembly.
Link to OneDrive folder: Genbank files

Table C. 3 Differentially expressed genes (*padj* ≤ 0.05, log2 fold change ≥ 0.5, < 1) between *ngo-lig E^erm^* and wild-type *Neisseria gonorrhoeae* MS11 (ASM15685v2, NCBI RefSeq assembly: GCF_000156855.2). Values with a log2 fold change ≥ 1 are shown in Table 5.2.

Locus tag	Gene annotation	Log2 fold change	*padj*	GO process	GO function	Other functional annotations
NGFG_RS04870	YdcH family protein	0.96852	0.00047	Uncharacterised	Function unknown	
NGFG_RS05730	Class II fumarate hydratase	0.72522	2.75E-06	GO:0006106 fumarate metabolic process	GO:0004333 fumarate hydratase activity	Energy production and conversion
NGFG_RS06725	Copper chaperone PCu(A)C	0.67093	0.02754		GO:0016531 copper chaperone activity	Inorganic ion transport and metabolism
NGFG_RS07810	Glycine cleavage system protein GcvH	0.71815	5.44E-10	GO:0019464 - glycine decarboxylation via glycine cleavage system		Amino acid transport and metabolism
NGFG_RS09450	FAD-binding domain-containing protein	0.62311	0.00728	Uncharacterised	Function unknown	Replication, recombination and repair

NGFG_RS12475	Hypothetical	0.82986	0.0073	Uncharacterised	Function unknown	
NGFG_RS16095	Hypothetical	0.68955	0.00199	Uncharacterised	Function unknown	Potential peptidase

Ngo-Lig E
Prediction: Signal Peptide (Sec/SPI)

Cleavage site between pos. 24 and 25.
Probability 0.975256

Protein type	Other	Signal Peptide (Sec/SPI)	Lipoprotein signal peptide (Sec/SPII)	TAT signal peptide (Tat/SPI)	TAT Lipoprotein signal peptide (Tat/SPII)	Pilin-like signal peptide (Sec/SPIII)
Likelihood	0.0002	0.9991	0.0002	0.0002	0.0002	0.0002

SignalP 6.0 prediction: Ngo-Lig E

OTHER
Sec/SPI n
Sec/SPI h
Sec/SPI c
CS

Probability

N N N N H H H H H H H H H H H H H H C C C C C C O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O O
M I K K I I G G I I P I F T A V F I P A S A G A A D L M L A Q E Y K G Q D I A G W A M S E K L D G V R A Y W D G K H L I S R Q G Y A F T P P

Protein sequence

Figure C. 4 Predicted cleavage site of the native Ngo-Lig E protein sequence between residues 24 and 25, determined via the SignalP 6.0 programme (Teufel et al., 2022).

Appendix D

Co-Authorship Forms

D.1 Co-authorship form for Chapter Two: The role of extracellular DNA in *Neisseria* biofilms

THE UNIVERSITY OF WAIKATO
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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter Two: Review: The role of extracellular DNA in Neisseria biofilms
To be submitted.

Nature of contribution by PhD candidate	Analysis of literature, manuscript writing and editing
Extent of contribution by PhD candidate (%)	95%

CO-AUTHORS

Name	Nature of Contribution
Adele Williamson	Analysis of literature, manuscript writing and editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Adele Williamson	[signature]	14/02/2025

July 2015

D. 2 Co-authorship form for Chapter Three: A potential role for the ATP-dependent DNA ligase Lig E of *Neisseria gonorrhoeae* in biofilm formation

THE UNIVERSITY OF WAIKATO
Te Whare Wānanga o Waikato

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Chapter Three: A role for the ATP-dependent DNA ligase lig E for Neisseria gonorrhoeae in biofilm formation
Published in BMC Microbiology (2024), Volume 24, Issue 29, DOI: https://doi.org/10.1186/s12866-024-03193-9

Nature of contribution by PhD candidate	Experimental design and processing, data analysis, manuscript writing and editing
Extent of contribution by PhD candidate (%)	80%

CO-AUTHORS

Name	Nature of Contribution
Avi Singh	DNA extraction and sequencing
Kyrin Hanning	Sequencing data analysis
Joanna Hicks	Supported experimental design and data anlysis, and manuscript editing
Adele Williamson	Experimental design, structure solving, supported data analysis, manuscript writing and editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Avi Singh	[signature]	10/02/2025
Kyrin Hanning	[signature]	12/02/2025
Joanna Hicks	[signature]	12/02/2025
Adele Williamson	[signature]	14/02/2025

July 2015

D.3 Co-authorship form for Chapter Four: Influence of the ATP-dependent DNA ligase, Lig E, on *Neisseria gonorrhoeae* microcolony and biofilm formation

Co-Authorship Form

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Email: SGR@waikato.ac.nz
Website: http://www.waikato.ac.nz/students/research-degree

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Chapter Four: Influence of the ATP-dependent DNA ligase, Lig E, on Neisseria gonorrhoeae microcolony and biofilm formation Submitted to Biofilm (2025).

Nature of contribution by PhD candidate	Experimental design and processing, data analysis, manuscript writing and editing, and funding acquisition
Extent of contribution by PhD candidate (%)	85%

CO-AUTHORS

Name	Nature of Contribution
Abdullah Albarrak	Guidance and assistance with experimental design and processing - CDC bioreactor and microscopy guidance
Joanna Hicks	Supported experimental design and data anlysis, and manuscript editing
David Williams	Supported experimental design and data analysis, manuscript writing and editing, and funding acquisiition
Adele Williamson	Experimental design, supported data analysis, manuscript writing and editing, and funding acquisition

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Abdullah Albarrak	A.Albarrak	7/2/25
Joanna Hicks	J Hicks	12/02/2025
David Williams	David Williams	6th Feb 2025
Adele Williamson	[illegible]	14/02/2025

July 2015

D.4 Co-authorship form for Chapter Five: DNA uptake during natural competence? A proposed location and role of the ATP-dependent DNA ligase, Lig E, in *Neisseria gonorrhoeae*

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Co-Authorship Form

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Chapter Five: DNA uptake during natural competence? A proposed location and role of the ATP-dependent DNA ligase, Lig E, in Neisseria gonorrhoeae
To be submitted.

Nature of contribution by PhD candidate	Experimental design and processing, data analysis, manuscript writing and editing
Extent of contribution by PhD candidate (%)	85%

CO-AUTHORS

Name	Nature of Contribution
Avi Singh	Golden gate cloning optimisation - generation of some DNA uptake constructs
Joanna Hicks	Supported experimental design and data anlysis, and manuscript editing
Adele Williamson	Experimental design, supported data analysis, manuscript writing and editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Avi Singh	[signature]	10/02/2025
Joanna Hicks	[signature]	12/02/2025
Adele Williamson	[signature]	14/02/2025

July 2015