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The Role of the Type IV Pilus Complex in DNA Transformation in *Neisseria gonorrhoeae*

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Abstract

The Role of the Type IV Pilus Complex in DNA Transformation in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea and is adapted to survive in humans, its only host. The *N. gonorrhoeae* cell wall is critical for maintaining envelope integrity, resisting immune cell killing, and production of cytotoxic peptidoglycan (PG) fragments. Deletion of the *N. gonorrhoeae* genes encoding two low-molecular-mass, penicillin-binding proteins (LMM PBPs), DacB and DacC, substantially altered the PG cross-linking. Loss of DacB peptidase resulted in global alterations to the PG composition, while loss of DacC affected a much narrower subset of PG peptide components. A double Δ*dacB*/Δ*dacC* mutant resembled the Δ*dacB* single mutant, but had an even greater level of cross-linked PG. While single Δ*dacB* or Δ*dacC* mutants did not show any major phenotypes, the Δ*dacB*/Δ*dacC* mutant displayed an altered cellular morphology, decreased resistance to antibiotics, and increased sensitivity to detergent mediated death. Loss of the two proteins drastically reduced the number of Type IV pili (Tfp), a critical virulence factor. The decreased piliation reduced transformation efficiency and correlated with increased growth rate. While these two LMM PBPs differentially alter the PG composition, their overlapping effects are essential to proper cell biology and expression of factors critical for pathogenesis.

Required for gonococcal infection, Tfp mediate many functions including adherence, twitching motility, defense against neutrophil killing, and natural transformation. Critical for immune escape, the gonococcal Tfp undergoes antigenic variation, a recombination event at the *pilE* locus that varies the surface exposed residues of the major pilus subunit PilE (pilin) in the

pilus fiber. This programmed recombination system has the potential to produce thousands of pilin variants and can produce strains with unproductive pilin molecules that are completely unable to form Tfp. Saturating mutagenesis of the 3' third of the *pilE* gene identified 68 unique single nucleotide mutations that each resulted in an underpiliated colony morphology. Notably, all isolates, including those with undetectable levels of pilin protein and no observable surface-exposed pili, retained an intermediate level of transformation competence not exhibited in Δ*pilE* strains. Site-directed, nonsense mutations revealed that only the first 38 amino acids of the mature pilin N-terminus (the N-terminal domain or Ntd) are required for transformation competence, and extended Tfp are not required for competence. The Ntd corresponds to the alternative product of S-pilin cleavage, a specific proteolysis unique to pathogenic *Neisseria*. Mutation of the S-pilin cleavage site demonstrated that S-pilin cleavage mediated release of the Ntd is required for competence when a strain produces unproductive pilin molecules that cannot assemble into a Tfp through mutation or antigenic variation. Attempts to identify the protease responsible for S-pilin cleavage were unsuccessful. We conclude that S-pilin cleavage evolved as a mechanism to maintain competence in nonpiliated antigenic variants and suggest there are alternate forms of the Tfp assembly apparatus that mediate various functions including transformation.

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List of Abbreviations

(AA) – Amino acid

(ATC) – Anhydrotetracycline

(CFU) – Colony forming units

(CRISPR) - Clustered, regularly interspaced, short palindromic repeat

(DC) – Double complement

(dsDNA) – Double-stranded DNA

(DUS) – DNA uptake sequence

(ELISA) – Enzyme-linked immunosorbent assay

(G4) – Guanine quadruplex

(GCB) – Gonococcal base

(HGT) – Horizontal gene transfer

(HLB) – Hydrophobic-Lipophilic-Balanced

(HMM) – High molecular mass

(HPLC) – High performance liquid chromatography

(HV_L) – Hyper-variable loop

(HV_T) – Hyper-variable tail

(LMM) – Low molecular mass

(LOS) – Lipooligosaccharide

(mRNA) – Messenger RNA

(ND) – Not determined

(Ntd) – N-terminal domain

(Opa) – Opacity

(PBP) – Penicillin binding proteins

(PG) – Peptidoglycan

(sRNA) – Small RNA

(ssDNA) – Single-stranded DNA

(SV) – Semi-variable region

(TEM) – Transmission electron microscopy

(TFA) – Trifluoracetic acid

(Tfp) – Type IV pilus

Dedication

To my family, both old and new, for their unwavering support. To my mother and father, Colleen and Mark Obergfell, for their constant love, my self-confidence, and their example and emphasis on hard work and the constant quest for knowledge. To my partner, best friend, and love of my life, Alexandra Kirsch, without whom none of this would have happened. Thank you for *challenging* and supporting me every day and being a constant source of happiness.

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Chapter 1: Introduction

Introduction

The majority of species in the genus *Neisseria* are commensal bacteria that colonize mucosal surfaces. These organisms are Gram-negative, coccal (except for *Neisseria elongata, Neisseria weaveri,* and *Neisseria bacilliformis*) members of the Neisseriaceae family along with *Moraxella, Kingella* and *Eikenella*. The two pathogenic species, *Neisseria gonorrhoeae* (the gonococcus) and *Neisseria meningitidis* (the meningococcus), are the causative agent of gonorrhea and the primary cause of bacterial meningitis in young adults, respectively. Both organisms are strict human pathogens with no known environmental reservoirs that have evolved from commensal organisms within the human population (Virji, 2009). Gonorrhea has plagued humanity for at least as long as we have had historical records, with Chinese medical texts from the year 2637 BCE describing inflammation and discharge from genitalia (Wain, 1947). Mistaken for involuntary seminal leakage, gonorrhea was name by the Greek physician Galen from the words *gonos* and *rhoia* meaning flowing seed. *N. gonorrhoeae* was finally identified as the causative agent of gonorrhea in 1879 by the 24-year-old Albert Ludwig Sigesmund Neisser (Neisser, 1879).

N. gonorrhoeae causes an estimated 106 million infections annually worldwide (Francis Ndowa, 2012). In 2015, 395,216 reported cases of gonorrhea occurred in the United States alone, but the Centers for Disease Control and Prevention (CDC) estimates that at least another 400,000 cases went unreported (Satterwhite *et al.*, 2013, Barton *et al.*, 2015). Nearly 70% of cases in the United states occur in people between the ages of 15 and 24. Disease incidence is also on the rise in the elderly, with the number of annual cases doubling between 2011 and 2015 in those over

the age of 65 (Barton *et al.*, 2015). *N. gonorrhoeae* typically colonizes the genitourinary tract resulting in non-complicated urethritis and cervicitis in men and women respectively. In men, the disease is characterized by purulent discharge, painful urination and dysuria. In women, symptoms often include vaginal discharge, dysuria, and pelvic pain. The spectrum of infection can vary dramatically with some individuals remaining asymptomatic. These asymptomatic cases are rarely treated and contribute to the spread of the disease. Infection can, in rare cases, result in severe sequelae, including pelvic inflammatory disease (PID), and epididymitis (Swasdio *et al.*, 1996, O'Brien *et al.*, 1983, Wasserheit, 1994). Mucosal contact can also result in infection of the rectum, conjunctiva, and pharynx (Wasserheit, 1994).

The study of the *Neisseria* is important for public health reasons, but also provides a defined system to study evolution of two highly related organisms that cause distinct diseases. A unique aspect of the pathogenic *Neisseria* is the presence of sophisticated genetic systems that contribute to pathogenesis including those that mediate the processes of DNA transformation and pilin antigenic variation.

Natural DNA transformation

There is a diverse set of more than 80 identified naturally transformable bacterial species that are able to recognize free DNA in the environment, import it across the envelope and recombine exogenous DNA with resident DNA molecules (Johnston *et al.*, 2014). Unlike the majority of naturally competent species, *Neisseria sp.* are constitutively competent, capable of transformation at all phases of growth (Sparling, 1966, Biswas *et al.*, 1977). Natural transformation is the primary means of horizontal gene transfer (HGT) in *Neisseria* with a documented flow of information amongst both commensal and pathogenic members of the genus

(Koomey, 1998, Sox *et al.*, 1978). Similar to the vast majority of Gram-negative bacteria, transformation in *Neisseria* is dependent on a type IV pilus (Tfp) complex (Chen & Dubnau, 2004). Pathogenic *Neisseria* often undergo HGT with recombination occurring so frequently that there is a marked inability to establish stable clonal lineages (Smith *et al.*, 1993). These genomic signatures suggest that mixed infections are common and this idea has been supported by some studies (Martin & Ison, 2003, Lynn *et al.*, 2005). Although *N. meningitidis* maintains limited lineage structure, both species tend toward linkage equilibrium, and this frequent genetic exchange is thought to contribute to the rapid spread of antibiotic resistance among *N. gonorrhoeae* strains (Gibbs & Meyer, 1996, Hobbs *et al.*, 1994, Snyder *et al.*, 2004, Buckee *et al.*, 2008). This HGT has led to clinical isolation of *N. gonorrhoeae* strains with resistance to multiple antibiotics (Goire *et al.*, 2014). Though no single strain has accumulated all of the resistance markers, there are resistant lineages to all currently recommended therapies (Goire *et al.*, 2014). The threat of untreatable gonorrhea has earned *N. gonorrhoeae* a spot on the Center for Disease Control's list of superbugs and its highest threat level reserved for only three organisms (Kirkcaldy *et al.*, 2011, Lewis, 2010, Prevention, 2013).

The Type IV pilus

Tfp are a critical virulence factor for many pathogens and also promote interactions of nonpathogens with their environments. Tfp are long, thin fibers that undergo dynamic cycles of extension and retraction and mediate twitching motility, cellular adherence, microcolony formation, and natural transformation in both *N. gonorrhoeae* and *N. meningitidis* (Craig *et al.*, 2004, Merz *et al.*, 2000, Swanson, 1973, Dietrich *et al.*, 2011, Freitag *et al.*, 1995). The expression of *Neisseria* Tfp correlates directly with transformation efficiency, an observation

that has been expanded to many Gram negative species (Sparling, 1966). In the presence of excess DNA, highly piliated strains of *N. gonorrhoeae* can achieve transformation efficiencies more than a million times higher than strains lacking the major Tfp pilin PilE (Long *et al.*, 2003). There is a complex Tfp assembly apparatus present in the bacterial envelope that is responsible for pilus expression and associated functions (***Figure 1***). Many of the Tfp complex proteins are required for transformation. Among the Tfp complex proteins that have a defined function, PilD is a periplasmic protease responsible for processing PilE into the mature form that can be assembled into the pilus fiber (Freitag *et al.*, 1995). PilD is required for pilus expression and transformation competence. Mutations in Tfp structural proteins including the inner membrane protein PilG and the pore forming secretin PilQ abrogate transformation (Drake & Koomey, 1995, Tonjum *et al.*, 1995). Additionally, transformation is dependent on the two cytoplasmic NTPases PilF and PilT (Freitag *et al.*, 1995, Wolfgang *et al.*, 1998a). PilF is thought to power pilus extension while PilT is required for pilus retraction and twitching motility. The requirement for many of the Tfp complex proteins in transformation has led to the generally accepted hypothesis that the pilus fiber mediates the binding and initial uptake of DNA into the periplasm as well as transport of DNA through the outer membrane during transformation (Biswas *et al.*, 1989, Aas *et al.*, 2002b, Berry *et al.*, 2013b). This has never been conclusively shown, and it is an open question whether Tfp or a pilus-like apparatus (pseudopilus) is actually responsible for DNA uptake across the outer membrane (Chen & Dubnau, 2003).

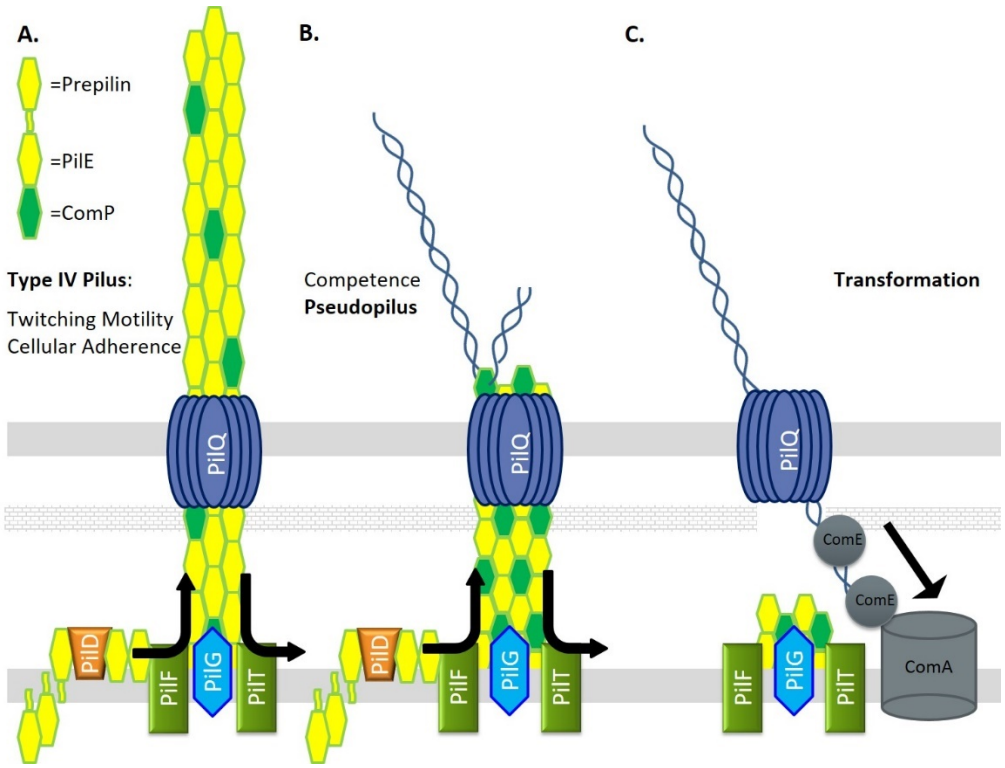


Figure 1. Type IV pilus and DNA uptake.

A.Type IV Pilus – The Tfp is a several micron long, 60 angstrom wide fiber anchored in the inner membrane by PilG that extends through the PilQ secretin pore. Composed mainly of the major pilin PilE (pilin), which is processed by a dedicated protease, PilD. The PilF and PilT NTPases mediate extension and retraction of the pilus through polymerization and depolymerization of the pilin subunits. **B.** Competence Pseudopilus – Hypothesized pseudopilus that could mediate transformation. Utilizes the Tfp complex including the PilQ pore but is not an extended fiber. Possible localization of ComP to the pseudopilus could mediate specific DNA binding. **C.** DNA Uptake Model - Retraction of the (pseudo)pilus mediated by PilT brings the initial length of DNA into the periplasm. DNA is then bound by a protein or protein complex which mediates import of the remaining length of DNA into the periplasm. The inner membrane protein ComA facilitates DNA entry into the cytoplasm.

Contributing to this uncertainty are the observations that small amounts and altered forms of pilin are sufficient for transformation. Pilin (*pilE* gene product) can exist in variant forms such as S-pilin (a short or secreted form) and L-pilin (long form). S-pilin results from a cleavage event and produces a soluble form that is secreted from the cell by an unknown mechanism (Haas *et al.,* 1987). Some S-pilin variants display intermediate piliation phenotypes but wild-type levels of transformation (Gibbs *et al.*, 1989, Long *et al.,* 1998). L-pilin variants result from a duplication of coding sequences in *pilE* that produces an oversized pilin monomer that cannot be assembled into pilus fibers, yet only reduces transformation efficiencies 35-fold (Haas *et al.*, 1987, Gibbs *et al.*, 1989). Alongside the pilin variants investigations, a 2003 study showed that when the level of pilin expression is reduced to the point where observable pili are extremely rare in a population of cells, the cells still exhibit considerable transformation efficiencies (Long *et al.*, 2003). The competence in these pilus-deficient gonococci was still dependent on PilT and PilQ, leading to the hypothesis that extended Tfp are not necessary for transformation; rather a pseudopilus apparatus, utilizing the Tfp complex of proteins, is sufficient for transformation. These observations form an alternative working model in which DNA is bound extracellularly by the (pseudo-)pilus (***Figure 1***). Retraction of the (pseudo-)pilus pulls the initial length of DNA into the periplasm. A single retraction event of either the pilus or pseudopilus would not be sufficient to import an entire length of DNA into the periplasm. Therefore, either concerted action of several pili together or non-specific binding of a complex possibly containing ComE then mediates import of the remaining length of DNA into the periplasm.

DNA uptake sequence

Although constitutively competent and lacking any apparent transformation regulation, *Neisseria* species preferentially transform self-DNA (Goodman & Scocca, 1988, Elkins *et al.*, 1991). This is accomplished through repeat sequences spread across their genomes that aid in efficient uptake and transformation of self-DNA. The initially identified DNA uptake sequence (DUS) is a 10 base sequence (DUS10 5′-GCCGTCTGAA), but an extended 12-mer DUS (DUS12 5′-ATGCCGTCTGAA) occurs at greater than 75% of DUS10 locations and slightly increases transformation efficiencies over the 10-mer DUS (Ambur *et al.*, 2007, Smith *et al.*, 1999, Goodman & Scocca, 1988). Remarkably, the 10 base sequence occurs about once every kb of the genome, a frequency a thousand times higher than chance predicts and is often located as an inverted repeat in putative Rho-independent transcriptional terminators (Goodman & Scocca, 1988, Smith *et al.*, 1999). It has been suggested that DUS sequences are more often found within DNA repair genes due to a role in genome maintenance, but this analysis does not take into account that DUS can act over several kb distances (Davidsen *et al.*, 2004). It is possible that the enrichment of DUS sequences in core genes (genes shared among all isolates) may indicate slow accumulation of the sequences with the oldest (most essential) genes accumulating the highest proportion of DUS (Treangen *et al.*, 2008). The 10 base DUS sequence was identified for its ability to competitively inhibit transformation (Goodman & Scocca, 1988) and to enhance both DNA uptake and transformation when added to a previously untransformable plasmid (Elkins *et al.*, 1991). DUS mediated transformation enhancement is both strain and strand specific in *N. gonorrhoeae*; DUS containing DNA only enhances transformation 20-fold in strain FA1090 while it increases efficiencies 150-fold in strain MS11 (Duffin & Seifert, 2010b). Because the DUS is non-palindromic, the two divergent single-stranded DUS sequences were investigated for

their relative effect on transformation and given identifiers Watson (5′-ATGCCGTCTGAA) and Crick (5′-TTCAGACGGCAT). The single-stranded Watson DUS12 increased transformation efficiencies with single-stranded DNA (ssDNA) 180-470 fold while the single-stranded Crick DUS12 only enhanced transformation 7 fold over non-DUS containing ssDNA (Duffin & Seifert, 2012). Notably, even with the Watson DUS12, ssDNA was 2-24 fold less efficient than DUS12 containing dsDNA in transformation assays. These data suggest that there may be different uses for the double-stranded and single-stranded uptake sequences.

While the Tfp and its components were exhaustively investigated for a role in specific binding of the DUS, the mediator of self-DNA recognition was not identified until a landmark 2013 study by the Pelicic lab (Mathis & Scocca, 1984, Dorward & Garon, 1989, Wolfgang *et al.*, 1999, Aas *et al.*, 2002a, Cehovin *et al.*, 2013, Chen & Gotschlich, 2001, Assalkhou *et al.*, 2007, Lang *et al.*, 2009, Benam *et al.*, 2011). This study identified the type IV minor pilin ComP as the DUS receptor in *N. meningitidis* (Cehovin *et al.*, 2013). The study investigated ComP due to its high level of sequence conservation amongst several *Neisseria* species (99%) suggesting an important function. They showed that ComP was the only known pilin component that bound dsDNA and had affinity for DUS-containing DNA. Through mutational analysis, it was also shown that an electronegative stripe on ComP that is predicted to be surface exposed on Tfp is responsible for the DNA binding ability of ComP (Cehovin *et al.*, 2013). While the DUS sequence is conserved in both *N. meningitidis* and *N. gonorrhoeae*, different members of the *Neisseria* genus and the broader *Neisseriaceae* family have slightly different DUS sequences termed dialects (Frye *et al.*, 2013). The efficiency of the DUS dialects for transformation of *N. meningitidis* was shown to be dependent on the expression of the cognate ComP protein (Berry

et al., 2013a). Notably, while the inner bases of the DUS are most critical for allowing high transformation efficiency and DNA binding by the ComP protein, not all residues that were important for full transformation efficiency were important for ComP binding (Berry *et al.*, 2013a). This result suggests that the DUS may act in a different step during transformation independent of ComP and could rely on the single stranded Watson DUS sequence shown to function in transformation (Duffin & Seifert, 2012)..

Processing and recombination

There are three gonococcal genes, *comL, tpc,* and *comA* important for transformation competence that are proposed to act during the transport of DNA into the cytoplasm (Fussenegger *et al.*, 1996a, Fussenegger *et al.*, 1996b, Chaussee & Hill, 1998). ComL and Tpc are both localized to the periplasm and have been implicated in DNA transport across the peptidoglycan layer possibly through the creation of localized breaks in the cell wall (Fussenegger *et al.*, 1996a, Fussenegger *et al.*, 1996b). ComA displays homology with ComEC of *B. subtilis* which is a polytopic membrane protein localized to the inner membrane that delivers ssDNA into the cytoplasm (Draskovic & Dubnau, 2005, Chaussee & Hill, 1998). Whether ComA also transports dsDNA into the cell is an unanswered question.

Following import into the cell, DNA is subject to restriction modification (Sox *et al.*, 1979). *N. gonorrhoeae* encodes a large array of methyltransferases and their corresponding endonucleases that form an effective restriction barrier to plasmid DNA, making plasmids 1,000 fold less transformable than chromosomal loci (Stein *et al.*, 1995, Eisenstein *et al.*, 1977). *N. meningitidis* is able to restrict transforming DNA through a unique CRISPR/Cas system that is not present in the sequenced gonococcal isolates (Zhang *et al.*, 2013). Clustered, regularly

interspaced, short palindromic repeat (CRISPR) loci confer sequence specific adaptive immunity based on CRISPR-associated (Cas) protein complexes ability to cleave incoming DNA (Barrangou, 2013). While generally thought to be an adaptation to protect against phage invasion and foreign plasmid conjugation, the type II CRISPR/Cas system of *N. meningitidis* was the first system shown to naturally prevent transformation (Zhang *et al.*, 2013). The meningococcal CRISPR/Cas pathway is the most streamlined system characterized to date and encodes sequences that may be able to restrict the transfer of certain virulence determinants amongst cells of different lineages. This may explain the increased ability of the meningococcus to form semi-clonal lineages in comparison to the gonococcus which lacks a CRISPR/Cas system.

Following entry into the cytoplasm, transforming DNA is integrated into the chromosome through homologous recombination mediated by the major recombinase, RecA. DprA, a cytoplasmic ssDNA binding protein, is required for transformation, likely to protect the DNA from degradation and promote RecA binding (Duffin & Barber, 2016). RecA binds ssDNA, locates the complement strand in a homologous DNA duplex, and catalyze D-loop formation (Koomey & Falkow, 1987, Chen *et al.*, 2008). RecA activity is limited by RecX which facilitates more efficient recombination (Gruenig *et al.*, 2010). Investigations into the role of DNA repair pathways in transformation showed the RecF-like pathway that mediates ssDNA gap repair in *E. coli* is not involved in transformation-mediated homologous recombination, but mutations in the RecBCD pathway show a 10 to 100-fold decrease in transformation efficiency (Mehr & Seifert, 1998, Stohl & Seifert, 2001). The RecBCD pathway is involved in dsDNA break repair in *E. coli* and would be predicted to only act on dsDNA transformation substrates (Dillingham & Kowalczykowski, 2008). It is likely that transformation proceeds mainly through ssDNA

intermediates, and the observation that the majority of transformation is independent of the RecBCD pathway confirms the ssDNA dependence. However, the amount of transformation that is RecBCD-dependent suggests that either there is some dsDNA transported into the cytoplasm or that a portion of the transported ssDNA is converted to dsDNA and then used for recombination. Finally, mutation of PriA helicase, which helps restart stalled replication forks in *E. coli*, lowers transformation efficiency. The helicase may act directly on the D-loop produced by RecA mediated ssDNA invasion of the duplex, or alternatively, may process a different intermediate formed during the recombination process that requires replication restart (Kline & Seifert, 2005a, Marians, 2000). There are no other naturally transformable organisms reported where PriA has been tested for a role in transformation processes, so it is unclear if this is a unique processing requirement in *Neisseria*. The details of DNA processing and recombination during transformation in the *Neisseria* is still open to further investigation.

Antigenic variation

Introduction

As obligate human pathogens, *N. gonorrhoeae* and *N. meningitidis* are constantly under immune surveillance. One vital mechanism for immune avoidance is antigenic variation, by which a pathogenic organism constantly modifies surface-exposed immunogenic molecules. This strategy can result in prolonged colonization or allow for reinfection of a previously infected host who has a potentially effective immune response that is made ineffectual by the variation (reviewed in (Vink *et al.*, 2012)). Phase variation is a related but distinct process of phenotypic variation where a cell switches between defined phases of expression, either between ON and OFF phases or between two variant forms. Both phase and antigenic variation processes generally occur at rates higher than the normal mutation rate of the organism. Phase variation

systems have the ability to reversibly switch between the phases and are usually mediated by polynucleotide repeat variation, invertible elements, or differential methylation (reviewed in (van der Woude, 2011)). In contrast, antigenic variation systems have the ability to stochastically express many different forms of a gene product and can be based on multigene phase variation or a recombination-based diversity generation system (see next section). Both phase and antigenic variation systems can have functional as well as immune system consequences.

Both pathogenic *Neisseria* species express three antigenically or phase variable major surface determinants: the opacity (Opa) outer membrane proteins, which act as adhesins; lipooligosaccharide (LOS), which decorates the outer membrane and is also involved in host interactions; and Tfp (Kline *et al.*, 2003). The commensal organisms express some of these molecules but do not undergo the variation processes. Both LOS and Opa proteins undergo antigenic variation through an ON/OFF phase variation mechanism, mediated by slipped-strand mispairing of tandem repeats in multiple genes (Stern *et al.*, 1986, Danaher *et al.*, 1995, Jennings *et al.*, 1995). In the case of the Opa gene family, there are 4-13 individual genes that phase vary ON and OFF independently through changes in a pentamer repeat found in the signal sequence coding region of each gene (Stern *et al.*, 1986). It is likely that the Opa protein antigenic variation process is mainly used to create functional variants to promote interactions with different human cellular receptors (Bos *et al.*, 1999). Five of the LOS biosynthetic glycosyltransferases are phase variable due to polynucleotide repeats in promoter or coding regions that when altered turn each gene ON or OFF (Gotschlich, 1994, Jennings *et al.*, 1995). The LOS structure is then defined by which set of these five genes is expressed in combination with the eight invariant biosynthetic genes. Pilus antigenic variation, in contrast, uses a complex,

programmed homologous recombination system to express antigenically distinct peptide sequences on the Tfp. While homologous recombination-based systems for antigenic variation are found in both prokaryotic and eukaryotic pathogens, the pilin antigenic variation in *Neisseria* has become a model system for these types of diversity generation systems.

Pilin antigenic variation

Pilin antigenic variation is mediated by non-reciprocal recombination events, where a sequence from a silent pilin copy is donated to the pilin expression locus but does not change in the reaction (***Figure 2***). Gonococci typically carry around 18 silent pilin copies in four to six *pilS* loci (Meyer *et al.*, 1982, Hamrick *et al.*, 2001). Lacking a promoter, ribosome binding site, and the coding sequence for the N-terminal alpha helix of pilin, the silent copies are not expressed (Haas & Meyer, 1986, Haas *et al.*, 1992, Segal *et al.*, 1986). The *pilE* gene encodes a 5' constant region followed by a semi-variable (SV) region, two highly conserved *cys* regions (containing the two-disulfide bond forming cysteines) sandwiched around the hypervariable loop, and finally the hypervariable tail. The amount of conservation and diversity in the different regions is due directly to the sequence variation in the silent copies (Haas & Meyer, 1986). The hypervariable regions of *pilE* correspond to the coding regions that display the largest diversity amongst the silent copies. Highlighting the functional role of pilin variation in creating antigenic diversity, the hypervariable regions correspond to the surface and antibody-exposed areas of pilin in the pilus fiber (Craig *et al.*, 2006, Forest *et al.*, 1996). During pilin antigenic variation a portion of a *pilS* copy is transferred into the expressed *pilE*. Because the amount of transferred sequence can range from a single nucleotide to the entire *pilS* copy and sequence can be donated from multiple

pilS copies along the length of *pilE*, this process can result in a remarkably large set of expressed pilin sequences (Figure 3A-C) (Criss *et al.*, 2005).

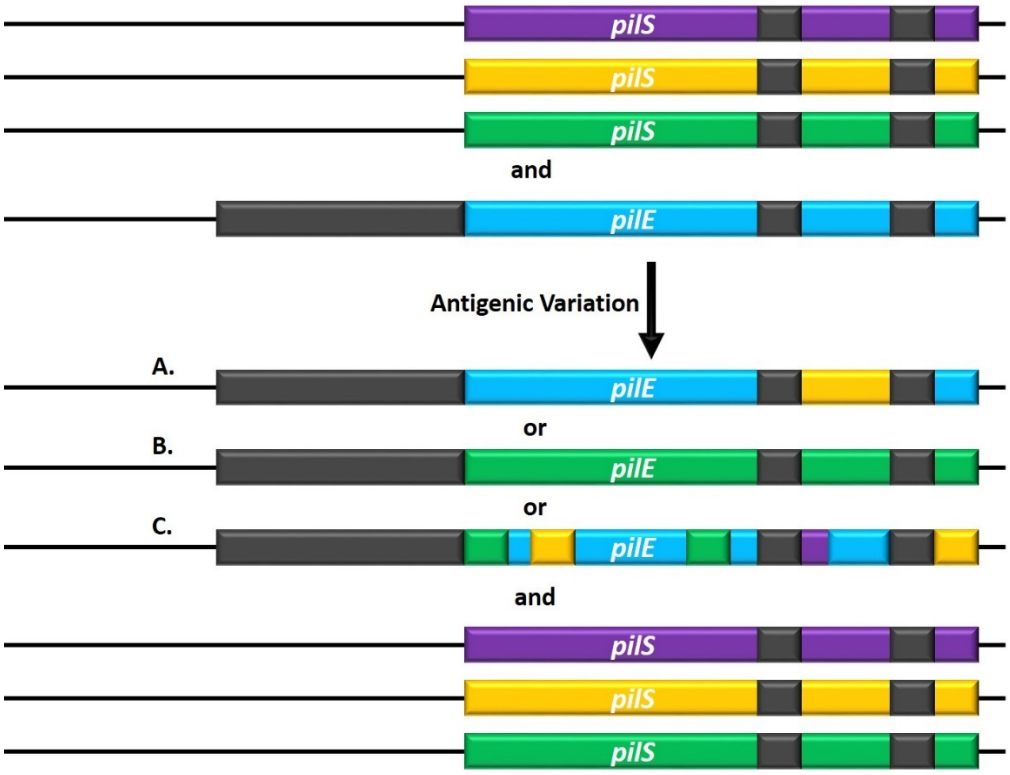


Figure 2. Molecular description of antigenic variation.

The *pilE* and *pilS* loci have regions of sequence microhomology (grey) and variability (colored). Sequence from a nonexpressed *pilS* loci copy is transferred into the expression locus with the *pilS* sequence not changing. Recombination can occur **A.** in just a section of the gene resulting in a *pilE-pilS* hybrid, **B.** across the entire *pilS* gene resulting in an entirely new variable region of *pilE,* or **C.** multiple times with different silent copies resulting in a new *pilE* sequence containing information from different silent copies throughout the variable regions.

The molecular process of antigenic variation results in a wide range of functional consequences. Noted as early as the 1960's, gonococcal colonies can exhibit a visible phase variation due to expression or lack thereof of pili (Kellogg *et al.*, 1963). While some pilus phase variation is due to ON/OFF switching of PilC or *pilE* deletion, antigenic variation events that introduce a premature stop codon from a silent copy or a non-favorable combination of silent copies can also prevent pilus expression (Jonsson *et al.*, 1991, Segal *et al.*, 1985, Hagblom *et al.*, 1985, Koomey *et al.*, 1987). While not fully understood, it is likely that such nonfavorable combinations of silent copies result in a pilin molecule that is unable to efficiently assemble into a pilus fiber. This is supported by the observation that strains with different *pilE* coding sequences exhibit different levels of piliation (Hagblom *et al.*, 1985). Although the lack of a suitable animal model means experimental data is lacking, it is likely that phase variation is critical for multiple reasons as all gonococcal isolates have this ability and multiple avenues to achieve it. Antigenic variation can also alter the sites of post-translational modification of the pilus which has been implicated in a variety of biological processes in *N. gonorrhoeae* including cellular adhesion and host-cell activation (Jennings *et al.*, 2011, Marceau *et al.*, 1998). In *N. meningitidis*, changes in the glycosylation status of the pilus can enhance transit across epithelial barriers, a critical step in pathogenesis (Chamot-Rooke *et al.*, 2011). In addition, variation of the exposed pilin residues has been implicated in controlling host cell response in *N. meningitidis* (Miller *et al.*, 2014). Engineered pilin variants demonstrated that the C-terminal domain of pilin is critical for colonization promoting host cell interactions, as well as that different pilin sequences conferred different host cell specificities (Miller *et al.*, 2014). These findings have direct implications on the pathogenesis of *N. meningitidis* and underscore the importance of the

diversity generation systems of *Neisseria* to provide both functional and immune avoidance capabilities to the organism.

Trans-acting factors important for pilin antigenic variation

A series of broad and directed genetic screens have identified many factors important for pilin antigenic variation, although the majority of their roles have been inferred from orthologous proteins rather than direct biochemical characterization (Mehr & Seifert, 1997, Sechman *et al.*, 2005, Cahoon & Seifert, 2009). The first protein identified as critical to antigenic variation is RecA, and mutations in the *recA* gene decreased pilus phase variation by 100-1000 fold (Koomey *et al.*, 1987). These results not only established RecA as a mediator of pilin antigenic variation but also demonstrated that antigenic variation is mediated by a homologous recombination-based process (Koomey *et al.*, 1987). *E. coli* RecA can complement a gonococcal RecA mutant and has similar biochemical properties (Stohl *et al.*, 2002, Stohl *et al.*, 2011). Interestingly, expression of the *E. coli* RecA in *N. gonorrhoeae* resulted in increased antigenic variation and this increased frequency was due to the co-transcription of the *E. coli* RecX protein (Stohl *et al.*, 2003). This work led to the discovery that in the gonococcus, RecX is required for efficient pilin antigenic variation and that *E. coli* RecX is a negative regulator of RecA filamentation (Stohl & Seifert, 2001, Gruenig *et al.*, 2010, Stohl *et al.*, 2003). Another protein that modulates RecA polymerization and activity named RdgC is also involved in promoting pilin antigenic variation presumably by also modulating RecA activity (Mehr *et al.*, 2000, Drees *et al.*, 2006).

The RecF-like recombination pathway has a central role in pilin antigenic variation (*Neisseria* do not encode a *recF* gene). In *E. coli*, the RecF pathway utilizes proteins both

pathway specific (RecF, RecR, RecO, RecQ) and non-specific (RecA, RecN). The pathway is mainly responsible for repairing single-stranded gaps in DNA, although it can repair dsDNA breaks when the primary RecBCD pathway is inactive (Hiom, 2009). In *N. gonorrhoeae*, mutational analysis of the pathway revealed that RecQ, and RecJ are required for some but not all antigenic variation events, but only the RecOR recombinase is required for all pilin antigenic variation (Skaar *et al.*, 2002, Mehr & Seifert, 1998). RecQ involvement was shown to be dependent on the helicase activity of two of the three HRDC domains at the C-terminus (Killoran *et al.*, 2009). The Rep protein, a 3'-5' helicase in *E. coli*, is also required for some events, and it is not known whether RecQ and Rep are partially redundant to one another (Lane & Denhardt, 1974, Kline & Seifert, 2005b). RecJ is a 5'-3' single-strand exonuclease whose role suggests that single-strand end resection is involved (Skaar *et al.*, 2002). RecR and RecO form a necessary recombinase that is the only known recombinase to act in the process of antigenic variation (Sechman *et al.*, 2005). RecN, whose role is not limited to the RecF pathway in *E. coli*, was not found to play a role in antigenic variation (Skaar *et al.*, 2002, Sechman *et al.*, 2005). The identification of the RecF-like pathway as being necessary for pilin antigenic variation suggests that there is a gapped intermediate that is required for one step of the recombination process, but the molecular description of the intermediate has not been reported.

The RecBCD recombination pathway was reported to not to be involved in pilin antigenic variation, but subsequent studies reported that a *recD* mutant showed an increased frequency of antigenic variation and that a *recB* mutant was deficient for pilin antigenic variation in one strain of *N. gonorrhoeae,* but not another (Chaussee *et al.*, 1999, Hill *et al.*, 2007, Mehr & Seifert, 1998). All of these studies relied on assays that scored only a subset of potential variants

and did not always account for the greatly reduced growth rate of the *recB, C,* and *D* mutants. A less biased sequencing assay, used to detect all pilin antigenic variation events, conclusively showed that insertional mutations in *recB, recC* and *recD* in both strains MS11 and FA1090 resulted in DNA repair phenotypes but not a pilin antigenic variation phenotype (Helm & Seifert, 2009). This study confirmed that the impaired growth of the *recB* and *recD* mutants resulted in a shift in the frequency of some, but not all, donor silent copies—possibly explaining the reason for the contrasting conclusion of a role for the RecBCD pathway in pilin antigenic variation.

Both the RuvABC and RecG Holliday Junction processing pathways are required for pilin antigenic variation (Sechman *et al.*, 2006, Sechman *et al.*, 2005). Mutations in either pathway prevent pilin antigenic variation, leading to the hypothesis that there are distinct substrates acted on by these pathways. Importantly, double mutants in *recG* and *ruvA,B, or C* created a partial synthetic lethality with an increase in *pilE* deletions in the surviving bacteria. This lethality could be rescued by a *recA* mutation as well as several other mutations that prevent antigenic variation (Sechman *et al.*, 2006, Cahoon & Seifert, 2009). This led to the hypothesis that antigenic variation involves two Holliday junctions, explaining the phenotype of the single mutations, and that the double *recG* and *ruvA,B,* or *C* mutations prevents the reversal of one or both of the Holliday junctions, thus locking the cell into a lethal antigenic variation intermediate. Preventing antigenic variation through processes such as *recA* mutation or *pilE* deletion prevents formation of the unresolvable lethal intermediate and rescues the synthetic lethality (Sechman *et al.*, 2006).

Required DNA sequences and structures

Several *cis*-acting sites have been identified as contributing to pilin antigenic variation. The 63 bp Sma/Cla repeat is located downstream of all pilin loci and often carries *Sma*I and *Cla*I restriction endonuclease sites (Haas *et al.*, 1992). Because of the similarity of the Sma/Cla sequence to recombinase binding sites, the Sma/Cla site was investigated, and it was found that deletion of the site downstream of the expressed pilin locus in strain MS11 reduced antigenic variation using a semi-quantitative assay that only detected transfer from two donor silent copies (Wainwright *et al.*, 1994). This observation has not been reported for other strains and remains unexplained. The *cys2* region of pilin is another conserved sequence involved in antigenic variation. Two studies have implicated the importance of *cys2* in antigenic variation as well as the spacing between the *cys1* and *cys2* region (Howell-Adams *et al.*, 1996, Howell-Adams & Seifert, 1999). There are two likely roles of these conserved regions in antigenic variation. First, the sequence conservation of the *cys2* region to that of the silent copies could serve as a shared region of homology to drive the recombination process. Secondly, the *cys2* region might serve as a binding site for *trans*-acting factors required for antigenic variation. Whatever the reason, it is clear that conserved sequences have a role in pilin antigenic variation and more investigation is needed to identify the critical sequences.

A breakthrough in the understanding of the mechanisms allowing pilin antigenic variation came from identification of a *cis*-acting DNA structure in the region of DNA immediately upstream of *pilE*. Transposon insertions isolated in a genetic screen prevented antigenic variation without disrupting pilin expression or an obvious open reading frame (Sechman *et al.*, 2005). A targeted mutagenesis of the genomic region carrying these transposon insertions identified twelve G-C base pairs that prevented antigenic variation when individually

mutated (***Figure 3A and B***) (Cahoon & Seifert, 2009). The sequence conforms to the definition of a guanine quadruplex (G4) forming motif (***Figure 3B***), which can adopt a four stranded, square planar structure utilizing non-traditional Hoogsteen base pairing (***Figure 3C***). Further investigation confirmed that the sequence forms a G4 structure *in vitro* and the individual mutations that abrogate antigenic variation also prevent G4 formation *in vitro*. Additionally, the point mutations that prevent G4 formation also decreased the detection of single-stranded nicks in both the G4 structure and in the C-rich strand opposite of the G4. These nicks are proposed to be required for the initiation of recombination and the subsequent gene conversion (Cahoon & Seifert, 2009). NMR analysis defined the *pilE* G4 structure, showing the sequence forms a three-layer, all-parallel stranded monomeric G4 with single residue double-chain-reversal loops (Figure 4C) (Kuryavyi *et al.*, 2012). Importantly, the *pilE* G4 structure (but not two other G4 forming sequences present in the *N. gonorrhoeae* genome) binds to RecA and does so with similar affinity as RecA for ssDNA (Kuryavyi *et al.*, 2012). A G4 structure present on a ssDNA substrate can stimulate RecA-mediated strand exchange *in vitro* (Kuryavyi *et al.*, 2012). Together these data suggest that the G4 serves to recruit RecA to the *pilE* locus and possibly serves as a nucleation site for RecA filamentation. Finally, the two extra HRDC domains of RecQ that were shown to be required for antigenic variation are also necessary to unwind the *pilE* G4 *in vitro*, suggesting that the effect of RecQ mutation on antigenic variation is due to the reduced capacity of the helicase to bind and unwind the G4 structure (Cahoon *et al.*, 2013, Killoran *et al.*, 2009).

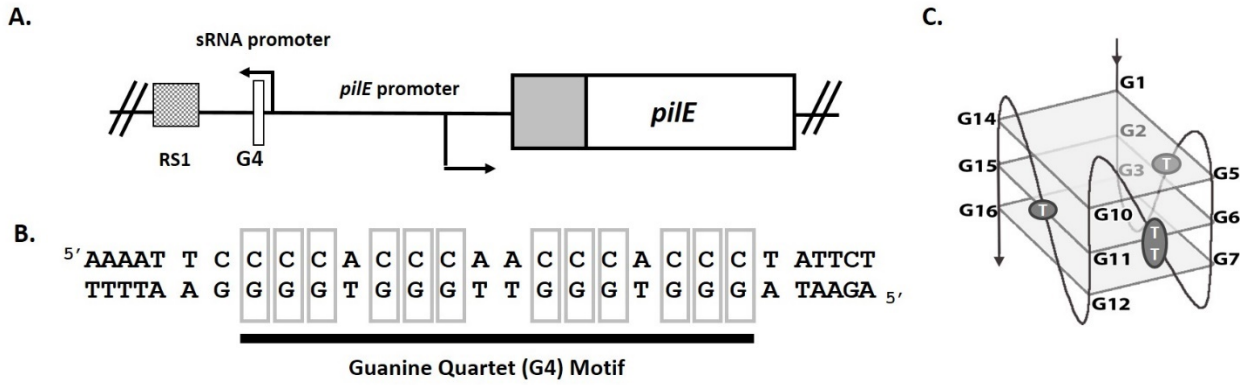


Figure 3. The *pilE* guanine quartet (G4).

A. Gene map showing the location of the *pilE*-associated G4 forming sequence and the sRNA promoter required for antigenic variation at the *pilE* locus. **B.** The sequence upstream of *pilE* that forms a G4. Mutation of the boxed guanine residues lead to loss of antigenic variation implicating the G4 in antigenic variation. **C.** The parallel G4 structure of the *pilE* G4 as solved by NMR analysis.

Although the G4 structure was shown to be required for antigenic variation, it was unclear what initiated G4 formation since the dsDNA must be melted to allow the G4 structure to form. Directed mutational analysis identified a promoter downstream of the G4-forming sequence that is required for pilin antigenic variation (Cahoon & Seifert, 2013). Transcription of a small RNA (sRNA) from this promoter was confirmed by 5'-RACE to start within the second set of Gs within the G4 forming sequence (***Figure 3A***). Expression of the sRNA at a distal chromosomal site did not restore antigenic variation in a promoter mutant showing that the sRNA was *cis*-acting. Together these data suggest that it is transcription of the sRNA at the G4 sequence and not some downstream role of the sRNA that initiates antigenic variation (Cahoon & Seifert, 2013). Throughout the investigation of pilin antigenic variation, a variety of models have been proposed to explain the phenomenon. In light of the most recent data, the feasibility of each model can be re-evaluated.

Antigenic variation models

Pilin antigenic variation is a gene conversion event, i.e., an apparent nonreciprocal recombination process that has been mainly studied in eukaryotic cells that possess two copies of their chromosomes. It was therefore proposed that the efficient DNA transformation system of the *Neisseria* could be used to allow for gene conversion from *pilS* to *pilE,* if the donor molecule was from a different cell than the recipient *(Gibbs et al., 1989, Seifert et al., 1988).* While there are data supporting or refuting transformation as a way to allow pilin antigenic variation, it is now generally accepted that the majority of pilin antigenic variation events do not involve transformation. (Gibbs *et al.*, 1989, Seifert *et al.*, 1988, Swanson *et al.*, 1990, Zhang *et al.*, 1992). With transformation ruled out as the main source for antigenic variation, all remaining

proposed models of antigenic variation involve intracellular recombination and necessitate at least two chromosomes. In most bacteria, two chromosomes only exist after replication, but it has been shown that both *N. gonorrhoeae* and *N. meningitidis* are polyploid, most likely diploid, and that *Neisseria lactamica*, which does not undergo pilin antigenic variation, has a single copy of its chromosome (Tobiason & Seifert, 2006, Stabler *et al.*, 2005). It has yet to be directly demonstrated that pilin antigenic variation relies on diploid homozygous chromosomes, but it remains a unique hypothesis.

One of the first models of antigenic variation proposed was the mini-cassette theory (Haas & Meyer, 1986). It postulated that there were seven defined mini- cassettes of variable sequence interspersed among regions of homology which were used to affect the segmental recombination that defines pilin antigenic variation. This model was discounted by sequencing data that established that antigenic variation events can change as few as one nucleotide or incorporate an entire *pilS* copy and can occur anywhere in the variable sequences where microhomology occurs between the recombining silent and expressed gene (Haas *et al.*, 1987, Seifert *et al.*, 1994a). There are three main models of pilin antigenic variation that have been proposed. The unequal crossing over model (or RecBCD-mediated double-chain-break repair model) (Kobayashi, 1992, Hill *et al.*, 2007) (***Figure 4A***) proposes that a double strand break occurs in the *pilE* locus, and after RecBCD-mediated end resection, the single-stranded 3' overhang invades the homologous *pilS* donor locus, presumably through RecA. The 3' end of the invading strand is extended by polymerase while the displaced donor strand is used as a template to repair the non-invading strand. Holliday junction resolution results in the donor DNA sequence replacing the recipient *pilE* sequence. Although not conclusively ruled out, the data

unambiguously showing RecBCD plays no role in antigenic variation make the unequal crossing-over model less likely (Helm & Seifert, 2009).

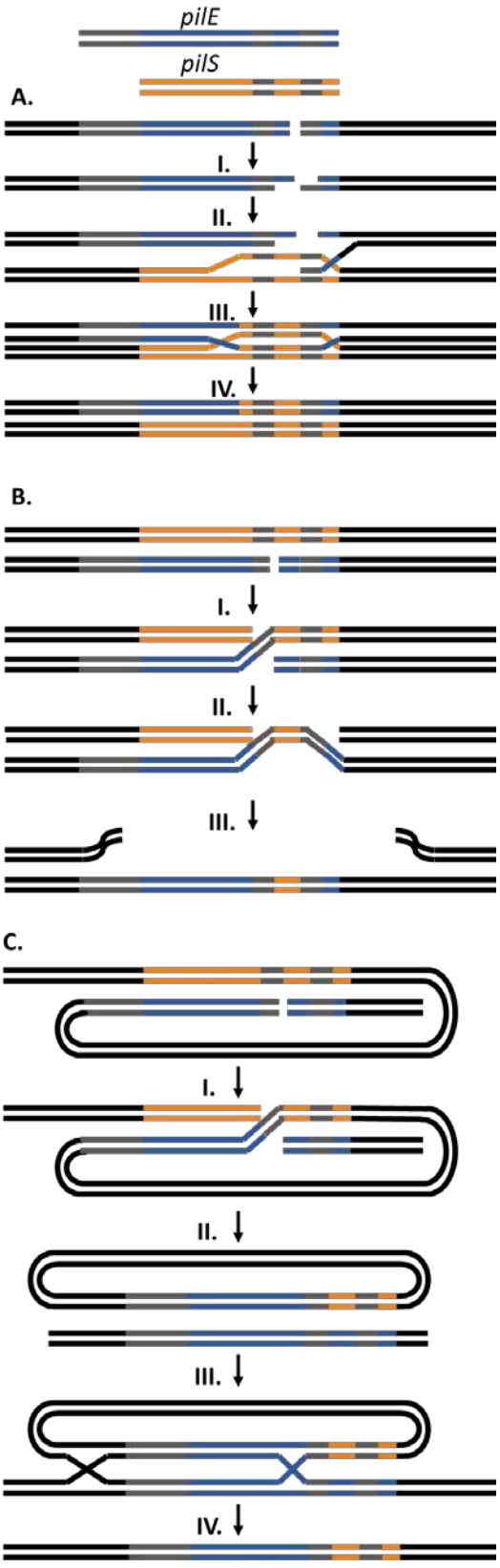

pilE
pilS
A.
I.
II.
III.
IV.
B.
I.
II.
III.
C.
I.
II.
III.
IV.

Figure 4. Proposed recombination pathways.

A. Unequal Crossing Over Model – A dsDNA break occurs at the *pilE* locus and **I.** the 5' ends are resected by RecBCD to leave 3' overhangs. **II.** A single 3' end mediated by RecA, invades the *pilS* locus forming a D-loop. **III.** The 3' ends are extended by DNA polymerase using the *pilS* gene as a template. **IV.** Resolution of the double Holliday junctions results in a new *pilE* sequence without altering the donor *pilS* sequence.

B. Successive Half Crossing Over Model – Recombination begins with a dsDNA break or single-stranded gap in *pilE* in a region of homology. **I.** A RecA and RecOR mediated half crossing over event occurs linking the *pilE* and a *pilS* locus on a sister chromosome. **II.** A second half crossing over event occurs in another region of microhomology downstream of the first event between the *pilE:pilS* hybrid and the original *pilE* locus. **III.** This recombination event leads to a new sequence at the *pilE* locus and destruction of the donor chromosome.

C. Hybrid Intermediate Model – Similar to the half crossing over model, recombination initiates with a double stranded break or single-stranded gap at *pilE* and **I.** a half crossing over event with a donor *pilS* on the same chromosome. **II.** This results in a *pilE:pilS* hybrid intermediate and the loss of the donor chromosome. **III.** The hybrid intermediate then undergoes two recombination events with the recipient *pilE* on a different chromosome. The first recombination event would occur in the extensive region of homology upstream of the genes while the second even would utilize microhomology within the variable regions of the genes. **IV.** Resolution of the Holliday junction intermediates leads to a new *pilE* sequence on the recipient chromosome.

The successive half crossing-over model also proposes an initiating double-strand break at the recipient *pilE* locus, but could also be initiated by a gapped substrate (***Figure 4B***) (Kobayashi, 1992, Mehr & Seifert, 1998). RecJ catalyzed 3' end resection at the *pilE* double-strand break or nick would provide a substrate for RecA, RecX and RecOR to mediate recombination with a donor *pilS* copy on a sister chromosome. This recombination event would create a *pilE-pilS* intermediate and link the sister chromosomes. A second half-crossing over event between the *pilS* region of the *pilE-pilS* 3' intermediate and the original *pilE* locus would result in the original *pilE* locus containing a hybrid *pilE-pilS* sequence and the destruction of the donor *pilS* chromosome. As a result of the first half-crossing over event the original *pilE* locus will not be intact, requiring some sort of yet uncharacterized tethering mechanism to keep the loose *pilE* end of the dsDNA break in close proximity to the donor *pilS* locus. The hybrid intermediate model is a variation of the half crossing over model that evolved from experimental observations (***Figure 4C***) (Howell-Adams *et al.*, 1996, Howell-Adams & Seifert, 2000). This model proposes that a recombination event occurs between *pilE* and *pilS* in a region of shared microhomology similar to that of the half-crossing over model but between genes located on the same chromosome. The crossover event would result in a circular *pilE-pilS* hybrid intermediate with the chromosomal sequences that existed between the two recombining loci also carried on the episomal circle and the resultant loss of the donor chromosome. The hybrid intermediate then requires two recombination events with a recipient *pilE* on a sister chromosome. One recombination event would occur in the extensive region of upstream homology and may utilize the homologous recombination factors while the second event occurs in a region of microhomology within the *pilE* coding sequence. Resolution of the resulting double Holliday

junction would create a new *pilE* sequence without any changes outside of the variable regions of *pilE*. While *pilE-pilS* hybrids can be isolated from the gonococcus and have been shown to undergo recombination at the *pilE* locus more readily than *pilS* loci, a hybrid intermediate consistent with all experimental data has yet to be defined (Howell-Adams & Seifert, 2000).

While the recombination event(s) that allow gene conversion in a bacterial chromosome remain undefined, the known antigenic variation proteins and the discovery of the G4 and *cis*-acting noncoding sRNA allows for the formation of a speculative, working model of the key events in antigenic variation (***Figure 5***). Initiation of antigenic variation occurs with transcription of the noncoding sRNA. The process of transcription melts the dsDNA and the occlusion of the C-rich strand by the formation of a DNA:RNA intermediate allows for the formation of the G4. The activation energy required for G4 formation may be lowered by a yet-unknown protein. The fact that G4 unwinding by RecQ (and possibly Rep) is required for pilin antigenic variation suggests that the G4 is resolved by RecQ either during or after antigenic variation. Formation of the G4 leads to local nicking of the DNA possibly induced by a stalled replication fork on the leading strand. The nicked substrate is likely processed by RecJ endonuclease and either the RecQ or Rep helicases. Based on RecA affinity for the *pilE* G4 structure, RecA may be recruited to the G4 structure to initiate RecA filamentation. A RecOR-assisted, RecA-mediated homologous pairing between the processed *pilE* and a *pilS* copy would create the half crossing over intermediate and the second half crossing over reaction. If the hybrid intermediate model is correct, these factors could be involved with the initial hybrid intermediate formation or recombination of the intermediate with the recipient *pilE*. The presence of microhomology at the ends of many pilin antigenic variation recombination tracts suggests there may be an annealing

process involved, but the identity of the protein that promotes annealing is unknown. Regardless, the models need to account for the requirement for both RuvABC and RecG and an intermediate that cannot be resolved if both pathways are inactivated. Though the actual nature of the recombination events is still unclear, the proposed working model provides predictions for conducting future studies.

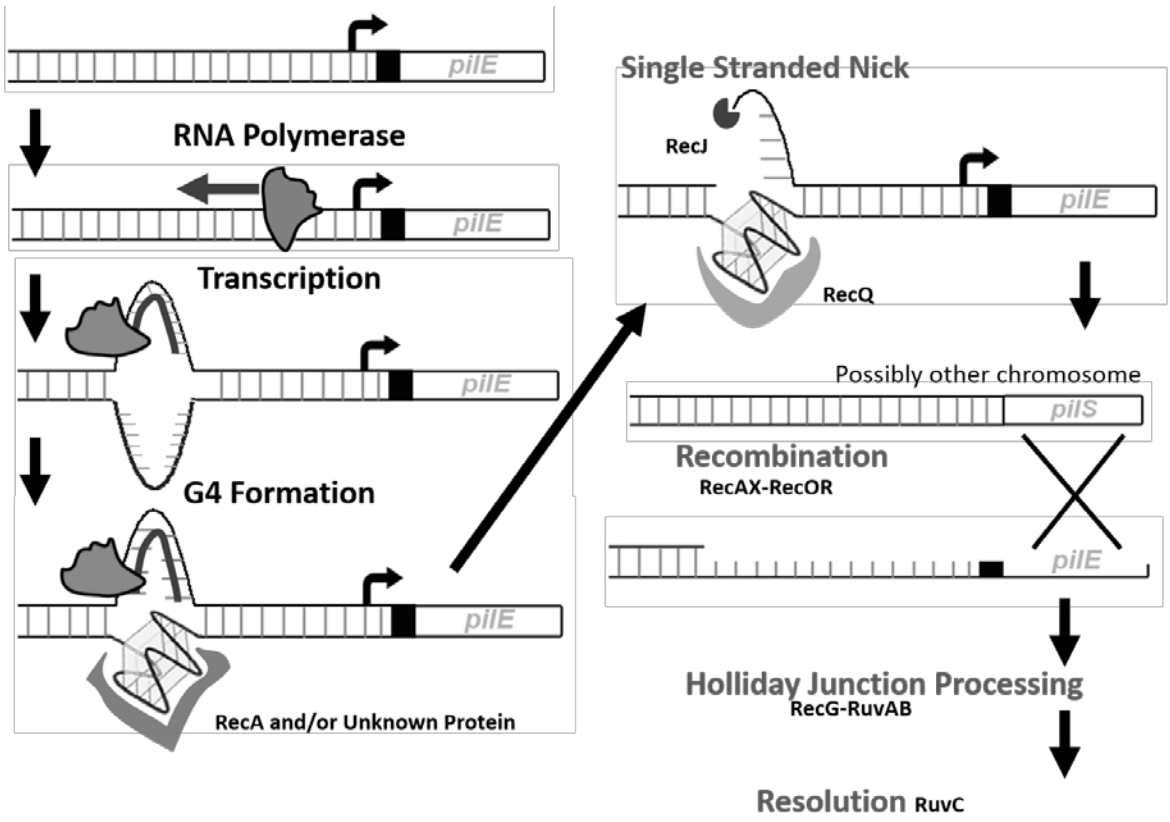


Figure 5. Proposed antigenic variation initiation pathway.

Transcription initiation at the sRNA upstream of *pilE* melts the DNA allowing the G4 structure to form. An unknown protein may bind the G4 to stabilize the structure. A single stranded nick may occur on the strand opposite the G4 due to a stalled replication fork. RecQ could unwind the G4 structure. RecJ resects the 5' nicked end allowing RecA to mediate recombination, possibly enhanced by binding the G4 structure, with RecOR using regions of homology between *pilE* and the donor *pilS*, possibly through a recombination mechanism detailed in Figure 5. RecG and RuvABC then process and resolve the recombination intermediate.

The pathogenic *Neisseria* undergo transformation with remarkable frequency and efficiency, and this genetic exchange is critical to allow the pathogens to survive as human restricted organisms. Additionally, the system of antigenic variation allows for high frequency productive gene conversion events without adverse effects on genomic stability. Although pilin antigenic variation has undergone the most thorough investigation of any antigenic diversity generating system, implicating specific DNA sequences and structures, as well as a multitude of DNA repair proteins, the detailed mechanisms of recombination are largely unresolved. Continued exploration of the molecular mechanisms will provide understanding of how directed recombination can occur as well as increasing our knowledge of a system thought to allow *N. gonorrhoeae* to escape immune detection, re-infect core populations, and cause the failure of attempted gonococcal vaccines.

Outstanding questions and introduction to dissertation work

While the pathogenic *Neisseria* serve as model organisms for the study of the Tfp complex and natural transformation, many questions remain to be answered. How does the Tfp complex mediate DNA uptake during transformation? Are extended, canonical Tfp required for this process, or is an alternative structure required? Is pilus retraction the main driving force for import of DNA into the periplasm? Additionally, there are outstanding questions specific to the Tfp complex of *Neisseria.* What is the functional role of S-pilin cleavage in gonorrheal biology and what protease(s) is responsible for cleavage of pilin? How does the process of antigenic variation alter Tfp complex properties? Is the pattern of conservation and variation of pilin sequence driven mainly by antigenic variation, Tfp stability, or both? In an attempt to better understand Tfp complex biology and how it is affected by antigenic variation, this dissertation

explores the role of the *Neisseria gonorrhoeae* Tfp complex in transformation. New factors critical for stable expression of Tfp are identified and the importance of the N-terminal domain of pilin in transformation following unproductive antigenic variation is detailed.

Chapter 2: The Low-Molecular-Mass, Penicillin-Binding Proteins DacB and DacC Combine to Modify Peptidoglycan Cross-Linking and Allow Stable Type IV Pilus Expression in *Neisseria gonorrhoeae*

Introduction

As a human restricted pathogen, *N. gonorrhoeae* has no known environmental reservoir and has evolved under constant pressure from the human immune system (Virji, 2009). This has led to exquisite adaptation to its niche with several virulence factors capable of undergoing antigenic variation to maintain function while avoiding immune surveillance (Zelewska *et al.*, 2016). One such factor is the Tfp, an organelle widespread amongst Gram-negative bacteria and required for productive *N. gonorrhoeae* infection (Swanson *et al.*, 1987). Tfp mediate an array of functions including microcolony formation, cellular adherence, modulation of host-cell signaling, and resistance to oxidative killing (Swanson, 1973, Dietrich *et al.*, 2011, Bottcher, 2011, Stohl *et al.*, 2013). The Tfp complex is also required for natural transformation, mediating at least the initial binding and uptake of DNA from the environment into the periplasmic space (Gangel *et al.*, 2014b, Obergfell & Seifert, 2015). In many Gram-negative bacteria, transformation efficiency is highly correlated with the expression of Tfp, and piliated strains of *N. gonorrhoeae* can undergo transformation more than a million times more frequently than a non-piliated strain (Long *et al.*, 2003, Sparling, 1966, Craig *et al.*, 2004).

The Tfp is a dynamic structure, undergoing cycles of extension and retraction that can result in pili extended several microns from the cell surface (Craig *et al.*, 2004, Stephens *et al.*, 1985). While these fibers are composed of thousands of polymerized pilin (PilE) subunits and are only six nm in width, they can exert significant force (Maier *et al.*, 2002). Tfp are produced from an assembly complex that spans the bacterial envelope. The ATPases that drive extension

and retraction (PilF and PilT, respectively) trade off occupying the inner membrane complex consisting of PilG, PilM, PilN, PilO (Berry & Pelicic, 2015, Chang *et al.*, 2016). PilP spans the periplasmic space from the inner-membrane complex to the secretin PilQ. PilQ multimerizes to form a pore in the outer membrane through which the pilus extends. Both TsaP and PilQ mediate proper anchoring of the complex through interactions with the outer membrane and peptidoglycan (PG) layer (Siewering *et al.*, 2014). Mpg, a zinc- dependent carboxy- and endopeptidase known to hydrolyze (PG) side chains, is required for stable pilus expression through an unknown mechanism (Stohl *et al.*, 2013).

The characteristic coccal shape of *N. gonorrhoeae* requires the cell-wall PG sacculus (Zapun *et al.*, 2008). In most bacteria, penicillin binding proteins (PBPs) polymerize and cross-link the cell-wall PG to form the large, macromolecular complex located in the periplasmic space (***Figure 6***) (Typas *et al.*, 2011). Transglycosylases polymerize the long glycan chains while transpeptidases establish the peptide cross-linking between the glycan strands. Along with hydrolase modification by carboxypeptidases and endopeptidases, these enzymes build a macromolecule, strong enough to withstand the osmotic pressure differential yet flexible enough to allow for cell growth and division. During *N. gonorrhoeae* cell-wall formation and maturation, PG fragments are released into the extracellular space that are cytotoxic and stimulate inflammation (Melly *et al.*, 1984, Mavrogiorgos *et al.*, 2014, Cloud-Hansen *et al.*, 2008). The *N. gonorrhoeae* cell wall also helps to protect the organism from neutrophil-mediated killing (Ragland *et al.*, 2017).

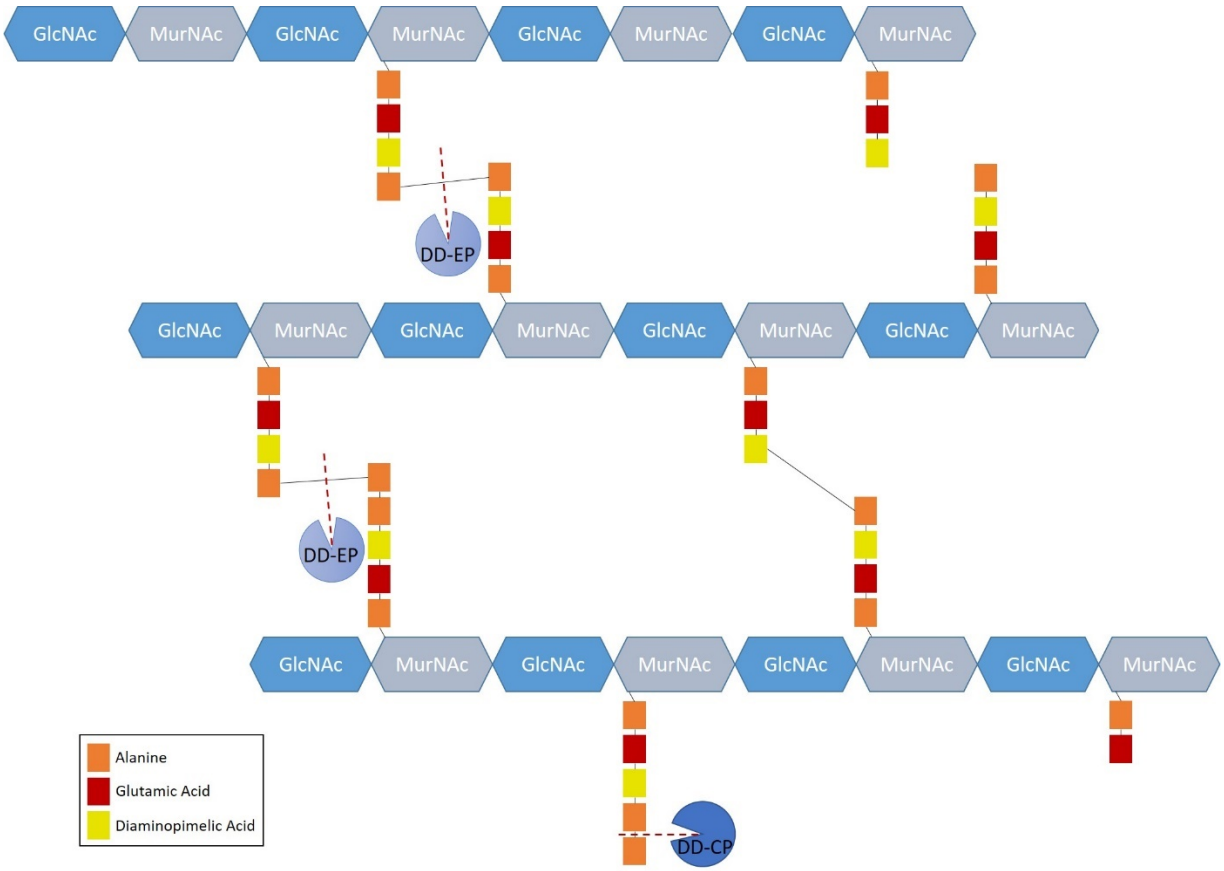


Figure 6. *N. gonorrhoeae* peptidoglycan structure and LMM PBPs

Representative structure of the *N. gonorrhoeae* cell wall. The glycan strands are composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) with a peptide side chain. The side chains consist typically consist of alanine, glutamic acid and diaminopimelic acid, with glycine sometimes substituting for the terminal alanine. Penta-tetra, tetra-tetra, and tetra-tri peptide cross-links connect the glycan strands. Peptide linkages hydrolyzed by predicted DD-carboxypeptidase (DD-CP) and DD-endopeptidase (DD-EP) activity of LMM PBPs are illustrated.

Similarly to other coccal bacteria, *N. gonorrhoeae* has a relatively small number of penicillin-binding proteins (PBPs) (Zapun *et al.,* 2008, Barbour, 1981). Of the four identified PBPs in *N. gonorrhoeae*, only the two high molecular mass (HMM) proteins, PBP1 and PBP2 are essential for cell viability (Barbour, 1981). HMM PBPs are largely responsible for the synthesis of new PG. PBP1 is a class A HMM PBP, homologous to *Escherichia coli* PBP1a, which is most probably responsible for transglycosylation and transpeptidation (Ropp & Nicholas, 1997). PBP2 is a class B HMM PBP, homologous to *E. coli* PBP3, which also catalyzes transpeptidation reactions (Spratt & Cromie, 1988, Zhang & Spratt, 1989, Dowson *et al.*, 1989). Mutations in the *penA* gene encoding PBP2 account for the majority of the widespread resistance to β-lactams (Ameyama *et al.,* 2002, Unemo & Nicholas, 2012).

Low molecular mass (LMM) PBPs tend to play non-essential roles in PG modification, PG recycling, and cell separation (Sauvage *et al.*, 2008). The functional outcome of activity of LMM PBPs is typically harder to determine as LMM PBPs tend to be more numerous and often have overlapping functions; however, only two LMM PBPs have been identified and characterized in *N. gonorrhoeae.* PBP3 binds radiolabeled penicillin and is homologous to *E. coli* PBP4 (DacB) (Stefanova *et al.*, 2003, Barbour, 1981). DacB exhibits both carboxypeptidase activity and endopeptidase activity as illustrated in ***Figure 6***. *N. gonorrhoeae* PBP4 does not bind radiolabeled penicillin but was identified through genetic analysis and is most similar to PBP7 of *E. coli* (Stefanova *et al.*, 2003, Stefanova *et al.*, 2004). PBP4 is a DD-carboxypeptidase with a preference for N^{ε}-acylated substrates. While initial observation of single knockout strains of PBP3 or PBP4 of *N. gonorrhoeae* revealed no gross abnormalities, loss of PBP3 and PBP4

together was reported to reduce cell growth and alter the cellular morphology (Stefanova *et al.*, 2003).

In this study, we mutated the genes encoding DacB (PBP3) and DacC, a third predicted LMM PBP not identified in radiolabeled penicillin binding assays, in *N. gonorrhoeae*. Inactivation of *dacB* dramatically altered the PG cross-linking, while mutation of *dacC* had much subtler effects on the PG composition. Loss of the two proteins in tandem produced highly cross linked PG, and resulted in significant cellular abnormalities. As these abnormalities were largely absent in the single knockout strains, our data suggest these two proteins have overlapping effects in *N. gonorrhoeae* physiology despite uniquely altering the PG profile.

Results

Δ*dacB*, Δ*dacC* and Δ*dacB*/Δ*dacC* strains have altered cell wall PG profiles

While *N. gonorrhoeae* only has two identified LMM PBPs, a third predicted LMM PBP is expressed (Zielke *et al.*, 2014). Here we investigated the role of DacB and the third predicted LMM PBP, encoded in locus NGO_0443 in *N. gonorrhoeae* physiology. NGO_0443 protein sequence aligns fully to COG1686 (DacC) with an E-value of 4.36e-128 and hereafter will be referred to as *dacC* (Altschul *et al.*, 1990, Marchler-Bauer *et al.*, 2017). DacC homologs are DD-carboxypeptidases and members of the S11 peptidase family (Kanehisa *et al.*, 2017). An alignment of *N. gonorrhoeae* DacC with DacC of *E. coli* shows that the three active sight motifs (SxxK, SxN, and KTG) are not conserved in *N. gonorrhoeae* (***Figure 7***)(Chen *et al.*, 2009). As DacB and DacC are confirmed and predicted LMM PBPs respectively, we constructed loss-of-function mutations in each gene to determine their role in bacterial physiology. The coding sequence of each gene was deleted and replaced with an antibiotic resistance cassette. A double mutant was also constructed. Both single mutants were viable as was the double mutant.

Figure 7. Alignment of *N. gonorrhoeae* and *E. coli* DacC.

Alignment of the amino acid sequence of DacC of *N. gonorrhoeae* (UniProt Entry A0A1D3HVP2) and *E. coli* (UniProt Entry P08506) (2017). Residues shaded in grey represent the predicted active sites (SxxK, SxN, and KTG) with the canonical active site sequences in bold. * (asterisk) indicates positions which have a single, fully conserved residue. **:** (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. **.** (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

To determine whether the DacB and DacC modify the cell-wall PG, high performance liquid chromatography (HPLC) analysis of the PG was conducted by our collaborators Dr. Ryan Schaub and Dr. Joe Dillard. Cell-wall PG was extracted from the Δ*dacB*, Δ*dacC*, or Δ*dacB*/Δ*dacC* mutants, digested by mutanolysin to cleave glycan strands, and the resulting di-saccharide, peptide fragments were separated by HPLC (***Figure 8***). Identities of the individual peaks were determined in comparison to those published by Antignac et al. (Antignac *et al.*, 2003). Identified peaks are currently a hypothesis and all identifies need to be confirmed by mass spectrometry. Loss of DacB resulted in global changes to almost all identifiable peptide fragment peaks. The PG had fewer non-cross linked di, tri, and tetra peptides and increased penta peptides and cross-linked tetra-tri and tetra-penta peptides (***Table 1***). These results confirm previous biochemical analysis characterization of DacB as a carboxypeptidase and endopeptidase (Stefanova *et al.*, 2003). Contrastingly, loss of DacC altered a smaller subset of PG peptides by a lesser degree. The mutant strain had fewer non-cross-linked peptides, an increase in cross-linked tetra-penta peptides, and a large increase in the larger cross-linked fragments with retention times longer than 100 min. The PG composition of the double mutant strain closely resembles that of the single Δ*dacB* mutant. Loss of the enzymes together results in PG that is much more heavily cross-linked than that of the parental strain. Loss of DacC in conjunction with DacB shifted the cross-linked fragments from tetra-tri towards tetra-penta and even larger fragments.

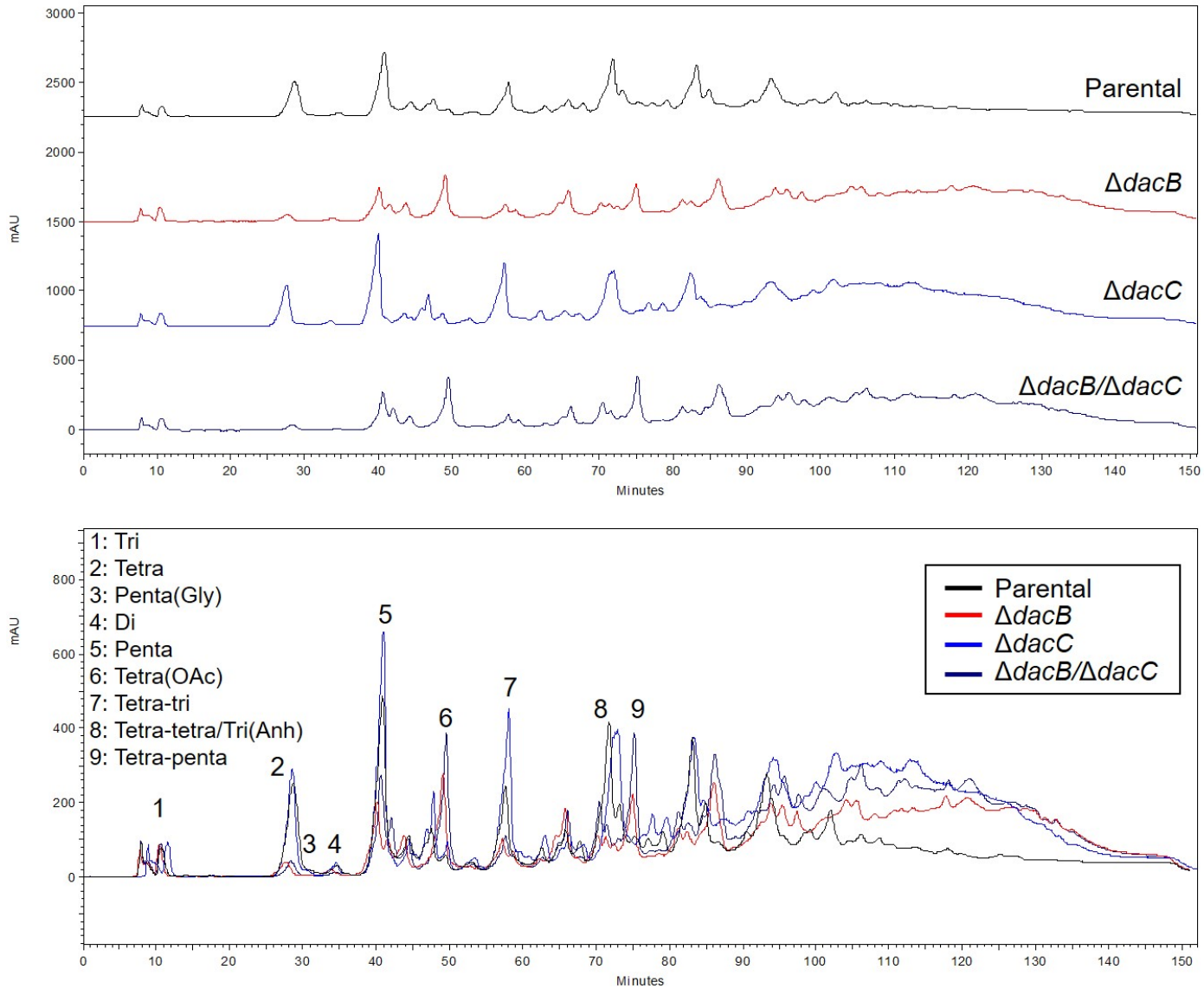

Parental
ΔdacB
ΔdacC
ΔdacB/ΔdacC
mAU
Minutes
1: Tri
2: Tetra
3: Penta(Gly)
4: Di
5: Penta
6: Tetra(OAc)
7: Tetra-tri
8: Tetra-tetra/Tri(Anh)
9: Tetra-penta
1
2
3
4
5
6
7
8
9

Figure 8. HPLC peptidoglycan profiling of Δ*dacB* and Δ*dacC* mutants.

Purified sacculi were digested with mutanolysin and soluble fragments were separated by HPLC. **A.** Representative PG profiles of the parental, Δ*dacB*, Δ*dacC*, or Δ*dacB*/Δ*dacC* strains. **B.** Representative PG profiles of parental, Δ*dacB*, Δ*dacC*, or Δ*dacB*/Δ*dacC* strains overlaid. Numbers correspond to identified peaks labeled in the upper left as determined by Antignac et al (Antignac *et al.*, 2003). Di: disaccharide dipeptide (disaccharide: N-acetylglucosamine-N-acetylmuramic acid); Tri: disaccharide tripeptide; Tetra: disaccharide tetrapeptide; Penta: disaccharide pentapeptide; Penta(Gly): disaccharide pentapeptide with a terminal glycine; OAc: O-acetylation on N-acetylmuramic acid. Anh: 1, 6-anhydromuramic acid.

	Tri	Tetra	Penta(Gly)	Di	Penta	Tetra(OAc)	Tetra-Tri	Tetra-tetra/ Tri(Anh)	Tetra-penta
Parental	5.20	9.28	0.00	2.77	2.59	5.41	2.48	10.20	1.83
Δ*dacB*	0.80	3.47	1.28	1.85	5.11	2.11	3.28	3.22	3.87
Δ*dacC*	3.33	7.21	0.00	1.16	2.37	5.60	1.34	7.50	2.16
Δ*dacB*/Δ*dacB*	0.58	2.92	1.39	1.15	4.73	1.50	1.95	4.00	5.93

Table 1: Percent area of peptidoglycan fragments separated by HPLC.

Quantification of percent area of peaks identified in (***Figure 8***) and normalized between strains. Reported area is the mean of three independent experiments. Di: disaccharide dipeptide (disaccharide: N-acetylglucosamine-N-acetylmuramic acid); Tri: disaccharide tripeptide; Tetra: disaccharide tetrapeptide; Penta: disaccharide pentapeptide; Penta(Gly): disaccharide pentapeptide with a terminal glycine; OAc: O-acetylation on N-acetylmuramic acid. Anh: 1, 6-anhydromuramic acid.

Mutation of *dacB* and *dacC* disrupts type IV pilus expression

The Δ*dacB*/Δ*dacC* double mutant strain, exhibited a non-piliated colony morphology similar to a *pilE* deletion strain (***Figure 9A***). Neither individual mutant displayed an altered colony morphology, and complementation with *dacB,* and *dacC* under control of their native promoters at an ectopic locus restored the piliated colony morphology. To determine the extent to which piliation was disrupted in the double mutant, we assayed the transformation efficiency of the strains. While loss of either DacB or DacC individually did not alter transformation efficiency, the double knockout strain exhibited a 94% reduction in transformation efficiency (***Figure 9B***). Although this represents a significant drop in transformation efficiency, the double mutant still exhibits relatively high levels of competence compared to the Δ*pilE* strain, which is non-transformable. The double complement strain fully restored transformation efficiency. As previously reported, *N. gonorrhoeae* strains with very low levels of pilin expression retain significant levels of transformation competence (Long *et al.*, 2003, Long *et al.*, 1998, Gibbs *et al.*, 1989, Rudel *et al.*, 1995). Loss of piliated colony morphology and transformation efficiency was not due to different expression levels of PilE as there are similar levels of full length PilE and S-pilin form expressed in the parental strain and double mutant strain (***Figure 9C***) (Haas *et al.*, 1987). These data show that loss of DacB and DacC together, reduces piliation but does not completely abrogate pilus function.

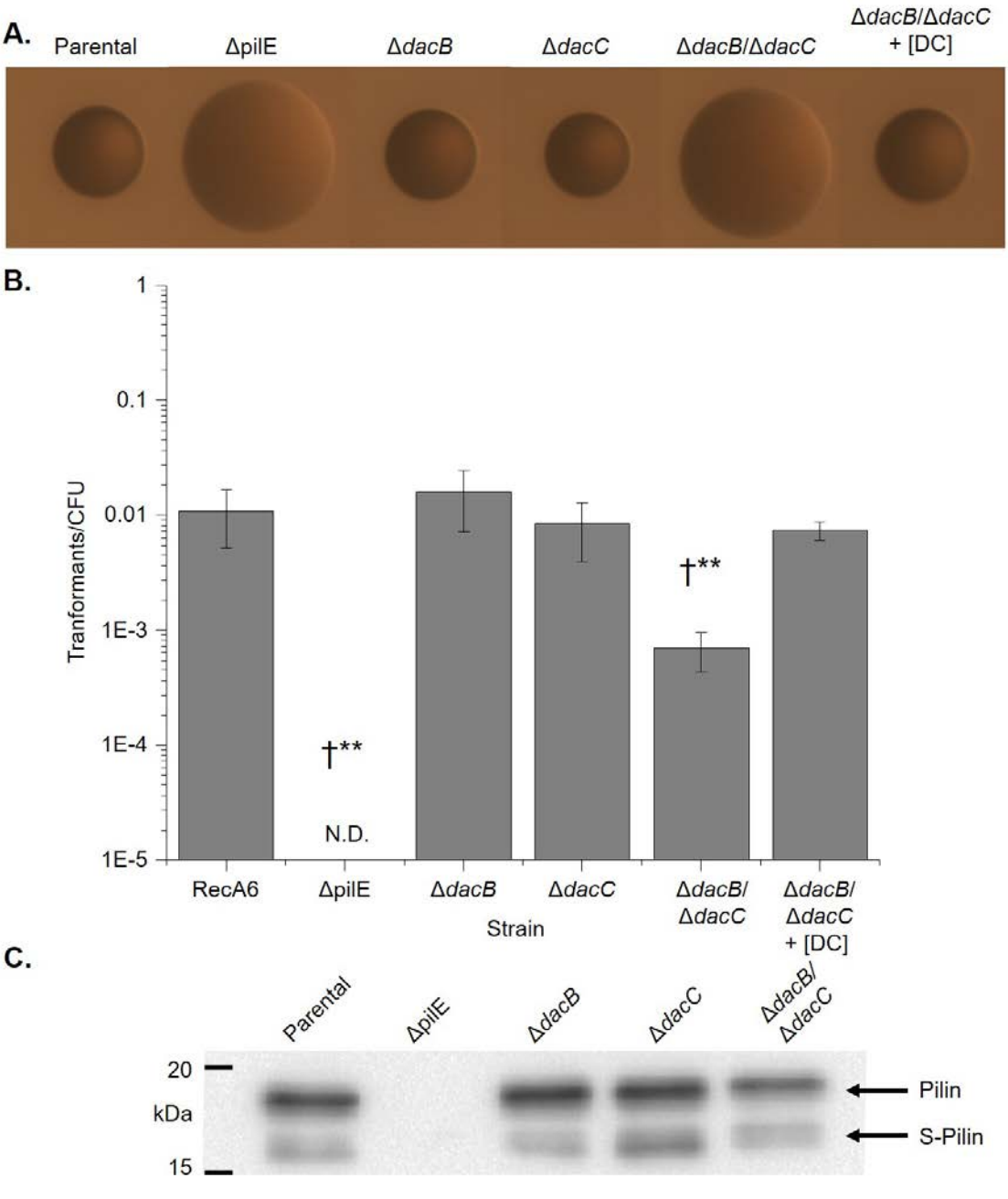

A.
Parental
ΔpilE
ΔdacB
ΔdacC
ΔdacB/ΔdacC
ΔdacB/ΔdacC + [DC]
B.
Tranformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
†**
N.D.
†**
RecA6
ΔpilE
ΔdacB
ΔdacC
ΔdacB/ ΔdacC
ΔdacB/ ΔdacC + [DC]
Strain
C.
Parental
ΔpilE
ΔdacB
ΔdacC
ΔdacB/ ΔdacC
20
kDa
15
Pilin
S-Pilin

Figure 9. Analysis of piliation in Δ*dacB*/Δ*dacC N. gonorrhoeae*.

A. Images of representative colonies grown on GCB solid media for 20hr and imaged using a stereo microscope. Non-piliated colonies are larger in diameter and are flatter rather than domed, resulting in different refraction of the light source. **B.** Transformation efficiencies of *dacB* and *dacC* mutant strains. Reported Transformation efficiencies are the mean ± standard deviation of at least four independent experiments. N.D. = transformants not detected. † represents statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain **$p<0.001$. [DC] = Double complement. **C.** PilE western blot of whole cell lysates of *dacB* and *dacC* mutant strains. Western blot analysis performed using the K36 anti-pilin peptide antibody.

To confirm the piliation status of the mutant strains, we imaged the strains using transmission electron microscopy (TEM). This technique allows qualitative analysis of the piliation state of the cell, but quantitative analysis is prevented by the inability to determine which cell a pilus originates from as well as the inability to differentiate individual pili form different sized pilus bundles. TEM analysis confirmed that both single mutants still expressed numerous pili, and the double mutant had few detectable per cell (***Figure 10***).

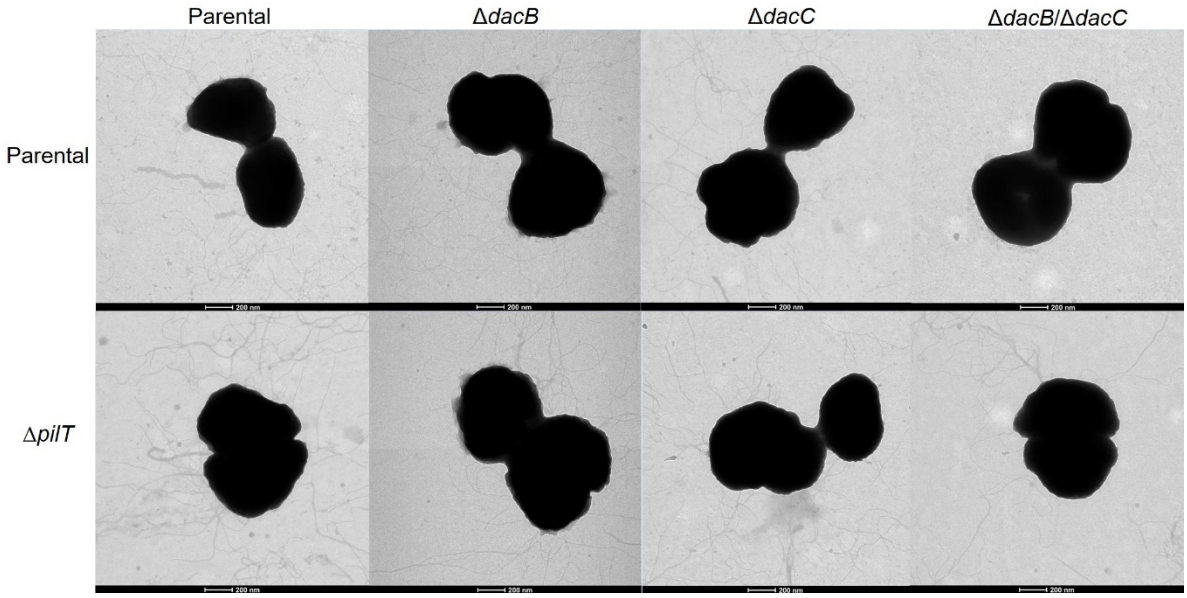


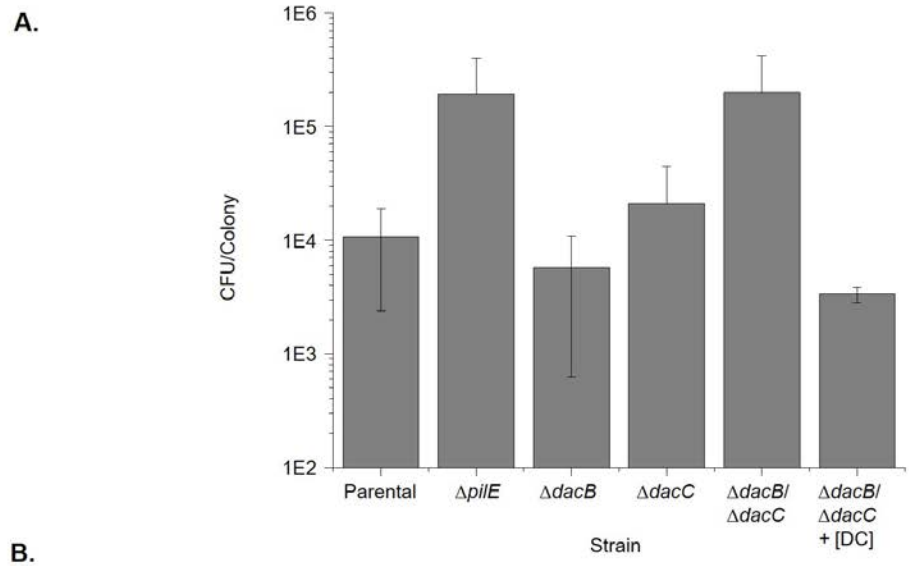
Figure 10. Electron micrographs of piliation in Δ*dacB* and Δ*dacC* mutants.

Micrographs of *N. gonorrhoeae* strains grown for 20 hours on solid medium, negatively stained with uranyl acetate, and imaged on a TEM. The top panel has images of the detailed parental strains while the lower panel images are of the detailed strains harboring a *pilT*::*erm* allele.

To determine whether loss of both DacB and DacC disrupts pilus biogenesis or stability, we introduced a *pilT* loss-of-function mutation into the single and double mutant strains. Pilus expression can be restored in many mutants that decrease the number of surface exposed pili by inactivating *pilT*, encoding the ATPase that is required for pilus retraction (Wolfgang *et al.*, 1998b, Wolfgang *et al.*, 1998a). Deletion of *pilT* resulted in increased piliation in all strains (***Figure 10***). Piliation of the Δ*dacB/*Δ*dacC* double mutant was restored to levels apparently similar to that of the parental strain, indicating that the loss of both DacB and DacC prevents stable pilus expression but that the pilus assembly process is not disrupted.

DacB and DacC alter cell fitness and morphology

To determine whether DacB and DacC play a role in general cell fitness, we assayed the growth of the mutant strains on solid medium by determining the colony forming units (CFU) per colony after 16hr (***Figure 11A***). While loss of DacB or DacC individually did not alter the growth, loss of both gene products resulted in approximately a twentyfold increase in the number of CFU/colony compared to the wild type strain. This increase in growth is the same magnitude as that displayed by a Δ*pilE* strain, suggesting that rather than directly altering growth, the increased growth in the Δ*dacB/*Δ*dacC* strain is likely due to the loss of stable pilus expression.


A.
1E6
1E5
1E4
1E3
1E2
CFU/Colony
Parental
ΔpilE
ΔdacB
ΔdacC
ΔdacB/ ΔdacC
ΔdacB/ ΔdacC + [DC]
Strain

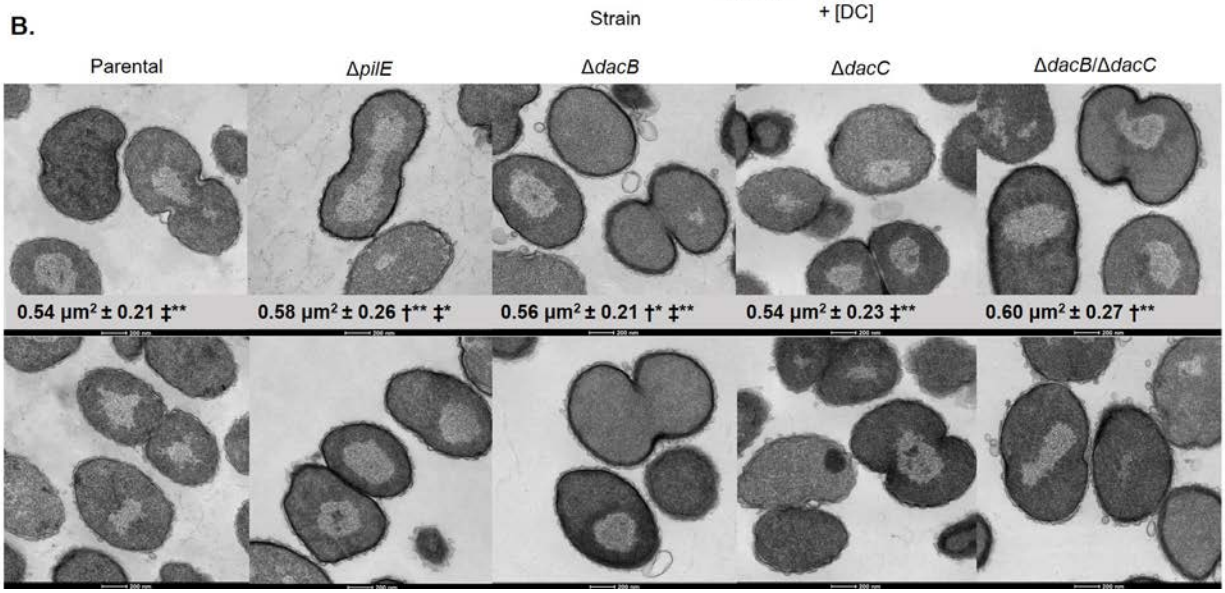

B.
Parental
ΔpilE
ΔdacB
ΔdacC
ΔdacB/ΔdacC
0.54 µm² ± 0.21 ‡**
0.58 µm² ± 0.26 †** ‡*
0.56 µm² ± 0.21 †* ‡**
0.54 µm² ± 0.23 ‡**
0.60 µm² ± 0.27 †**

Figure 11. Cell growth and morphology in Δ*dacB* and Δ*dacC* mutants.

A. Growth of *N. gonorrhoeae* strains grown on GCB solid media. At 16hr post inoculation, six individual colonies per strain were picked from plates, diluted in liquid culture, and plated in triplicate to enumerate CFU/colony. Data points are the mean of at least three independent experiments. Error bars represent standard deviation. One-way analysis of variance (ANOVA) indicates statistically significant difference amongst the strains, but Tukey's multiple-comparison test shows no statistical differences between paired strains. [DC] = Double complement. **B.** Representative electron micrographs of thin sections of *N. gonorrhoeae* strains. Mean cell cross sectional area ± standard deviation of at least 1300 individual cells is shown on each micrograph. †‡ symbols represent statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain (†) and Δ*dacB*/Δ*dacC* strain (‡). Asterix indicate degree of significance *$p<0.05$ **$p<0.001$.

As the murein sacculus is the scaffold on which the cell is shaped, we analyzed cellular morphology using TEM of thin-sections of the parental, Δ*pilE*, Δ*dacB*, Δ*dacC*, and Δ*dacB*/Δ*dacC* strains (***Figure 11B***). While loss of either protein did not result in any gross morphologic abnormalities, the micrographs show increased cross-sectional area of the Δ*dacB*/Δ*dacC* cells. To analyze the change in cross-sectional area as a proxy for change in cell volume, we quantified the cross-sectional area of at least 1,300 cells per strain using ImageJ particle analysis. The analysis was gated on cells with a cross-sectional area of at least of $0.3\mu m^2$ to prevent analysis of slices that transited near the edge of cells. While all cross-sections do not necessarily transect the thickest part of the cell, analyzing over 1,300 cells allows for an appropriate comparison of the average cellular volume. The Δ*dacB*/Δ*dacC* strain exhibited a cross-sectional area 11% larger than the parental strain, which translates to a calculated 16% increase in total cell volume. While the Δ*pilE* strain also showed an increase in cross-sectional area, the magnitude of increase was less than that of the Δ*dacB*/Δ*dacC* strain. The Δ*dacB* strain also showed a very small but statistically significant increase in cell size. Together these data demonstrated that PG modification mediated by DacB and DacC constrains the cell size in *N. gonorrhoeae* independent of their effect on piliation.

Antibiotic sensitivity is increased in the Δ*dacB*/Δ*dacC* strain

To determine the effect of inactivating DacB and DacC on antibiotic sensitivity, we determined the minimum inhibitory concentration of an array of antibiotics representing eight major classes (***Table 2***). Cell wall synthesis inhibitors included Ampicillin (Penicillins), Ceftazimide (Cephalosporins) and Imipenem (Carbapenems). Protein synthesis inhibitors included Spectinomycin (Aminoglycosides) and Erythromycin (Macrolides). Naldixic acid

(Fluoroquinolones) inhibits DNA gyrase. RNA synthesis was inhibited by Rifampicin and cell membranes structure was disrupted with Polymyxin B. Loss of DacC did not alter antibiotic sensitivity of *N. gonorrhoeae* to any of the tested antibiotics. However, loss of DacB slightly increased the susceptibility to cell wall synthesis inhibitor, Ceftazidime. In contrast, the Δ*dacB/*Δ*dacC* double mutant strain was much more susceptible to all three classes of cell wall synthesis inhibitors tested. These data demonstrate that PG modification by either DacB or DacC serves to make *N. gonorrhoeae* more resistant to antibiotics that target PG. Moreover, the Δ*dacB/*Δ*dacC* double mutant strain was also more resistant to Erythromycin, Rifampicin, and Polymixin B. Complementation of the double mutant restored all antibiotic sensitivities to those displayed by the parental strain.

Antibiotic	Parental	Δ*pilE*	Δ*dacB*	Δ*dacC*	Δ*dacB*/Δ*dacC*	Δ*dacB*/Δ*dacC* + [DC]
Ampicillin	0.19-0.25	0.125-0.25	0.125-0.38	0.19-0.38	0.064-.125	0.19-0.25
Ceftazidime	0.064-0.125	0.047-0.094	0.047-0.064	0.064-0.125	0.016-0.032	0.064-0.094
Imipenem	0.064-0.094	0.064	0.064-0.094	0.064	0.023-0.032	0.064-0.094
Spectinomycin	12-16	12-16	12-16	12-24	12-16	16
Erythromycin	0.094-0.125	0.094	0.094-0.125	0.094-0.125	.064-0.094	N.D.
Naldixic Acid	0.25-0.38	0.19-0.38	0.19-0.38	0.25-0.38	0.19-0.38	0.25-0.38
Rifampicin	0.023-0.032	0.023-0.032	0.023-0.032	0.023-0.032	0.012-0.023	0.023-0.032
Polymixin B	64-96	64-96	64	64-96	48-64	64-96

Table 2. Antibiotic sensitivities of Δ*dacB* and Δ*dacC* mutant strains.

Minimum inhibitory concentrations (MIC) in μg/mL for *N. gonorrhoeae* strains grown on solid media and exposed to antibiotic E-test strips. MICs are represented as a range of at least three independent experiments. N.D. Not determined due to erythromycin resistance gene present in this strain.

We also tested the sensitivity of the mutant strains to the metal-dependent, aminoquinone antibiotic Streptonigrin. Streptonigrin requires metal ions for activity and has been used as measure of free-iron available within bacterial cells (Yeowell & White, 1982, Cohen *et al.*, 1987). All strains tested showed different sensitivities to Streptonigrin (***Figure 12***). The Δ*pilE* and strain exhibited the greatest sensitivity to Streptonigrin killing. The parental strain was the most resistant to Streptonigrin with the mutant strains displaying intermediate phenotypes. Each single mutant appears to have a non-redundant effect on Streptonigrin sensitivity as the double mutant has an additive, increased sensitivity. As the single mutants do not display pilus expression deficiencies, these data suggest that both pilus expression and proper PG composition is important for resistance to Streptonigrin.

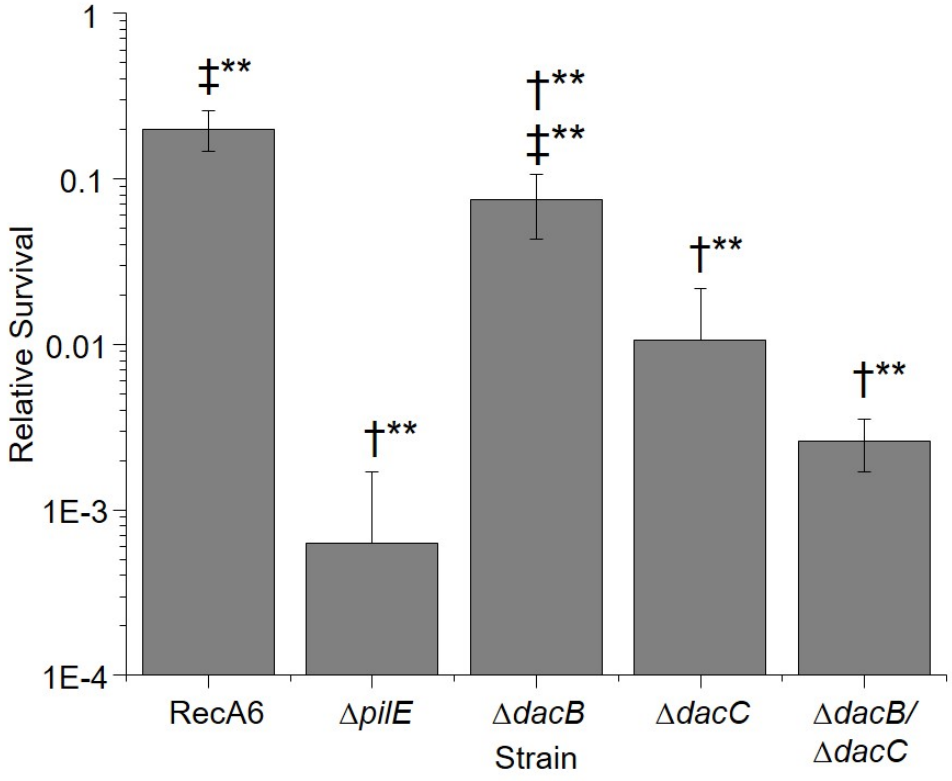


Figure 12. Streptonigrin sensitivity of Δ*dacB and* Δ*dacC* mutants.

Relative survival of strains exposed to 1μM Streptonigrin in DMSO for 1hr. The reported ratio represents the number of CFU enumerated from cultures treated with streptonigrin to the number of CFU from cultures treated with DMSO alone. Ratio represents the mean of at least three independent experiments. †‡ symbols represent statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain (†) and Δ*dacB*/Δ*dacC* strain (‡). **p<0.001.

The Δ*dacB/ΔdacC* double mutant shows increased detergent sensitivity

When performing western blot analysis, we observed there was less total protein present in lysates from the Δ*dacB/*Δ*dacC* strain relative to the single mutants or the parental strain. This was despite normalizing the input cells in each sample. We hypothesized that the increased cross-linking observed in the Δ*dacB/*Δ*dacC* strain was interfering with SDS-mediated lysis in the bacterial cells. To test this hypothesis, lysozyme was added to the SDS lysis buffer to independently digest the PG cross-links. Δ*dacB/*Δ*dacC* cells lysed in the absence of lysozyme resulted in 15% less total protein as determined by densitometry of the coomassie stained gels as compared to cells lysed in the presence of lysozyme (***Figure 13A and B***). No significant difference was observed in the parental and single mutant strains between the two conditions, and the Δ*dacB/*Δ*dacC* cells lysed in the presence of lysozyme resulted in the same total protein amounts as the other strains in both conditions.

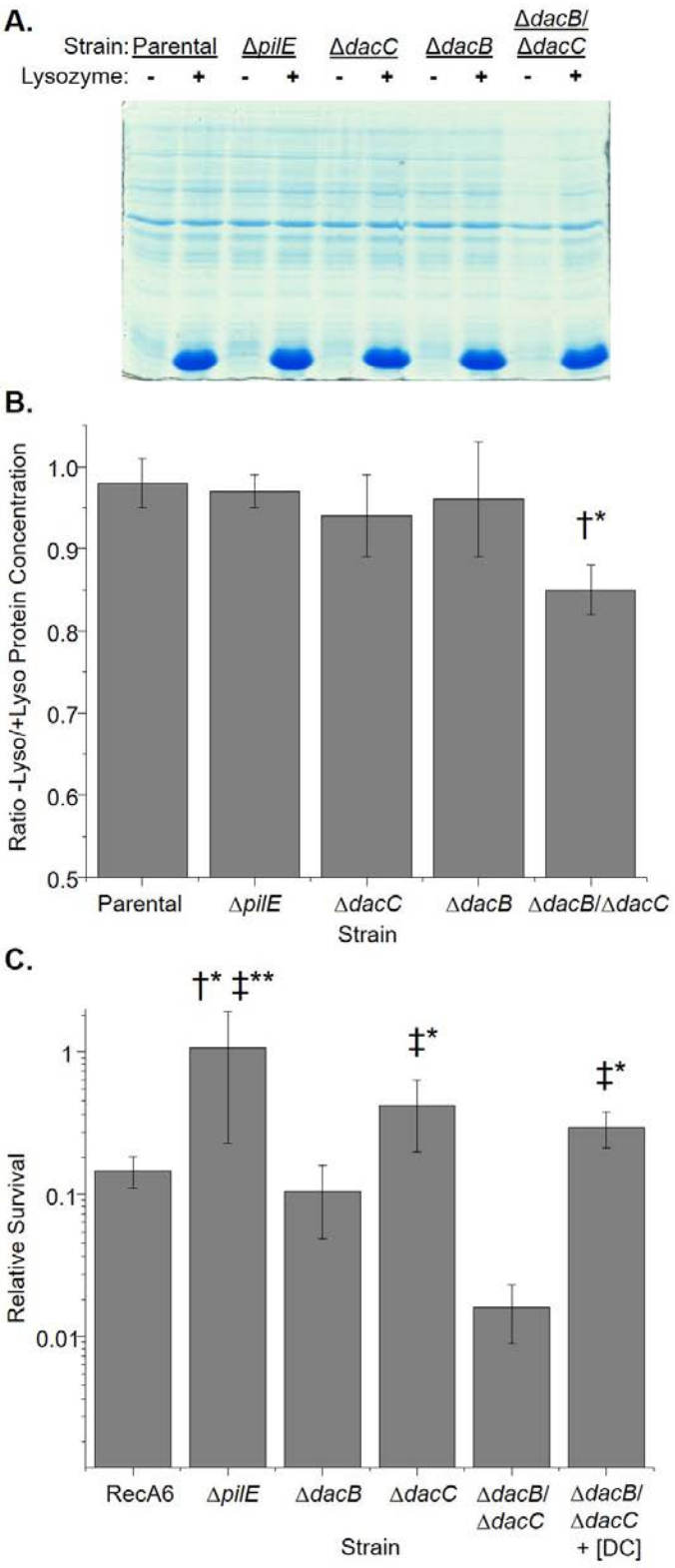

A.
Strain: Parental ΔpilE ΔdacC ΔdacB ΔdacB/ ΔdacC
Lysozyme: - + - + - + - + - +
B.
Ratio -Lyso/+Lyso Protein Concentration
1.0
0.9
0.8
0.7
0.6
0.5
†*
Parental
ΔpilE
ΔdacC
ΔdacB
ΔdacB/ΔdacC
Strain
C.
†* ‡**
‡*
‡*
Relative Survival
1
0.1
0.01
RecA6
ΔpilE
ΔdacB
ΔdacC
ΔdacB/ ΔdacC
ΔdacB/ ΔdacC + [DC]
Strain

Figure 13. Detergent sensitivity of Δ*dacB* and Δ*dacC* mutants.

A. Representative coomassie stained SDS-PAGE gel of protein samples of *dacB* and *dacC* mutants lysed by SDS sample buffer in the presence or absence of lysozyme. **B.** Ratio of total protein in samples lysed in SDS sample in the absence of lysozyme to samples lysed in the presence of lysozyme. Total protein was quantified by using Image Studio to analyze total protein run in a lane of a SDS-PAGE gel and stained by Coomassie Brilliant Blue. Ratio represents the mean of at four independent experiments. **C.** Relative survival of strains exposed to 0.001% SDS for 1hr. The reported ratio represents the number of CFU enumerated from cultures treated with SDS to the number of CFU enumerated from cultures treated with vehicle alone. Ratio represents the mean of at least three independent experiments. †‡ symbols represent statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain (†) and Δ*dacB*/Δ*dacC* strain (‡). Asterix indicate degree of significance *$p<0.05$ **$p<0.001$. [DC] = Double complement

To investigate if the observed resistance to SDS mediated lysis was biologically relevant, we assayed the sensitivity of the strains to SDS killing. When liquid cultures were exposed to 0.001% SDS for 1hr, the Δ*dacB*/Δ*dacC* strain was ten times more sensitive to SDS-mediated killing than the parental and Δ*dacB* strains (***Figure 13C***). Contrastingly, the Δ*pilE* strain was not killed by SDS at this concentration, and the Δ*dacC* was killed less than the parental strain although all strains could be fully killed by 0.01% SDS. Complementation restored resistance to SDS killing to parental levels. Taken together, these results demonstrate that while increased cross-linking of the PG can hold the proteinaceous content of the cell together when exposed to detergent, the increased cross-linking observed in a Δ*dacB*/Δ*dacC* strain makes *N. gonorrhoeae* more susceptible to killing by SDS.

Discussion

We have shown through loss-of-function mutations of *dacB* and *dacC* alone or in combination that mutants deficient for these proteins have distinct PG composition. Characterization of cell-wall PG from the single mutants revealed increased PG cross-linking. The decreased activity in the double mutant resulted in an even further cross-linked murein sacculus, confirming that these are not redundant activities but that each protein results in modification of specific PG subsets. These results confirm that DacB acts as a carboxypeptidase and endopeptidase and show that even though DacC was not identified in radiolabeled penicillin binding assays, that it should be classified as a LMM PBP.

The analysis of the Δ*dacC* mutant PG showed that while there were obvious changes in the PG cross-linking, the PG structure was more similar to that found with the parental PG. In contrast, the Δ*dacB* mutant PG showed extensive increases in PG cross-links. This suggests that DacB acts as a global modifier of PG peptides while the presence of DacC results in the

alteration of a more specific subset of peptides. The PG profile observed in the DacB mutant closely resembles the profile observed when *N. gonorrhoeae* is treated with sub-minimum-inhibitory-concentration doses of beta-lactams (Garcia-Bustos & Dougherty, 1987). While loss of DacB drives the majority of the characteristic changes observed in the double mutant PG, concomitant loss of DacC is necessary for any large phenotypic changes. This is notable as the lack of conserved catalytic residues in *N. gonorrhoeae* DacC suggests the protein should be enzymatically inactive (***Figure 7***). Our data clearly demonstrate, however, that expression of the *dacC* gene product results in an alteration of the PG. If the protein is indeed catalytically inactive, it may modify the PG profile through direct interaction with PG or an additional PG modifying enzyme. Taken together these data demonstrate that DacB and DacC each have unique activities within the cell, and result in the modification of specific subsets of PG peptides. However, they have largely overlapping effects on cellular biology, as loss of both is required for phenotypes that demonstrate the importance of proper PG processing for normally cellular activities.

Because the study of cell division and PG synthesis has been mainly performed in rod-shaped bacteria, mechanistic insight into coccal growth and cell division is still relatively scarce (Pinho *et al.*, 2013). Therefore, it is hard to predict how loss of DacB and DacC increases the overall cell volume, but it may involve regulation of the septal synthetic machinery at the division site (Zapun *et al.*, 2008). Consistent with DacC playing a specific role in coccal growth, an alignment of DacC from different *Neisseria* species shows that the rod-shaped *Neisseria* have DacC orthologues that retain the catalytic residues while most coccal *Neisseria* species have at least one defective active site motif (***Figure 14***). If the coccal forms were derived from rod-

shaped precursors through the modification of PG processing factors, then the loss of DacC peptidase activity may have occurred during the transition from rod to coccus (Veyrier *et al.*, 2015). We cannot determine whether the remaining DacC activity in the coccal *Neisseria* is shared with the DacC from the rod-shaped members of this genus, or whether there were other mutations that altered DacC function in the coccal organisms. The sequence conservation of the NGO_0443 gene product with prototypical members of the DacC family, support continued labeling of the gene product as DacC, but the lack of conserved catalytic residues show that it is not functional homolog. A detailed structure/function analysis will be required to determine exactly how the different DacC orthologues function in their representative cell types.


N.gonorrhoeae MTAHKI----------LPVLLPIILGVSHATAASP 25
N.meningitdis MTAHKI----------LPVLLSIILGVSHATAASP 25
N.flavescens MKKTLLSLIFAALLNT----PALA----------ADPVASAPVVQSEA 34
N.polysaccharea MTAHKI----------LPVLLSIILGVSHATAASP 25
N.sicca MKKTLSVLIAAMMIAAAQAAPQAAK----------N--NAAPAVAASEPAPAAAS 43
N.mucosa MKKTLLSLIFAALLNT----HALA----------ADPVASAPAVQGEA 34
N.cinerea MTANKA----------LSIFLSVILGSSPATADSF 25
N.lactamica MTAHKA----------LSILIPIILAASPAPAALS 25
N.weaveri MKKHLIALTVSSL----------IIGSAVASPQPAAPAAAPSTAAAP 37
N.bacilliformis MKKYLVLTALAAALSFQTASAAHYKKR-HSRYKRAAVAAAPAAAGAAAAAASAPEQAASA 59
N.enlongata MKKYLTVAAFAVLLSFQTADAARSKHKYKHRYKRPVAAATAA--AATAAASAVPGENAAS 58

N.gonorrhoeae APNRPTVH-AAPTLQTPETLTAAHIVIDLQSRQTLSAKNTNTPVEPAALTQLMTAYLVFK 84
N.meningitdis APNRPTAH-AAPTFQTPETLTAAHIVIDLQSKQILSAKNINTPVEPAALTQLMTAYLVFK 84
N.flavescens QTQPLLHS-INSPTTPPEIAATAYIVTDLHSKQTLASNNADTPIEPAALTQMMTAYLAFK 93
N.polysaccharea APNKLTVH-AAPTLQTPETLTAAHIVIDLQSRQTLSAKNINTPVEPAALTQLMTAYLVFK 84
N.sicca QPEAMMPS-INSPDAPPAIAAAAYIVTDLQSHQVLASGNIDTQIEPASLTKMMTAYLAFK 102
N.mucosa QPQPLLHS-INSPTTPPEIAATAYIVTDLHSKQTLASNNADAPIEPAALTQMMTAYLAFK 93
N.cinerea FPNRLPAN-IPSSPQTPETLTAAHIVTDLQSKQTLSAKNINAPVEPAALTQLMTAYLVFK 84
N.lactamica -SNRLPVR-AASSPQTPETLAAAHVVIDLQSRQTLSAKNIDAPAEPAALAQLMTAYLVFK 83
N.weaveri APAASQPAISPLQAGVPEIAATAYVIKDVQSGQILAQKELNTQIEPASLTKLMTAYLAFK 97
N.bacilliformis PEDVLPDEPKPQAAAAPEIAAAAYLVQDLQSGQILTGRDLDKPVEPASLTKLMTAYLAFK 119
N.enlongata EPDVLPPEPVPQASTAPEIAAAAYLVQDLQSGQILTGKNLDKQIEPASLTKLMTAYLAFK 118

N.gonorrhoeae NMKSGNIQSEENLKIPESAWASEGSRMFVRPGDTVSTDKLLKGMIALCANDAALTLADRL 144
N.meningitdis NMKSGNIRSEENLKIPESAWASEGSRMFVRPGDTVSTDKLLKGMIALSANDAALTLAGRL 144
N.flavescens ALENGTLEAGQMLTVSNAAWKVEGSRMFLDPKVPVSVSSLIKGTTIQSANDAAITLAEAL 153
N.polysaccharea NMKSGNIQSEENLKIPESAWASEGSRMFVRPGDTVSTDKLLKGMIALSANDAALTLAGRL 144
N.sicca ALENGTLRADQMLTVSDAGWKIEGSRMFLSPKVPASVSDLIKGMIVQSGNDAATTLAEAM 162
N.mucosa ALENGTLEAGQMLTVSNAAWKVEGSRMFLDPKVPVSVSSLIKGTTIQSANDAAITLAEAI 153
N.cinerea NLKSGNIRPEETLKIRESAWKSDGSRMFVRPGNLIRTDDLLKGMIALSANDAALTLADKL 144
N.lactamica NLKSGNIRPEETLKIRESAWKSEGSRMFVRPGGSVRTDTLLKGMIVLSANDAALTLAGRL 143
N.weaveri ALDNGTLKPEQMLTVSERGWKAEGSRMFLDVRKPASVSDLLKGLIVQSGNDAAITLAEAI 157
N.bacilliformis ALESGKLNATDTLTPSEKAWRAEGSRMFLNAGKPVAVGDLLKGLIVQSGNDAAITLAEAL 179
N.enlongata ALEEGRLKAETMLTPSETAWRAEGSRMFLNAGKPVAVGDLLKGLIVQSGNDAAVTLAEAL 178

N.gonorrhoeae GNGSIENFVQQMNKEARRLGMKNTVFKNPTGLGREGQVSTAKDLSLLSEALMRDFPEYYP 204
N.meningitdis GNGSIENFVQQMNKEARRLGMKNTVFKNPTGLSREGQVSTAKDLAQLSEALMRDFPEYYP 204
N.flavescens GNGSIDEFVKQMNEEAKRLGMKHTHFNNPTGISSNGHVSTVGDLAILAAALINDYPKYYP 213
N.polysaccharea GNGSIENFVQQMNKEARRLGMKNTVFKNPTGLSREGQVSTAKDLALLSEALMRDFPEYYP 204
N.sicca GGGSVDAFVQQMNDEAKRLGMTKTHFKNPTGLAAEGHVSTVGDLAILSAALIHDYPKYYP 222
N.mucosa GNGSIDEFVKQMNEEAKRLGMKHTHFNNPTGISSNGHVSTVGDLAILAAALINDYPKYYP 213
N.cinerea GNGSIENFIKQMNEEARRLGMKDTLFKNPTGLSQERQVSTARDLALLSEALILDFPEYYP 204
N.lactamica GNGSIENFVKQMNEEARRLGMKNTLFRNPTGLSQERQVSTARDLALLAEALMRDFPEYYP 203
N.weaveri GG-SEEGFADLMNAEAKRLGMNQTVFHNSTGLTSDGHLTSVNDLVILAEAIIKDFPKYYP 216
N.bacilliformis GG-SEEGFAKMMNAEAKRLGMNGTHFTNATGLPGDGHFTTVRDLAILAAAIIRDYPKYYP 238
N.enlongata GG-SEVGFADMMNAEAKRLGMSKTHFVNATGLPSDGHMSTVRDLAILSAAIIKDYPKYYP 237

N.gonorrhoeae LFSIKSFKFENIEQNNRNILLYRDNNVNGLKAGHTESGGYNLAVSYSGNGRHILVITLGS 264
N.meningitdis LFSIKSFKFKNIEQNNRNILLYRDNNVNGLKAGHTESGGYNLAVSYSGNGRHILVITLGS 264
N.flavescens LFANKSFKYNNIEQPNRNLLLYRDSSIDGLKTGYSEGAGYHLAASSKRNNRRIVSILAGA 273
N.polysaccharea LFSIKSFKFKNIEQNNRNILLYRDNNVNGLKAGHTESGGYNLAVSYSGNGRHILVITLGS 264
N.sicca VFSIKSFKYNNVEQPNRNLLLYRDSNVDGLKTGHTESAGYNLAASSKRNGRRIVSIVVGT 282
N.mucosa LFANKSFKYNNIEQPNRNLLLYRDSSIDGLKTGYSEGAGYHLAASSKRNNRRIVSILAGA 273
N.cinerea LFSIKSFKFKNIEQNNRNILLYRDSGVNGLKAGHTESGGYNLAVSYTGNGRHILVITLGS 264
N.lactamica LFSIKSFKFENIEQNNRNILLYRDNNVNGLKAGHTESGGYNLAVSYSGNGRHILVITLGS 263
N.weaveri IYSIKSFKYNNIEQPNRNLLLYRDADVDGLKTGHTSSAGYNLVASSKRNGRRVISVVVGT 276
N.bacilliformis IYSMQSFKYNNIEQPNRNLLLYRDPNVDGLKTGHTNSAGYNLVASSKRNGRRVVSVVVGT 298
N.enlongata IYSMQSFKYNNIEQPNRNLLLYRDPNVDGLKTGHTNTAGYNLIASSKRNGRRVVSVVVGT 297

N.gonorrhoeae ESAETRASDNSKLLNRALQAFDTPKIYPKGKTVAQIQISGGSKKTVRAGFLKEAYITLPH 324
N.meningitdis ESAETRASDNSKLLNWALQAFDTPKIYPKGKTVAQIQISGGSKKTVRAGFLKEAYITLPH 324
N.flavescens ESTEARASESSKLLNWSLQAFDTPKLYNGGEVISQVKVYKGSTKAVDIGFLDDVYITIPH 333
N.polysaccharea ESAETRASDNSKLLNWALQAFDTPKIYPKGKTVAQIQISGGSKKTVRAGFLKEAYITLPH 324
N.sicca DSTEARASESGKLLNWALQAFDTPKLYNGGEIISKVKVYKGSSKSVNVGFLEDVYITIPH 342
N.mucosa ESTEARASESSKLLNWSLQAFDTPKLYNGGEVISQVKVYKGSTKAVDIGFLDDVYITIPH 333
N.cinerea ESAEIRASDNSKLLNWALQSFDTPKIHSAGEIVTKIQVLGGSKKNVHAGFPKGVYITLPH 324
N.lactamica ESAETRASDNSKLLNRALQAFDTPKIYPKGKTVAQIQISGGSKKTVRAGFLKEVYITLPH 323
N.weaveri GSTEARAAESSKLLNWALQSFDTPKLYEANQTISQVKVYKGASNAVNVGFIDAAYITLPR 336
N.bacilliformis ESIEARAGESSKLLNWALQAFDTPKAYDAGKAVSAAQVYKGKADTVNVGFAEPVYVTIPH 358
N.enlongata ESIEARAGESSKLLNWALQAFDTPKAYDANTPVSSVKVYKGQEDNVNIGFLEPVYLTIPH 357

N.gonorrhoeae KEAKMAEQILETIQPIPAPVKKGQILGKIKIRQNGHTIAEKEIVALENVEKRSRWQRLWT 384
N.meningitdis KEAKMAEQILETIQPIPAPVKKGQILGKIKIRQNGYTIAEKEIVALENVKKRSRWQRLWA 384
N.flavescens DTGKNVKPILETLQPVLAPIEKGQVLGKLKVMKDGKIIAEKDVVALTGVEEGSWLRRMWD 393
N.polysaccharea KEAKTAEQILETIQPIPAPVKKGQILGKIKIRQNGYTVAEKEIVALENVEKRSRWQRLWT 384
N.sicca DAGQNIKPILETVQPVIAPIRKGQTLGKLKIVKDGKVITEKNVVALHSVEEGSWFRRMWD 402
N.mucosa DTGKNVKPILETLQPVLAPIEKGQVLGKLKVMKDGKIIAEKDVVALNGVEEGSWLRRMWD 393
N.cinerea TQAKTAEQILETIQPLRAPVRKGQVLGKIKIRQNGYTIAEKEIVALENVEKKNWWKKLWA 384
N.lactamica KEAKTAEQILETIQPIPAPVKKGQILGKIKIRQNGYTLAEKEIAALENVEKRSRWQRLWT 383
N.weaveri GEGKNIKPILETVQPVLAPVHKGQVLGKLKIMNGDKVLAEKEVTALNAVEEAGWFGRLYD 396
N.bacilliformis GEGSKVKTVLETVQPVVAPVEKGQVLGKLKISYDGKVLAERPVVALDAVPEAGFFGRLWD 418
N.enlongata GEGNKVKPVLETVQPVLAPIEKGQVLGKLKITYNGQVLAERKVVALTAVDEAGFFGRLWD 417

N.gonorrhoeae RLTGQ---------- 389
N.meningitdis CLTGQ---------- 389
N.flavescens AIVLWFKSLFS---- 404
N.polysaccharea RLTGQ---------- 389
N.sicca DIVLWFKGLFGSSSK 417
N.mucosa AIVLWFKGLFS---- 404
N.cinerea RLTGQ---------- 389
N.lactamica RLTGQ---------- 388
N.weaveri GIVLWFKNLFADE-- 409
N.bacilliformis SVKLWFKNMFADN-- 431
N.enlongata SIKLWFKNMFADE-- 430

Figure 14. Alignment of DacC of coccal and rod-shaped Neisseria.

Alignment of the amino acid sequence of DacC of *N. gonorrhoeae* (UniProt Entry A0A1D3HVP2), *N. meningitidis* (UniProt Entry X5F8K2), *Neisseria flavescens* (UniProt Entry C5TJQ4), *Neisseria polysaccharea* (UniProt Entry E2PCI4), *Neisseria sicca* (UniProt Entry I2NMQ5), *Neisseria mucosa* (UniProt Entry E5UIX3), *Neisseria cinerea* (UniProt Entry D0W0V3), *Neisseria lactamica* (UniProt Entry D0W745), *Neisseria weaveri* (UniProt Entry G2DNW0), *Neisseria bacilliformis* (UniProt Entry F2BGK7), *Neisseria elongata* (UniProt Entry D4DUP2) (2017). The non-shaded sequences are from coccal shaped *Neisseria,* while the sequences shaded in light grey are from rod-shaped *Neisseria*. Residues shaded in dark grey represent the predicted active sites (SxxK, SxN, and KTG) with canonical active site sequences in bold.

Alternatively, the increase in overall cell volume may be due to abnormal PG cross-linking may interfere with tethering of the outer membrane to the PG. A recent study in *Salmonella enterica* demonstrated that outer-membrane lipoprotein tethering to the PG is important for proper periplasmic length, and loss of this tethering disrupted osmolalic balance between the cytoplasm and periplasm (Cohen *et al.*, 2017). We did observe outer membrane disruption and blebbing in thin section electron micrographs of the double mutant (***Figure 11B***), although not to the extent observed in a lipoprotein mutant in *S. enterica.*

DacB and DacC expression is important for resistance to multiple classes of antibiotics. The increased susceptibility of the Δ*dacB*/Δ*dacC* strain to cell wall targeting antibiotics is likely due to the Δ*dacB*/Δ*dacC* induced cell wall perturbations making the cell less able to resist additional insults to the cell wall synthesis machinery. This indicates that DacB and DacC play a functionally redundant role in protecting *N. gonorrhoeae* from beta-lactams. Of particular note is that the double mutant was also more sensitive to non-cell wall targeting antibiotics Erythromycin, Rifampicin, and Polymixin B. We presume that the altered cell wall in the Δ*dacB*/Δ*dacC* double mutant influences steady state levels of these compounds in the cell. The simplest explanation for increase cytoplasmic level of antibiotics is that the barrier function of the PG sacculus is disrupted, but if this was correct, the sensitivity to most antibiotics should have been increased. An alternative hypothesis is that expression or activity of outer membrane porins or efflux pumps is disrupted (Goire *et al.*, 2014). It is also possible that the expression or availability of the targets of these antibiotics is altered in the double mutant, but we consider this less likely.

Resulting changes to the peptidoglycan cross-linking from loss of DacB and DacC dramatically reduced the expression of stable Tfp, a virulence factor required for pathogenesis. The decreased piliation also reduced transformation, the mechanism of HGT. Taken together, these data demonstrate that the individual activates of these enzymes have overlapping cellular functions essential to *N. gonorrhoeae* biology and expression of critical pathogenesis mediators.

Chapter 3: The Pilin N-terminal Domain Maintains *Neisseria gonorrhoeae* Transformation Competence during Pilus Phase Variation

Summary

Neisseria gonorrhoeae, the bacterium responsible for the disease gonorrhea, is capable of undergoing natural DNA transformation, a critical mechanism by which bacteria can take up DNA from the environment. Uptake of foreign DNA can lead to adaptation to a changing environment and allow the spread of antibiotic resistance, a particularly relevant issue in *N. gonorrhoeae*. It has been proposed that the Tfp directly mediates transformation, however, our data show that the extended pilus is not required for transformation. Only a portion (the Ntd) of the pilin protein is required to maintain transformation ability and this domain is released by a unique proteolysis event termed S-pilin cleavage. Release of the Ntd allows cells to maintain competence during pilin antigenic variation, a process vital for immune escape that frequently produces cells that cannot form Tfp. While these cells were previously thought to be incapable of DNA transformation, our data suggest that S-pilin cleavage evolved as a mechanism that allows DNA transformation and horizontal gene transfer to occur in these cells lacking pili. We propose that a structurally similar but distinct pseudopilus mediates transformation.

Introduction

The process of DNA transformation in *N. gonorrhoeae* and the closely related pathogen *Neisseria meningitidis* has been well-studied with the majority of contributing factors having been identified. All *N. gonorrhoeae* strains are naturally competent for DNA transformation, and competence is not regulated as the organism is able to undergo transformation during all phases of growth, which contributes to the spread of antibiotic resistance (Biswas *et al.*, 1977, Gibbs & Meyer, 1996). Transformation in *Neisseria* is mediated by the Tfp complex. The Tfp is a major virulence factor involved in cellular adherence, microcolony formation, resistance to neutrophil mediated killing, twitching motility, and transformation (Craig *et al.*, 2004, Merz *et al.*, 2000, Stohl *et al.*, 2013, Swanson, 1973, Dietrich *et al.*, 2011, Freitag *et al.*, 1995). Similar to *Haemophilus influenza*e, *N. gonorrhoeae* preferentially takes up its own DNA that contains a 10 or 12 base DNA uptake sequence (DUS) (5’ ATGCCGTCTGAA 3’) (Goodman & Scocca, 1988, Ambur *et al.*, 2007). The DUS occurs frequently within Neisserial genomes and significantly increases efficiency of DNA transformation compared to the same DNA lacking the sequence (Duffin & Seifert, 2010b).

Tfp are several microns long, six nm wide, dynamic structures undergoing cycles of extension and retraction and exert one of the largest forces known for a biological machine (Stephens *et al.*, 1985, Maier *et al.*, 2002). It has been assumed that Tfp directly bind DNA and retraction of the Tfp mediates DNA uptake into the periplasm; however, there is a lack of direct data supporting this model, and transporting DNA through the secretin that already hosts a pilus is problematic (Obergfell & Seifert, 2015). What is known is that the major pilin subunit (PilE or pilin), along with many of the proteins of the Tfp complex such as the prepilin peptidase PilD are

required for competence (Drake & Koomey, 1995, Freitag *et al.*, 1995). PilT, a traffic ATPase that mediates retraction of pili, is required for transformation but is not required for piliation (Wolfgang *et al.*, 1998a). The minor pilin ComP is responsible for recognition and binding of DNA and is responsible for DUS recognition (Cehovin *et al.*, 2013). Expression of ComP promotes transformation while expression of the minor pilin PilV decreases transformation efficiency by antagonizing ComP (Aas *et al.*, 2002a).

The pilin structure is characteristic of Tfp pilin with a 7 AA leader peptide that is cleaved by the PilD peptidase from the N-terminal hydrophobic α-helices that form the core of the pilus structure. The α-helices are connected to a variable C-terminal domain consisting of the αβ loop, a β sheet, and the D Region containing a critical disulfide bond (***Figure 15A***). The globular C-terminal head contains the residues that are surface exposed in the pilus fiber (Chen & Dubnau, 2004). Some pilin variants mediate release of the C-terminal globular head in a soluble form called S-pilin that is the product of a specific proteolysis unique to the pathogenic *Neisseria* (Haas *et al.*, 1987, Koomey *et al.*, 1991). No role for S-pilin in pathogenesis has been conclusively established. Pilin variants that produce fewer pili tend to produce larger amounts of S-pilin, but the ratio of pilin to S-pilin is not directly correlated to the number of elaborated pili (Haas *et al.*, 1987, Long *et al.*, 1998).

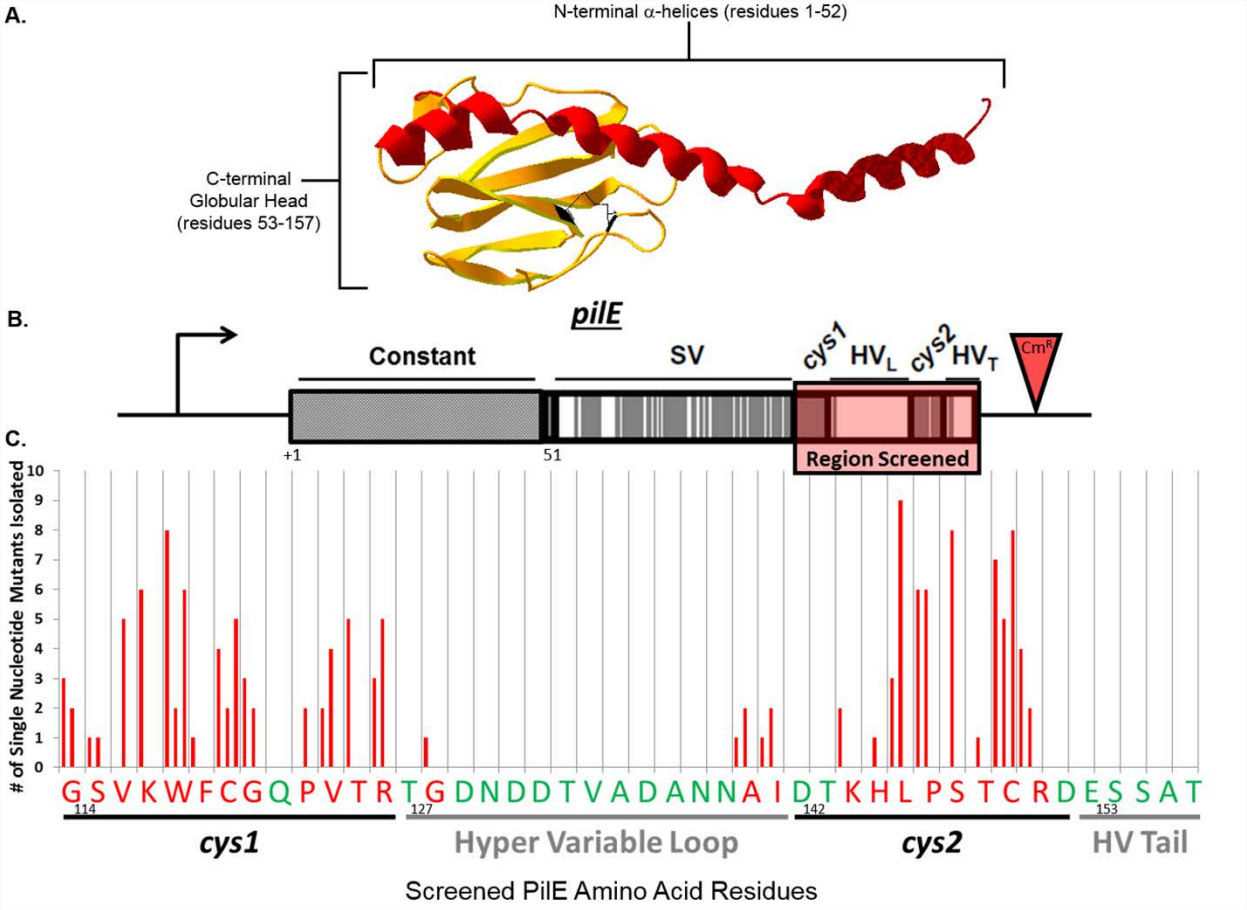


Figure 15. Single nucleotide mutations in *pilE* that result in a P- phenotype.

A. Picture of the PilE pilin structure Protein Data Bank accession no. 2HI2 (Craig *et al.*, 2006).

B. Gene map of *pilE* showing the regions of sequence conservation (grey) and variation (white) with the screened region boxed in light red. Triangle labeled Cm^R indicates location of linked chloramphenicol resistance cassette used to select for transformants. **C.** Graph depicting the location of mutations isolated in the screen that result in a P- phenotype. The x-axis depicts the amino acid residues corresponding to the *pilE* sequence with each residue divided into the 3 segments representing the coding nucleotides. Amino acids in red lettering resulted in a P- phenotype when mutated. The y-axis depicts the number of times a mutated residue was isolated.

Both eukaryotic and prokaryotic pathogens utilize an array of molecular tactics in order to avoid recognition by host immune systems. Diversity generation systems are one of the most widely used of these tactics to avoid immune detection. From the V(D)J recombination mediated generation of antigen receptors in vertebrate adaptive immune responses to phase variation of antigenic determinants in bacteria, many different diversity generation systems exist throughout nature that are vital to an organism's survival (Gellert *et al.*, 1999, van der Woude & Baumler, 2004). Pathogen antigenic variation, or the modification of immunogenic surface molecules, forces the host to continuously alter its humoral immune response. Antigenic variation can provide a pathogen with the capability to persist within a host for an extended time or to continually re-infect core populations.

The pathogenic *Neisseria* mediate a high-frequency, DNA-based, homologous recombination process termed pilin antigenic variation, that varies the *pilE* coding sequence resulting in many different variant pilins (Obergfell & Seifert, 2015). During pilin antigenic variation, sequences from unexpressed silent pilin loci (*pilS)* donate variable coding sequences to the expression locus in a gene conversion event. The *pilE* gene and pilin protein can be divided into variable regions (semi-variable region (SV), hyper-variable loop (HV_L), hyper-variable tail (HV_T)) and conserved regions (N-terminal domain, *cys1*, *cys2*) (***Figure 15B***). The regions with variable amino acid residues correspond to the surface exposed regions of the mature pilus fiber and are thought to mediate immune escape and add to the ineffectiveness of Tfp-based vaccine candidates. In addition, the process of pilin antigenic variation can result in pilin molecules that are inefficiently assembled into pilus fibers or pilin phase variants that cannot form pili (Criss *et al.*, 2005). All gonococcal strains maintain the ability to phase vary, which may contribute to

immune escape by preventing pilus expression, but can additionally mediate the detachment of the bacterium from epithelial surfaces and a switch from a sessile biofilm state to a free-living planktonic state. Due to the observation that *pilE* deletion strains are nonfunctional for pilus-dependent processes (Seifert *et al.*, 1990, Jonsson *et al.*, 1994, Stohl *et al.*, 2013), it has been assumed that when pilus expression is disrupted by the pilin antigenic variation process that all pilus associated functions are also disrupted.

In this chapter, we show that *pilE* mutations that prevent pilus formation do not necessarily abolish transformation competence. Mutations that disrupt the N-terminal domain (Ntd) prevent transformation, while those past the S-pilin cleavage site retain transformation competence. Moreover, we show that S-pilin proteolysis is required to release the Ntd to mediate transformation when unproductive pilin monomers are produced by antigenic variation. Our data demonstrate that a proteolytic process maintains transformation competence even when pilus expression is disrupted by pilin antigenic variation. These findings suggest that the extended pilus fiber is not required for transformation and that maintaining continual transformation competence is extremely important for this strict human pathogen.

Results

Targeted genetic screen to identify C-terminal pilin residues required for piliation

To determine the amino acid residues critical for proper Tfp formation and function we conducted a random, saturating mutagenesis screen on the 3’ region of the *pilE* coding sequence encoding the *cys1*, HV_L, *cys2*, and HV_T regions (***Figure 12B***). A pool of random mutant plasmids was constructed using a series of degenerate mega-primers to introduce all possible mutations into every nucleotide position in the 3’ region of the gene. The plasmid pool was used

to transform *N. gonorrhoeae* selecting for recombination at the native *pilE* locus using a linked, downstream chloramphenicol resistance marker (Cm^R) (***Figure 15B***). Transformants with underpiliated (P-) colony morphologies were isolated and the *pilE* coding region was sequenced to determine the causative mutation. Only mutants containing a single nucleotide change in the *pilE* coding region were analyzed further.

68 unique mutations were isolated that result in a P- colony morphology in the targeted 132 3'-base pairs of *pilE* (**Figure *12C***). Nine of these mutations created early stop codons, 13 changed either of the two absolutely conserved cysteine residues that form the critical disulfide bond, and the remaining 46 were missense mutations. This mutagenesis screen provides a comprehensive analysis of which amino acid residues in the carboxy-terminus of this highly variable protein in this strain of *N. gonorrhoeae* are essential for piliation. Residues in which a mutation produced a P- phenotype were concentrated in the highly conserved *cys1* and *cys2* regions of *pilE*. There were, however, multiple amino acid residues in the hypervariable loop that prevent piliation when mutated. No mutations were isolated in the hypervariable tail consisting of the five most C-terminal amino acids (***Figure 15C***).

Phenotypic analysis of P- *pilE* mutants

To further characterize representative mutant isolates for Tfp formation and function, pilin protein levels were assayed by western blot, surface exposed fibers were imaged by transmission electron microscopy (TEM), and transformation efficiencies were measured (***Figure 16***). While the missense mutants expressed varying levels of pilin, the nonsense and cysteine mutants had no detectable pilin by western blot analysis, presumably due to degradation of improperly folded pilin (***Figure 16A***). A complete summary of the phenotypic

characterization of every isolate from the screen can be found in (***Table 3***). In addition, we were unable to detect any surface exposed pili by TEM with selected nonsense and cysteine mutants (***Figure 16B***). Several Tfp defects can be suppressed in a Δ*pilT* background, which prevents pilus retraction (Wolfgang *et al.*, 1998b), yet a representative set of nonsense and cysteine mutants did not display any observable pili by TEM in a Δ*pilT* background (***Figure 16B and Figure 17***). Surprisingly, while a Δ*pilE* mutant is not competent, all of the *pilE* mutants were transformable including the nonsense and cysteine mutants completely lacking detectable pilin or surface exposed pili (***Figure 16C***). The P- isolates demonstrated a wide range of transformation efficiencies, with mutants that express some detectable pilin protein on Western blot analysis generally exhibiting a higher level of transformation competence (***Table 3***).

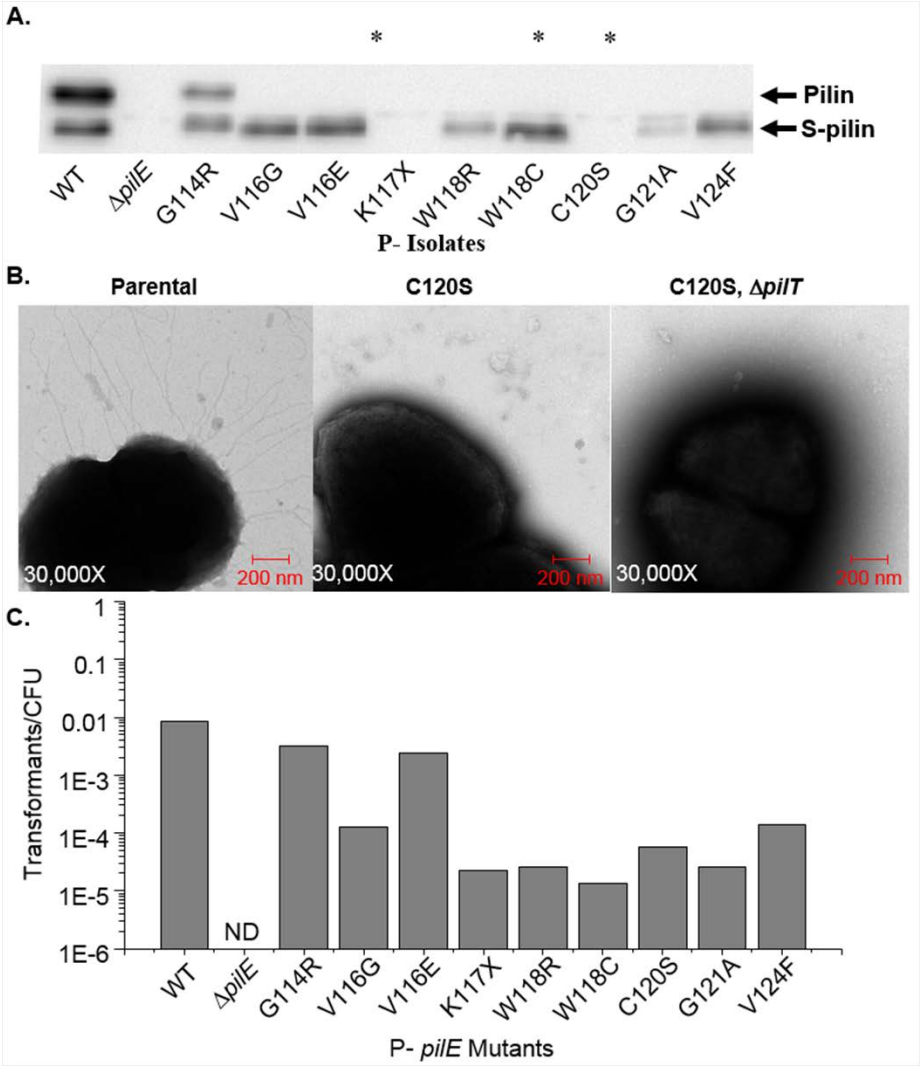

A.
*
*
*
Pilin
S-pilin
WT
ΔpilE
G114R
V116G
V116E
K117X
W118R
W118C
C120S
G121A
V124F
P- Isolates
B.
Parental
C120S
C120S, ΔpilT
30,000X
200 nm
30,000X
200 nm
30,000X
200 nm
C.
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
ND
WT
ΔpilE
G114R
V116G
V116E
K117X
W118R
W118C
C120S
G121A
V124F
P- pilE Mutants

Figure 16. Phenotypic characterization of P- *pilE* mutants.

A. Representative PilE western blot of whole cell lysates of P- isolates. The upper band is full-length PilE while the lower band is S-pilin. Lanes marked with an * are nonsense or cysteine mutants. Western blot analysis performed using the K36 peptide anti-pilin antibody. **B.** Representative TEM images of the parental strain (left), a C120S mutant (middle), and a C120S, *pilT* double mutant (right). **C.** Transformation efficiencies of representative P- isolates from the screen including nonsense, cysteine mutants, and missense mutants. X = nonsense mutation, ND = transformants not detected.

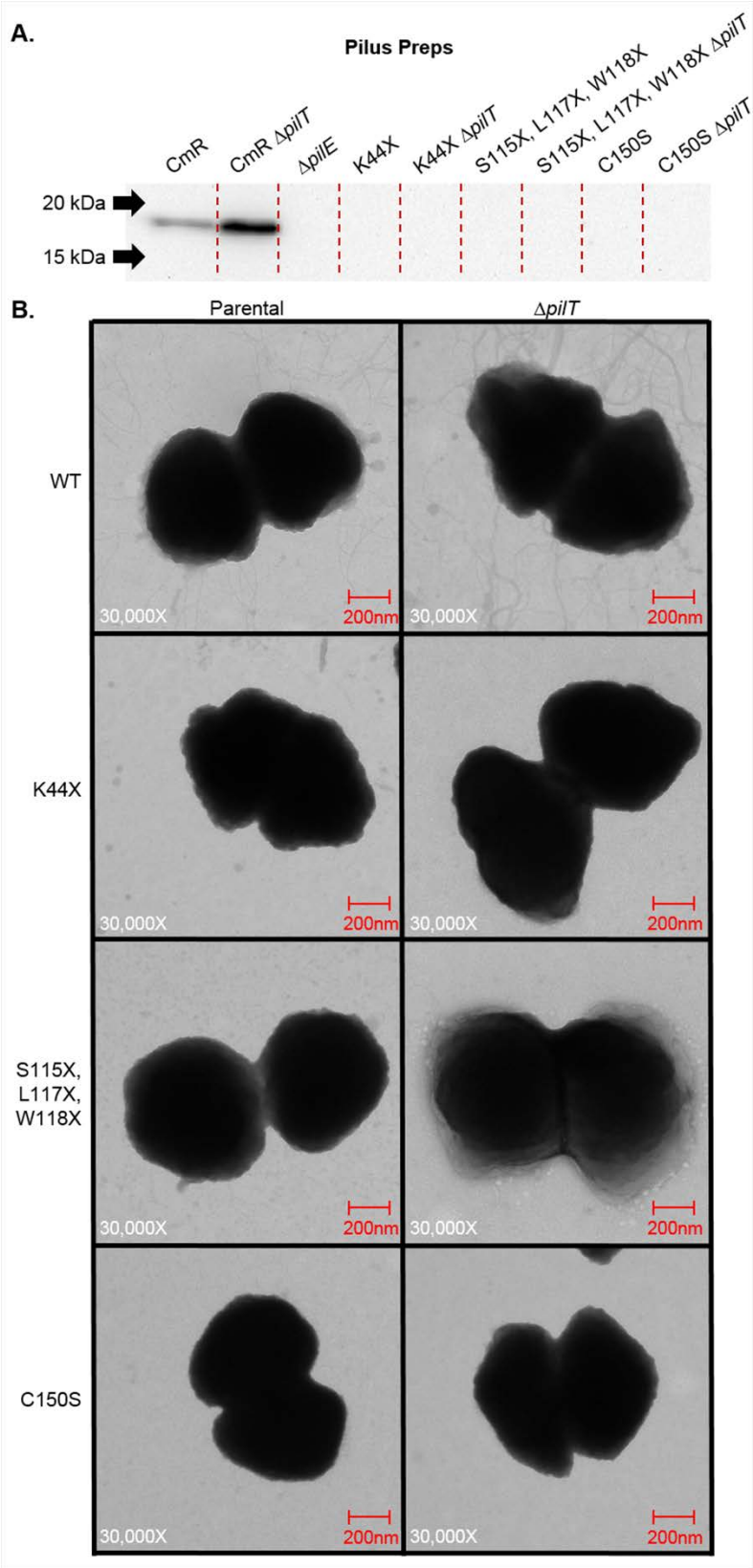

A.
Pilus Preps
CmR
CmR ΔpilT
ΔpilE
K44X
K44X ΔpilT
S115X, L117X, W118X
S115X, L117X, W118X ΔpilT
C150S
C150S ΔpilT
20 kDa
15 kDa
B.
Parental
ΔpilT
WT
K44X
S115X, L117X, W118X
C150S
30,000X
200nm

Figure 17. Characterization of piliation of *pilE* mutants.

A. PilE western blot of pilus filament purification using monoclonal anti-PilE MAb IE8G8 at a 1:1,000 dilution. Pili were purified from the parental strain (CmR), Δ*pilE*, and *pilE* mutants in both a WT and Δ*pilT* strain background using an equal number of bacteria per strain. **B.** Representative electron micrographs of indicated strains in both a WT and Δ*pilT* strain background. X = nonsense mutation, CmR = Cm^R parental strain with 1-81-S2 *pilE* variant.

[a]Mutated Base	[b]AA Change	[c]Transformation Efficiency	[d]Pilin Protein	[e]S-pilin Protein	[f]Colony Morphology
361	Gly_{114} to Arg	++++	++	++	-
362	Gly_{114} to Ala	++++	++	++	+
364	Ser_{114} to Pro	++++	+	++	+
365	Ser_{115} to Stop	++	-	-	--
368	Val_{116} to Glu	+++	-	++	-
368	Val_{116} to Gly	++	-	++	-
370	Lys_{117} to Stop	+	-	-	-
373	Trp_{118} to Arg	+	-	+	-
373	Trp_{118} to Arg	+	-	++	-
373	Trp_{118} to Gly	++	-	+++	-
374	Trp_{118} to Ser	++	-	++	-
374	Trp_{118} to Leu	++	-	+	-
375	Trp_{118} to Stop	+	-	-	-
375	Trp_{118} to Cys	+	-	+++	-
375	Trp_{118} to Cys	++	-	++	-
376	Phe_{119} to Val	+++	-	++	-
379	Cys_{120} to Ser	++	-	-	-
379	Cys_{120} to Arg	+	-	-	-
379	Cys_{120} to Gly	++	-	-	+
380	Cys_{120} to Ser	++	-	-	-
380	Cys_{120} to Phe	++	-	-	-
381	Cys_{120} to Stop	++	-	-	-
381	Cys_{120} to Trp	++	-	-	+
382	Gly_{121} to Arg	++	-	+	-
382	Gly_{121} to Stop	++	-	-	-
383	Gly_{121} to Glu	++	-	+	--
383	Gly_{121} to Ala	+	-	+	-
389	Pro_{123} to Arg	+++	-	++	--
391	Val_{124} to Leu	+++	-	+++	-
391	Val_{124} to Phe	++	-	++	-
392	Val_{124} to Gly	++	-	++	-
394	Thr_{125} to Pro	++	-	+++	-
397	Arg_{126} to Ser	+++	-	++	-
397	Arg_{126} to Cys	+	-	++	-
398	Arg_{126} to His	++	-	++	-
398	Arg_{126} to Pro	+++	-	+++	+
398	Arg_{126} to Leu	+++	-	+	-

403	Gly_{128} to Cys	+++	+	++	+
439	Ala_{140} to Thr	++++	+	++	+
440	Ala_{140} to Asp	+++	+	++	-
442	Ile_{141} to Leu	+++	++	++	-
443	Ile_{141} to Thr	++	-	++	-
443	Ile_{141} to Ser	++	-	++	-
451	Lys_{144} to Stop	++	-	-	-
455	His_{145} to Pro	++	-	+	-
457	Leu_{146} to Val	++	-	+	--
458	Leu_{146} to Pro	++	-	+	-
458	Leu_{146} to Gln	++	-	+	-
460	Pro_{147} to Thr	++	-	+	-
460	Pro_{147} to Ala	+++	-	++	-
460	Pro_{147} to Ser	+++	-	++	-
461	Pro_{147} to Leu	++	-	+	-
461	Pro_{147} to Gln	+++	-	+	--
461	Pro_{147} to Arg	++	-	-	-
464	Ser_{148} to Stop	++	-	-	-
464	Ser_{148} to Stop	++	-	-	+
467	Thr_{149} to Asn	++++	++	++	+
469	Cys_{150} to Ser	++	-	-	+
469	Cys_{150} to Arg	+	-	-	-
469	Cys_{150} to Gly	++	-	-	-
470	Cys_{150} to Tyr	++	-	-	-
470	Cys_{150} to Ser	++	-	-	-
470	Cys_{150} to Phe	++	-	-	-
471	Cys_{150} to Stop	++	-	-	-
471	Cys_{150} to Trp	++	-	-	-
472	Arg_{151} to Gly	++	-	-	-
472	Arg_{151} to Cys	+++	+	++	-
473	Arg_{151} to Pro	++	-	++	+

Table 3. Phenotypic characterization of P- isolates.

[a]location of mutation in *pilE* resulting in a P- colony morphology

[b]resultant amino acid change. Red shading indicates a nonsense mutation. Yellow shading indicates a cysteine mutant.

[c]Transformation competence relative to the WT strain (++++) and the non-competent Δ*pilE* strain

[d]Pilin protein levels relative to the WT strain (++) and the Δ*pilE* strain (-)

[e]S-pilin protein levels relative to the WT strain (++) and the Δ*pilE* strain (-)

[f]Pilus related colony morphology relative to WT (++) and the Δ*pilE* strain (--)

Although all experiments were performed in IPTG inducible RecA strain background to prevent antigenic variation, it was possible that the observed transformation was due to a low level of pilin antigenic variation restoring full-length pilin. We ruled out this possibility as all transformants obtained from the mutants retained their non-piliated colony morphology and sequencing of the *pilE* locus from a representative set of transformants showed no changes to the *pilE* sequence. We tested whether the competence of the P- isolates might be due to translational miscoding allowing a small amount of full-length pilin to be expressed from the mutants, since it has previously been shown that only a small amount of pilin expression is sufficient for competence (Long *et al.*, 2003, Rudel *et al.*, 1992). If translational miscoding were responsible for the competence, insertion of consecutive nonsense mutations and cysteine mutations would lower transformation efficiency beyond that of a single mutant. However, upon introduction of two or three nonsense and cysteine mutants into the 3' region of the gene, all mutant strains maintained a similar level of transformation as the individual nonsense and cysteine mutants isolated in the screen (***Figure 18***). Consistent with the lack of mutants isolated in the HV_T from the P- screen, insertion of consecutive nonsense mutations in the HV_T did not affect transformation efficiency (***Figure 18***). These data show that the full-length pilin protein is not required for a reduced but biologically relevant level of transformation competence.

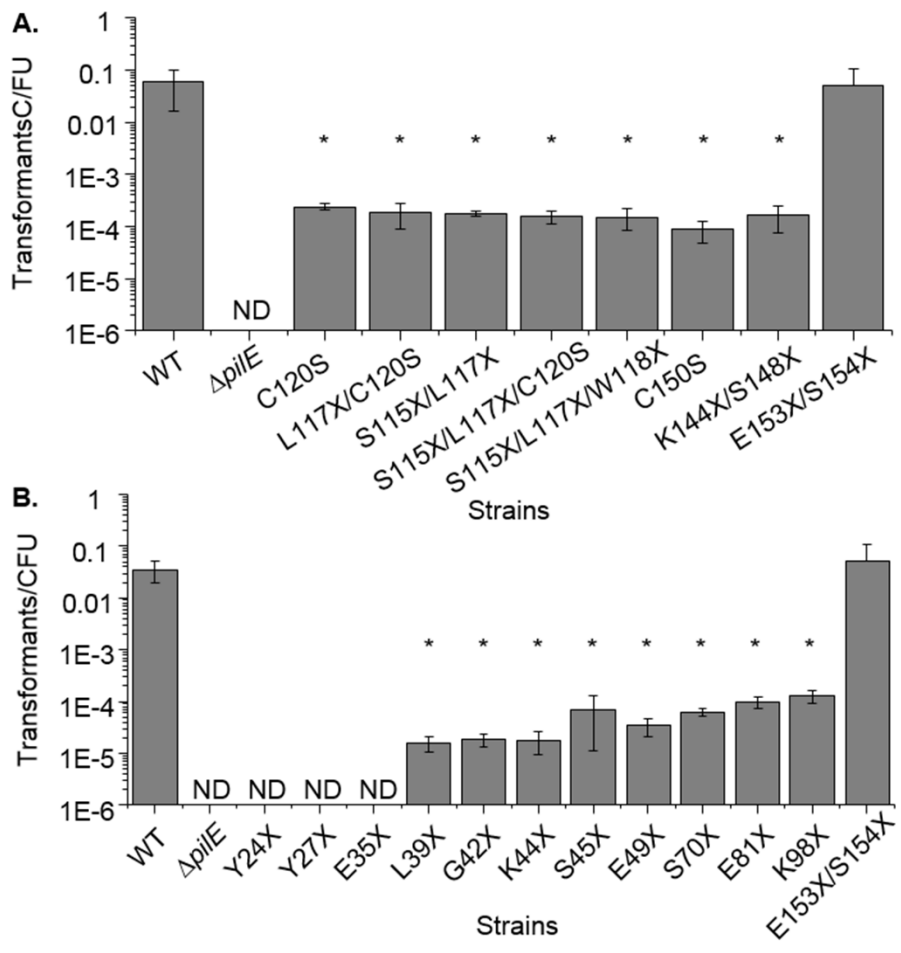


Figure 18. *pilE n*onsense mutants necessary for transformation.

A. Transformation efficiencies of strains with multiple site-directed nonsense and/or cysteine mutations of *pilE* designated by the original amino acid and the resultant amino acid or nonsense mutation (represented by the X). **B.** Transformation efficiencies of site-directed nonsense mutants of *pilE*. WT is the parental FA1090 strain and Δ*pilE* is a deletion derivative of that strain. X = nonsense mutation, ND = transformants not detected, *p<0.05 Student's T-test, each mutant compared to the WT strain.

PilE Ntd-mediated transformation

Since a Δ*pilE* mutant was non-competent for transformation, but the *pilE* truncation mutants retained competence, we predicted that only the N-terminal portion of pilin is required to maintain competence. Nonsense mutations inserted along the length of the *pilE* coding region showed that strains with nonsense mutations after AA38 of mature pilin retained competence, while nonsense mutations prior to, or at AA34, abolished detectable transformation (***Figure 18***). For the remainder of the experiments in this report, we selected three representative non-piliated *pilE* mutations: K44X, a short nonsense mutant expressing only the N-terminal domain (Ntd); S115X/L117X/W118X, a triple C-terminal nonsense mutant; and C150S, a C-terminal cysteine mutant. All three of these mutants retain competence but completely lack pili as shown by pilus purification and electron microscopy, even when *pilT* was inactivated (***Figure 17***). Additionally, while Tfp could be detected in a parental strain, no short pilus filaments, or alternate filaments formed by the Ntd, were detected in the Ntd expressing K44X strain by whole-cell enzyme-linked immunosorbent assay (ELISA) or immuno-gold TEM using a polyclonal anti-Ntd antibody (***Figure 19***). Taken together, these results confirm that Tfp are not required for competence and only the pilin Ntd is required to allow transformation competence.

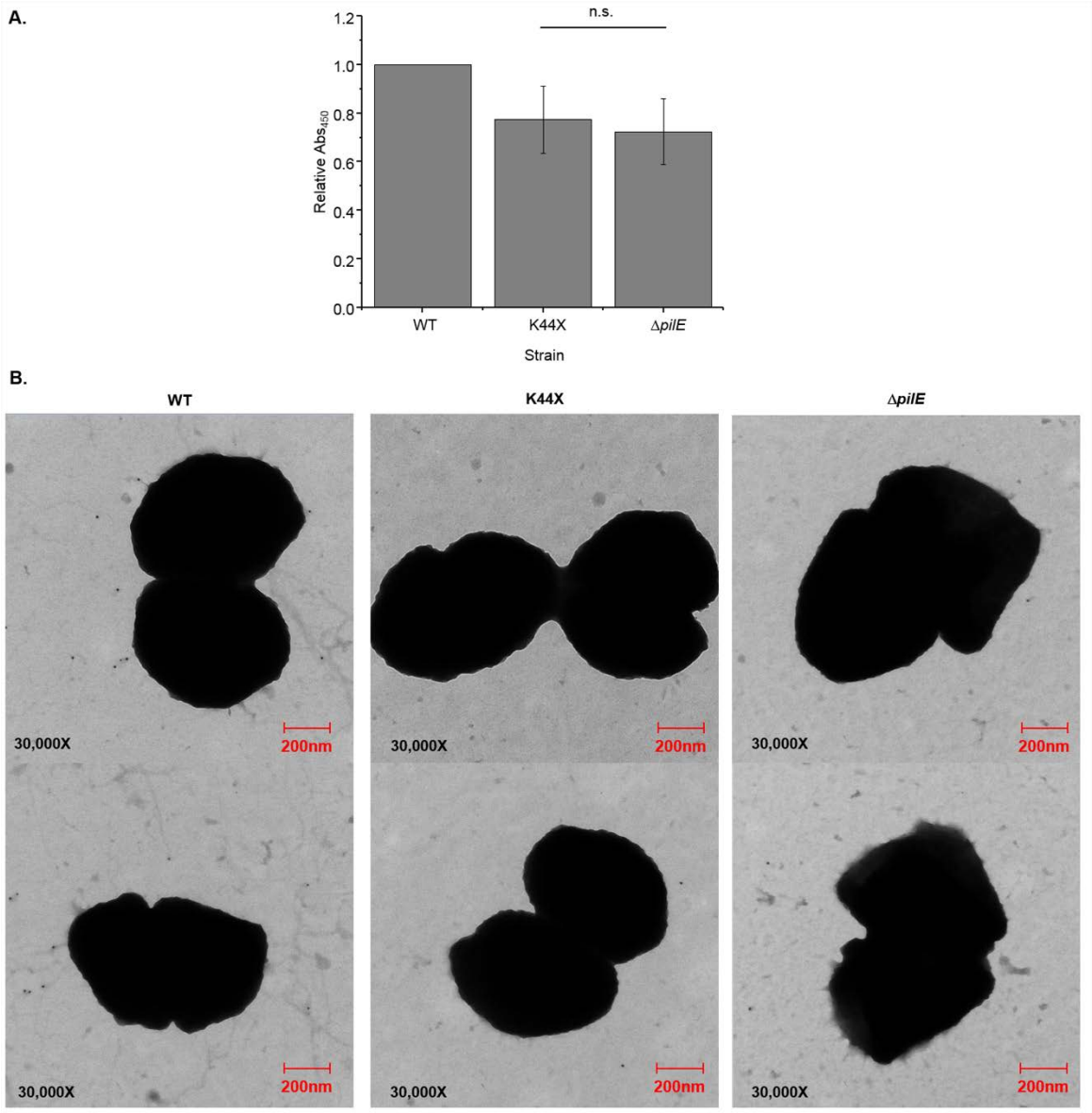

A.
n.s.
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Abs450
WT
K44X
ΔpilE
Strain
B.
WT
K44X
ΔpilE
30,000X
200nm
30,000X
200nm
30,000X
200nm
30,000X
200nm
30,000X
200nm
30,000X
200nm

Figure 19. Analysis of piliation in Ntd expressing strain.

A. Quantification of pilus filament formation of strains using whole cell ELISA with a polyclonal, anti-Ntd antibody at 1:4,000 dilution. The Abs_{450} is plotted relative to the signal in the WT strain. n.s. – not significant indicated by Student's T-test calculated p value above 0.05.

B. Representative Immuno-gold TEM images of negatively stained *N. gonorrhoeae* strains with labeling of pilus filaments using a polyclonal, anti-Ntd antibody at 1:500 dilution. X = nonsense mutation.

To determine whether Ntd-mediated transformation was similar or distinct from the normal transformation process, we assayed for transformation efficiency of Ntd strains in several genetic backgrounds with established effects on transformation. The role of PilD was tested by introducing a $G_{-1}S$ mutation in PilE that prevents PilD cleavage (Koomey *et al.*, 1991) (***Figure 20A***). We also investigated the role of the DNA binding minor pilin, ComP (***Figure 20B***), and the competing minor pilin PilV (***Figure 20C***), by creating insertional mutants in the respective genes. The requirement for a DUS was assayed by using matched constructs for transformation that only differ in the presence or absence of a DUS (***Figure 20D***) (Duffin & Seifert, 2010b). Transformation in *pilE* mutants has the same genetic determinants as the normal process as all strains required PilD cleavage, ComP and a DUS for full transformation efficiency, while the PilV knockouts showed increased frequencies of transformation. The reliance on PilD cleavage also suggests that the Ntd must be present in the inner membrane or periplasm to mediate transformation as PilD cleavage occurs following transport of the pilin molecule through the inner membrane (Nunn & Lory, 1991).

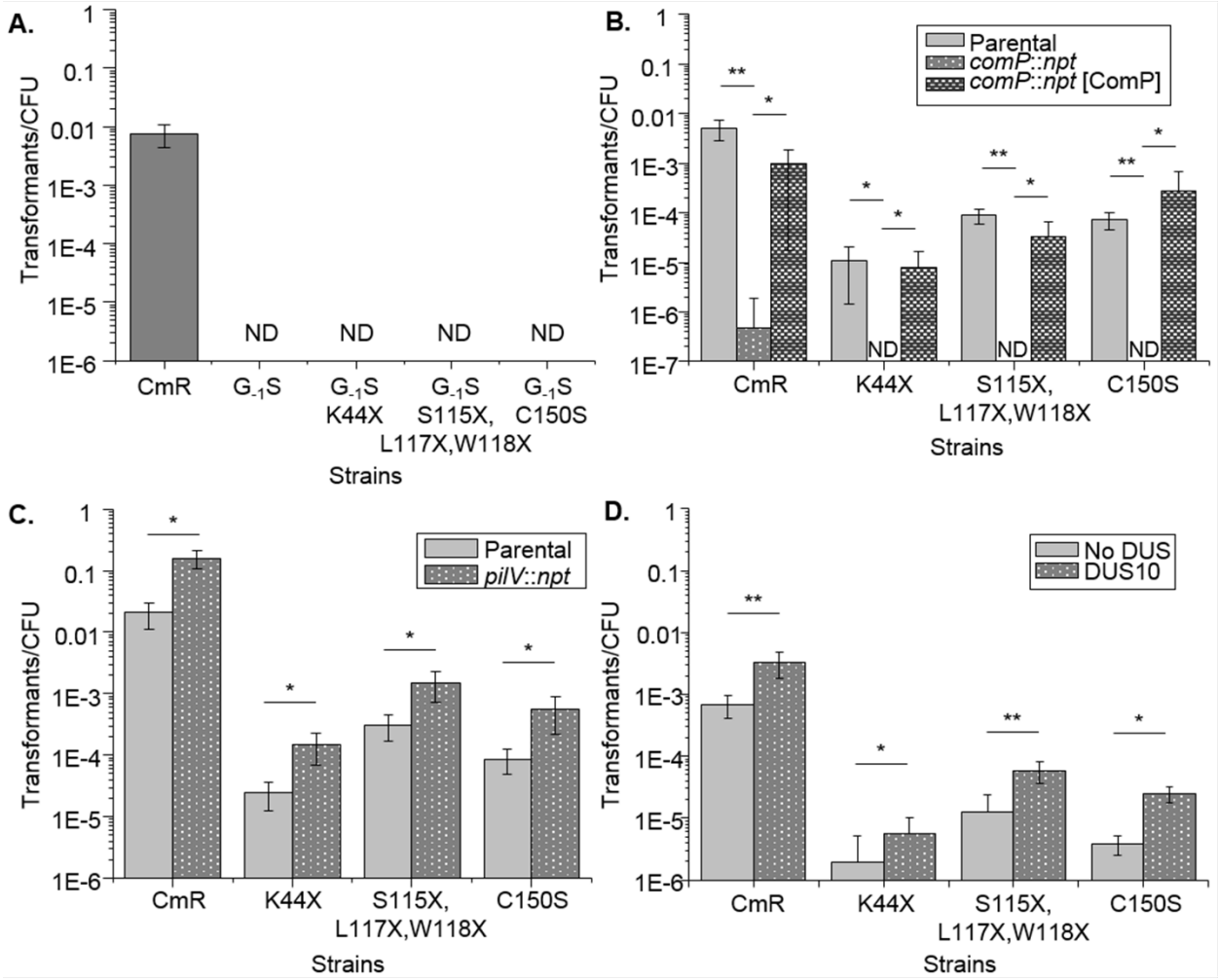

A.
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
ND
ND
ND
ND
CmR
G-1S
G-1S K44X
G-1S S115X, L117X,W118X
G-1S C150S
Strains
B.
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
1E-7
Parental
comP::npt
comP::npt [ComP]
**
*
*
*
**
*
**
*
ND
ND
ND
CmR
K44X
S115X, L117X,W118X
C150S
Strains
C.
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
Parental
pilV::npt
*
*
*
*
CmR
K44X
S115X, L117X,W118X
C150S
Strains
D.
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
No DUS
DUS10
**
*
**
*
CmR
K44X
S115X, L117X,W118X
C150S
Strains

Figure 20. Pilin processing and role of minor pilins in Ntd-mediated transformation.

A. Transformation efficiencies of strains with a *pilE* $G_{-1}S$ mutation that prevents PilD processing coupled with representative C-terminal nonsense mutations preventing piliation. **B.** Transformation efficiencies of *comP::npt* loss-of-function strains with *pilE* C-terminal nonsense mutations preventing piliation. [ComP] complements have an anhydrotetracycline (ATC) inducible copy of *comP* at an ectopic locus. **C.** Transformation efficiencies of *pilV***::***npt* strains with C-terminal *pilE* nonsense mutations preventing piliation. **D.** Transformation efficiencies of strains with C-terminal *pilE* nonsense mutations preventing piliation using as transforming DNA a plasmid either containing a 10-mer DUS (DUS10) or a scrambled DUS (No DUS). CmR = Cm^R parental strain, X = nonsense mutation, ND = transformants not detected, *p<0.05 **p<0.001 Student's T-test.

S-pilin Cleavage and the Ntd

It was striking that the minimal N-terminal fragment necessary for transformation competence was 38 amino acids long, as S-pilin cleavage occurs between amino acids 39 and 40 of the mature pilin protein. We reasoned that the C-terminal PilE mutants unable to assemble pilus fibers were undergoing S-pilin cleavage to allow release of the Ntd to mediate transformation. Site-directed mutagenesis was used to make a set of double mutants in which a C-terminal mutation that abrogates piliation was coupled with a $L_{38}L_{39}A_{40}$-AAM mutation at the S-pilin cleavage site that was previously described to prevent the majority of S-pilin cleavage, with the caveat that this mutation reduces overall piliation (***Figure 21A***) (Aas *et al.*, 2007). This previously reported phenotype in strain MS11 was confirmed in newly constructed $L_{38}L_{39}A_{40}$-AAM mutants in strain FA1090 (***Figure 21B***). The C-terminal mutant strains each showed a drastic decrease in competence when the S-pilin cleavage site was mutated (***Figure 21C***). As expected, the $L_{38}L_{39}A_{40}$-AAM mutation did not completely abrogate transformation as the mutation does not completely prevent S-pilin cleavage (***Figure 21C***).

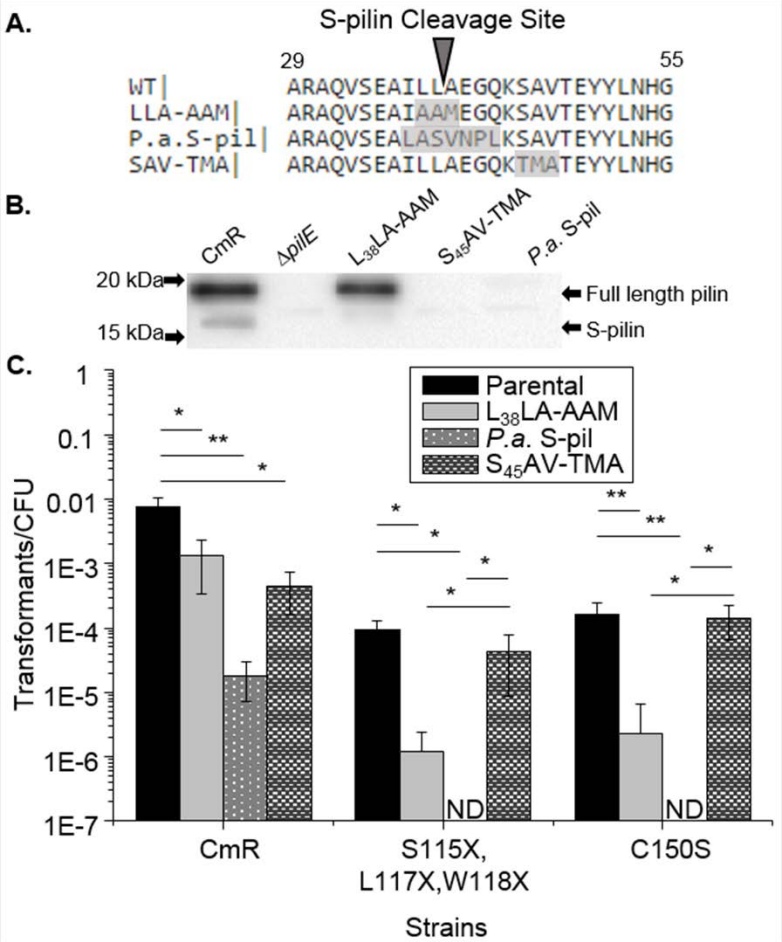

A.
S-pilin Cleavage Site
29
55
WT| ARAQVSEAILLAEGQKSAVTEYYLNHG
LLA-AAM| ARAQVSEAIAAMEGQKSAVTEYYLNHG
P.a.S-pil| ARAQVSEALASVNPLKSAVTEYYLNHG
SAV-TMA| ARAQVSEAILLAEGQKTMATEYYLNHG
B.
CmR
ΔpilE
L38LA-AAM
S45AV-TMA
P.a. S-pil
20 kDa
15 kDa
Full length pilin
S-pilin
C.
Parental
L38LA-AAM
P.a. S-pil
S45AV-TMA
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
1E-7
ND
ND
CmR
S115X, L117X,W118X
C150S
Strains

Figure 21. S-pilin cleavage and transformation competence in *pilE* mutant strains.

A. Alignment of the PilE primary sequence surrounding the S-pilin cleavage site of the parental strain and the S-pilin cleavage mutant strains. **B.** PilE western blot of parental strain (CmR), Δ*pilE* mutant, *pilE* $L_{38}L_{39}A_{40}$-AAM S-pilin cleavage mutant, a strain with *P. aeruginosa* PilA sequence at residues 37-43, and the S-pilin control mutation $S_{45}A_{46}V_{47}$-TMA. Upper band is full-length pilin. Lower band is the processed S-pilin form. Western blot analysis performed using the K36 peptide anti-pilin antibody. **C.** Transformation efficiencies of a *pilE* C-terminal mutations coupled with the S-pilin cleavage site mutation ($L_{38}L_{39}A_{40}$-AAM), *P. aeruginosa pilA* sequence (*P.a.* S-pil), or the control mutation ($S_{45}A_{46}V_{47}$-TMA). X = nonsense mutation, CmR = Cm^R parental strain, *p<0.05 **p<0.001 Student's T-test. ND = transformants not detected.

In the same report describing the $L_{38}L_{39}A_{40}$-AAM, Aas et al. also replaced the S-pilin cleavage region with the corresponding seven amino acid region from *Pseudomonas aeruginosa* pilin, PilA (***Figure 21A***)(Aas *et al.*, 2007). While this replacement mutation reduced transformation efficiency and destabilized Tfp in a WT background, it also prevented all production of S-pilin (***Figure 21B and Figure 22***). Full-length pilin protein can be detected in this *P. aeruginosa* S-pilin (*P.a.* S-pil: I_{37}LLAEGQ-LASVNPL) strain using a monoclonal antibody IE8G8 whose binding epitope is not altered by the *P. aeruginosa* sequence (***Figure 22A***). When we constructed this mutation in strains harboring a C-terminal *pilE* mutation, transformation was completely abolished (***Figure 21C***). The decrease in transformation efficiency observed with either of the S-pilin cleavage mutations was specific to mutation of the S-pilin cleavage site as a similar mutation ($S_{45}A_{46}V_{47}$-TMA) just downstream of the S-pilin cleavage site did not decrease transformation efficiency when coupled with a C-terminal mutation. Moreover, this control mutation, in a WT *pilE* background, resulted in a P- colony morphology and completely destabilized full length pilin protein with only S-pilin being detected in concentrated cell supernatants (***Figure 21B and C and Figure 22***). These data demonstrate that while any destabilizing mutation in the C-terminal region will reduce the transformation efficiency, when the S-pilin cleavage site is disrupted, the retained competence is lost.

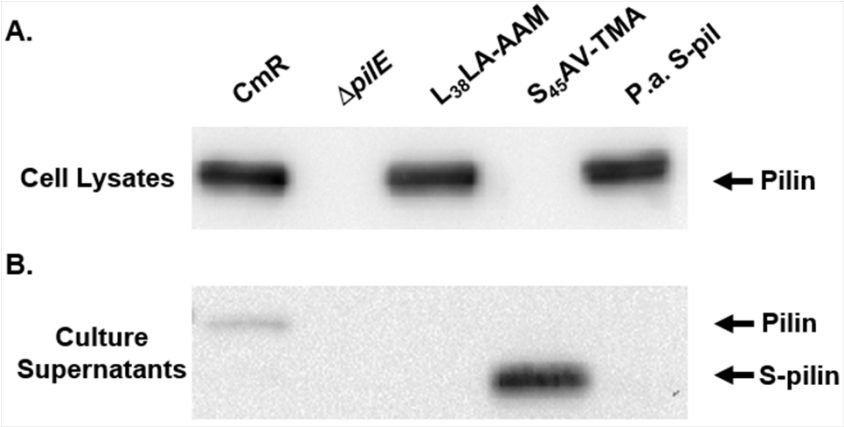


Figure 22. Pilin production by S-pilin mutant strains.

PilE western blots of whole cell lysates (**A.**) and concentrated cell supernatants (**B.**) of parental strain (CmR), Δ*pilE* mutant, *pilE* $L_{38}L_{39}A_{40}$-AAM S-pilin cleavage mutant, a strain with *P. aeruginosa* PilA sequence at residues 37-43, and the S-pilin control mutation $S_{45}A_{46}V_{47}$-TMA. Upper band is full-length pilin. Lower band is the processed S-pilin form. Western blot analysis performed using monoclonal anti-PilE MAb IE8G8 at a 1:500 dilution.

Complementation of a Δ*pilE* strain was accomplished with a copy of *pilE* or the Ntd with an anhydrotetracycline (ATC) inducible promoter at an ectopic site. These constructs restored transformation efficiency in a Δ*pilE* background although not to parental levels (***Figure 23***). The ability of the complementation constructs to express *pilE* transcripts relative to parental levels was measured by quantitative RT-PCR (***Figure 23A***). *pilE* transcript levels were reduced by about 1.5 logs in the complementation strains and the transformation efficiencies of the Δ*pilE iga::pilE* and Δ*pilE iga*::$pilE_{Ntd}$ strains were reduced by a similar amount in comparison to the CmR and L39X strains respectively (***Figure 23***). The transformability of the Δ*pilE iga*::$pilE_{Ntd}$ strain demonstrates that the Ntd is sufficient to mediate transformation in a PilE deletion strain. This result, along with the transformation data from the S-pilin cleavage mutants and the demonstrated loss of competence of any nonsense mutation upstream of the S-pilin cleavage site proves that release of the Ntd by S-pilin cleavage is required to maintain transformation competence.

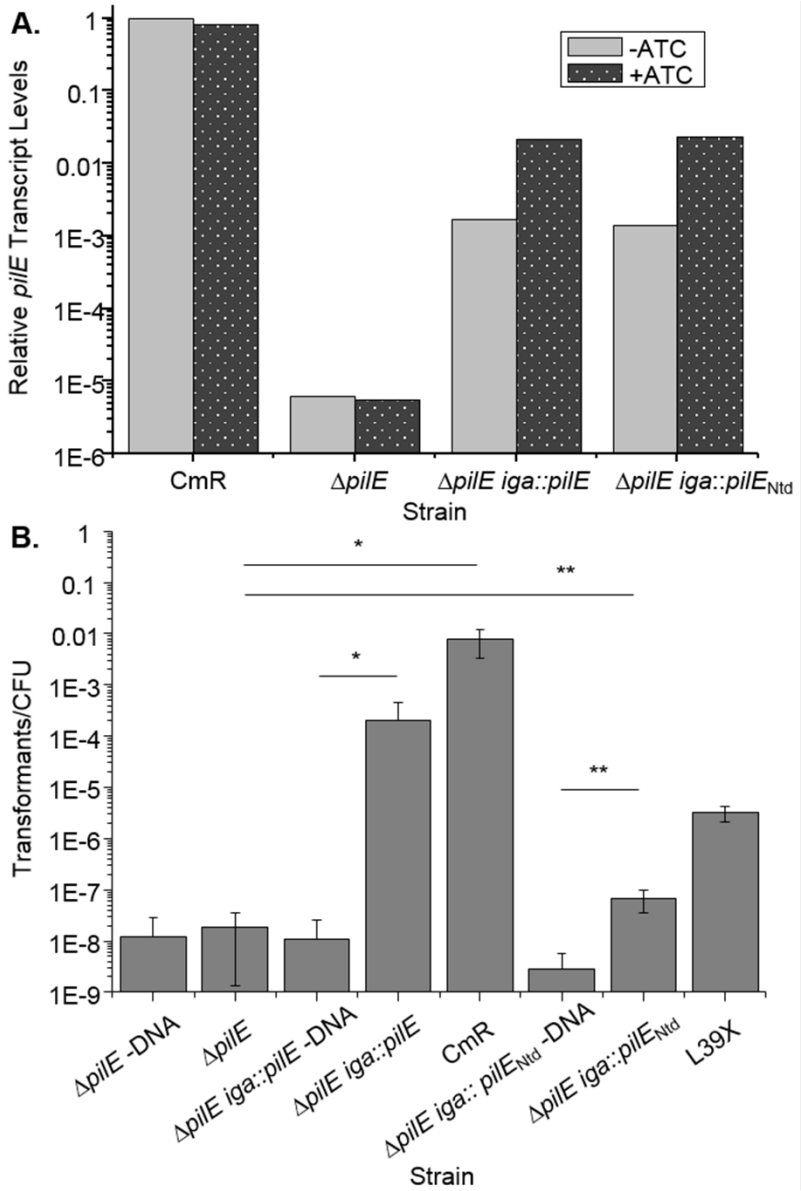

A.
Relative pilE Transcript Levels
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
-ATC
+ATC
CmR
ΔpilE
ΔpilE iga::pilE
ΔpilE iga::pilE Ntd
Strain
B.
Transformants/CFU
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
1E-9
*
**
*
**
ΔpilE -DNA
ΔpilE
ΔpilE iga::pilE -DNA
ΔpilE iga::pilE
CmR
ΔpilE iga:: pilE Ntd -DNA
ΔpilE iga::pilE Ntd
L39X
Strain

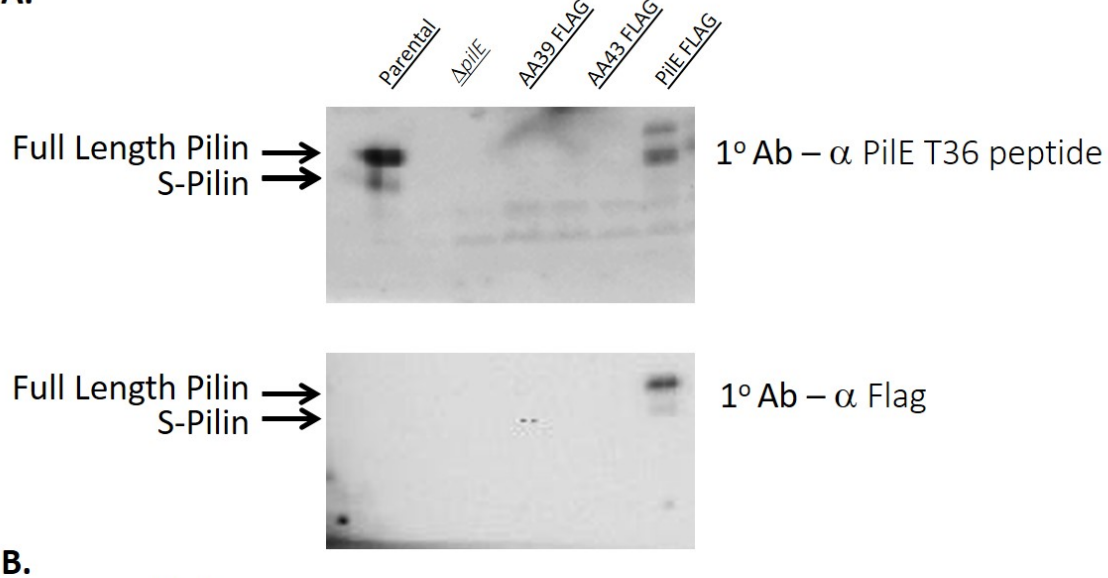
Figure 23. Complementation of Δ*pilE* strain with the Ntd.

A. Relative mRNA levels of *pilE* in the presence or absence of ATC as measured by quantitative RT-PCR. **B.** Transformation efficiencies of Δ*pilE* complementation strains in the presence of ATC. Strains Δ*pilE iga::pilE* and Δ*pilE iga*::$pilE_{Ntd}$ have an ATC inducible copy of *pilE* or the Ntd (PilE L39X) respectively inserted at the *iga* locus. X = nonsense mutation, CmR = Cm^R parental strain, -DNA = no transforming DNA added to reaction, *$p<0.05$, **$p<0.001$ Student's T-test.

Attempts to biochemically detect the Ntd

The genetic data presented here demonstrates the role of the Ntd in mediating transformation. To complement this genetic evidence, we sought to biochemically detect and quantify the Ntd in *N. gonorrhoeae* cells. We first attempted to detect the Ntd by attaching Flag or C-Myc epitope tags to PilE truncated after mature amino acid 39, 43, and 53 and performing western blots with tag specific primary antibodies. When assayed by western blot, full-length, tagged PilE protein could be detected, but no tagged, truncated PilE could be detected with tag specific antibodies (***Figure 24A***). Attachment of an epitope tag to truncated PilE also prevented transformation (***Figure 24B***). These results were not tag specific as His6x, HA, HSV, and VSV-g tags all also abolished transformation.

A.


Parental
ΔpilE
AA39 FLAG
AA43 FLAG
PilE FLAG
Full Length Pilin
S-Pilin
1° Ab – α PilE T36 peptide
Full Length Pilin
S-Pilin
1° Ab – α Flag

B.

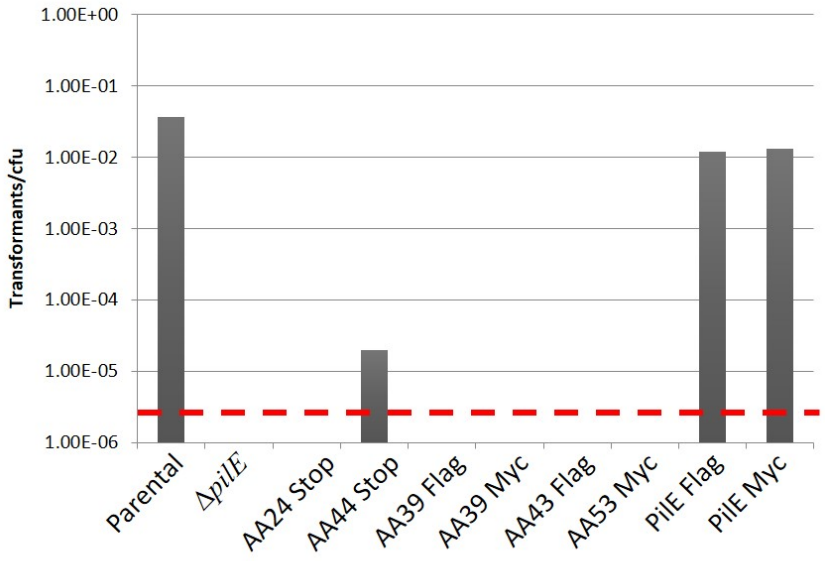

1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
Transformants/cfu
Parental
ΔpilE
AA24 Stop
AA44 Stop
AA39 Flag
AA39 Myc
AA43 Flag
AA53 Myc
PilE Flag
PilE Myc

Figure 24. Analysis of epitope tagged PilE truncations.

A. Western blots of cell lysates of Flag-tagged PilE strains. Upper panel shows a western blot using α-PilE T36 primary antibody. Lower panel shows a western using α-Flag primary antibody. **B.** Transformation efficiencies of Flag-tagged and C-Myc-tagged PilE strains. Red line indicates limit of detection. Preliminary experiment only performed once with no statistical analysis.

We next attempted to detect the Ntd through western blots using an Ntd specific primary antibody. A rabbit polyclonal antibody was raised against a peptide included in the Ntd - YQDYTARAQVSEAILLC (AA24-39 of mature PilE) and affinity purified by GenScript. The affinity purified antibody failed to detect even full-length PilE. The sera did contain antibody that when adsorption purified by incubation with acetone powder of protein from a Δ*pilE* strain recognized full length PilE but not S-pilin (***Figure 25***). This is consistent with the antibody targeting the Ntd of PilE. No specific bands were detected that corresponded to the predicted molecular weight of the Ntd in cell lysates, however. Synthesized Ntd (FTLIELMIVIAIVG ILAAVALPAYQDYTARAQVSEAILL) was detected by this antibody but only if high amounts of peptide (≥10ng) was loaded on gels (***Figure 25***). The extreme hydrophobic nature likely hampered detection as even the purified peptide did not run as a discrete band.

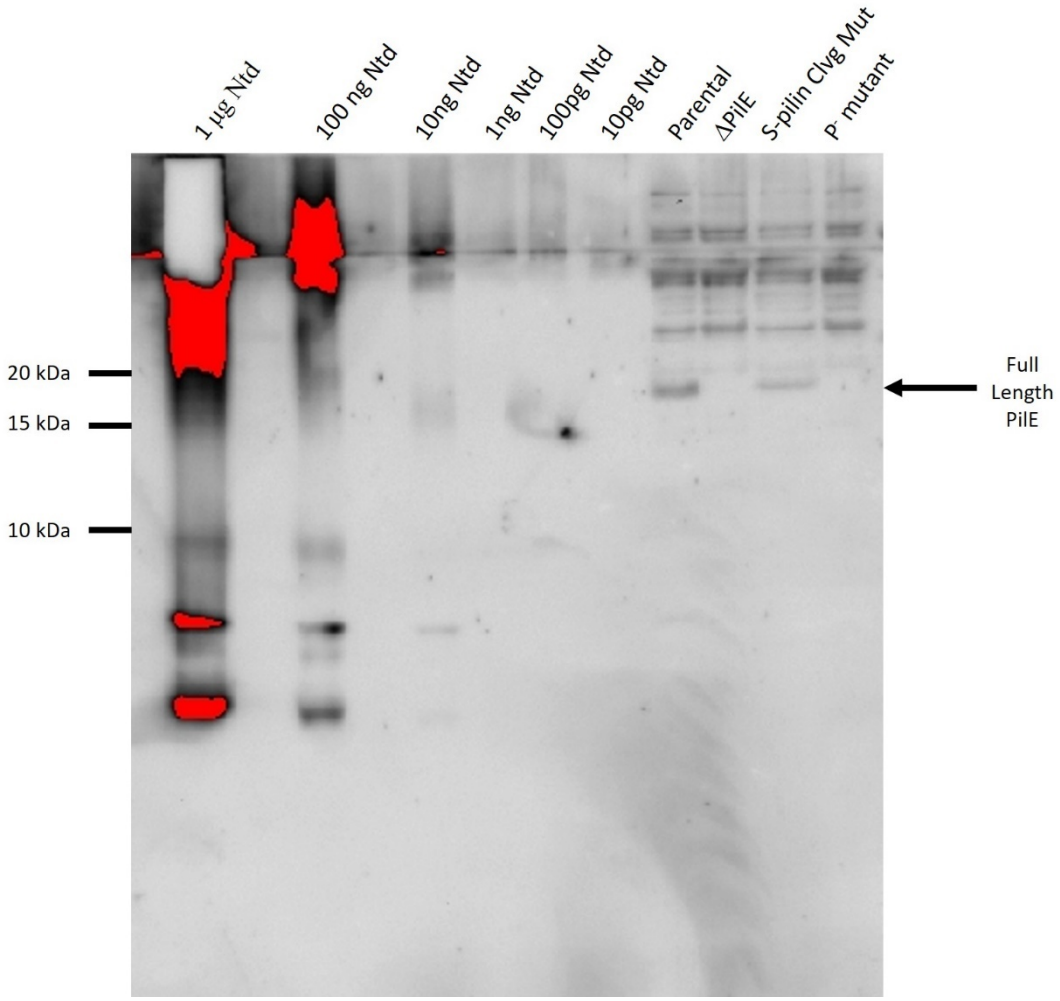


Figure 25. Ntd western blot.

Western blot of synthetic Ntd and cell lysates using polyclonal α-Ntd serum. Left lanes were loaded with increasing dilutions of synthetic Ntd. Final four lanes were loaded with cell lysates from the indicated strain. Primary anti-serum was used at a 1:1000 dilution.

The final technique employed to attempt to detect the Ntd was HPLC. We hypothesized that the Ntd could be purified from cell lysate if an HPLC column could be identified that bound and eluted the Ntd under the proper conditions. Cell lysates from a parental strain and a Δ*pilE* could be purified and ran over the column to identify a peak corresponding to the Ntd in the parental strain that was lacking in the Δ*pilE* strain. The elution fraction corresponding to the specific Ntd peak could be collected and sent for mass spectrometric analysis to confirm the Ntd was present. To this end, two reverse-phase HPLC columns were tested for their ability to bind and elute synthetic Ntd. Both an XBridge Peptide BEH C18 column and a PLRP-S column bound the Ntd, but proper, reproducible elution conditions could not be identified. The extreme hydrophobicity of the Ntd likely resulted in the Ntd binding too tightly, as some Ntd could be found in fractions of pure organic solvent used to regenerate the columns after each use.

Based on this analysis we employed an XBridge BEH C4 column typically used only to purify full length protein because the packing material only weakly binds hydrophobic domains. Synthetic Ntd, solubilized in formic acid, bound the column and was eluted consistently after 20 minutes using an increasing gradient of 0.1% Trifluoracetic acid (TFA) in acetonitrile as detected by absorbance at 280nm (***Figure 26A***). Synthetic Ntd could be passed through solid phase extraction Hydrophobic-Lipophilic-Balanced (HLB) cartridges, eluted efficiently with 50% Acetonitrile with 0.1% TFA, and detected following separation by the BEH C4 column (***Figure 26B***). When synthetic Ntd was mixed with cell lysate from a Δ*pilE* strain that had been purified through solid phase extraction on HLB cartridges, the Ntd could be detected by HPLC analysis on the BEH C4 column (***Figure 26C***). However, a high amount of Ntd (~2μg) was required for peak detection when mixed with the purified cell lysate. When the same conditions were used to

run cell lysates from a piliated strain and the Δ*pilE* strain on the BEH C4 column, no unique peaks were detected in the piliated strain. Additionally, no significant peak corresponding to the elution time of the Ntd was detected (***Figure 26D***). Having failed to detect the Ntd due to various technical difficulties likely related to the extreme hydrophobicity and short nature of the Ntd, no further attempts were made to biochemically detect the Ntd.

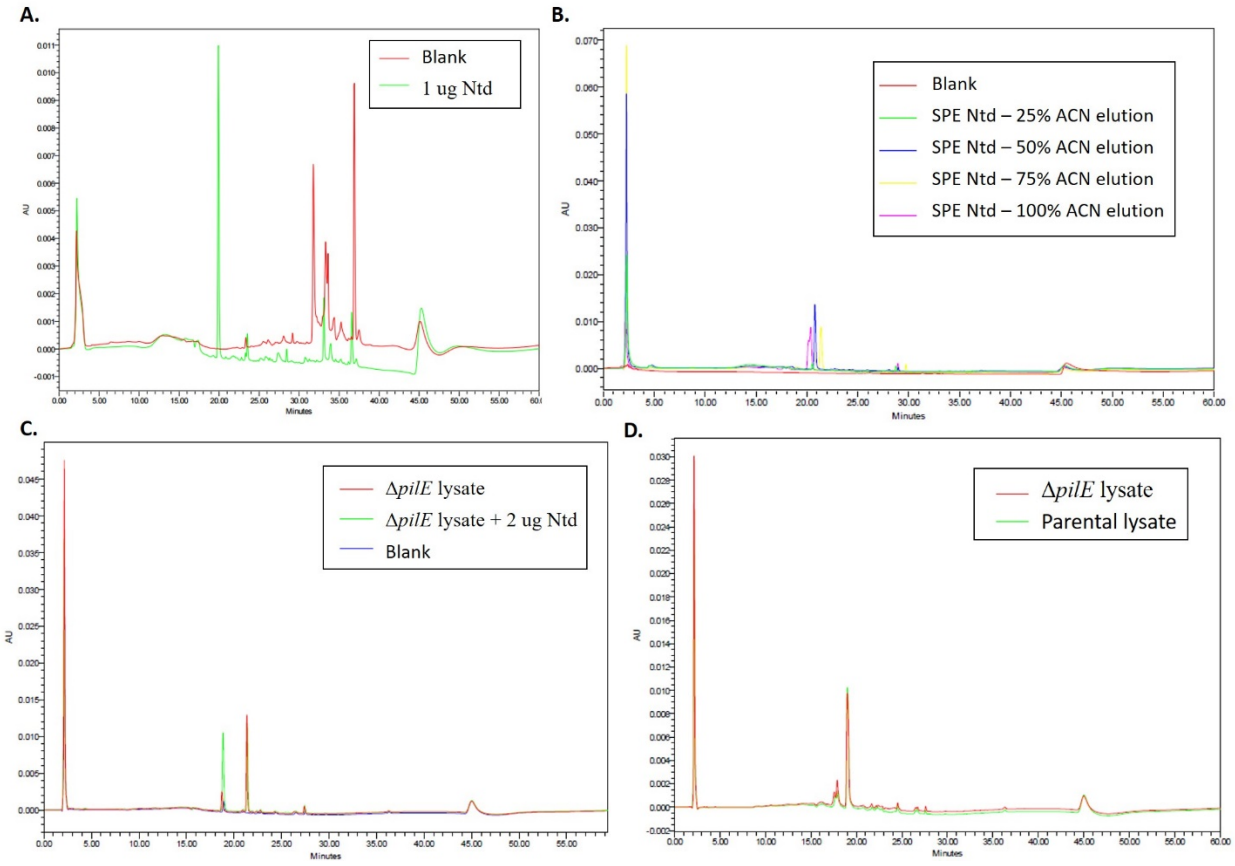

A.
Blank
1 ug Ntd
AU
Minutes
B.
Blank
SPE Ntd – 25% ACN elution
SPE Ntd – 50% ACN elution
SPE Ntd – 75% ACN elution
SPE Ntd – 100% ACN elution
AU
Minutes
C.
ΔpilE lysate
ΔpilE lysate + 2 ug Ntd
Blank
AU
Minutes
D.
ΔpilE lysate
Parental lysate
AU
Minutes

Figure 26. HPLC detection of the Ntd.

Reverse phase HPLC analysis of 10μL samples separated on a BEH C4 column with protein eluents detected by absorbance measurement at 280nm on a UV/Visible detector. **A.** 1μg synthetic Ntd solubilized in formic acid and diluted in 50% acetonitrile (ACN) with 0.1% TFA. **B.** Synthetic Ntd solubilized in formic acid, solid phase extracted using HLB cartridges, and eluted in varying concentrations of acetonitrile with 0.1% TFA. **C.** Δ*pilE* cultures lysed and solid phase extracted using HLB cartridges and eluted with 50% acetonitrile with 0.1% TFA. The purified lysates were mixed with vehicle alone or 2ug synthetic Ntd per 10μL of purified lysate. **D.** Δ*pilE* or parental strain cultures lysed and solid phase extracted using Oasis HLB cartridges and eluted with 50% acetonitrile with 0.1% TFA.

The role of Ntd-mediated transformation during antigenic variation

One of the many possible outcomes of pilin antigenic variation is phase variation or the creation of pilin molecules that cannot efficiently assemble into pilus fibers (Swanson *et al.*, 1986). Some pilus phase variants are the outcome of frameshifts encoded in silent copies that result in early stop codons similar to the nonsense mutations isolated in our screen, but with additional amino acid changes between the frame shift and the stop codon (Criss *et al.*, 2005). To determine whether naturally occurring pilus phase variants retained competence, we allowed the parental strain to undergo antigenic variation and isolated several unique *pilE* frameshift phase variants which resulted in nonsense mutations. We tested these naturally occurring nonpiliated variants for transformation efficiency, and each retained considerable competence (***Figure 27A***). Notably, the P- pilin antigenic variant strains exhibited higher transformation efficiencies than the strains with similar nonsense mutations created by site-directed mutagenesis. qRT-PCR of the *pilE* transcript showed that the Cm^R used to select the mutants actually decreased the *pilE* transcript to a third of wild-type levels resulting in lower PilE levels (***Figure 28A***). This decrease in mRNA corresponded with a decrease in transformation efficiencies as mutants isolated without a selection marker displayed greater competence than strains with the same mutation coupled to the Cm^R (***Figure 28B***).

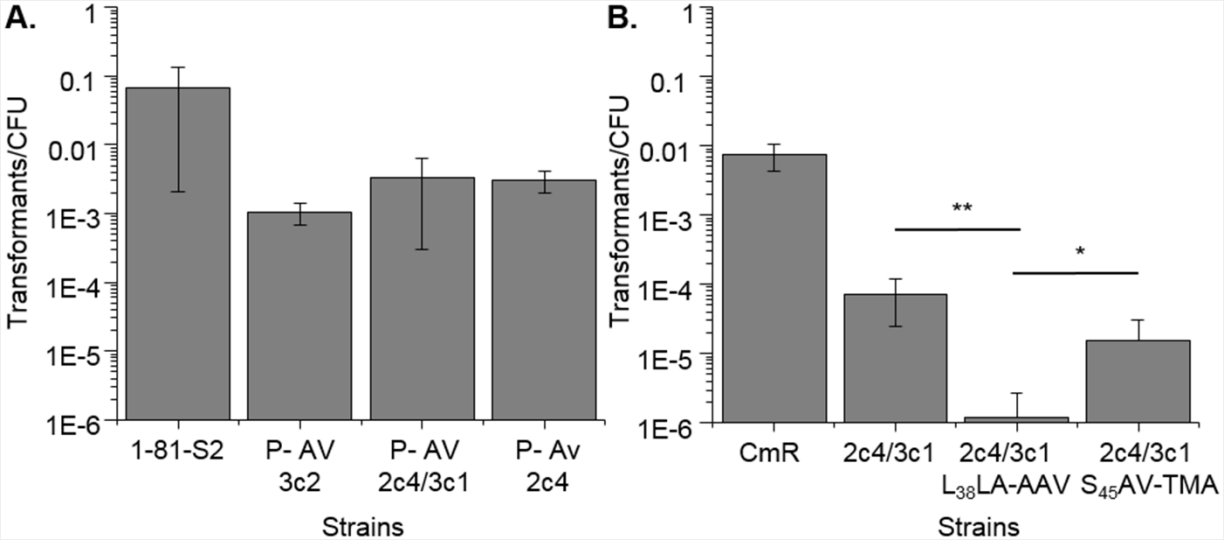


Figure 27. Transformation competence of pilus phase variants.

A. Transformation efficiencies of naturally occurring P- antigenic variants with *pilE* variants that encode frameshift mutations resulting in early stop codons. **B.** Transformation efficiencies of P- strains resulting from antigenic variation events from donor silent loci 2c4 and 3c1 coupled with either the S-pilin cleavage mutation ($L_{38}L_{39}A_{40}$-AAM) or the control mutation ($S_{45}A_{46}V_{47}$-TMA). 3c2, 2c4/3c1, 2c4 indicate silent loci which donated sequence to the variant. CmR = Cm^R parental strain, *$p<0.05$ **$p<0.001$ Student's T-test.

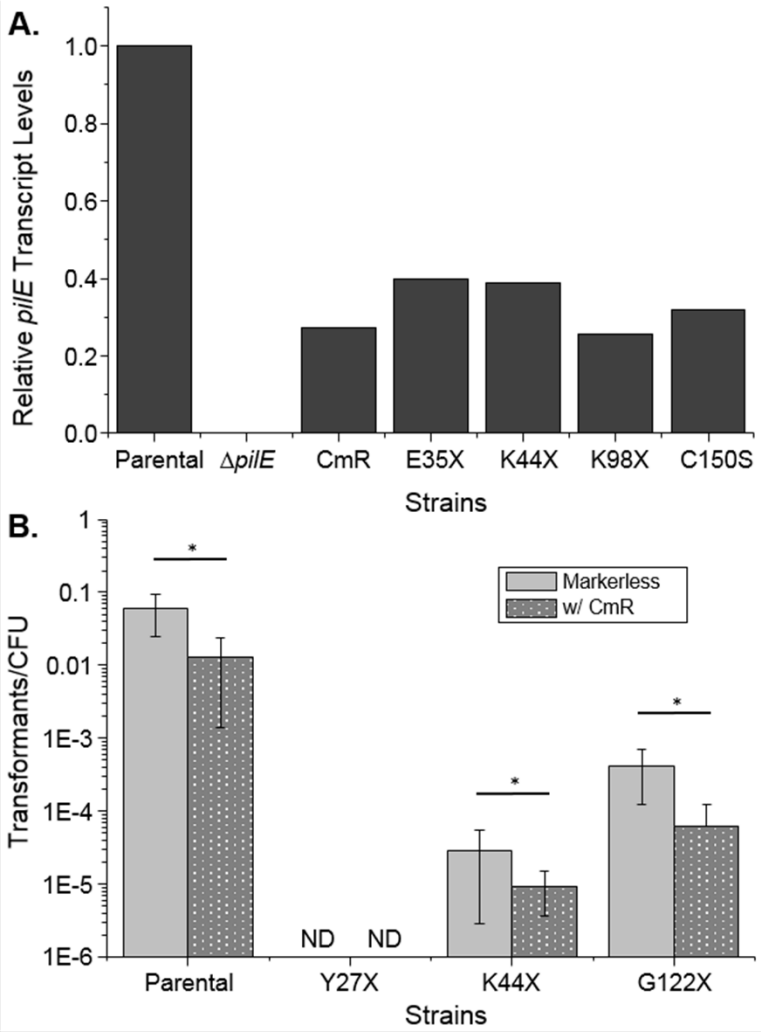


Figure 28. Effects of chloramphenicol resistance cassette inserted downstream of *pilE*.

A. Relative mRNA levels of *pilE* as measured by quantitative RT-PCR. Strains CmR, E35X, K44X, K98X, and C150S contain a Cm^R. **B.** Transformation efficiencies of strains either with or without the Cm^R downstream of *pilE*. X = nonsense mutation, ND = transformants not detected, *p<0.05 Student's T-test.

Finally, we tested whether the $L_{38}L_{39}A_{40}$-AAM mutation, when introduced into a natural P- phase variant also reduced transformation efficiency (***Figure 27B***). Consistent with the strains harboring other C-terminal mutations, the S-pilin cleavage mutation drastically reduced transformation efficiency of the phase variant, demonstrating that S-pilin cleavage is required for transformation in natural antigenic variants with unproductive pilin molecules. Taken together, these results strongly suggest that the Ntd is released by S-pilin proteolysis and can substitute for full-length pilin by producing a form of the pilus assembly apparatus that is active for DNA transformation during the normal process of pilus phase variation.

Non-varying class II pilins

While all *N. gonorrhoeae* strains contain an antigenically varying class I pilin, some *N. meningitidis* strains have a class II pilin that does not undergo antigenic variation (Gault *et al.*, 2015). The only observation of S-pilin cleavage in *N. meningitidis* that we are aware of was in strain MS11, which contains a class I variable pilin (Marceau & Nassif, 1999). An alignment of pilin amino acid sequences from class I and class II pilins reveals that there are amino acid changes around the S-pilin cleavage site in class II pilins (***Figure 29***). If release of the Ntd is only required to mediate transformation during unproductive antigenic variation, we hypothesized that these sequence differences may not allow for S-pilin cleavage. The strains harboring class II pilins would not need to undergo S-pilin cleavage as they don't undergo antigenic variation. To test this hypothesis, *N. gonorrhoeae pilE* was mutated to replace the *N. gonorrhoeae* region near the S-pilin cleavage site with the *N. meningitidis* class II pilin sequence ($I_{37}L_{38}$-LT). The $I_{37}L_{38}$-LT strain exhibited a slight decrease in piliation as observed by colony morphology (***Figure 30A***). PilE western blot analysis revealed that the $I_{37}L_{38}$-LT mutation did

not prevent S-pilin cleavage (***Figure 30B***). Notably, while the mutation appears to decrease piliation, the class II pilin sequence increased the transformation efficiency of the strain (***Figure 30C***). Together these data indicate that the $I_{37}L_{38}$-LT mutation does alter pilus related functions but does not prevent S-pilin cleavage. Further investigation would be necessary to conclusively determine whether or not class II pilins undergo S-pilin cleavage.

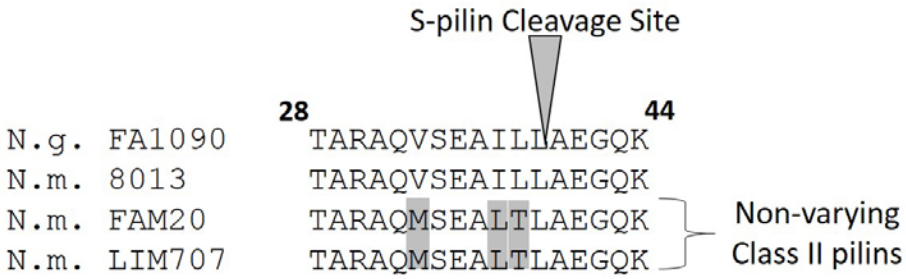


Figure 29. Alignment of class I and class II PilE sequences.

Alignment of AA 28-44 of *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains 8013, FAM20 and LIM707. Sequence deviations are highlighted in grey. Strains FA1090 and 8013 have class I pilin sequences while strains FAM20 and LIM707 have class II pilins that do not undergo antigenic variation.

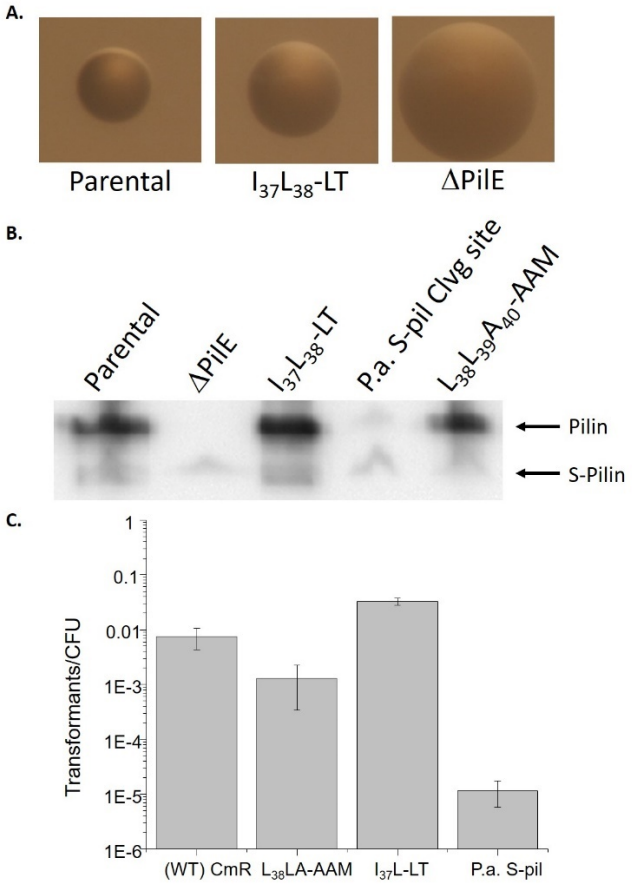


Figure 30. Analysis of *pilE* I37L38-LT mutant.

A. Colony morphology of the parental, $I_{37}L_{38}$-LT, and nonpiliated Δ*pilE* strains. **B.** PilE western blot, using the K36 anti-peptide antibody, of parental strain (CmR), Δ*pilE* mutant, $I_{37}L_{38}$-LT class II mutant, a strain with *P. aeruginosa* PilA sequence at residues 37-43, $L_{38}L_{39}A_{40}$-AAM S-pilin cleavage mutant. Upper band is full-length pilin. Lower band is the processed S-pilin form. **C.** Transformation efficiencies of S-pilin cleavage site mutants.

Discussion

We have shown in a directed mutational analysis of the 3' *pilE* coding region that a variety of *pilE* mutations that either alter or completely abolish pilus elaboration on the bacterial cell surface do not prevent transformation. A series of site-directed *pilE* mutants demonstrated that this transformation competence is not due to translational read-through, but that the maintenance of competence requires the pilin Ntd, a putative cleavage product of S-pilin proteolysis. Moreover, naturally occurring pilin variants that have pilin molecules incapable of forming an extended pilus fiber also retain competence. In these variants, S-pilin cleavage is required for transformation to mediate release of the Ntd from the mutated C-terminal head. We propose that the process of S-pilin cleavage releases the Ntd to maintain competence in cells undergoing antigenic variation, a diversity-generation system critical for immune escape during infection.

In comparing naturally occurring pilin phase variants to site-directed *pilE* mutants, we determined that our method for selecting *pilE* mutants with a Cm^R in the 3' Sma/Cla region had the unintended consequence of suppressing *pilE* mRNA levels. We assume the Cm^R marker has interrupted an mRNA stability element, but further investigation will be required to define the mechanism that this insertion affects mRNA levels. It is important to note that most of the reported transformation efficiencies in this study are lowered by the action of the Cm^R cassette on *pilE* transcript. Without the Cm^R cassette, Ntd mediated transformation can still be remarkably efficient with more than one in a thousand cells being transformed (***Figure 27 and Figure 28***). While this level of transformation is significantly lower than that of the fully piliated parental strain, it is still greater or equal to the competence efficiencies reported for the related

bacterium *N. meningitidis* (Davidsen & Tonjum, 2006) and other naturally competent bacterial species (Antonova & Hammer, 2015, Poje & Redfield, 2003). Additionally, there was a notable drop in transformation efficiency as the site-directed truncations approached amino acid 38, which is either due to instability of the shorter peptide or loss of residues important for interaction with other factors. However, we cannot substantiate this conclusion as the extreme hydrophobic nature of the α1-N domain of the Ntd (residues 1 to 28) has prevented detection by western blot and HPLC (Giltner *et al.*, 2012) and multiple epitope tagged versions of the Ntd were not stable and prevented transformation competence. However, the requirement of at least a 38 amino acid long *pilE* gene product for competence demonstrates that the Ntd is stable enough to supply substantial biological function.

Although the $L_{38}L_{39}A_{40}$-AAM pilin cleavage site mutant inhibits S-pilin cleavage but does not completely prevent cleavage, and the *Pseudomonas* S-pilin mutation abrogates S-pilin production but destabilizes full-length pilin; transformation assays with these two strains demonstrate that release of the Ntd is critical for maintenance of competence when unproductive pilin molecules with C-terminal mutations are expressed. While we cannot presently determine whether S-pilin cleavage is required for transformation with a fully functional pilin, the fact that the $L_{38}L_{39}A_{40}$-AAM mutation did decrease transformation efficiency in the parental strain leaves open the possibility that S-pilin cleavage has a role regardless of the functional status of pilin. However, this reduction in competence may also be due to decreased ability of full-length, $L_{38}L_{39}A_{40}$-AAM pilin to polymerize into pili (Aas *et al.*, 2007). Regardless of the mechanism, these data show that the proteolytic cleavage that releases S-pilin also releases the reciprocal Ntd product to maintain competence.

To our knowledge, the production of S-pilin and the Ntd has only been described for the pathogenic *Neisseria*, organisms also notable for producing many different piliation states through the process of antigenic variation, but not other Tfp producing bacteria. We propose that S-pilin/Ntd proteolysis evolved as a mechanism to release the Ntd to maintain competence in the face of pilus phase variation. These organisms undergo high frequency pilin variation that not only modulates the immune epitopes on the pilus fiber but also mediates phase variation, which can allow for detachment of phase variants from cell surfaces or biofilms. The observation that *N. meningitidis* strains that do not undergo pilus antigenic variation have variable sequence surrounding the S-pilin cleavage site and have not yet been reported to undergo S-pilin cleavage supports this hypothesis. Further investigation into whether the strains undergo S-pilin cleavage should be conducted.

The role of S-pilin/Ntd cleavage in compensating for the consequences of pilin antigenic variation adds another layer of adaptation to the already complex and highly evolved system. This complexity further underscores the importance of this diversity generation system to *N. gonorrhoeae* pathogenesis. Furthermore, because these organisms protect competence during antigenic variation and undergo horizontal gene transfer so frequently that there is an inability to establish clonal lineages (Smith *et al.*, 1993), it appears that horizontal gene transfer is a vital function for these human specific organisms. It is not settled why these and other naturally-competent, human-restricted organisms require continual horizontal gene transfer, but our discovery that there is a specific mechanism to maintain competence in an easily reversible non-piliated state supports the notion that there is strong selection to maintain competence.

Chapter 4: Search for the S-pilin Protease

Introduction

N. gonorrhoeae produces two pilin length variants that cannot assemble into pilus fibers: L-pilin and S-pilin. Truncated versions of pilin were observed even earlier, but S-pilin was identified as a soluble, secreted form in a 1987 report (Swanson *et al.*, 1985, Haas & Meyer, 1987). S-pilin is soluble due to a proteolytic cleavage event (S-pilin cleavage) that cleaves the hydrophobic Ntd from the globular head. Loss of the first 39 amino acids of the mature pilin molecule (Ntd) results in the soluble S-pilin variant that can be found in culture supernatants. S-pilin can be produced from a broad spectrum of functional as well as nonfunctional pilin sequences. Changes in the pilin sequence can alter S-pilin production as well as pilus expression, although the relationship is not understood (Long *et al.*, 1998). Post-translation modification of pilin by glycosylation of serine 63 increases S-pilin production in both *N. gonorrhoeae* and *N. meningitidis* (Marceau & Nassif, 1999). While more few pilin sequences need to be investigated to make a firm conclusion, the published data suggest that glycosylation of Ser63 is required for S-pilin production in *N. meningitidis* but not in *N. gonorrhoeae*.

The reasons for S-pilin production are not understood. The production of S-pilin was hypothesized to be a mechanism to rid the cell of toxic pilin monomers that are non-functional in pilus assembly (Haas *et al.*, 1987, Jonsson *et al.*, 1992). This scenario appears unlikely as the production of S-pilin is not directly linked to the production of non-functional pilin monomers (Long *et al.*, 1998). It has been demonstrated that S-pilin interacts with human tissue but not rat or mouse tissue (Rytkonen *et al.*, 2001). This suggested a specific interaction that might help

account for the human tropism of the organism, and further analysis demonstrated that S-pilin could bind the human cell surface glycoprotein CD46, once thought to be the essential pilus receptor for *Neisseria* (Kallstrom et al., 1997). However, a subsequent study was published showing that piliated cells do not bind to epithelial cells in a CD46 dependent manner and questioning the role of CD46 as the pilus receptor (Kirchner *et al.*, 2005). Thus, a role for S-pilin has yet to be established.

As discussed in Chapter 3 of this dissertation, S-pilin production also results in the release of the reciprocal cleavage product, the Ntd (Obergfell & Seifert, 2016). Release of the Ntd can maintain transformation competence in the event of unproductive antigenic variation which results in the loss of piliation. Investigation of the role of the Ntd in transformation as well as possible functional analysis of S-pilin is hampered by the fact that the protease responsible for S-pilin cleavage has not been identified. In an attempt to further study S-pilin cleavage and its effect on transformation, two strategies were unsuccessfully employed to identify the S-pilin cleavage protease(s).

Results

A Candidate Approach

To screen gene candidates for S-pilin cleavage activity, putative S-pilin proteases were bioinformatically identified and each protease was mutated individually and in several combinations.

Serine protease inhibitors block S-pilin cleavage

To determine the class of the protease responsible for S-pilin cleavage, *N. gonorrhoeae* cultures were grown in liquid in the presence of a variety of different protease inhibitors. Each

protease inhibitor prevents cleavage of specific subsets of proteases. AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride) is a sulfonyl fluoride that irreversibly inhibits serine proteases (Powers *et al.*, 2002). E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane) is an epoxide which inhibits an array of cysteine proteases (Barrett *et al.*, 1982). Leupeptin (N-Acetyl-L-leucyl-L-leucyl-L-argininal hemisulfate salt) inhibits a subset of serine, cysteine and threonine peptidases (Aoyagi *et al.*, 1969). cOmplete EDTA-free Protease Inhibitor Cocktail from Roche inhibits a large array of proteases including serine and cysteine type inhibitors but not metalloproteases. EDTA is typically used to inhibit metalloproteases, but was not used here as the bacteria were not able to survive in the presence of EDTA. Growth in the presence of AEBSF and the inhibitor cocktail both prevented S-pilin cleavage as demonstrated by a decrease in the amount of S-pilin observed by a PilE western blot (***Figure 31***). Quantification of the relative intensities of the full-length pilin band to the truncated S-pilin band showed that incubation with AEBSF and the inhibitor cocktail increased the ratio of full-length pilin to S-pilin (***Table 4***). As AEBSF specifically inhibits serine proteases, we narrowed the list of candidate S-pilin proteases to serine proteases.

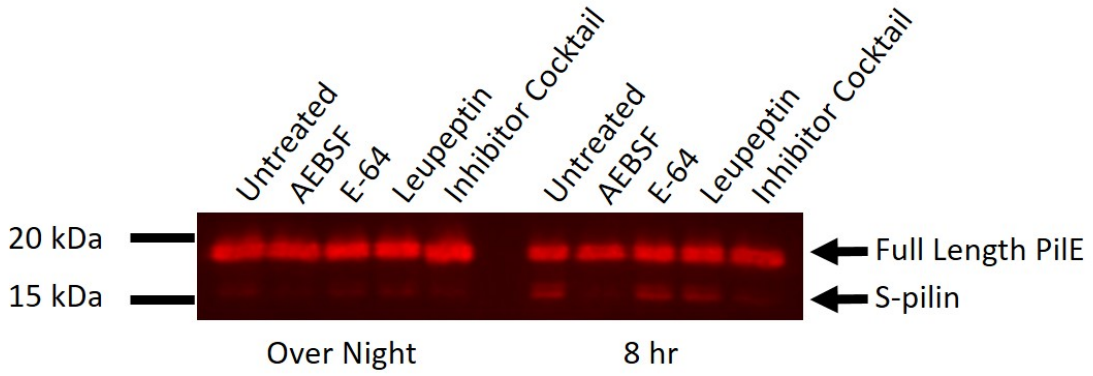


Figure 31. Protease inhibitor western blot.

N. gonorrhoeae grown in liquid medium in the presence of four protease inhibitors (Untreated-PBS, 10mM AEBSF, 0.1mM E-64, 1mM Leupeptin, cOmplete Mini, EDTA-free Protease Inhibitor Cocktail). Bacteria were either grown in the presence of the inhibitors over night at 30°C (left section of the image) or for 8hr at 37°C (right section of the image). Upper band is full-length pilin. Lower band is the processed S-pilin form. Blots were developed with K36 anti-PilE peptide primary antibody at 1:1,000 dilution and IRDye 800CW Goat anti-Rabbit IgG at a dilution of 1:1,000 as the secondary antibody.

	Full Length Pilin:S-pilin Ratio	
Protease Inhibitor	**Over Night**	**8 hr**
Untreated	13.0	2.9
AEBSF	44.1	21.4
E-64	17.7	2.7
Leupeptin	14.1	3.3
Inhibitor Cocktail	24.4	21.7

Table 4. S-pilin cleavage inhibition by various protease inhibitors.

Ratio of full length pilin to S-pilin based as quantified from the western blot in (**Figure *28***) using LiCOR Image Studio.

Export signal prediction

Based on the observation that S-pilin production is dependent on pilin maturation mediated on the periplasmic side of the inner membrane by the prepilin peptidase, PilD, we hypothesized that S-pilin cleavage must occur in the periplasm (Obergfell & Seifert, 2016). Therefore, the protease responsible for S-pilin cleavage should require an export signal for translocation from the cytoplasm to the periplasmic space. We used this reasoning to narrow a list of all possible *N. gonorrhoeae* serine proteases assembled from the MEROPS and KEGG databases to only those with an export signal (Rawlings *et al.*, 2016, Kanehisa & Goto, 2000, Kanehisa *et al.*, 2016, Kanehisa *et al.*, 2017). Each serine protease was analyzed by SingalIP 4.1 to predict whether the peptidase contained a signal motif directing the protein for export to the periplasm (Petersen *et al.*, 2011). This analysis resulted in the list of possible candidate S-pilin proteases detailed in ***Table 5***.

Protease	Protease Family
NGO_2105	S6 peptidase (IgA1 protease)
NGO_0275	S6 peptidase (IgA1 protease)
NGO_0138	DegP type trypsin-like
NGO_0572	S41 peptidase
NGO_0443	Serine-type carboxypeptidase (S11)
NGO_0107	Serine-type carboxypeptidase (S13)
NGO_1274	Muramoyltetrapeptide carboxypeptidase (S66)
NGO_0327	Serine endopeptidase (S11)

Table 5. Predicted *N. gonorrhoeae* periplasmic serine proteases.

The left column displays the *N. gonorrhoeae* gene loci corresponding to predicted periplasmic serine proteases. The right column details the protease family to which the predicted proteases belong.

Assaying S-pilin production in protease knockout strains

Each predicted periplasmic serine protease was mutated by deleting the coding region and inserting an antibiotic resistance cassette, and production of S-pilin cleavage was assayed by PilE western blot (***Figure 32***). No single mutant decreased production of S-pilin. Loss of NGO_0572 and NGO_0443 slightly increased production of S-pilin cleavage. As none of the single mutant strains showed a reduction in S-pilin amounts, a set of double and triple knockouts were created. The candidate proteases were divided into a set of predicted PG modifying peptidases (NGO_0443, 0107, 0327, 1274) and non-PG modifying enzymes (NGO_2105, 0275, 0138, 0572). All possible combinations of double and triple knockouts were created for the non-PG modifying enzymes and combinations of double knockouts were created for the PG modifying enzymes NGO_0443, 0107, and 1274. None of these strains showed a reduction in S-pilin. No further attempts by candidate knockout were made to identify the protease responsible for S-pilin cleavage.

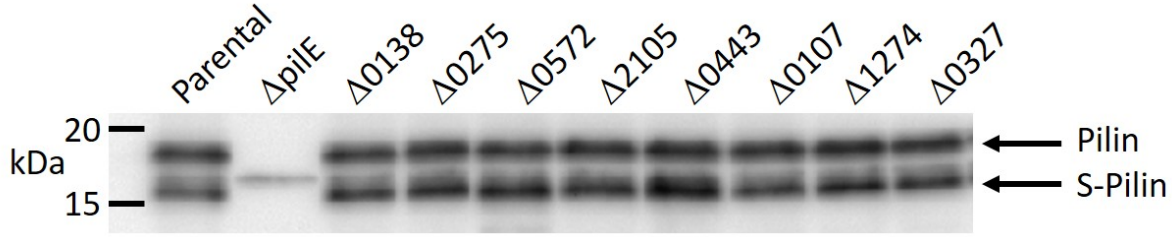


Figure 32. PilE western blot of serine protease mutant strains.

PilE western blot of parental strain, Δ*pilE* mutant, and eight knockout strains of predicted periplasmic serine proteases. Upper band is full-length pilin. Lower band is the processed S-pilin form. Western blot analysis performed using the K36 peptide anti-pilin antibody.

Developing an S-pilin cleavage assay

As the candidate gene knockout approach failed to identify the protease responsible for S-pilin cleavage, we sought to develop a biochemical assay that could detect S-pilin cleavage. If such an assay could be developed, cell lysates could be strategically purified to identify the fraction containing the protein with the S-pilin cleavage activity. Mass spectrometry could then be employed to identify the enzyme responsible for S-pilin cleavage. Towards this end, a chemiluminescent sensor was developed using the Promega ProteaseGlo modified firefly luciferase (Fan *et al.*, 2008). A set of firefly luciferase sensors containing various lengths of the amino acid sequence from PilE that include the S-pilin cleavage site was created (***Figure 33***). Theoretically, if the inserted sequence is cleaved, a domain of the sensor should be released to associate with another domain, resulting in high luminescence.

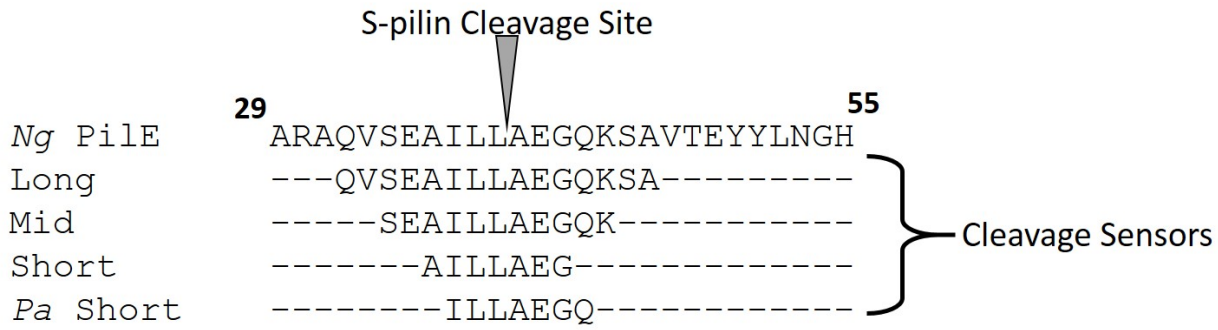


Figure 33. Sequences of cleavage site of modified luciferase sensors.

Alignment of the *N. gonorrhoeae* PilE AA sequence (top) and the four different AA sequences inserted into modified firefly luciferase to serve as cleavage sensors.

The four, modified firefly luciferase sensors were produced by cell-free transcription and translation (Promega, 2016). Cell lysates from *N. gonorrhoeae* FA1090 *recA6* were incubated with each sensor at 37°C, but no increase in luminescence was observed for any of the substrates suggesting that there was no active protease activity against these sensor substrates in the lysate (***Figure 34A***). The control cleavage sensor with a TEV cleavage site did produce luminescence when incubated with TEV protease demonstrating the cell-free transcription and translation and the luminescence assay was successful. As whole cell lysates are a complex mixture that could contain interfering compounds or nonspecific proteases, increasing concentrations of ammonium sulfate were used to precipitate protein fractions from cell lysates. Protein was purified from both the parental strain and a Δ*pilE* strain as there would be less pilin protein to compete with the cleavage sensor as an S-pilin protease substrate. Following resuspension and dialysis, these ammonium sulfate protein fractions were incubated at 37°C with only the Long cleavage sensor, but again, no increase in fluorescence was detected (***Figure 34B***).

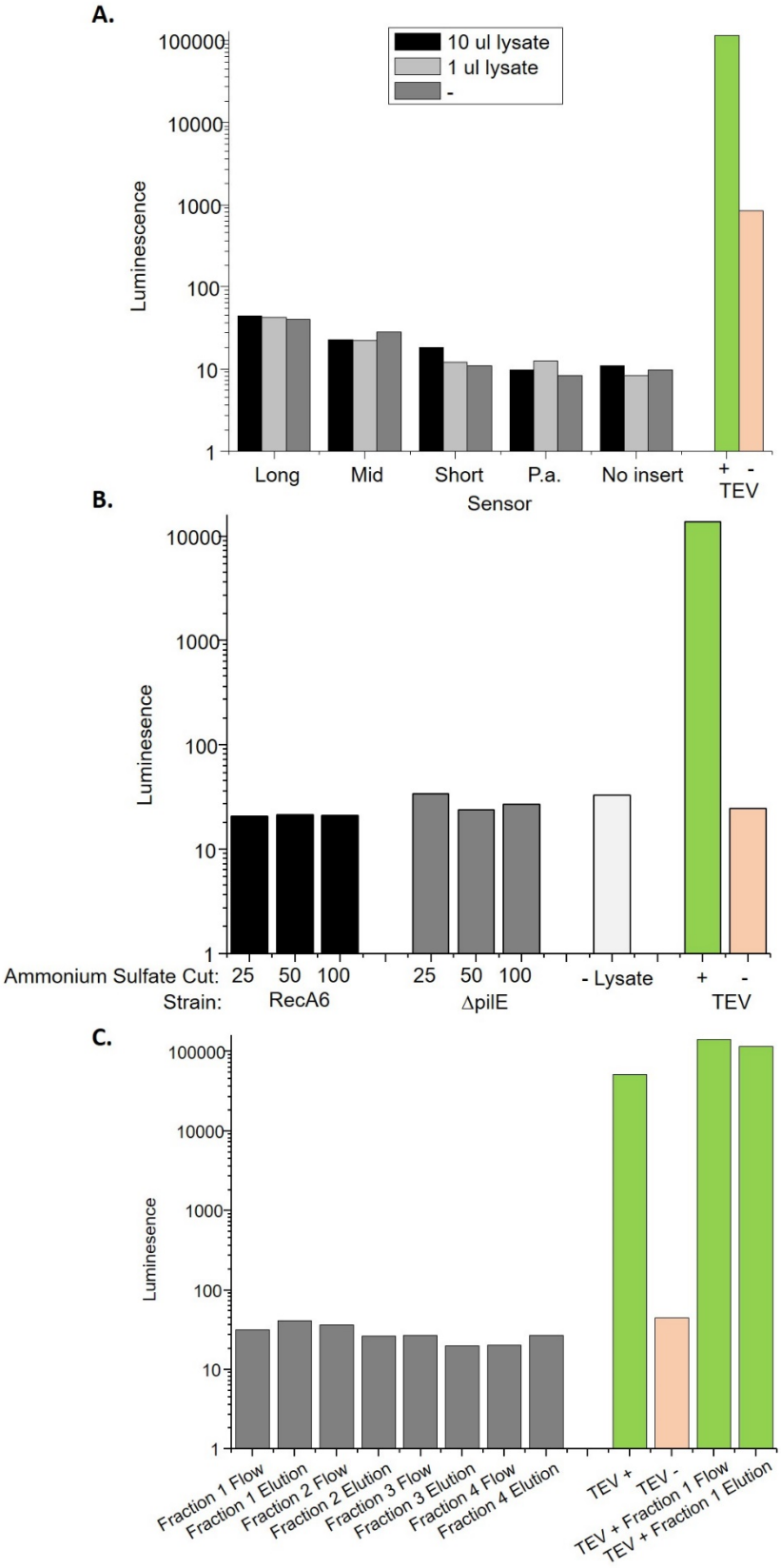

A.
10 ul lysate
1 ul lysate
-
Luminescence
100000
10000
1000
100
10
1
Long
Mid
Short
P.a.
No insert
+
-
TEV
Sensor
B.
Luminesence
10000
1000
100
10
1
Ammonium Sulfate Cut: 25 50 100 25 50 100 - Lysate + -
Strain: RecA6 ΔpilE TEV
C.
Luminesence
100000
10000
1000
100
10
1
Fraction 1 Flow
Fraction 1 Elution
Fraction 2 Flow
Fraction 2 Elution
Fraction 3 Flow
Fraction 3 Elution
Fraction 4 Flow
Fraction 4 Elution
TEV +
TEV -
TEV + Fraction 1 Flow
TEV + Fraction 1 Elution

Figure 34. S-pilin cleavage assay optimization.

Several different reaction conditions were tested to determine if S-pilin cleavage activity could be sensed by modified firefly luciferase. Cleavage sensors were incubated with various *N. gonorrhoeae* cell lysate derivatives and luminescence was measured over a one second integration on a luminometer using Bright-Glo assay reagent. Positive control reactions were performed with TEV protease cleavage sensor with(+) and without(-) TEV protease. **A.** 10µL or 1µL of whole cell lysates or buffer alone were incubated with 4 different S-pilin cleavage sensors (Long, Mid, Short, Pa Short). **B.** Protein precipitated from lysates of the parental (RecA6) and Δ*pilE* strains by ammonium sulfate at concentrations of 25%, 50% and 100% saturation was incubated with the Long cleavage sensor. **C.** Serine proteases were purified from cell lysates on a benzamidine sepharose column. Elution fractions from the column were then ran over PD10 desalting columns. The PD10 column flow through and elution fractions were concentrated and incubated with the Long cleavage sensor.

As the S-pilin protease is predicted to be a serine type protease, we reasoned that if all the serine proteases could be purified from the cell, the enzyme(s) responsible for S-pilin cleavage should be amongst those purified. To test this hypothesis, serine proteases were purified from cell lysates by a benzamidine-sepharose column. As a reversible trypsin and serine protease inhibitor, benzamidine binds serine proteases. When coupled to sepharose, benzamidine can be used to specifically remove serine proteases from a complex solution. Elution fractions from the benzamidine-sepharose column were desalted on PD10 columns and incubated with the Long cleavage sensor. No cleavage was detected (***Figure 34C***). To test whether the purified serine proteases were non-specifically digesting the modified firefly luciferase sensors, the purified proteases were supplemented in the TEV positive control reaction. Luminescence was still detected in the presence of the purified proteases indicating that the sensor was not being non-specifically degraded. No further attempts were made to develop a S-pilin protease cleavage sensor or detect cleavage in cell lysate derivatives. Neither the candidate knockout approach nor the development of an S-pilin cleavage allowed for the identification of the protease(s) responsible for S-pilin cleavage.

Discussion

Identification of the protease or proteases responsible for S-pilin cleavage would be an important step forward in understanding *N. gonorrhoeae* biology. If identified, S-pilin protease knockouts could be used to answer significant questions. Is S-pilin cleavage required for transformation competence in a cell with functional pilin molecules? Answering this question would be a substantial step toward understanding whether competence pseudopili are present in cells with functional pilin molecules and whether full length pilin molecules rather than just the

Ntd can be used to form pseudopili. Does production of S-pilin have any function role during interaction with host cells during pathogenesis? Investigation into functional differences between a parental strain and the S-pilin protease knockout in many infection assays and systems could provide critical insight into the pathogenesis of this organism.

The experiments undertaken in this study failed to identify the S-pilin protease, and thus, these questions remain unanswered for now. Several different factors may account for the failure of the knockout candidate approach for identifying the S-pilin protease. Our assumption that the S-pilin protease is a serine protease could be incorrect. While incubation of *N. gonorrhoeae* with serine protease inhibitors blocked S-pilin cleavage, this could have been through indirect inhibition. If a process upstream of S-pilin cleavage requires a cytoplasmic, serine type protease, the serine protease inhibitors would still have blocked S-pilin cleavage. Secondly, our assumption that S-pilin cleavage occurs in the periplasm may not be accurate, or the SignalIP prediction may have ruled out candidates that are exported to the periplasm through unconventional mechanisms. Additionally, the possibility of functional redundancy amongst proteases could have still prevented detection if the right combination of proteases was not mutated. With the advancement of multiplex cloning techniques, it may be worth interrogating more candidate proteases in greater combinations.

Development of the S-pilin cleavage assay could have failed for a wide array of reasons that can be separated into two categories. First, the modified firefly luciferase sensors may not actually serve as a substrate for the S-pilin protease under any conditions. As many residues can be mutated around the S-pilin cleavage site without preventing cleavage, the sequence surrounding the cleavage site may not be sufficient for S-pilin protease interaction (Aas *et al.*,

2007). The protease may bind a different PilE domain, or the three-dimensional structure of the pilin monomer could be critical for protease recognition. In support of this, post-translational modification of pilin at a residue more than 20 amino acids from the cleavage site has been shown to alter S-pilin production (Marceau & Nassif, 1999). Second, even if the constructed sensors can serve as a substrate for the S-pilin protease, the tested reactions may not have contained active S-pilin protease or may not have had the right conditions to allow cleavage to occur. While the conditions employed in this study were based on previous serine protease cleavage reactions, time and resource consuming trial and error may be the only way to identify proper cleavage conditions. As we are not even sure that we have active S-pilin protease in the cleavage reactions, troubleshooting the reaction conditions is likely not worth the resources. Additionally, S-pilin cleavage may be a self-proteolytic event; although, there is no evidence for pilin possessing a catalytic domain.

Chapter 5: Discussion

Summary

These studies investigated the interplay between the Tfp complex and *N. gonorrhoeae* cell biology. Loss of function mutations in LMM PBPs DacB and DacC demonstrated that normal PG modification is necessary for proper Tfp complex stability and expression. Loss of PG modification resulted in cell morphology and growth rate abnormalities as well as underpiliated cells and a decrease in transformation efficiency. A mutational screen on the 3' end of the *pilE* gene revealed that extended Tfp are not strictly necessary for transformation to occur. Site-directed mutants demonstrated that only the Ntd, a product of S-pilin cleavage, is necessary for transformation. Release of the Ntd allows for the bacteria to continue to undergo transformation following antigenic variation that produces unproductive pilin molecules. Pursuits to biochemically detect the Ntd and identify the protease(s) responsible for S-pilin cleavage were unsuccessful.

Discussion

DacB or DacC expression allows for normal levels of horizontal gene transfer through DNA transformation. Although there is still much debate around the nature of evolutionary benefits of horizontal gene transfer, this organism has evolved to exhibit remarkably high levels of transformation and developed mechanisms to remain competent at all stages of growth (Obergfell & Seifert, 2015). This genetic competence has allowed the spread of antibiotic resistance worldwide with the specter of untreatable gonorrhea infections looming in the near future. Our data demonstrate that pilus functions critical for productive gonococcal infection are dependent on proper expression of either *dacB* or *dacC*. These findings contrast to the recent observation that *Pseudomonas aeruginosa* Tfp do not require PG-remodeling enzymes for proper

pilus expression (Carter *et al.*, 2017). *Pseudomonas aeruginosa*, which is rod shaped and only has polar pili, targets and preinstalls the pilus complexes into nascent poles. As *N. gonorrhoeae* is coccoid and has peritrichous pili, a different strategy appears to be necessary for pilus elaboration. Rather than insertion of the complex into nascent poles prior to PG formation, our data suggest that the peritrichous nature of *N. gonorrhoeae* pili requires PG remodeling for proper pilus elaboration.

The Δ*dacB*/Δ*dacC* strain exhibited both an increased cell volume and increased growth relative to the parental strain or either individual mutant. We presume these phenotypes are partially dependent on the loss of stable pilus expression since the Δ*pilE* strain also shows similar changes, but we do not presently have a direct link to the pilus phenotype. One possibility to explain these phenotypes is that there is an increase in ATP availability when pili are lost. Assembly and retraction of the pilus requires the sequential insertion or removal of pilin subunits and each subunit step is predicted to require hydrolysis of two ATP molecules (Chang *et al.*, 2016, McCallum *et al.*, 2017). As pili consist of thousands of subunits per fiber, extension and retraction of large numbers of pili per cell would be energetically expensive. Hence, cells not having to waste energy on pilus dynamics could put more energy towards cell growth and division.

Since the single mutants have no phenotype with respect to pilus expression, either DacB or DacC activity is sufficient to module the cell wall to allow stable pilus expression. A third *N. gonorrhoeae* carboxypeptidase/endopeptidase that acts on PG cross-links is also required for stable Tfp expression, Mpg (Stohl *et al.*, 2013). This metalloprotease also acts on the PG side chains, but there is no change in bulk PG, suggesting a localized activity. As with the

Δ*dacB*/Δ*dacC* mutant, the piliation defect observed in the *mpg* mutant strain can also be rescued by mutation of the *pilT* gene. Pilus expression can be restored in a subset of pilus complex mutations that decrease the number of surface exposed pili by inactivating *pilT.* As pili are undergoing frequent cycles of extension and retraction, deletion of the ATPase responsible for retraction can increase the number of pili per cell by locking all elaborated pili in the extended position. Non-stable pili are also retracted through the action of PilT, and thus, deletion of the ATPase prevents retraction of non-stable pilus fibers and results in an increased number of surface exposed pili. Taken together the data from these studies as well as the Mpg studies clearly demonstrate a critical role for PG modification in pilus biogenesis in *N. gonorrhoeae*. One possible explanation for the requirement for PG modification is that it is necessary to create holes in the PG layer through which the macromolecular Tfp complex can assemble. This would be consistent with the lack of PG-modifying enzymes required in *P. aeruginosa*, as the complex can form prior to cell wall formation (Carter *et al.*, 2017). However, as neither loss of Mpg cleavage nor loss of DacB and DacC abrogated all piliation and a large number of pili could be restored in Δ*pilT* strains, it is unlikely that the sole function of PG modification in pilus biogenesis by these peptidases is to cut holes for pilus complex assembly.

While limiting the amount of cross-linking probably plays a role in allowing for type IV complexes to assemble through the cell wall, PG modification may also be important for proper pilus assembly complex stability. The Tfp retraction motor is the most powerful molecular motor described, capable of generation of force greater than 100pN (Maier *et al.*, 2002). Thus, proper anchoring of the Tfp complex is essential for maintaining function. The type IV complex structure is predicted to be robustly anchored to the cell wall through PG binding domains such

as AMIN and LysM (de Souza *et al.*, 2008, Joris *et al.*, 1992). A single Tfp complex is predicted to contain 36 PilQ AMIN domains and 12 TsaP LysM domains (Chang *et al.*, 2016). Disruption of PG remodeling by loss of DacB and DacC as well as Mpg may alter the PG structure in a manner that disrupts complex anchoring. This loss of stability would result in fewer pili per cell due to increased PilT-mediated retraction and help account for the restoration of piliation in the *pilT* mutant. As loss of these peptidases results in an increasingly cross-linked cell wall, loss of rigidity of the cell wall would not be the cause of decreased pilus stability. Rather the increased cross-linking could interfere with the proper binding of TsaP and/or PilQ domains to the PG. In support of this hypothesis, binding of AMIN domains to PG has been suggested to be dependent on the local murein configuration and cross-linking density (de Souza *et al.*, 2008). Regardless of the mechanism, it is clear that PG modification is necessary for proper type IV complex expression.

These studies demonstrate that Tfp expression is not absolutely required for transformation competence and suggest that the prevailing model of extended Tfp-mediated DNA binding and uptake is not necessary. We propose that that there is a pseudopilus structure (Chen & Dubnau, 2004, Chen & Dubnau, 2003), distinct from the pilus, which helps mediate transformation. Similar to the pseudopilus present in Gram positive bacteria that spans the thick peptidoglycan layer, a Gram-negative, competence pseudopilus would span from the anchor in the inner membrane, across the periplasmic space and through the outer membrane (Chen & Dubnau, 2004). In the pilin mutants described here, the pilin Ntd could form the pseudopilus within the Tfp complex to present the competence minor pilin ComP near the cell surface but not produce a fiber that extends beyond the immediate cell surface. Formation of a functional fiber

by the Ntd is plausible as the Ntd forms the core of the Tfp structure, and extremely short type IV pilins can form Tfp such as the sixty-one amino acid version in *Geobacter sulfurreducens* (Craig *et al.*, 2006, Reardon & Mueller, 2013). Alternatively, the Ntd may not be included in the pseudopilus fiber, but rather serve as a structural or signaling component that is present in the inner membrane to allow for pseudopilus formation. Consistent with this hypothesis, several minor pilins (pilH-K) required for Tfp biogenesis are included in the pilus fibers at very low amounts possibly acting as an initiation complex that primes pilus assembly (Winther-Larsen *et al.*, 2005, Giltner *et al.*, 2010, Giltner *et al.*, 2012).

The composition and formation of the pseudopilus may be differentiated from a pilus fiber by the profile of minor pilins. The data showing that loss of PilV increases transformation in the Ntd producing mutants suggests that both PilV and ComP compete for access to the Tfp complex. Notably, neither of these two minor pilins is required for formation of the canonical Tfp. If this distinct transformation apparatus exists, it is possible that both full-length PilE and the PilE-Ntd could form the core of the pseudopilus as all phenotypes observed with Ntd mediated transformation in this study were consistent with transformation mediated by full-length pilin. The decrease in transformation efficiency exhibited by PilE nonsense mutations may indicate that full-length PilE more efficiently forms a pseudopilus, but this efficiency difference may be due to a lower protein stability in Ntd mutant strains. If full-length pilin can complete the pseudopilus, this model could account for transformation competence amongst all competent species that express Tfp. The pathogenic *Neisseria* may have further evolved the process of S-pilin cleavage to compensate for the nonfunctional pilin molecules produced by antigenic variation. Whether the processed Ntd or the full-length pilin protein is required for

promoting transformation in piliated cells, the data presented here clearly show that an extended pilus is not required for transformation. It is also possible that extended Tfp mediate transformation during favorable conditions but that the alternative pseudopilus structure only mediates transformation when Tfp cannot be formed.

Regardless of whether extended Tfp or pseudopili are responsible for specific binding of extracellular DNA, retraction of the Tfp or pseudopilus is likely not sufficient to account for the difficulties of transporting DNA across the outer membrane. The PilQ pore is only 6nm in width, the same width as the predicted Tfp structure (Collins *et al.*, 2001). This leaves no room for concomitant transport of other substrates, much less the doubled up DNA structure that would result from binding at a mid-strand site. Taking into consideration that lengths of DNA several times longer than the cell are routinely transformed, a single pilus retraction event would not bring the entire DNA molecule into the periplasm (Hamilton & Dillard, 2006). Therefore, DNA transport across the outer membrane requires a mechanism more complicated than pilus elaboration, DNA binding, and a single retraction event. Successive cycles of pilus extension and retraction could be responsible for pulling long DNA molecules across the outer membrane, but evidence suggests that the import of DNA is processive and occurs at a constant velocity (Maier *et al.*, 2004, Hepp & Maier, 2016). This could be explained by the cooperative retraction of several pili in succession but there is no experimental evidence to support this hypothesis (Burton & Dubnau, 2010).

Further decreasing the likelihood that Tfp are responsible for the entire process of import is recent evidence showing that the speed at which DNA is brought into the periplasm is slower than that observed for Tfp retraction (Hepp & Maier, 2016). Tfp retract at an approximate

velocity of 2 μm/s. As the length of a Tfp regularly exceeds 1 μm, it would be expected that DNA would be taken into the cell at speeds of 2 μm/s for periods that could exceed 1 second. However, observed rates of DNA uptake do not exceed 400 nm/s with an average velocity of about 200nm/s. Therefore, it is highly unlikely that depolymerization of extended Tfp is involved in the DNA uptake process.

An alternative hypothesis revolves around the gonococcal protein ComE. Although not surface localized, ComE is required for DNA uptake and transformation and binds DNA non-specifically (Chen & Gotschlich, 2001). Four copies of ComE are encoded in the gonococcal genome and deletion of individual *comE* genes results in an additive negative effect on transformation. A study in *Vibrio cholerae* showed that the ComE homolog of *V. cholerae,* ComEA, is required for DNA uptake into the periplasm and that ComEA binding of DNA can potentially prevent retrograde transport of DNA through the PilQ pore (Seitz *et al.*, 2014). The authors proposed that ComEA is responsible for pulling DNA into the periplasm through a ratcheting mechanism reliant on non-specific DNA binding and entropic forces similar to what has been proposed to drive dsDNA transport through the nuclear pore complex in eukaryotic cells (Salman *et al.*, 2001). Subsequent studies in *N. gonorrhoeae* have demonstrated that the rate of DNA uptake and DNA carrying capacity of the periplasm are directly correlated with the level of ComE in the cell (Hepp & Maier, 2016, Gangel *et al.*, 2014a).

Taken together with the experimental evidence contained within this report, a speculative model of DNA uptake during transformation can be built (***Figure 35***). DNA is present in the extracellular milieu, either near the cell but non-cell associated or non-specifically bound to the PilQ pore. DNA uptake is initiated by sequence specific binding of the DNA by ComP. This

binding occurs in the context of a competence pseudopilus rather than an extended Tfp fiber. Depolymerization of the competence pseudopilus is required to transmit the initial length of DNA into the periplasm as the retraction ATPase PilT is required. Once in the periplasm, non-specific, reversible binding of ComE to the DNA acts as ratchet mechanism to bias the direction of DNA translocation into the periplasm. The more ComE molecules present in the periplasm, the greater the bias towards import into the cell.

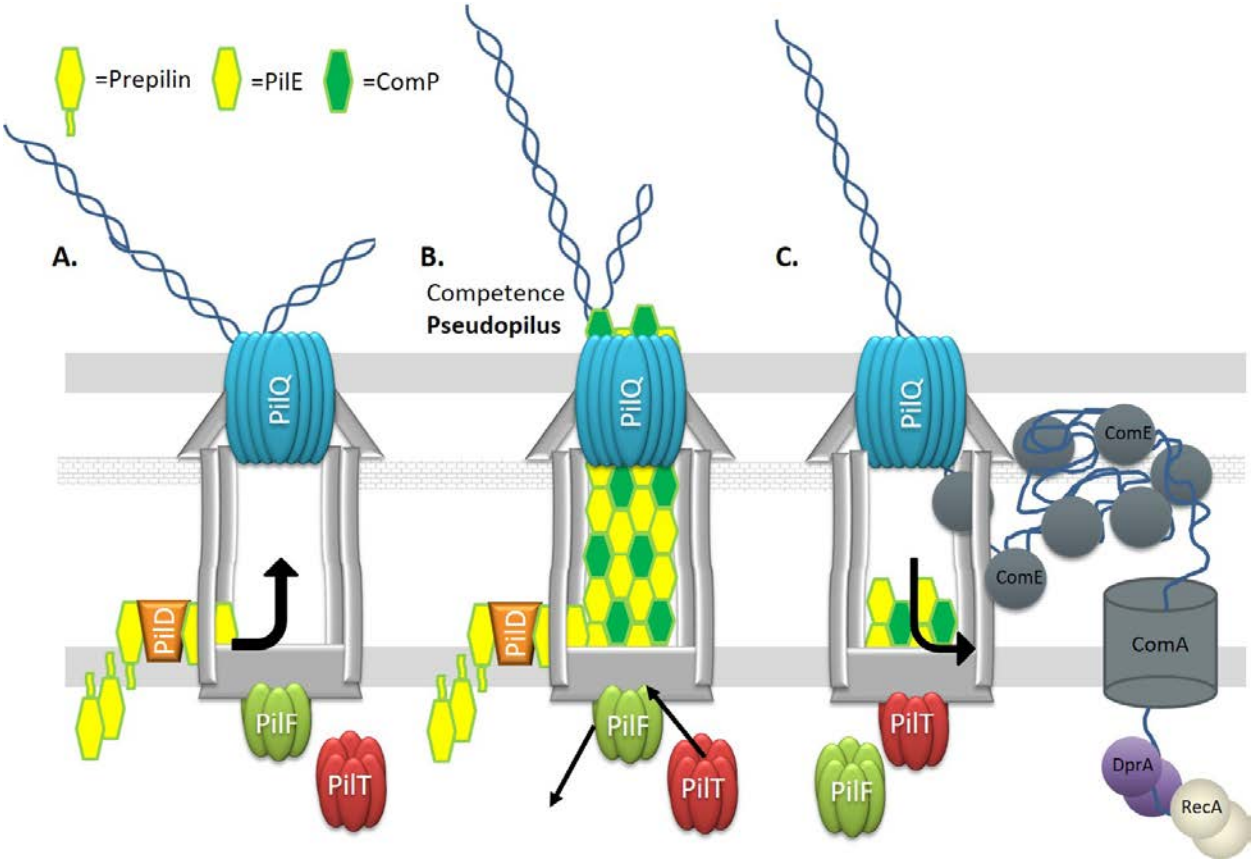


Figure 35. Model of *N. gonorrhoeae* transformation.

A. DNA binds non-specifically to the PilQ outer membrane pore. **B.** Polymerization of the competence pseudopilus presents ComP on the cell surface where it specifically binds the DNA uptake sequence. **C.** Depolymerization of the competence pseudopilus initiates transport of the DNA into the periplasmic space. ComE non-specifically binds the DNA, serving as a ratchet to bias the translocation of the DNA into the periplasm. The DNA is then transported into the cytoplasm through the ComA pore, possibly through a second ratcheting mechanism mediated by DprA and RecA.

An interesting remaining question involves the mechanism of transport of DNA from the periplasmic space into the cytoplasm. If reversible ComE binding is indeed responsible for DNA uptake into the periplasm, then DNA transport into the cytoplasm would need to be mediated by a process capable of generating greater force than that which is generated by reversible ComE binding. It is possible that the ssDNA binding proteins such as DprA and RecA that coat DNA entering the cytoplasm to protect it from degradation and promote homologous recombination also act as a translocation ratchet. Either a greater excess of DNA binding proteins in the cytoplasm or stronger binding kinetics than ComE could bias the equilibrium towards transport of DNA from the periplasm to the cytoplasm. Alternatively, the process that mediates formation of ssDNA from dsDNA may be critical for transport of DNA into the cytoplasm. While it is largely unclear when during transformation DNA is converted from dsDNA to ssDNA there is some evidence that ssDNA is formed transiently in the periplasm in *Neisseria* (Chaussee & Hill, 1998). The formation of ssDNA would lower the binding affinity of ComE and allow for easier transport into the cytoplasm. This working model can serve as a basis to further investigate the process of Tfp complex mediated transformation in this organism which undergoes HGT at a remarkable frequency.

The fundamental question of what benefit HGT provides to any organism remains controversial (Johnston *et al.*, 2014). Several doubts have been raised about whether HGT is the evolutionary basis of competence due to the unpredictable results of genetic transfer with critics supporting a model where competence evolved as a nutrient acquisition system (Mell & Redfield, 2014). An *in-silico* study, however, suggests that even if extracellular DNA comes from dead cells with more deleterious alleles than the recipient cell, HGT can actually allow a

population to escape the predicted irreversible accumulation of harmful alleles (Takeuchi *et al.*, 2013). This in turn suggests that HGT may be important for long term genomic maintenance rather than having deleterious effects. Another possibility is that transformation competence allows a species to have a larger gene pool to draw upon (Baumdicker *et al.*, 2012). While this simulative study does not end the controversy, there is no doubt that natural transformation plays a significant role in genetic exchange and nutrient uptake. Further, it is clear that *N. gonorrhoeae* has been driven by evolutionary pressures to develop a remarkable ability to constitutively undergo transformation at high frequencies. Thus, HGT is an important area for continued investigation. With the HGT-mediated spread of antibiotic resistance, the specter of untreatable gonorrheal infections is a looming reality.

Future Directions

Continuation of these studies could address several significant aspects of Tfp biology in *N. gonorrhoeae.* The requirement of PG modification mediated by DacB and DacC as well as Mpg for proper Tfp complex function raises many avenues to pursue. Is PG modification required for proper anchoring of the Tfp complex to the cell wall? Is this mediated by the PilQ AMIN domains? This could be tested by purification of the PilQ AMIN domain and incubation with PG purified from the parental strain and the Δ*dacB/*Δ*dacC* strain in a PG binding assay. Preferential binding of the AMIN domain to PG from the parental strain would indicate that PG modification through DacB and DacC is important for proper Tfp anchoring. This experiment could be complimented by mutation of the PG binding residues in the PilQ AMIN domain. If the Tfp tethering hypothesis is correct, mutation would be predicted to result in reduced piliation in a parental strain background, but no further pilus related deficiencies should be observed in a

Δ*dacB/*Δ*dacC* strain background. Is the increased cell morphology and growth rate observed in the Δ*dacB/*Δ*dacC* strain due to the lack of piliation, and is it a result of increased ATP availability? Performing an ATP assay to measure ATP availability in the parental, Δ*pilE,* and Δ*dacB/*Δ*dacC* strains could answer this. If the increased growth rate is indeed due to ATP availability, it would suggest the piliation deficiency is due to defects in pilus biogenesis rather than stability. Mutations that prevent pilus elaboration would prevent the large amount of ATP hydrolysis required for pilus elaboration and retraction. Mutations that alter stability would still allow for draining of the ATP pool as the cell extends and then subsequently retracts the unstable pili.

In the course of investigating the role of the Ntd in transformation, several mutations were isolated that completely prevent extended Tfp elaboration. Notably, many of these mutants had varying levels of transformation efficiency. Transformation efficiency decreased as the nonsense mutations occurred closer to the S-pilin cleavage site, presumably due to reduced stability of the shorter peptide or loss of residues important for interaction with other factors. Regardless, it can be assumed that the strains with higher transformation efficiencies form more stable pseudopili. These mutants can be used to separate the functions of the hypothesized pseudopilus and extended Tfp through comparison of a parental strain, a PilE C-terminal nonsense mutant, and a nonsense mutant at the site of S-pilin cleavage in assays of functions previously attributed to Tfp. If the two nonsense mutants behave identically in the assay but different from the parental strain in the assay, stability of the pseudopilus is not important for that biological function. Rather, extended Tfp are likely to mediate the function. However, if the

two nonsense mutants have different phenotypes (as is observed in transformation assays), it can be assumed that the pseudopilus, rather than extended Tfp mediate the function.

The most pressing question that emerges from this work is whether the transformation and pilus apparatuses are indeed distinct. To answer this question, microscopic experiments need to be conducted to localize the Ntd and ComP relative to other pilus components. Two complementary techniques could be utilized. First we possess a large array of antibodies that detect different pilus components including the Ntd as well as several epitope-tagged pilus components. Using these reagents to perform fluorescent super resolution microscopy via Nikon Stochastic Optical Reconstruction Microscopy (N-STORM) that can achieve resolutions as high as 20nm, several questions can be addressed. Does the Ntd localize to the pilus assembly apparatus in the periplasm? Does ComP co-localize with full length PilE and/or the Ntd? Does the Ntd localize with a subset of assembly apparatuses that also mediate DNA uptake? The spatial relationship between the minor pilins and PilE, the Ntd, and assembly apparatuses could also be defined by this approach. As a complementary method, 3D electron cryotomography (ECT) could be employed to map the structural components of the Tfp complexes as recently done in both *Myxococcus xanthus* and *V. cholerae* (Chang *et al.*, 2016, Chang *et al.*, 2017). ECT performed on a full-length pilin expressing strain and a Ntd expressing strain could differentiate transformation and pilus apparatuses.

As mentioned previously, identification of the S-pilin protease is a worthwhile pursuit. As a transposon insertion screen is impractical without a more high-throughput experimental readout than a western blot, a candidate approach is most likely to yield success. As genetic tools continue to advance, the cost in time and resources to create a library of knockouts of all non-

essential genes continues to fall. If such a library were created, all protease mutants could be screened for loss of S-pilin cleavage. Such a library would be invaluable to the field of *N. gonorrhoeae* biology and used in any number of studies. The set of mutants already constructed for this study could be used to characterize the effects serine proteases have on the periplasmic proteome. Additional insight into S-pilin biology could also be derived from investigation into S-pilin production in *N. meningitidis.* A wide variety of class I and class II pilin expressing strains *N. meningitidis* could be assayed for S-pilin production. If only class I expressing strains produce S-pilin, further mutational analysis of class I and class II *pilE* should be performed to determine what sequence elements are required for S-pilin production.

Conclusion

While S-pilin cleavage mediated release of the Ntd may be unique to *Neisseria*, a functional role for the N-terminal alpha helix apart from the entire pilin molecule has been previously demonstrated. Esquivel et al showed that the H-domain of the archaeal type IV pilin regulates motility in *Haloferax volcanii* (Esquivel & Pohlschroder, 2014). Additionally, other studies in organisms such as *Thermus thermophilus* have demonstrated that mutations in multiple Tfp complex proteins separate the functions of Tfp and natural transformation (Burkhardt *et al.*, 2012, Salzer *et al.*, 2014). Although it remains to be seen if the Ntd of pilins in other organisms can have functional roles outside the context of the entire pilin molecule, it is clear that the paradigm of Tfp-mediated transformation needs to be reexamined. Though this study focuses on transformation, it stands to reason that the remarkable diversity of pilus-mediated functions coupled with a variety of minor pilins corresponding to different functions may allow formation of multiple alternate arrangements of the Tfp apparatus, each mediating a distinct process.

Chapter 6: Materials and Methods

Bacterial Strains and Growth

All studies were performed using strain FA1090 PilE variant 1-81-S2 (Seifert *et al.*, 1994b) and its derivatives which contain an IPTG inducible *recA6* allele to control pilin antigenic variation (Seifert, 1997). The Δ*pilE* mutant allele consists of a 924-bp deletion that includes the promoter and ribosome binding site of *pilE* as previously described (Chen *et al.*, 2004). The Δ*pilT* experiments were performed in strains with an IPTG-regulatable *pilT* allele without IPTG induction (Wolfgang *et al.*, 1998a) or a *pilT*::*erm* allele described previously (Long *et al.*, 2003). *N. gonorrhoeae* strains were grown on GC Medium Base (Difco) plus Kellogg supplements I and II (GCB) at 37°C in 5% CO_2 and liquid medium was GCB Liquid Broth (GCBL: 1.5% peptone protease no. 3 [Difco], 0.4% K2HPO4 [Fisher], 0.1% KH2PO4 [Fisher], 0.1% NaCl [Fisher]) plus Kellogg supplements I and II and 0.042% sodium bicarbonate (GCBL+). Antibiotics and their concentrations used for selection in GCB were: Chloramphenicol (Cm) 1 ug/ml, Kanamycin (Kan) 50 ug/ml, Naldixic Acid (Nal) 0.75 ug/ml, and Erythromycin (Erm) 2 ug/ml. Plasmids were propagated in One Shot TOP10 Electrocomp *E. coli* (Invitrogen), DH5α *E. coli,* or E. cloni 10G ELITE Electrocompetent *E. coli* (Lucigen). *E. coli* strains were grown on Luria-Bertani (LB) solid media containing 15g/L agar or in broth at 37°C. Antibiotics and their concentrations used for selection in LB were: Kanamycin (Kan) 50 ug/ml, Ampcillin (Amp) 100 ug/ml, Chloramphenicol (Cm) 20 ug/ml, Tetracycline (Tet) 12 ug/ml.

Construction of Δ*dacB*, Δ*dacC*, Δ*dacB*/Δ*dacC*

PCR was used to amplify the upstream and downstream region of NGO_0443 (*dacC)* and NGO_0107(*dacB)* using primers KP264-267 and KP272-275 respectively. The 3' primer of the upstream region and 5' primer of the downstream region contained complementary sequence including a KpnI restriction site. This region of complementarity was used in a splicing overlap extension PCR (SOE-PCR) to combine the upstream and downstream regions with a KpnI restriction site in-between. This construct was cloned into pSMART LCAmp (Lucigen) following manufacturer's instructions and introduced in *E. coli* by electroporation. Following confirmation by sequencing, the resulting plasmids (pKP152, 154) were isolated using the QIAprep Spin Miniprep Kit (Qiagen), digested with KpnI (NEB), and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The Kan resistant *nptII* gene was digested from plasmid pBSL86 using KpnI and gel purified. The *nptII* allele was cloned into the KpnI digested plasmids using T4 DNA ligase (NEB), electroporated into *E. coli* and selected for on Amp and Kan. For use in the double mutant, a Cm resistance cassette was digested from plasmid (pKP133) using KpnI and cloned into the NGO_0443 upstream and downstream containing plasmid (pKP152) resulting in plasmid (pKP157). Sequencing confirmed the plasmid sequences and the *nptII* containing plasmids were used to transform the parental strain with selection on Kanamycin. Deletion was confirmed by PCR using primers KP293-294 (*ΔdacC*) and KP297-298(*ΔdacB*). The double mutant was made by transforming the Kan resistant *ΔdacB* with the *dacC*::*cat* containing plasmid (pKP157).

Construction of Δ*dacB*/Δ*dacC* double complement

NGO_0443(*dacC)* and the flanking region was PCR amplified using primers KP309 and KP310 containing an AatII and a SpeI restriction site, respectively. NGO_0107(*dacB)* and the

flanking region was PCR amplified using primers KP311 and KP312 containing a SpeI and a PacI restriction site, respectively. Each amplicon was cloned separately into pSMART LCKan and electroporated into *E. coli*. The sequence confirmed *dacC* containing plasmid (pKP178) was digested with AatII and SpeI, the *dacB* containing plasmid (pKP179) was digested with SpeI and PacI. The *dacC* and *dacB* containing fragments were gel purified and cloned into AatII and PacI cut pGCC2 in a single ligation reaction (Mehr *et al.*, 2000). The ligation reaction was electroporated into DH5α *pcnB*. The resulting plasmid pKP180 was confirmed by PCR amplification and sequencing and and was transformed into the Δ*dacB*/Δ*dacC* strain with selection on Erm for correct chromosomal insertion between *lctP and aspC*. Transformants were verified by PCR amplification and sequencing.

Construction of pKP11 and pKP37

PCR was used to amplify the *pilE* gene from strain FA1090 1-81-S2 *recA6*. KOD DNA polymerase (Novagen) was used following manufacturers protocols using kinase treated primers (T4 polynucleotide kinase, NEB) KP001 and KP002 for pKP11 and KP040 and KP002 for pKP37. Gel purified products (QIAquick Gel Extraction Kit, Qiagen) were cloned into pSMART LCAmp (Lucigen) following manufacturer's instructions and electroporated in *E. coli* E.cloni 10g elite cells (Lucigen). Positive clones were confirmed by DNA sequencing using primers SL1 and SR2. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen), digested with SmaI (NEB), and CIP treated (NEB). The Cm resistance cassette was PCR amplified with primers KP005 and KP006 to add a 12-mer DUS to the sequence and blunt cloned into the SmaI digested plasmids using T4 DNA ligase (NEB). The reaction was electroporated into *E. coli* Top10 cells and positive clones were confirmed by DNA sequencing.

Single, Degenerate, Mega-Primer Mutagenesis

The pKP11 plasmid containing the *pilE* coding sequence was mutagenized by linear amplification using two synthetic 90-mer oligonucleotides (KP013 and KP014) with 12 base conserved flanking regions of homology and a central 66 base degenerate stretch (IDT) as megaprimers targeting the 3' 132 coding nucleotides of *pilE*. The degenerate stretch was made by doping the synthesis reaction with 0.5% of each of the incorrect nucleotide resulting in the inclusion on average of one wrong base per oligonucleotide. The reactions were composed of KOD DNA polymerase 0.02 U/ul, $MgSO_4$ 2.0 mM, dNTPs 0.2 mM each, pKP11 50 ng, and degenerate mega-primer 0.4 μM in 1X KOD reaction buffer. Following initial denaturation at 96°C for 2 min, linear amplification consisted of 18 cycles of 96°C for 1 min, 55°C for 1 min and 68°C for 8 min. Reactions were purified using the QIAquick PCR Purification Kit (Qiagen) and the template DNA was digested using 30U DpnI (NEB) overnight at 37°C. Reactions were dialyzed using 0.025 μm VSWP membrane discs (Millipore) and electroporated into *E. coli* E.cloni cells. Positive clones were selected on LB plates containing Amp and Cm, and mutant pools of several hundred were isolated by Miniprep reactions (Qiagen). DNA sequencing of select clones confirmed that ~10% of isolates contained a single nucleotide mutation in the region of interest. Mutant plasmid pools were used to transform FA1090 1-81-S2 *recA6*, and Cm^R, P- transformants were isolated.

Construction of *pilE* Mutants

All site-directed PilE mutants were made through single-primer mutagenesis of pKP11 and pKP37 depending on the location of the desired mutation. To mutate the plasmid, a linear amplification step was carried out with primers (KP016,021,023,024,026,043-051,054-

056,155,159,162, or 227) homologous to the region of interest with the nucleotide change required to mutate the desired site. The primer was used in a linear amplification step with subsequent processing and electroporation into *E. coli* in the same reaction manner as described for the degenerate mega-primer mutagenesis. Selection of correct clones was accomplished through PCR amplification of the *pilE* sequence of Cm^R isolates using primers KP001 and KP010 for pKP11 and KP040 and KP010 for pKP37 and subsequent DNA sequencing to select those plasmids incorporating the desired mutation. Double and triple mutants were constructed by repeating this process using additional mutagenic primer(s).

Markerless PilE mutant strains were made by PCR amplifying the *pilE* coding region of strains containing the desired mutation using primers KP173 and KP174, which contain a DUS but do not amplify the region with the inserted Cm^R originally used to make the mutation. The PCR reaction was performed using KOD polymerase following manufacturer's conditions and purified using the QIAquick PCR Purification Kit. The resulting DNA was used to spot transform FA1090 1-81-S2 RecA6 and possible transformants were screened using visual assays for the P- colony morphology. All P- colonies were isolated and selection of correct clones whose P- phenotype was due to harboring the desired *pilE* mutation was accomplished through PCR amplification of the *pilE* and subsequent DNA. Complementation of the Δ*pilE* strain was accomplished by inserting a copy of *pilE,* amplified from the parental strain (*iga::pilE)* or the markerless L39X strain (*iga::pilE*$_{Ntd}$) using primers KP221 and KP222, under an anhydrotetracycline inducible promoter at the *iga* protease locus using plasmid pMR69 (Ramsey *et al.*, 2012).

Epitope tagging PilE

Constructs to attach epitope tags to PilE and various PilE truncations was accomplished through splicing overlap extension PCR (SOE-PCR). The following epitope tags were placed following mature amino acids 23, 39, 43, 53 and full length PilE: FLAG, MYC, His6X, HA, HSV, VSV-G. PilE DNA was amplified from pKP37 which includes a DUS site and a Cm cassette inserted at the SmaI site just downstream of the stop codon. The upstream amplicon was amplified with primer KP040 and a 3' primer complementary to the region of truncation preceded by the sequence for the epitope tag (KP059-063, 065, 068-071, 089-093, 095-099, 101-105, 107-111). The downstream region was amplified with a 5' tag specific primer that had the sequence of the epitope tag followed by sequence complementary to the *pilE* stop codon and the downstream region of the gene (KP057, 058, 088, 094, 100, 106) and primer KP002. Following gel purification, the upstream and downstream amplicons were used in SOE-PCR utilizing the epitope tag sequence as the region of complementarity to connect the two sequences. This resulted in a tag being inserted at the correct truncation site while removing the *pilE* sequence between the truncation site and the stop codon. The gel purified SOE-PCR product was cloned into pSMART LCAmp following manufacturer's instructions and electroporated into E. cloni cells. Plasmid sequences were confirmed through sequencing. The resulting plasmids (pKP40-65) were then transformed into *N. gonorrhoeae* using Cm to select for transformants that were confirmed by sequencing.

Construction of *comP::npt* and *pilV::npt*

comP and *pilV* sequences were amplified from strain FA1090 using kinase treated primers KP176 and KP177 (*comP)*, and KP178 and KP179 (*pilV*) using KOD DNA polymerase. Gel purified products were cloned into pSMART LCAmp and electroporated into *E. coli* BH10B

cells. Positive clones were confirmed by DNA sequencing and the labeled pSM9 for the *pilV* construct and pSM18 for the *comP* construct. For the *pilV* construct pSM9, site directed mutagenesis was carried out using primers KP180 and KP181 to insert KpnI digestion sites. The resultant construct was named pSM12. pSM12 and pSM18 were digested by KpnI and PstI respectively and both were ligated using T4 DNA ligase with the nptII cassette digested from pBSL86 to insert the Kan^R gene into the coding sequence of *pilV* and *comP*. The resulting plasmids were electroporated into *E. coli* BH10B cells and positive clones were confirmed by DNA sequencing. The sequencing confirmed constructs pSM14 (*pilV*::*npt*) and pSM19 (*comP*::*npt*) were transformed into their native loci in FA1090 1-81-S2 *recA6* and its derivatives, selected for Kan^R and confirmed by DNA sequencing. Complementation of the *comP*::*npt* strain was accomplished by inserting a copy of *comP,* amplified using primers KP157 and KP158, under an anhydrous tetracycline inducible promoter at the *iga* protease locus using plasmid pMR69 (Ramsey *et al.*, 2012).

Construction of *0138::npt, 0275::npt, 0327::npt, 0572::npt, 1274::npt, 2105::npt*

PCR was used to amplify the upstream and downstream region of each gene and using primers KP204-207(NGO_2105), KP189,190,209,210(NGO_0275), KP192-195(NGO_0572), KP196-199(NGO_0138), KP276-279(NGO_1274), KP305-308(NGO_0327). The 3’ primer of the upstream region and 5’ primer of the downstream region contained complementary sequence including a KpnI restriction site. This region of complementarity was used in a splicing overlap extension PCR (SOE-PCR) to combine the upstream and downstream regions with a KpnI restriction site in-between. This construct was cloned into pSMART LCAmp (Lucigen) following manufacturer’s instructions and electroporated in *E. coli*. Following confirmation by

sequencing the resulting plasmids, were isolated using the QIAprep Spin Miniprep Kit (Qiagen), digested with KpnI (NEB), and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The Kan resistant *nptII* allele was digested from plasmid pBSL86 using KpnI and gel purified. The *nptII* allele was cloned into the KpnI digested plasmids using T4 DNA ligase (NEB), electroporated into *E. coli* and selected for on Amp and Kan. Sequencing confirmed the plasmid sequences and the *nptII* containing plasmids were used to transform the parental strain with selection on Kanamycin.

Spot transformation of *N. gonorrhoeae*

Constructs were transformed into *N. gonorrhoeae* through coculture on solid media (spot transformation). Strains to be transformed were plated from frozen stocks on GCB solid media and grown for 18 hr. Several isolated colonies were streaked as lawns onto GCB solid media supplemented with 1mM IPTG, 10μL of DNA containing solution was mixed with 10μL GCBL containing supplements I and II and 5 mM $MgSO_4$, and was immediately spotted onto the lawn. Following incubation for 20hr at 37°C, the bacteria from the area where the DNA solution was spotted were suspended in GCBL and dilutions were plated on GCB solid media containing the proper selective antibiotics.

Cell wall PG characterization by HPLC

Triplicate cultures of each strain were grown to exponential phase in amended GCBL. PG was isolated as described previously (Dillard & Hackett, 2005). Briefly, cells were spun down at 3,800g for 10min at 4°C and washed twice with cold 25mM sodium phosphate buffer pH6. Following resuspension in cold, 10mL phosphate buffer, the cells were added drop wise to an

equal volume of boiling 8% SDS solution, boiled for 1hr, and spun down for at 30,000g for 30min at 15°C. Boiling and centrifugation were repeated. The pellet was washed 5 times in 10mL of phosphate buffer following centrifugation at 30,000g for 30min. PG was collected through ultracentrifugation for 30min at 162,000g with resuspension in 3mL phosphate buffer. Cell wall PG was digested for 48 hours in 20ug/mL mutanolysin (Sigma) and Amico-Ultra 10kDA filters were used to eliminate insoluble fragments. Soluble PG was reduced with 10 mg/mL sodium borohydride in 0.5 M borate buffer pH=8 for 20 minutes with the reaction stopped by lowering sample pH to 2 using o-phosphoric acid. HPLC separation of soluble PG fragments was performed as previously described (Dougherty, 1985).

Imaging of colony morphology

Strains were grown on GCB solid media for 20 hr and imaged using a Nikon DS-Fi1 camera attached to a Nikon SMZ-10A stereo microscope.

Quantification of average *N. gonorrhoeae* cross-sectional area

Thin-section TEMs were imaged at 1900X magnification. Images were imported into ImageJ, converted to binary format, inverted, and particle size was analyzed with exclusion on the edges and gating on a cross-sectional area between 0.3 and 2.0um^2 to eliminate sections through the edges of cells and cell clusters. Reported cell cross-sectional area is the mean ± standard deviation of at least 1300 cells imaged.

Antibiotic sensitivity testing

Minimum inhibitory concentrations (MIC) were assayed using Etest strips (Biomerieux). *N. gonorrhoeae* was grown in lawn cultures for 20hr and suspended in GCBL to an OD_{550} of approximately 1.0. Cell suspensions were diluted 1:4 into 48°C GCB-Top agar (23.2g GCB in a 1:5 dilution of GCBL). 4mL of GCB-Top-agar, cell suspensions were spread onto 37°C GCB agar plates and allowed to solidify at RT for 15min. A single E-test strip was placed in the center of each plate. Following incubation at 37°C, 5% CO_2 for 20hr the zone of clearing was used to determine the MIC for each strain and each antibiotic. Reported MICs are the mean of at least three independent experiments.

Measuring growth on solid media

Cells were plated on GCB from frozen stocks and grown for 20 hr. Several isolated colonies were suspended in GCBL and dilutions were plated on GCB for isolated colony growth. At 16hr post inoculation, 6 single colonies per strain were picked using a sterile 6 mm filter disk (GE Healthcare) and dispersed in 500uL GCBL by vortexing. 10uL of serial dilutions were spot plated in triplicate and the resulting colonies enumerated.

Assaying detergent mediated lysis

Lawn growths of strains were grown for 20hr on GCB solid media and suspended in 1mL PBS. Cells were centrifuged at 14,000*g* and suspended in 1mL PBS two times. A 50µL aliquot was taken for each strain and used to determine the total protein concentration using a bicinchoninic acid assay (Peirce BCA Assay). Cells were then centrifuged at 14,000*g* and resuspended to a normalized concentration based upon the BCA results. Cells were then lysed in SDS lysis buffer ± 1mg/mL chicken egg white lysozyme for 2hr rotating at 4°C. Samples were

then passed through a small bore needle to shear genomic DNA, boiled for 5min and equal volumes were loaded and electrophoresed on 10% SDS-PAGE gels. Total protein was visualized using Coomassie Brilliant Blue and quantified using Image Studio (LiCor).

SDS sensitivity testing

Strains were grown for 22hr on GCB solid media and 20 colonies were picked and struck for lawn growth on fresh GCB solid media. Lawns were grown for 8hr, suspended in 6mL amended GCBL, and incubated at 30°C overnight in a rotating drum. Liquid cultures were back diluted 1:2 in fresh amended GCBL and grown for 2.5hr at 37°C in a rotating drum. Cultures were back diluted to an OD_{550} of 0.07 and grown for 4hr at 37°C in a rotating drum. 900µL of culture from each strain was incubated with either 100µL PBS or 0.001% SDS in PBS for 1hr at 37°C in a rotating drum. Cultures were serially diluted in GCBL and 10µL of serial dilutions were spotted on GCB solid media in triplicate. CFUs were enumerated and sensitivity was reported as the ratio of CFUs of SDS treated culture to CFUs of PBS treated cultures. The reported ratio represents the mean of at least three independent experiments.

Streptonigrin Sensitivity

Strains were grown for 22hr on GCB solid media and 20 colonies were picked and struck for lawn growth on fresh GCB solid media. Lawns were grown for 8hr, suspended in 6mL amended GCBL, and incubated at 30°C overnight in a rotating drum. Liquid cultures were back diluted 1:2 in fresh amended GCBL and grown for 2.5hr at 37°C in a rotating drum. Cultures were back diluted to an OD_{550} of 0.07 and grown for 4hr at 37°C in a rotating drum. 1mL of culture from each strain was incubated with either 1µL DMSO or 1µL of 1mM Streptonigrin in

DMSO for 1hr at 37°C in a rotating drum. Cultures were serially diluted in GCBL and 10µL of serial dilutions were spotted on GCB solid media in triplicate. CFUs were enumerated and sensitivity was reported as the ratio of CFUs of Streptonigrin treated culture to CFUs of DSMO treated cultures. The reported ratio represents the mean of at least three independent experiments.

Transformation Assays

N. gonorrhoeae strains were grown for 20 hours on GCB plates and resuspended in liquid transformation media (GCBL, 1mM IPTG, 5mM $MgSO_4$ and Kellogg supplements I and II, pH 7.2) at high density. 20 µl of the cell suspension was added to 200µl transformation media containing 150ng pSY6 DNA (Stein *et al.*, 1991). For DUS experiments, 150 ng of either *gyrB1*DUS0 or *gyrB1*DUS10 (Duffin & Seifert, 2010a), were used as the transforming DNA. After 20 min incubation at 37°C, the transformation reactions were diluted into 2ml 37°C transformation media and incubated at 37°C in the presence of 5% CO_2 for 4 h. Reactions were then serially diluted and spotted onto GCB plates in the presence and absence of Nal. Transformation efficiencies are reported as antibiotic resistant CFU (transformants) divided by total CFU, and are the mean of at least three replicates.

Western Blots

Protein isolation from cell lysates was accomplished after growth of strains on GCB plates for 18 hours. Cells were swabbed into 1 mL PBS and pelleted at 4,000 x g for 5 minutes and washed with 500 µL PBS. Bacteria were resuspended in PBS to 520 µL total volume. 20 µl was reserved for BCA analysis (Pierce) to determine protein concentration and 5x SDS sample buffer was added to the remaining 500 µl. For DacB and DacC western blots, chicken egg white

lysozyme was added to the cell suspension and rotated for 2hr at 4°C. To aid in loading of the sample, genomic DNA was sheared through repeated passage of the sample through a small bore needle and stored at -20°C. For western blots of concentrated pilin protein from cell supernatants (**Figure *19***), strains were grown as lawns on GCB for 8 hours prior to inoculation of 5 ml of amended GCBL. (GCBL + 0.042% sodium bicarbonate). Following overnight growth at 30°C with rotation, cells were pelleted by ultracentrifugation at 200,000 x g for 1 hr at 4°C. Pilin protein in the supernatants was concentrated using trichloroacetic acid as described (Haas *et al.*, 1987) and suspended in PBS and 5x SDS sample buffer. For western blot analysis equal amounts of protein were loaded onto 15% SDS-PAGE gels and run at 150 V using standard technique. Gels were blotted using CAPS buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.0); 10% methanol] to 0.45 μm polyvinylidene difluoride (PVDF) membrane using a Bio-Rad transfer cell at 100 v for 1 h at 4°C. Antibodies were used at the following dilutions: K36 (anti-PilE peptide) 1:50,000, IE8G8 (anti-PilE monocolonal Ab) 1:1,000 and 1:500, Peroxidase-conjugated AffinPure Gt α-Rabbit IgG (Jackson ImmunoResearch) 1:10,000. Western blots were developed using the Enhanced Chemiluminescence (ECL) Kit (GE Healthcare) following manufacturer's instructions.

Ntd Large Format Western Blots

Western blots to detect the N-terminal domain in cell lysates were performed using large format urea-Tricine-SDS-PAGE gels as detailed (Schagger, 2006). Cell lysate samples were prepared as above with the exception of Urea Sample Buffer (USB) replacing SDS page lysis buffer. Synthetic Ntd (Lifetein, LT141104-LT422899 Sequence: FTLIELMIVIAIVGILAA

VALPAYQDYTARAQVSEAILL) was suspended in DMSO and diluted in USB for loading. Samples were loaded onto large format gels (4% stacking layer, 10% 1cm sharpening band, and 16%, 6M urea separating gel) and ran at 30V overnight using Tris-HCl anode buffer (0.1M Tris, 25mM HCL, pH8.9) and Tris-Tricine cathode buffer (0.1M Tris, 0.1M Tricine, 0.1% SDS pH8.25). Blots were developed as above using Rb2 α-Ntd sera at 1:1000 dilution as the primary antibody and peroxidase-conjugated AffinPure Gt α-Rabbit IgG 1:10,000 as the secondary antibody.

Protease Inhibitor Assay

Strains were grown from frozen stocks on solid GCB media for 20hr and 20 colonies per strain were restruck for lawn growth. Following incubation for 8hr, 5mL of amended GCBL was inoculated for the ON treatment condition with 500 μL of the following additions: PBS, 10mM AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride - Sigma), 0.1mM E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane – Sigma), 1mM Leupeptin (N-Acetyl-L-leucyl-L-leucyl-L-argininal hemisulfate salt – Sigma), cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche - 1 tablet dissolved in 1 mL PBS). The cultures were incubated at 30°C overnight in a rotating drum and harvested. For the 8hr treatment condition, 5mL of amended GCBL was inoculated and grown ON at 30°C overnight in a rotating drum. Cultures were back diluted 1:2 in fresh amended GCBL and grown for 2.5hr at 37°C in a rotating drum. Cultures were back diluted to an OD_{550} of 0.07 in 5mL amended GCBL with the same additions as detailed for the ON treatment condition. Protease inhibitor treated cultures were grown for 8hr at 37°C in a rotating drum and harvested.

To harvest cells, liquid cultures were spun at 4,200*g* for 10min at 4°C. Cell pellets were then treated as described for western blot analysis with modification for use with the LiCor Odyssey Imaging System. Protein samples were blotted onto Amersham Hybond Low Fluorescence 0.2um PVDF membranes (GE Healthcare). Blots were developed using K36 (anti-PilE peptide) 1:1,000 as the primary antibody and IRDye 800CW Goat anti-Rabbit IgG 1:1,000 (LI-COR) as the secondary antibody. Fluorescent signal was imaged on the LICOR Odyssey Imaging system and signal was quantified using the LICOR Image Studio software.

Antibody Adsorption

α-ComP, α -PilV, K36 α -PilE, and α -Ntd antibodies were cleaned up to remove background binding by antibody adsorption with acetone powder. For each antibody, the corresponding knockout strain (i.e. a Δ*comP* strain was used for the α-ComP antibody) was grown for lawn growth for 20 hrs. 20 plates of growth per strain was swabbed into 200mL GCBL and was spun down at 10,000*g* for 10 min. The pellet was weighed and resuspended to a concertation of 1g/mL in cold 0.9% NaCl. The suspension was diluted 1:5 in -20°C acetone and incubated on ice for 30min with occasional vigorous mixing. The acetone solution was centrifuged at 10,000*g* for 10 min and the pellet was resuspended in fresh -20°C acetone and incubated on ice for 10min with occasional vigorous mixing. The acetone solution was centrifuged at 10,000*g* for 10 min, and the pellet was transferred to filter paper, dried overnight, and stored at -20°C.

To adsorb non-target specific antibodies, the acetone powder from the corresponding knockout strain was added to the antibody solution to a concentration of 1% at rotated for 30min

at 4°C. The powder was spun down at 10,000*g* for 10 min and the adsorbed sera was collected. This process was repeated 2 additional times and the purified antibody was stored at 4°C.

Pilus Filament Purification

Purification of Tfp was performed based on previously described methods (Wolfgang *et al.*, 1998a). Bacteria were grown as lawns on GCB plates for 20h. Bacteria from 30 plates were suspended in 20 ml of 0.15 M ethanolamine pH 10.5 and pili were sheered for 30 seconds in a blender at high speed. Bacterial cells were pelleted by centrifugation at 17,000g, 4°C for 15 minutes. The supernatant containing the pilus filaments was precipitated with one tenth volume of ammonium sulfate saturated 0.15 M ethanolamine on ice for 30 minutes. Pili were pelleted by centrifugation at 17,000g, 4°C for 15 minutes. Supernatants were discarded and pellets were twice washed in 10 ml 0.05 M Tris buffered saline followed by centrifugation at 17,000g, 4°C for 15 minutes. Pili were solubilized in 100 µl PBS.

Transmission Electron Microscopy

Strains were plated on GCB solid media for isolated colonies and grown for 18 hrs. 300-mesh nickel grids with carbon support films (Ladd Research) were touched to medium density colonies to pick up bacterial cells, fixed for 10 min in PBS, 4% PFA and 0.2% gluteraldehyde; washed 5X in sterile water for 5 min each; and negatively stained with 1-3% uranyl acetate for at least 1 min prior to imaging. Immuno-gold labeled samples were fixed for 10 min in PBS, 4% PFA and 0.2% gluteraldehyde; washed 3X in PBS, 1% bovine serum albumin (BSA); blocked for 30 min in PBS, 5% BSA and 0.1% gelatin; incubated with rabbit polyclonal anti-Ntd antibody (1:500 dilution); washed 3X in PBS, 1% BSA; blocked for 30 min in PBS, 5% BSA

and 0.1% gelatin; incubated with 12nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch) (1:50 dilution); washed 5X in sterile water for 5 min each; and negatively stained with 1% uranyl acetate for at least 1 min prior to imaging. Thin-section micrographs were taken from samples prepared by Northwestern's Center for Advanced Microscopy. Strains were plated for lawn growth on GCB solid media and grown for 20hr. Two plates per strain were swabbed into 1mL phosphate buffered saline pH7.4 (PBS) and pelleted at 14,000g for 1min. The pellet was suspended in 1% paraformaldehyde in PBS and kept at room temperature (RT) for 1hr. Cell culture samples were fixed in 0.1 M sodium cacodylate buffer (pH 7.3) containing 2% paraformaldehyde and 2.5% glutaraldehyde and were postfixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer. They were then rinsed with distilled water, en bloc stained with 3% uranyl acetate, rinsed a second time with distilled water, dehydrated in ascending grades of ethanol, transitioned with propylene oxide, embedded in the resin mixture of the EMbed 812 kit, and cured in a 60°C oven. Samples were sectioned on a Leica Ultracut UC6 ultramicrotome. 70 nm thin sections were collected on 200 mesh copper grids, post stained with 3% uranyl acetate and Reynolds lead citrate. All imaging was done on a FEI Tecnai Spirit G2 120-kV at Northwestern's Center for Advanced Microscopy.

ELISA

Whole-cell ELISAs were carried out as described (Imhaus & Dumenil, 2014) with slight modifications. Strains were grown on GCB solid media as lawns for 20 hrs. Cells were swabbed into four ml PBS and diluted to an OD_{550} of 0.2. 2X serial dilution were used to inoculate a 96-well flat bottomed plate (Sarstedt) with 100 μL of culture per well with six repeats per condition. The plate was spun down at 3,220 x g for 10 min and the 75 μL of supernatant was removed. The

remaining liquid was allowed to dry at 50 °C until all liquid evaporated and the cells were fixed for 10 min at RT in 100 μL PBS, 4% PFA. The wells were washed 3X with PBS, and blocked for 10 min in 1% BSA in PBS 0.1% tween. Blocking solution was removed and 50 μL rabbit polyclonal anti-Ntd antibody (1:4,000 dilution in blocking solution) was added for 1 hr. Primary antibody was removed and wells were washed 3X with PBS and 50 μL peroxidase-conjugated AffinPure Gt α-Rabbit IgG (Jackson ImmunoResearch) (1:1,000 dilution in blocking solution) was added for 1 hr. Secondary antibody was removed and the wells were washed 3X in PBS. Assay was developed using 100 μL 3,3',5,5'-tetramethylbenzidine (TMB) substrate (10mg/ml TMB in DMSO diluted 1:100 in .01% hydrogen peroxide, citrate acetate buffer pH 6.0,) for 10 minutes and stopped by addition of 25 μL of 2M sulfuric acid as stop solution. Absorbance was measured 450nm with corrective absorbance at 570nm. Results are the average absorbance of four serial dilutions and three independent experiments.

qRT-PCR

pilE transcript levels were determined by quantitative RT-PCR in strains grown in liquid culture, and total RNA was isolated and cDNA was amplified as previously described (Anderson & Seifert, 2013). Relative transcript abundance of *pilE* was determined using the comparative Ct method (Schmittgen & Livak, 2008) with the *omp3* transcript serving as the internal control using primers KP170 and KP171 (*pilE*), and KP182 and KP183 (*omp3*).

Ntd HPLC

For experiments in which cell lysates were used as a substrate for HPLC, strains were grown on GCB solid media for 20h, swabbed into PBS and lysed with 2% SDS. Lysates were

spun down at 15,000*g* for 1 min, and the supernatant was passed through a 0.45μm filter to remove particulates. Formic acid was added to the lysate to a final concentration of 1%. The cell lysate was then flowed over an Oasis HLB SPE column (Waters). The column was washed with several column volumes of PBS and proteinaceous content was eluted with 50% acetonitrile, 0.1% TFA in water. For experiments in which synthetic Ntd was used as a substrate for HPLC, the peptide was dissolved in formic acid and diluted to a final concentration of 1% formic acid. Acidic Ntd solutions were either directly loaded onto the HPLC column or flowed over an Oasis HLB SPE column and eluted in the same manner as the cell lysates. For experiments to determine the optimal concentration of organic solvent for elution from the SPE columns, solutions of 0.1% TFA in 25%, 50%, 75%, and 100% acetonitrile were used.

HPLC was performed using a XBridge Protein BEH C4 Column (300Å, 3.5 μm, 2.1 mm X 150 mm, 10K - 500K) from Waters. 5, 10 and 20 μL samples were injected using a Waters 2707 Autosampler. A Waters 1525 Binary HPLC Pump was used to pump an increasing gradient of organic solvent at 0.25mL/min. Solution A was 0.1% TFA in acetonitrile while solution B was 0.1%TFA in water. The initial flow was 5% solution A, 95% solution B. To elute sample from the column over a 25min period, the concentration of solution A was linearly increased to 70%. The concentration was then ramped to 100% solution A over the next 5 min. 100% solution A was pumped for 5 min to regenerate the column. The concentration of solution A was then linearly lowered to 5% over the next 5min. 5% solution A was pumped through the column for 20 minutes to equilibrate the column for the next run. Sample elution from the column was detected by a Waters 2489 UV/Visible detected at 210 and 280nm.

S-pilin Cleavage Assay

Luminescence cleavage reporters were developed using the Protease-Glo Assay Kit (Promega). Cleavage reporters were constructed following manufacturer's instructions with the following sequences inserted at the cloning site to sense specific protease cleavage: Long-QVSEAILLAEGQKSA, Mid-SEAILLAEGQK, Short-AILLAEG, P.a.-ILLAEGQ, or Null(no insert). The resulting plasmids were propogated in *E. cloni* cells as pKP163-167 respectively. Plasmids were purified and used as template in a cell-free translation/transcription reaction to produce the sensor according to manufacturer's instructions. Briefly, 2ug of plasmid was incubated with 30µL TNT SP6 High-Yield Wheat Germ Master Mix in a total volume of 50µL for 25°C for 2h. A control sensor with a TEV protease cleavage site was also produced using the pGloSensor-10F[TEV] plasmid. 10µL of each S-pilin cleavage sensor was incubated with 10µL of cell lysate, ammonium sulfate purified protein, or benzamidine purified serine proteases (see below) for 30min at 37°C. For the control TEV reaction, 10U of TEV protease was incubated with the TEV sensor in ProTEV buffer with 2mM DTT for 30min at 30°C. Luminescence was detected using the Bright-Glo Assay Reagent (Promega). Protease reactions were diluted 1:20 in nuclease-free water and 100µL were added in triplicate to an opaque 96-well plate. 100µL of Bright-Glo Reagent was added to each well and incubated for 3min at RT. Luminescence was measured on a SpectraMax M5 Microplate Reader with a 1-second integration.

Cell lysates for use in the protease assay were obtained by growing lawn cultures of FA1090 RecA6 1-81-S2 or the Δ*pilE* derivative strain on GCB solid media for 20hr. 2 plates per strain were swabbed into cold 50 mM Tris-HCl, pH 8.0 (Protease Buffer A) and resuspended to a density of 0.1g/mL. 1mL of culture was lysed on a MP Biomedicals FastPrep-24 Classic using Lysing Matrix B at a rate of 6.0m/s for 40sec. Lysates were chilled, spun down at 10,000g for

30sec and the supernatant was collected and used in the protease reaction. Alternatively, cell lysates were further enriched for protein content through ammonium sulfate fractionation. Cell lysates were mixed with Ammonium Sulfate (Sigma) at a concentration of 25% w/v and incubated on ice for 30 min. The precipitate was collected by centrifugation, resuspended and the suspensison was exchanged with Protease Buffer A through 3X dialysis with 3,000 KDa dialysis tubing. This process was repeated with the non-precipitated lysate using 50% ammonium sulfate w/v and again with saturating conditions. Ammonium Sulfate fractions were then used in the protease reaction.

Serine proteases were purified from cell lysates generated as described above using a benzamidine-Sepharose column following the protocol detailed previously (Gadwal *et al.*, 2014). Lysate generated from 0.4g of bacteria was centrifuged at 2X at 15,000*g* to remove large particulates, diluted to a volume of 10mL in Protease Buffer A with 450mM NaCl, and then passed through a 0.45uM filter. Filter lysate was then subjected to affinity chromatography at a flow rate of 1mL/min on a benzamidine-Sepharose column (GE Healthcare) preconditioned with Protease Buffer A. The column was washed with 20 column volumes of Protease Buffer A with 450mM NaCl and then serine proteases were eluted using 10mL Protease Buffer A with 100mM benzamidine (Sigma). Elution fractions were pooled and the benzamidine was removed through buffer exchange on PD10 columns (GE Healthcare). Protein was concentrated using Amicon Ultra Centrifugation Filters with a 3,000 d molecular weight cut-off (EMD Millipore) and used in the protease reaction.

References

(2017) UniProt: the universal protein knowledgebase. *Nucleic Acids Res* **45**: D158-d169.

Aas, F.E., C. Lovold & M. Koomey, (2002a) An inhibitor of DNA binding and uptake events dictates the proficiency of genetic transformation in *Neisseria gonorrhoeae*: mechanism of action and links to Type IV pilus expression. *Mol. Microbiol.* **46**: 1441-1450.

Aas, F.E., H.C. Winther-Larsen, M. Wolfgang, S. Frye, C. Lovold, N. Roos, J.P. van Putten & M. Koomey, (2007) Substitutions in the N-terminal alpha helical spine of *Neisseria gonorrhoeae* pilin affect Type IV pilus assembly, dynamics and associated functions. *Mol. Microbiol.* **63**: 69-85.

Aas, F.E., M. Wolfgang, S. Frye, S. Dunham, C. Lovold & M. Koomey, (2002b) Competence for natural transformation in *Neisseria gonorrhoeae*: components of DNA binding and uptake linked to type IV pilus expression. *Mol. Microbiol.* **46**: 749-760.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers & D.J. Lipman, (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Ambur, O.H., S.A. Frye & T. Tonjum, (2007) New functional identity for the DNA uptake sequence in transformation and its presence in transcriptional terminators. *J. Bacteriol.* **189**: 2077-2085.

Ameyama, S., S. Onodera, M. Takahata, S. Minami, N. Maki, K. Endo, H. Goto, H. Suzuki & Y. Oishi, (2002) Mosaic-Like Structure of Penicillin-Binding Protein 2 Gene (penA) in Clinical Isolates of *Neisseria gonorrhoeae* with Reduced Susceptibility to Cefixime. *Antimicrob. Agents Chemother.* **46**: 3744-3749.

Anderson, M.T. & H.S. Seifert, (2013) Phase variation leads to the misidentification of a *Neisseria gonorrhoeae* virulence gene. *PloS one* **8**: e72183.

Antignac, A., J.C. Rousselle, A. Namane, A. Labigne, M.K. Taha & I.G. Boneca, (2003) Detailed structural analysis of the peptidoglycan of the human pathogen *Neisseria meningitidis*. *J. Biol. Chem.* **278**: 31521-31528.

Antonova, E.S. & B.K. Hammer, (2015) Genetics of Natural Competence in Vibrio cholerae and other Vibrios. *Microbiol Spectr* **3**: 0010-2014.

Aoyagi, T., S. Miyata, M. Nanbo, F. Kojima & M. Matsuzaki, (1969) Biological activities of leupeptins. *J. Antibiot. (Tokyo).* **22**: 558-568.

Assalkhou, R., S. Balasingham, R.F. Collins, S.A. Frye, T. Davidsen, A.V. Benam, M. Bjoras, J.P. Derrick & T. Tonjum, (2007) The outer membrane secretin PilQ from *Neisseria meningitidis* binds DNA. *Microbiology* **153**: 1593-1603.

Barbour, A.G., (1981) Properties of penicillin-binding proteins in *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* **19**: 316-322.

Barrangou, R., (2013) CRISPR-Cas systems and RNA-guided interference. *Wiley Interdiscip Rev RNA* **4**: 267-278.

Barrett, A.J., A.A. Kembhavi, M.A. Brown, H. Kirschke, C.G. Knight, M. Tamai & K. Hanada, (1982) L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **201**: 189-198.

Barton, J., J. Braxton, D. Davis, A.d. Voux, E. Flagg, L. Grier, A. Harvey, S. Kidd, R. Kirkcaldy, K. Kreisel, E. Llata, R. Presley, V. Ramirez, F. Rivers, M. Rose, C. Smith, M. Stenger, T.

Tilert, E. Torrone, H. Weinstock & E. Weston, (2015) Sexually Transmitted Disease Surveillance 2015. In. C.f.D.C.a. Prevention (ed). Atlanta, Georgio, pp.

Baumdicker, F., W.R. Hess & P. Pfaffelhuber, (2012) The infinitely many genes model for the distributed genome of bacteria. *Genome Biol Evol* **4**: 443-456.

Benam, A.V., E. Lang, K. Alfsnes, B. Fleckenstein, A.D. Rowe, E. Hovland, O.H. Ambur, S.A. Frye & T. Tonjum, (2011) Structure-function relationships of the competence lipoprotein ComL and SSB in meningococcal transformation. *Microbiology* **157**: 1329-1342.

Berry, J.L., A. Cehovin, M.A. McDowell, S.M. Lea & V. Pelicic, (2013a) Functional analysis of the interdependence between DNA uptake sequence and its cognate ComP receptor during natural transformation in *Neisseria* species. *PLoS Genet* **9**: e1004014.

Berry, J.L., A. Cehovin, M.A. McDowell, S.M. Lea & V. Pelicic, (2013b) Functional analysis of the interdependence between DNA uptake sequence and its cognate ComP receptor during natural transformation in *Neisseria* species. *PLoS Genet* **9**: 19.

Berry, J.L. & V. Pelicic, (2015) Exceptionally widespread nanomachines composed of type IV pilins: the prokaryotic Swiss Army knives. *FEMS Microbiol. Rev.* **39**: 134-154.

Biswas, G.D., S.A. Lacks & P.F. Sparling, (1989) Transformation-deficient mutants of piliated *Neisseria gonorrhoeae*. *J. Bacteriol.* **171**: 657-664.

Biswas, G.D., T. Sox, E. Blackman & P.F. Sparling, (1977) Factors affecting genetic transformation of *Neisseria gonorrhoeae*. *J. Bacteriol.* **129**: 983-992.

Bos, M.P., D. Hogan & R.J. Belland, (1999) Homologue scanning mutagenesis reveals CD66 receptor residues required for neisserial Opa protein binding. *J. Exp. Med.* **190**: 331-340.

Bottcher, J.P., (2011) Piliated *Neisseria gonorrhoeae* induce host cell signaling to stabilize extracellular colonization and microcolony formation. In: Mathematics and Natural Sciences. Humboldt University of Berlin, pp. 144.

Buckee, C.O., K.A. Jolley, M. Recker, B. Penman, P. Kriz, S. Gupta & M.C. Maiden, (2008) Role of selection in the emergence of lineages and the evolution of virulence in *Neisseria meningitidis*. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 15082-15087.

Burkhardt, J., J. Vonck, J.D. Langer, R. Salzer & B. Averhoff, (2012) Unusual N-terminal alphaalphabetaalphabetabetaalpha fold of PilQ from Thermus thermophilus mediates ring formation and is essential for piliation. *J. Biol. Chem.* **287**: 8484-8494.

Burton, B. & D. Dubnau, (2010) Membrane-associated DNA transport machines. *Cold Spring Harbor perspectives in biology* **2**: a000406.

Cahoon, L.A., K.A. Manthei, E. Rotman, J.L. Keck & H.S. Seifert, (2013) The *Neisseria gonorrhoeae* RecQ helicase HRDC domains are essential for efficient binding and unwinding of the *pilE* guanine quartet structure required for pilin Av. *J. Bacteriol.*

Cahoon, L.A. & H.S. Seifert, (2009) An alternative DNA structure is necessary for pilin antigenic variation in *Neisseria gonorrhoeae*. *Science* **325**: 764-767.

Cahoon, L.A. & H.S. Seifert, (2013) Transcription of a *cis*-acting, noncoding, small RNA is required for pilin antigenic variation in *Neisseria gonorrhoeae*. *PLoS Pathog* **9**: e1003074.

Carter, T., R.N. Buensuceso, S. Tammam, R.P. Lamers, H. Harvey, P.L. Howell & L.L. Burrows, (2017) The Type IVa Pilus Machinery Is Recruited to Sites of Future Cell Division. *mBio* **8**.

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

Chamot-Rooke, J., G. Mikaty, C. Malosse, M. Soyer, A. Dumont, J. Gault, A.F. Imhaus, P. Martin, M. Trellet, G. Clary, P. Chafey, L. Camoin, M. Nilges, X. Nassif & G. Dumenil, (2011) Posttranslational modification of pili upon cell contact triggers *N. meningitidis* dissemination. *Science* **331**: 778-782.

Chang, Y.W., A. Kjaer, D.R. Ortega, G. Kovacikova, J.A. Sutherland, L.A. Rettberg, R.K. Taylor & G.J. Jensen, (2017) Architecture of the Vibrio cholerae toxin-coregulated pilus machine revealed by electron cryotomography. *Nature microbiology* **2**: 16269.

Chang, Y.W., L.A. Rettberg, A. Treuner-Lange, J. Iwasa, L. Sogaard-Andersen & G.J. Jensen, (2016) Architecture of the type IVa pilus machine. *Science* **351**: aad2001.

Chaussee, M.S. & S.A. Hill, (1998) Formation of single-stranded DNA during DNA transformation of *Neisseria gonorrhoeae*. *J. Bacteriol.* **180**: 5117-5122.

Chaussee, M.S., J. Wilson & S.A. Hill, (1999) Characterization of the *recD* gene of *Neisseria gonorrhoeae* MS11 and the effect of *recD* inactivation on pilin variation and DNA transformation. *Microbiology* **145**: 389-400.

Chen, C.J., D.M. Tobiason, C.E. Thomas, W.M. Shafer, H.S. Seifert & P.F. Sparling, (2004) A mutant form of the *Neisseria gonorrhoeae* pilus secretin protein PilQ allows increased entry of heme and antimicrobial compounds. *J. Bacteriol.* **186**: 730-739.

Chen, I. & D. Dubnau, (2003) DNA transport during transformation. *Front. Biosci.* **8**: s544-556.

Chen, I. & D. Dubnau, (2004) DNA uptake during bacterial transformation. *Nature reviews. Microbiology* **2**: 241-249.

Chen, I. & E.C. Gotschlich, (2001) ComE, a competence protein from *Neisseria gonorrhoeae* with DNA-binding activity. *J. Bacteriol.* **183**: 3160-3168.

Chen, Y., W. Zhang, Q. Shi, D. Hesek, M. Lee, S. Mobashery & B.K. Shoichet, (2009) Crystal structures of penicillin-binding protein 6 from Escherichia coli. *J. Am. Chem. Soc.* **131**: 14345-14354.

Chen, Z., H. Yang & N.P. Pavletich, (2008) Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *Nature* **453**: 489-484.

Cloud-Hansen, K.A., K.T. Hackett, D.L. Garcia & J.P. Dillard, (2008) *Neisseria gonorrhoeae* uses two lytic transglycosylases to produce cytotoxic peptidoglycan monomers. *J. Bacteriol.* **190**: 5989-5994.

Cohen, E.J., J.L. Ferreira, M.S. Ladinsky, M. Beeby & K.T. Hughes, (2017) Nanoscale-length control of the flagellar driveshaft requires hitting the tethered outer membrane. *Science* **356**: 197-200.

Cohen, M.S., Y. Chai, B.E. Britigan, W. McKenna, J. Adams, T. Svendsen, K. Bean, D.J. Hassett & P.F. Sparling, (1987) Role of extracellular iron in the action of the quinone antibiotic streptonigrin: mechanisms of killing and resistance of *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* **31**: 1507-1513.

Collins, R.F., L. Davidsen, J.P. Derrick, R.C. Ford & T. Tonjum, (2001) Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. *J. Bacteriol.* **183**: 3825-3832.

Craig, L., M.E. Pique & J.A. Tainer, (2004) Type IV pilus structure and bacterial pathogenicity. *Nature reviews. Microbiology* **2**: 363-378.

Craig, L., N. Volkmann, A.S. Arvai, M.E. Pique, M. Yeager, E.H. Egelman & J.A. Tainer, (2006) Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol. Cell* **23**: 651-662.

Criss, A.K., K.A. Kline & H.S. Seifert, (2005) The frequency and rate of pilin antigenic variation in *Neisseria gonorrhoeae*. *Mol. Microbiol.* **58**: 510-519.

Danaher, R.J., J.C. Levin, D. Arking, C.L. Burch, R. Sandlin & D.C. Stein, (1995) Genetic basis of *Neisseria gonorrhoeae* lipooligosaccharide antigenic variation. *J. Bacteriol.* **177**: 7275-7279.

Davidsen, T., E.A. Rodland, K. Lagesen, E. Seeberg, T. Rognes & T. Tonjum, (2004) Biased distribution of DNA uptake sequences towards genome maintenance genes. *Nucleic Acids Res* **32**: 1050-1058.

Davidsen, T. & T. Tonjum, (2006) Meningococcal genome dynamics. *Nature reviews. Microbiology* **4**: 11-22.

de Souza, R.F., V. Anantharaman, S.J. de Souza, L. Aravind & F.J. Gueiros-Filho, (2008) AMIN domains have a predicted role in localization of diverse periplasmic protein complexes. *Bioinformatics* **24**: 2423-2426.

Dietrich, M., S. Bartfeld, R. Munke, C. Lange, L.A. Ogilvie, A. Friedrich & T.F. Meyer, (2011) Activation of NF-kappaB by *Neisseria gonorrhoeae* is associated with microcolony formation and type IV pilus retraction. *Cell Microbiol* **13**: 1168-1182.

Dillard, J.P. & K.T. Hackett, (2005) Mutations affecting peptidoglycan acetylation in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Infect. Immun.* **73**: 5697-5705.

Dillingham, M.S. & S.C. Kowalczykowski, (2008) RecBCD enzyme and the repair of double-stranded DNA breaks. *Microbiol. Mol. Biol. Rev.* **72**: 642-671.

Dorward, D.W. & C.F. Garon, (1989) DNA-binding proteins in cells and membrane blebs of *Neisseria gonorrhoeae*. *J. Bacteriol.* **171**: 4196-4201.

Dougherty, T.J., (1985) Analysis of *Neisseria gonorrhoeae* peptidoglycan by reverse-phase, high-pressure liquid chromatography. *J. Bacteriol.* **163**: 69-74.

Dowson, C.G., A.E. Jephcott, K.R. Gough & B.G. Spratt, (1989) Penicillin-binding protein 2 genes of non-beta-lactamase- producing, penicillin-resistant strains of *Neisseria gonorrhoeae*. *Mol. Microbiol.* **3**: 35-41.

Drake, S.L. & M. Koomey, (1995) The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. *Mol. Microbiol.* **18**: 975-986.

Draskovic, I. & D. Dubnau, (2005) Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds. *Mol. Microbiol.* **55**: 881-896.

Drees, J.C., S. Chitteni-Pattu, D.R. McCaslin, R.B. Inman & M.M. Cox, (2006) Inhibition of RecA protein function by the RdgC protein from *Escherichia coli*. *J. Biol. Chem.* **281**: 4708-4717.

Duffin, P.M. & D.A. Barber, (2016) DprA is required for natural transformation and affects pilin variation in *Neisseria gonorrhoeae*. *Microbiology* **162**: 1620-1628.

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

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

Duffin, P.M. & H.S. Seifert, (2012) Genetic transformation of *Neisseria gonorrhoeae* shows a strand preference. *FEMS Microbiol. Lett.* **334**: 44-48.

Eisenstein, B.I., T. Sox, G. Biswas, E. Blackman & P.F. Sparling, (1977) Conjugal transfer of the gonococcal penicillinase plasmid. *Science* **195**: 998-1000.

Elkins, C., C.E. Thomas, H.S. Seifert & P.F. Sparling, (1991) Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. *J. Bacteriol.* **173**: 3911-3913.

Esquivel, R.N. & M. Pohlschroder, (2014) A conserved type IV pilin signal peptide H-domain is critical for the post-translational regulation of flagella-dependent motility. *Mol. Microbiol.* **93**: 494-504.

Fan, F., B.F. Binkowski, B.L. Butler, P.F. Stecha, M.K. Lewis & K.V. Wood, (2008) Novel genetically encoded biosensors using firefly luciferase. *ACS chemical biology* **3**: 346-351.

Forest, K.T., S.L. Bernstein, E.D. Getzoff, M. So, G. Tribbick, H.M.X. Geysen, C.D. Deal & J.A. Tainer, (1996) Assembly and antigenicity of the *Neisseria gonorrhoeae* pilus mapped with antibodies. *Infect. Immun.* **64**: 644-652.

Francis Ndowa, M.L.-N., (2012) Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. In.: World Health Organization, pp.

Freitag, N.E., H.S. Seifert & M. Koomey, (1995) Characterization of the *pilF-pilD* pilus-assembly locus of *Neisseria gonorrhoeae*. *Mol. Microbiol.* **16**: 575-586.

Frye, S.A., M. Nilsen, T. Tonjum & O.H. Ambur, (2013) Dialects of the DNA uptake sequence in *Neisseriaceae*. *PLoS Genet* **9**: 18.

Fussenegger, M., D. Facius, J. Meier & T.F. Meyer, (1996a) A novel peptidoglycan-linked lipoprotein (ComL) that functions in natural transformation competence of *Neisseria gonorrhoeae*. *Mol. Microbiol.* **19**: 1095-1105.

Fussenegger, M., A.F. Kahrs, D. Facius & T.F. Meyer, (1996b) Tetrapac (*tpc*), a novel genotype of *Neisseria gonorrhoeae* affecting epithelial cell invasion, natural transformation competence and cell separation. *Mol. Microbiol.* **19**: 1357-1372.

Gadwal, S., K.V. Korotkov, J.R. Delarosa, W.G.J. Hol & M. Sandkvist, (2014) Functional and Structural Characterization of Vibrio cholerae Extracellular Serine Protease B, VesB. *The Journal of Biological Chemistry* **289**: 8288-8298.

Gangel, H., C. Hepp, S. Muller, E.R. Oldewurtel, F.E. Aas, M. Koomey & B. Maier, (2014a) Concerted spatio-temporal dynamics of imported DNA and ComE DNA uptake protein during gonococcal transformation. *PLoS Pathog* **10**: e1004043.

Gangel, H., C. Hepp, S. Müller, E.R. Oldewurtel, F.E. Aas, M. Koomey & B. Maier, (2014b) Concerted Spatio-Temporal Dynamics of Imported DNA and ComE DNA Uptake Protein during Gonococcal Transformation. *PLOS Pathogens* **10**: e1004043.

Garcia-Bustos, J.F. & T.J. Dougherty, (1987) Alterations in peptidoglycan of *Neisseria gonorrhoeae* induced by sub-MICs of beta-lactam antibiotics. *Antimicrob. Agents Chemother.* **31**: 178-182.

Gault, J., M. Ferber, S. Machata, A.F. Imhaus, C. Malosse, A. Charles-Orszag, C. Millien, G. Bouvier, B. Bardiaux, G. Pehau-Arnaudet, K. Klinge, I. Podglajen, M.C. Ploy, H.S. Seifert, M. Nilges, J. Chamot-Rooke & G. Dumenil, (2015) *Neisseria meningitidis* Type IV Pili Composed of Sequence Invariable Pilins Are Masked by Multisite Glycosylation. *PLoS Pathog* **11**: e1005162.

Gellert, M., J.E. Hesse, K. Hiom, M. Melek, M. Modesti, T.T. Paull, D.A. Ramsden & D.C. van Gent, (1999) V(D)J recombination: links to transposition and double-strand break repair. *Cold Spring Harbor Symp. Quant. Biol.* **64**: 161-167.

Gibbs, C.P. & T.F. Meyer, (1996) Genome plasticity in *Neisseria gonorrhoeae*. *FEMS Microbiol. Lett.* **145**: 173-179.

Gibbs, C.P., B.Y. Reimann, E. Schultz, A. Kaufmann, R. Haas & T.F. Meyer, (1989) Reassortment of pilin genes in *Neisseria gonorrhoeae* occurs by two distinct mechanisms. *Nature* **338**: 651-652.

Giltner, C.L., M. Habash & L.L. Burrows, (2010) Pseudomonas aeruginosa minor pilins are incorporated into type IV pili. *J. Mol. Biol.* **398**: 444-461.

Giltner, C.L., Y. Nguyen & L.L. Burrows, (2012) Type IV pilin proteins: versatile molecular modules. *Microbiol. Mol. Biol. Rev.* **76**: 740-772.

Goire, N., M.M. Lahra, M. Chen, B. Donovan, C.K. Fairley, R. Guy, J. Kaldor, D. Regan, J. Ward, M.D. Nissen, T.P. Sloots & D.M. Whiley, (2014) Molecular approaches to enhance surveillance of gonococcal antimicrobial resistance. *Nature reviews. Microbiology* **12**: 223-229.

Goodman, S.D. & J.J. Scocca, (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. U. S. A.* **85**: 6982-6986.

Gotschlich, E.C., (1994) Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. *J. Exp. Med.* **180**: 2181-2190.

Gruenig, M.C., E.A. Stohl, S. Chitteni-Pattu, H.S. Seifert & M.M. Cox, (2010) Less is more: *Neisseria gonorrhoeae* RecX protein stimulates recombination by inhibiting RecA. *J. Biol. Chem.* **285**: 37188-37197.

Haas, R. & T.F. Meyer, (1986) The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell* **44**: 107-115.

Haas, R. & T.F. Meyer, (1987) Molecular principles of antigenic variation in *Neisseria gonorrhoeae*. *Antonie Van Leeuwenhoek* **53**: 431-434.

Haas, R., H. Schwarz & T.F. Meyer, (1987) Release of soluble pilin antigen coupled with gene conversion in *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. U. S. A.* **84**: 9079-9083.

Haas, R., S. Veit & T.F. Meyer, (1992) Silent pilin genes of *Neisseria gonorrhoeae* MS11 and the occurrence of related hypervariant sequences among other gonococcal isolates. *Mol. Microbiol.* **6**: 197-208.

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

Hamilton, H.L. & J.P. Dillard, (2006) Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. *Mol. Microbiol.* **59**: 376-385.

Hamrick, T.S., J.A. Dempsey, M.S. Cohen & J.G. Cannon, (2001) Antigenic variation of gonococcal pilin expression in vivo: analysis of the strain FA1090 pilin repertoire and identification of the *pilS* gene copies recombining with *pilE* during experimental human infection. *Microbiology* **147**: 839-849.

Helm, R.A. & H.S. Seifert, (2009) Pilin antigenic variation occurs independently of the RecBCD pathway in *Neisseria gonorrhoeae*. *J. Bacteriol.* **191**: 5613-5621.

Hepp, C. & B. Maier, (2016) Kinetics of DNA uptake during transformation provide evidence for a translocation ratchet mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **113**: 12467-12472.

Hill, S.A., T. Woodward, A. Reger, R. Baker & T. Dinse, (2007) Role for the RecBCD recombination pathway for *pilE* gene variation in repair-proficient *Neisseria gonorrhoeae*. *J. Bacteriol.* **189**: 7983-7990.

Hiom, K., (2009) DNA Repair: Common Approaches to Fixing Double-Strand Breaks. *Curr. Biol.* **19**: R523-R525.

Hobbs, M.M., A. Seiler, M. Achtman & J.G. Cannon, (1994) Microevolution within a clonal population of pathogenic bacteria: recombination, gene duplication and horizontal genetic exchange in the opa gene family of *Neisseria* meningitidis. *Mol. Microbiol.* **12**: 171-180.

Howell-Adams, B. & H.S. Seifert, (1999) Insertion mutations in *pilE* differentially alter gonococcal pilin antigenic variation. *J. Bacteriol.* **181**: 6133-6141.

Howell-Adams, B. & H.S. Seifert, (2000) Molecular models accounting for the gene conversion reactions mediating gonococcal pilin antigenic variation. *Mol. Microbiol.* **37**: 1146-1158.

Howell-Adams, B., L.A. Wainwright & H.S. Seifert, (1996) The size and position of heterologous insertions in a silent locus differentially affect pilin recombination in *Neisseria gonorrhoeae*. *Mol. Microbiol.* **22**: 509-522.

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

Jennings, M.P., D.W. Hood, I.R. Peak, M. Virji & E.R. Moxon, (1995) Molecular analysis of a locus for the biosynthesis and phase-variable expression of the lacto-N-neotetraose terminal lipopolysaccharide structure in *Neisseria meningitidis*. *Mol. Microbiol.* **18**: 729-740.

Jennings, M.P., F.E. Jen, L.F. Roddam, M.A. Apicella & J.L. Edwards, (2011) *Neisseria gonorrhoeae* pilin glycan contributes to CR3 activation during challenge of primary cervical epithelial cells. *Cell Microbiol* **13**: 885-896.

Johnston, C., B. Martin, G. Fichant, P. Polard & J.P. Claverys, (2014) Bacterial transformation: distribution, shared mechanisms and divergent control. *Nature reviews. Microbiology* **12**: 181-196.

Jonsson, A.B., D. Ilver, P. Falk, J. Pepose & S. Normark, (1994) Sequence changes in the pilus subunit lead to tropism variation of *Neisseria gonorrhoeae* to human tissue. *Mol. Microbiol.* **13**: 403-416.

Jonsson, A.B., G. Nyberg & S. Normark, (1991) Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. *EMBO J.* **10**: 477-488.

Jonsson, A.B., J. Pfeifer & S. Normark, (1992) *Neisseria gonorrhoeae* PilC expression provides a selective mechanism for structural diversity of pili. *Proc. Natl. Acad. Sci. U. S. A.* **89**: 3204-3208.

Joris, B., S. Englebert, C.P. Chu, R. Kariyama, L. Daneo-Moore, G.D. Shockman & J.M. Ghuysen, (1992) Modular design of the Enterococcus hirae muramidase-2 and Streptococcus faecalis autolysin. *FEMS Microbiol. Lett.* **70**: 257-264.

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

Kanehisa, M., M. Furumichi, M. Tanabe, Y. Sato & K. Morishima, (2017) KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* **45**: D353-d361.

Kanehisa, M. & S. Goto, (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **28**: 27-30.

Kanehisa, M., Y. Sato, M. Kawashima, M. Furumichi & M. Tanabe, (2016) KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* **44**: D457-462.

Kellogg, D.S., Jr., W.L. Peacock, Jr., W.E. Deacon, L. Brown & D.I. Pirkle, (1963) *Neisseria gonorrhoeae*. I. Virulence Genetically Linked to Clonal Variation. *J. Bacteriol.* **85**: 1274-1279.

Killoran, M.P., P.L. Kohler, J.P. Dillard & J.L. Keck, (2009) RecQ DNA helicase HRDC domains are critical determinants in *Neisseria gonorrhoeae* pilin antigenic variation and DNA repair. *Mol. Microbiol.* **71**: 158-171.

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

Kirkcaldy, R.D., R.C. Ballard & D. Dowell, (2011) Gonococcal resistance: are cephalosporins next? *Curr Infect Dis Rep* **13**: 196-204.

Kline, K.A., E.V. Sechman, E.P. Skaar & H.S. Seifert, (2003) Recombination, repair and replication in the pathogenic *Neisseriae*: the 3 R's of molecular genetics of two human-specific bacterial pathogens. *Mol. Microbiol.* **50**: 3-13.

Kline, K.A. & H.S. Seifert, (2005a) Mutation of the *priA* gene of *Neisseria gonorrhoeae* affects DNA transformation and DNA repair. *J. Bacteriol.* **187**: 5347-5355.

Kline, K.A. & H.S. Seifert, (2005b) Role of the Rep helicase gene in homologous recombination in *Neisseria gonorrhoeae*. *J. Bacteriol.* **187**: 2903-2907.

Kobayashi, I., (1992) Mechanisms for gene conversion and homologous recombination: the double-strand break repair model and the successive half crossing-over model. *Adv. Biophys.* **28**: 81-133.

Koomey, J.M. & S. Falkow, (1987) Cloning of the *recA* gene of *Neisseria gonorrhoeae* and construction of gonococcal *recA* mutants. *J. Bacteriol.* **169**: 790-795.

Koomey, M., (1998) Competence for natural transformation in *Neisseria gonorrhoeae*: a model system for studies of horizontal gene transfer. *APMIS. Suppl.* **84**: 56-61.

Koomey, M., S. Bergstrom, M. Blake & J. Swanson, (1991) Pilin expression and processing in pilus mutants of *Neisseria gonorrhoeae*: critical role of Gly_{-1} in assembly. *Mol. Microbiol.* **5**: 279-287.

Koomey, M., E.C. Gotschlich, K. Robbins, S. Bergstrom & J. Swanson, (1987) Effects of *recA* mutations on pilus antigenic variation and phase transitions in *Neisseria gonorrhoeae*. *Genetics* **117**: 391-398.

Kuryavyi, V., L.A. Cahoon, H.S. Seifert & D.J. Patel, (2012) RecA-binding *pilE* G4 sequence essential for pilin antigenic variation forms monomeric and 5' end-stacked dimeric parallel G-quadruplexes. *Structure* **20**: 2090-2102.

Lane, H.E. & D.T. Denhardt, (1974) The *rep* mutation. III. Altered structure of the replicating *Escherichia coli* chromosome. *J. Bacteriol.* **120**: 805-814.

Lang, E., K. Haugen, B. Fleckenstein, H. Homberset, S.A. Frye, O.H. Ambur & T. Tonjum, (2009) Identification of neisserial DNA binding components. *Microbiology* **155**: 852-862.

Lewis, D.A., (2010) The Gonococcus fights back: is this time a knock out? *Sex. Trans. Inf.* **86**: 415-421.

Long, C.D., R.N. Madraswala & H.S. Seifert, (1998) Comparisons between colony phase variation of *Neisseria gonorrhoeae* FA1090 and pilus, pilin, and S-pilin expression. *Infect. Immun.* **66**: 1918-1927.

Long, C.D., D.M. Tobiason, M.P. Lazio, K.A. Kline & H.S. Seifert, (2003) Low-level pilin expression allows for substantial DNA transformation competence in *Neisseria gonorrhoeae*. *Infect. Immun.* **71**: 6279-6291.

Lynn, F., M.M. Hobbs, J.M. Zenilman, F.M. Behets, K. Van Damme, A. Rasamindrakotroka & M.C. Bash, (2005) Genetic typing of the porin protein of *Neisseria gonorrhoeae* from

clinical noncultured samples for strain characterization and identification of mixed gonococcal infections. *J. Clin. Microbiol.* **43**: 368-375.

Maier, B., I. Chen, D. Dubnau & M.P. Sheetz, (2004) DNA transport into *Bacillus subtilis* requires proton motive force to generate large molecular forces. *Nat Struct Mol Biol* **11**: 643-649.

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

Marceau, M., K. Forest, J.L. Beretti, J. Tainer & X. Nassif, (1998) Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and pilus-mediated adhesion. *Mol. Microbiol.* **27**: 705-715.

Marceau, M. & X. Nassif, (1999) Role of glycosylation at Ser63 in production of soluble pilin in pathogenic Neisseria. *J. Bacteriol.* **181**: 656-661.

Marchler-Bauer, A., Y. Bo, L. Han, J. He, C.J. Lanczycki, S. Lu, F. Chitsaz, M.K. Derbyshire, R.C. Geer, N.R. Gonzales, M. Gwadz, D.I. Hurwitz, F. Lu, G.H. Marchler, J.S. Song, N. Thanki, Z. Wang, R.A. Yamashita, D. Zhang, C. Zheng, L.Y. Geer & S.H. Bryant, (2017) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* **45**: D200-d203.

Marians, K.J., (2000) PriA-directed replication fork restart in *Escherichia coli*. *Trends Biochem. Sci.* **25**: 185-189.

Martin, I.M. & C.A. Ison, (2003) Detection of mixed infection of *Neisseria gonorrhoeae*. *Sex. Transm. Infect.* **79**: 56-58.

Mathis, L.S. & J.J. Scocca, (1984) On the role of pili in transformation of *Neisseria gonorrhoeae*. *J. Gen. Microbiol.* **130**: 3165-3173.

Mavrogiorgos, N., S. Mekasha, Y. Yang, M.A. Kelliher & R.R. Ingalls, (2014) Activation of NOD receptors by *Neisseria gonorrhoeae* modulates the innate immune response. *Innate Immun* **20**: 377-389.

McCallum, M., S. Tammam, A. Khan, L.L. Burrows & P.L. Howell, (2017) The molecular mechanism of the type IVa pilus motors. *Nature communications* **8**: 15091.

Mehr, I.J., C.D. Long, C.D. Serkin & H.S. Seifert, (2000) A homologue of the recombination-dependent growth gene, *rdgC,* is involved in gonococcal pilin antigenic variation. *Genetics* **154**: 523-532.

Mehr, I.J. & H.S. Seifert, (1997) Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Mol. Microbiol.* **23**: 1121-1131.

Mehr, I.J. & H.S. Seifert, (1998) Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol. Microbiol.* **30**: 697-710.

Mell, J.C. & R.J. Redfield, (2014) Natural competence and the evolution of DNA uptake specificity. *J. Bacteriol.* **31**: 31.

Melly, M.A., Z.A. McGee & R.S. Rosenthal, (1984) Ability of monomeric peptidoglycan fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube mucosa. *J. Infect. Dis.* **149**: 378-386.

Merz, A.J., M. So & M.P. Sheetz, (2000) Pilus retraction powers bacterial twitching motility. *Nature* **407**: 98-102.

Meyer, T.F., N. Mlawer & M. So, (1982) Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. *Cell* **30**: 45-52.

Miller, F., G. Phan, T. Brissac, C. Bouchiat, G. Lioux, X. Nassif & M. Coureuil, (2014) The Hypervariable Region of Meningococcal Major Pilin PilE Controls the Host Cell Response via Antigenic Variation. *mBio* **5**: 01024-01013.

Neisser, A., (1879) Ueber eine der Gonorrhoe eigentümliche Micrcoccenform. *Ueber eine der Gonorrhoe eigentümliche Micrcoccenform*: 497-500.

Nunn, D.N. & S. Lory, (1991) Product of the Pseudomonas aeruginosa gene pilD is a prepilin leader peptidase. *Proc. Natl. Acad. Sci. U. S. A.* **88**: 3281-3285.

O'Brien, J.P., D.L. Goldenberg & P.A. Rice, (1983) Disseminated gonococcal infection: a prospective analysis of 49 patients and a review of pathophysiology and immune mechanisms. *Medicine (Baltimore).* **62**: 395-406.

Obergfell, K.P. & H.S. Seifert, (2015) Mobile DNA in the pathogenic *Neisseria*. *Microbiol Spectr* **3**: 0015-2014.

Obergfell, K.P. & H.S. Seifert, (2016) The Pilin N-terminal Domain Maintains *Neisseria gonorrhoeae* Transformation Competence during Pilus Phase Variation. *PLoS Genet* **12**: e1006069.

Petersen, T.N., S. Brunak, G. von Heijne & H. Nielsen, (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**: 785-786.

Pinho, M.G., M. Kjos & J.W. Veening, (2013) How to get (a)round: mechanisms controlling growth and division of coccoid bacteria. *Nature reviews. Microbiology* **11**: 601-614.

Poje, G. & R.J. Redfield, (2003) Transformation of Haemophilus influenzae. *Methods Mol Med* **71**: 57-70.

Powers, J.C., J.L. Asgian, O.D. Ekici & K.E. James, (2002) Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem. Rev.* **102**: 4639-4750.

Prevention, C.f.D.C.a., (2013) Antibiotic resistance threats in the United States, 2013. In. Atlanta: CDC, pp.

Promega, (2016) Protease-Glo Assay Technical Manual. In: Instructions for Use of Products G9451 and G9461. pp.

Ragland, S.A., R.E. Schaub, K.T. Hackett, J.P. Dillard & A.K. Criss, (2017) Two lytic transglycosylases in *Neisseria gonorrhoeae* impart resistance to killing by lysozyme and human neutrophils. *Cell Microbiol* **19**.

Ramsey, M.E., K.T. Hackett, C. Kotha & J.P. Dillard, (2012) New complementation constructs for inducible and constitutive gene expression in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Appl. Environ. Microbiol.* **78**: 3068-3078.

Rawlings, N.D., A.J. Barrett & R. Finn, (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res* **44**: D343-350.

Reardon, P.N. & K.T. Mueller, (2013) Structure of the type IVa major pilin from the electrically conductive bacterial nanowires of Geobacter sulfurreducens. *J. Biol. Chem.* **288**: 29260-29266.

Ropp, P.A. & R.A. Nicholas, (1997) Cloning and characterization of the ponA gene encoding penicillin-binding protein 1 from *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *J. Bacteriol.* **179**: 2783-2787.

Rudel, T., D. Facius, R. Barten, I. Scheuerpflug, E. Nonnenmacher & T.F. Meyer, (1995) Role of pili and the phase-variable PilC protein in natural competence for transformation of *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 7986-7990.

Rudel, T., J.P. van Putten, C.P. Gibbs, R. Haas & T.F. Meyer, (1992) Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. *Mol. Microbiol.* **6**: 3439-3450.

Rytkonen, A., L. Johansson, V. Asp, B. Albiger & A.B. Jonsson, (2001) Soluble pilin of *Neisseria gonorrhoeae* interacts with human target cells and tissue. *Infect. Immun.* **69**: 6419-6426.

Salman, H., D. Zbaida, Y. Rabin, D. Chatenay & M. Elbaum, (2001) Kinetics and mechanism of DNA uptake into the cell nucleus. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 7247-7252.

Salzer, R., M. Herzberg, D.H. Nies, F. Joos, B. Rathmann, Y. Thielmann & B. Averhoff, (2014) Zinc and ATP binding of the hexameric AAA-ATPase PilF from Thermus thermophilus: role in complex stability, piliation, adhesion, twitching motility, and natural transformation. *J. Biol. Chem.* **289**: 30343-30354.

Satterwhite, C.L., E. Torrone, E. Meites, E.F. Dunne, R. Mahajan, M.C. Ocfemia, J. Su, F. Xu & H. Weinstock, (2013) Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008. *Sex. Transm. Dis.* **40**: 187-193.

Sauvage, E., F. Kerff, M. Terrak, J.A. Ayala & P. Charlier, (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* **32**: 234-258.

Schagger, H., (2006) Tricine-SDS-PAGE. *Nat. Protocols* **1**: 16-22.

Schmittgen, T.D. & K.J. Livak, (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* **3**: 1101-1108.

Sechman, E.V., K.A. Kline & H.S. Seifert, (2006) Loss of both Holliday junction processing pathways is synthetically lethal in the presence of gonococcal pilin antigenic variation. *Mol. Microbiol.* **61**: 185-193.

Sechman, E.V., M.S. Rohrer & H.S. Seifert, (2005) A genetic screen identifies genes and sites involved in pilin antigenic variation in *Neisseria gonorrhoeae*. *Mol. Microbiol.* **57**: 468-483.

Segal, E., E. Billyard, M. So, S. Storzbach & T.F. Meyer, (1985) Role of chromosomal rearrangement in *N. gonorrhoeae* pilus phase variation. *Cell* **40**: 293-300.

Segal, E., P. Hagblom, H.S. Seifert & M. So, (1986) Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proc. Natl. Acad. Sci. U. S. A.* **83**: 2177-2181.

Seifert, H.S., (1997) Insertionally inactivated and inducible recA alleles for use in *Neisseria*. *Gene* **188**: 215-220.

Seifert, H.S., R.S. Ajioka, C. Marchal, P.F. Sparling & M. So, (1988) DNA transformation leads to pilin antigenic variation in *Neisseria gonorrhoeae*. *Nature* **336**: 392-395.

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

Seifert, H.S., C.J. Wright, A.E. Jerse, M.S. Cohen & J.G. Cannon, (1994a) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J. Clin. Invest.* **93**: 2744-2749.

Seifert, H.S., C.J. Wright, A.E. Jerse, M.S. Cohen & J.G. Cannon, (1994b) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J. Clin. Invest.* **93**: 2744-2749.

Seitz, P., H. Pezeshgi Modarres, S. Borgeaud, R.D. Bulushev, L.J. Steinbock, A. Radenovic, M. Dal Peraro & M. Blokesch, (2014) ComEA Is Essential for the Transfer of External DNA into the Periplasm in Naturally Transformable *Vibrio cholerae* Cells. *PLoS Genet* **10**: 2.

Siewering, K., S. Jain, C. Friedrich, M.T. Webber-Birungi, D.A. Semchonok, I. Binzen, A. Wagner, S. Huntley, J. Kahnt, A. Klingl, E.J. Boekema, L. Sogaard-Andersen & C. van der Does, (2014) Peptidoglycan-binding protein TsaP functions in surface assembly of type IV pili. *Proc. Natl. Acad. Sci. U. S. A.* **111**: E953-961.

Skaar, E.P., M.P. Lazio & H.S. Seifert, (2002) Roles of the *recJ* and *recN* genes in homologous recombination and DNA repair pathways of *Neisseria gonorrhoeae*. *J. Bacteriol.* **184**: 919-927.

Smith, H.O., M.L. Gwinn & S.L. Salzberg, (1999) DNA uptake signal sequences in naturally transformable bacteria. *Res. Microbiol.* **150**: 603-616.

Smith, J.M., N.H. Smith, M. O'Rourke & B.G. Spratt, (1993) How clonal are bacteria? . *Proc. Natl. Acad. Sci. U. S. A.* **90**: 4384-4388.

Snyder, L.A., J.K. Davies & N.J. Saunders, (2004) Microarray genomotyping of key experimental strains of *Neisseria gonorrhoeae* reveals gene complement diversity and

five new neisserial genes associated with Minimal Mobile Elements. *BMC Genomics* **5**: 23.

Sox, T.E., W. Mohammed, E. Blackman, G. Biswas & P.F. Sparling, (1978) Conjugative plasmids in *Neisseria gonorrhoeae*. *J. Bacteriol.* **134**: 278-286.

Sox, T.E., W. Mohammed & P.F. Sparling, (1979) Transformation-derived *Neisseria gonorrhoeae* plasmids with altered structure and function. *J. Bacteriol.* **138**: 510-518.

Sparling, P.F., (1966) Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. *J. Bacteriol.* **92**: 1364-1371.

Spratt, B.G. & K.D. Cromie, (1988) Penicillin-binding proteins of gram-negative bacteria. *Rev. Infect. Dis.* **10**: 699-711.

Stabler, R.A., G.L. Marsden, A.A. Witney, Y. Li, S.D. Bentley, C.M. Tang & J. Hinds, (2005) Identification of pathogen-specific genes through microarray analysis of pathogenic and commensal *Neisseria* species. *Microbiology* **151**: 2907-2922.

Stefanova, M.E., J. Tomberg, C. Davies, R.A. Nicholas & W.G. Gutheil, (2004) Overexpression and enzymatic characterization of *Neisseria gonorrhoeae* penicillin-binding protein 4. *Eur. J. Biochem.* **271**: 23-32.

Stefanova, M.E., J. Tomberg, M. Olesky, J.V. Holtje, W.G. Gutheil & R.A. Nicholas, (2003) *Neisseria gonorrhoeae* penicillin-binding protein 3 exhibits exceptionally high carboxypeptidase and beta-lactam binding activities. *Biochemistry (Mosc).* **42**: 14614-14625.

Stein, D.C., R.J. Danaher & T.M. Cook, (1991) Characterization of a gyrB mutation responsible for low-level nalidixic acid resistance in *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* **35**: 622-626.

Stein, D.C., J.S. Gunn, M. Radlinska & A. Piekarowicz, (1995) Restriction and modification systems of *Neisseria gonorrhoeae*. *Gene* **157**: 19-22.

Stephens, D.S., A.M. Whitney, J. Rothbard & G.K. Schoolnik, (1985) Pili of *Neisseria meningitidis*. Analysis of structure and investigation of structural and antigenic relationships to gonococcal pili. *J. Exp. Med.* **161**: 1539-1553.

Stern, A., M. Brown, P. Nickel & T.F. Meyer, (1986) Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell* **47**: 61-71.

Stohl, E.A., L. Blount & H.S. Seifert, (2002) Differential cross-complementation patterns of *Escherichia coli* and *Neisseria gonorrhoeae* RecA proteins. *Microbiology* **148**: 1821-1831.

Stohl, E.A., J.P. Brockman, K.L. Burkle, K. Morimatsu, S.C. Kowalczykowski & H.S. Seifert, (2003) *Escherichia coli* RecX inhibits RecA recombinase and coprotease activities in vitro and in vivo. *J. Biol. Chem.* **278**: 2278-2285.

Stohl, E.A., E.M. Dale, A.K. Criss & H.S. Seifert, (2013) *Neisseria gonorrhoeae* metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H2O2- and neutrophil-mediated killing. *mBio* **4**.

Stohl, E.A., M.C. Gruenig, M.M. Cox & H.S. Seifert, (2011) Purification and characterization of the RecA protein from *Neisseria gonorrhoeae*. *PloS one* **6**: 0017101.

Stohl, E.A. & H.S. Seifert, (2001) The *recX* gene potentiates homologous recombination in *Neisseria gonorrhoeae*. *Mol. Microbiol.* **40**: 1301-1310.

Swanson, J., (1973) Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J. Exp. Med.* **137**: 571-589.

Swanson, J., S. Bergstr:om, O. Barrera, K. Robbins & D. Corwin, (1985) Pilus- gonococcal variants. Evidence for multiple forms of piliation control. *J. Exp. Med.* **162**: 729-744.

Swanson, J., S. Bergstrom, K. Robbins, O. Barrera, D. Corwin & J.M. Koomey, (1986) Gene conversion involving the pilin structural gene correlates with pilus+ in equilibrium with pilus- changes in *Neisseria gonorrhoeae*. *Cell* **47**: 267-276.

Swanson, J., S. Morrison, O. Barrera & S. Hill, (1990) Piliation changes in transformation-defective gonococci. *J. Exp. Med.* **171**: 2131-2139.

Swanson, J., K. Robbins, O. Barrera, D. Corwin, J. Boslego, J. Ciak, M. Blake & J.M. Koomey, (1987) Gonococcal pilin variants in experimental gonorrhea. *J. Exp. Med.* **165**: 1344-1357.

Swasdio, K., S. Rugpao, T. Tansathit, C. Uttavichai, P. Jongusuk, T. Vutayavanich, A. Oranratanachai, N. Pruthitada, S. Peerakom, W. Ittipunkul, P.J. Rowe & M.E. Ward, (1996) The association of Chlamydia trachomatis/gonococcal infection and tubal factor infertility. *J. Obstet. Gynaecol. Res.* **22**: 331-340.

Takeuchi, N., K. Kaneko & E.V. Koonin, (2013) Horizontal Gene Transfer Can Rescue Prokaryotes from Muller's Ratchet: Benefit of DNA from Dead Cells and Population Subdivision. *G3* **17**: 009845.

Tobiason, D.M. & H.S. Seifert, (2006) The obligate human pathogen, *Neisseria gonorrhoeae*, is polyploid. *PLoS Biol* **4**.

Tonjum, T., N.E. Freitag, E. Namork & M. Koomey, (1995) Identification and characterization of *pilG*, a highly conserved pilus-assembly gene in pathogenic *Neisseria*. *Mol. Microbiol.* **95**: 451-464.

Treangen, T.J., O.H. Ambur, T. Tonjum & E.P. Rocha, (2008) The impact of the neisserial DNA uptake sequences on genome evolution and stability. *Genome Biol* **9**: 2008-2009.

Typas, A., M. Banzhaf, C.A. Gross & W. Vollmer, (2011) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature reviews. Microbiology* **10**: 123-136.

Unemo, M. & R.A. Nicholas, (2012) Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future microbiology* **7**: 1401-1422.

van der Woude, M.W., (2011) Phase variation: how to create and coordinate population diversity. *Curr. Opin. Microbiol.* **14**: 205-211.

van der Woude, M.W. & A.J. Baumler, (2004) Phase and antigenic variation in bacteria. *Clin. Microbiol. Rev.* **17**: 581-611, table of contents.

Veyrier, F.J., N. Biais, P. Morales, N. Belkacem, C. Guilhen, S. Ranjeva, O. Sismeiro, G. Pehau-Arnaudet, E.P. Rocha, C. Werts, M.K. Taha & I.G. Boneca, (2015) Common Cell Shape Evolution of Two Nasopharyngeal Pathogens. *PLoS Genet* **11**: e1005338.

Vink, C., G. Rudenko & H.S. Seifert, (2012) Microbial antigenic variation mediated by homologous DNA recombination. *FEMS Microbiol. Rev.* **36**: 917-948.

Virji, M., (2009) Pathogenic *Neisseriae*: surface modulation, pathogenesis and infection control. *Nature reviews. Microbiology* **7**: 274-286.

Wain, B.S., (1947) The Unconquered Plague. *New York: International University Press*.

Wainwright, L.A., K.H. Pritchard & H.S. Seifert, (1994) A conserved DNA sequence is required for efficient gonococcal pilin antigenic variation. *Mol. Microbiol.* **13**: 75-87.

Wasserheit, J.N., (1994) Effect of changes in human ecology and behavior on patterns of sexually transmitted diseases, including human immunodeficiency virus infection. *Proc Natl Acad Sci U S A* **91**: 2430-2435.

Winther-Larsen, H.C., M. Wolfgang, S. Dunham, J.P. van Putten, D. Dorward, C. Lovold, F.E. Aas & M. Koomey, (2005) A conserved set of pilin-like molecules controls type IV pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. *Mol. Microbiol.* **56**: 903-917.

Wolfgang, M., P. Lauer, H.S. Park, L. Brossay, J. Hebert & M. Koomey, (1998a) *pilT* mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. *Mol. Microbiol.* **29**: 321-330.

Wolfgang, M., H.S. Park, S.F. Hayes, J.P. van Putten & M. Koomey, (1998b) Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in pilT, a twitching motility gene in *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. U. S. A.* **95**: 14973-14978.

Wolfgang, M., J.P. van Putten, S.F. Hayes & M. Koomey, (1999) The *comP* locus of *Neisseria gonorrhoeae* encodes a type IV prepilin that is dispensable for pilus biogenesis but essential for natural transformation. *Mol. Microbiol.* **31**: 1345-1357.

Yeowell, H.N. & J.R. White, (1982) Iron requirement in the bactericidal mechanism of streptonigrin. *Antimicrob. Agents Chemother.* **22**: 961-968.

Zapun, A., T. Vernet & M.G. Pinho, (2008) The different shapes of cocci. *FEMS Microbiol. Rev.* **32**: 345-360.

Zelewska, M.A., M. Pulijala, R. Spencer-Smith, H.A. Mahmood, B. Norman, C.P. Churchward, A. Calder & L.A. Snyder, (2016) Phase variable DNA repeats in *Neisseria gonorrhoeae* influence transcription, translation, and protein sequence variation. *Microbial genomics* **2**: e000078.

Zhang, Q.Y., D. DeRyckere, P. Lauer & M. Koomey, (1992) Gene conversion in *Neisseria gonorrhoeae*: evidence for its role in pilus antigenic variation. *Proc. Natl. Acad. Sci. U. S. A.* **89**: 5366-5370.

Zhang, Q.Y. & B.G. Spratt, (1989) Nucleotide sequence of the penicillin-binding protein 2 gene of *Neisseria meningitidis*. *Nucleic Acids Res* **17**: 5383.

Zhang, Y., N. Heidrich, B.J. Ampattu, C.W. Gunderson, H.S. Seifert, C. Schoen, J. Vogel & E.J. Sontheimer, (2013) Processing-independent CRISPR RNAs limit natural transformation in *Neisseria meningitidis*. *Mol. Cell* **50**: 488-503.

Zielke, R.A., I.H. Wierzbicki, J.V. Weber, P.R. Gafken & A.E. Sikora, (2014) Quantitative proteomics of the *Neisseria gonorrhoeae* cell envelope and membrane vesicles for the discovery of potential therapeutic targets. *Molecular & cellular proteomics : MCP* **13**: 1299-1317.

Curriculum Vitae

Kyle P. Obergfell

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Education

Northwestern University Evanston, IL
PhD, Microbiology and Immunology May 2017
GPA: 3.97

University of Southern California Los Angeles, CA
B.S., Chemical Engineering (Biochemical Emphasis) May, 2011
magna cum laude GPA: 3.88

Research Experience

Northwestern University Feinberg School of Medicine Chicago, IL
Department of Microbiology and Immunology 2011-2017
Laboratory of Dr. Hank Seifert
Doctoral Researcher
Projects:

- The Type IV Pilus Complex and Natural Transformation in *Neisseria gonorrhoeae*
- Identification of the S-pilin protease in *N. gonorrhoeae*
- Role of DacB and DacC Mediated Peptidoglycan Remodeling in Pilus Biogenesis
- Effects of Heme-like Molecules on G4 formation and Antigenic Variation

Northwestern University Feinberg School of Medicine Chicago, IL
Department of Microbiology and Immunology Spring 2012
Laboratory of Dr. Allen Hauser
Graduate Rotation Student
Project:

- The Role of ExoS in a Murine Pneumonia Model of *Pseudomonas aeruginosa*

Northwestern University Feinberg School of Medicine Chicago, IL
Department of Surgery Winter 2012
Laboratory of Dr. Jason Wertheim
Graduate Rotation Student
Project:

- Evaluation of Growth Factor Content in Decellularized Kidneys

Northwestern University Feinberg School of Medicine Chicago, IL
Department of Microbiology and Immunology Fall 2011
Laboratory of Dr. Wyndham Lathem
Graduate Rotation Student
Project:

- sRNA Ysr29 and Pathogenesis of *Yersinia pestis* and *Yersinia pseudotuberculosis*

University of Southern California Los Angeles, CA
School of Dentistry 2010-2011
Laboratory of Dr. Steven D. Goodman
Undergraduate Research Assistant
Project:

- Role of Integration Host Factor in Biofilm Stability

Nationwide Children's Hospital, Ohio State University Columbus, OH
Center for Microbial Pathogenesis Summer 2010
Laboratory of Dr. Lauren Bakaletz
Research Assistant
Projects:

- Dispersal of NTHI Biofilms in the Chinchilla Middle Ear
- Role of Integration Host Factor in Biofilm Stability

University of Southern California Los Angeles, CA
Department of Aerospace and Mechanical Engineering 2007-2010
Laboratory of Dr. Paul Ronney
Merit Research Scholar
Project:

- Optimization of Microbial Fuel Cells

Publications

Obergfell KP, Seifert HS. The Pilin N-terminal Domain maintains *Neisseria gonorrhoeae* Transformation Competence during Pilus Phase Variation. PLoS Genet. 2016;12(5): e1006069

Obergfell KP, Seifert HS. Mobile DNA in the pathogenic *Neisseria*. Microbiol Spectr. 2015;3(3):0015-2014.

Caralt M, Uzarski JS, Iacob S, **Obergfell KP**, Berg N, Bijonowski BM, et al. Optimization and critical evaluation of decellularization strategies to develop renal extracellular matrix scaffolds as biological templates for organ engineering and transplantation. Am J Transplant. 2015;15(1):64-75.

Goodman SD, **Obergfell KP**, Jurcisek JA, Novotny LA, Downey JS, Ayala EA, et al. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. Mucosal Immunol. 2011;4(6):625-37.

Presentations and Posters

The Gonococcal Pilin N-Terminal Domain maintains transformation competence during pilus phase variation. Oral Presentation, International Pathogenic Neisseria Conference, September 9th, 2016

Retaining Competence During Gonococcal Antigenic Variation. Oral Presentation, Northwestern University Immunology and Microbial Sciences Training Symposium, August 15th, 2016

Research-in-Progress Talk, Cellular and Molecular Basis of Disease Training Grant Research-in-Progress Series, June 16, 2015

Research Chalk Talk, Driskill Graduate Program Research Club, January 20, 2015

Research-in-Progress Talk, Northwestern University Bacteriology Journal Club, October 30, 2015

The Gonococcal Pilin N-Terminal Domain maintains transformation competence during pilus phase variation. Chicago Biomedical Consortium 13th Annual Symposium Poster Session. October 26, 2015

The Gonococcal Pilin N-Terminal Domain maintains transformation competence during pilus phase variation. Cold Spring Harbor Laboratory Meeting on Microbial Pathogenesis and Host Response Poster Session. September 8-12, 2015

Neisseria gonorrhoeae can Maintain Competence during Pilin Antigenic Variation through Ntd-Mediated Transformation, Northwestern University Feinberg School of Medicine Department of Pathology Inflammation Research-in-Progress Seminar Series, February 5th, 2015

Research-in-Progress Talk, Cellular and Molecular Basis of Disease Training Grant Research-in-Progress Series, January 15, 2015

Neisseria gonorrhoeae can maintain Competence during Pilin Antigenic Variation through Ntd-Mediated Transformation, Northwestern University Feinberg School of Medicine Department of Microbiology-Immunology Seminar Series, January 6, 2015

A Targeted Genetic Screen of *Neisseria gonorrhoeae pilE* and the role of the Ntd in Transformation, 21st Annual Midwest Microbial Pathogenesis Conference Poster Session, September 12-14, 2014

A Targeted Genetic Screen of *Neisseria gonorrhoeae pilE* and its role in Transformation, Research Day for the Immunology-Molecular Pathogenesis and Allergy-Immunology Training Grants Poster Session, July 10, 2014

Research-in-Progress Talk, Northwestern University Bacteriology Journal Club, April 11, 2014

Research-in-Progress Talk, Cellular and Molecular Basis of Disease Training Grant Research-in-Progress Series, October 9, 2013

A Targeted Genetic Screen of *Neisseria gonorrhoeae pilE* and its role in Pilus Expression and Function, Northwestern University Microbiology-Immunology Retreat Research Talk, September 8-9, 2013

A Targeted Genetic Screen of *Neisseria gonorrhoeae pilE* and its role in Pilus Expression and Function, 20th Annual Midwest Microbial Pathogenesis Conference Poster Session, August 23-25, 2013

Research-in-Progress Talk, Northwestern University Bacteriology Journal Club, March 1, 2013

Evaluation of Growth Factor Content in Perfusion Decellularized Kidneys for Renal Tissue Engineering, 8th Annual Lewis Landsberg Research Day Poster Session, April 5, 2012

Biofilms Formed in Vitro by Bacterial Strains That Cause Otitis Media Are Eradicated by Treatment with Antibodies Directed Against Integration Host Factor Protein, 10th International Symposium on Recent Advances in Otitis Media Poster Session, June 5-9, 2011

Honors and Awards

Dr. John N. Nicholson Fellowship, Full Tuition and Stipend Scholarship 2016-2017

Katten Muchin Rosenman Travel Scholarship 2016

Cellular and Molecular Basis of Disease Training Grant Appointment 2013-2016

Northwestern Graduate School Conference Travel Grant (2x) 2015, 2016

DGP Travel Award 2015

Conference Outstanding Poster Runner-up, 10th International Symposium on Recent Advances in Otitis Media 2011

B.S. Chemical Engineering, *magna cum laude* 2011

USC Trustee Scholar, Full Tuition Scholarship 2007-2011

USC Merit Research Scholar 2007-2011

National Merit Scholarship 2007-2008, renewed 2008-2009, 2009-2010, and 2010-2011

Teaching and Service

Elected Student Council Member 2016-2017
Northwestern University Driskill Graduate Program in Life Sciences
Advocated on behalf on the graduate student body with faculty and administration and raised funds to improve student quality of life as a student elected representative

Instructor of Record, Genetics and Evolution Spring 2016
Northwestern University
Conceived, developed and taught a distribution course for undergraduate students on genetics and evolution

Center for the Integration of Research, Teaching, and Learning Practitioner 2016
Northwestern University
Applied the priciples of evidence-based teaching through the development and execution of a classroom-based research question in a Teaching-As-Research project

Searle Teaching Certificate Recipient 2015-2016
Northwestern University Searle Center for Advanced Learning and Teaching
Completed yearlong certificate program combining seminars, workshops, mentoring, and discipline-specific discussions to prepare for college teaching responsibilities

An Introduction to Evidence-Based Undergraduate STEM Teaching 2015-2016
Center for the Integration of Research, Teaching and Learning
Completed a Massive, Open, Online Course, designed to prepare STEM future and current faculty to be more effective teachers

Northwestern University Science Club Mentor and Volunteer 2013-2017
Boys and Girls Clubs of Chicago
Providing mentorship to underprivileged middle school students through engaging and challenging science instruction, doubling amount of students' science instruction

Retreat Organizer 2014, 2015
1st and 2nd Annual Cellular and Molecular Basis of Disease Training Grant Retreat
Developed, organized, and directed the first and second annual retreat for the CMBD training grant members

DGP Student Assisted Mentoring Program Founding Member 2014-2105
Driskill Graduate Program in Life Sciences
Provided incoming and second-year students with one-on-one mentoring to help incoming students adapt to the difficulties of graduate school

Graduate Teaching Assistant
Northwestern University, Department of Biology
BIOL SCI 220-0 Genetic and Molecular Processes Laboratory Fall 2013
Led a laboratory section of 24 students with responsibilities including developing and delivering introductory lectures, laboratory instruction, and crafting and grading quizzes

Elective Courses

Northwestern University Kellogg School of Business Evanston, IL
MORS 468, Managerial Leadership Winter 2015
MORS 476, Bargaining Spring 2015

References

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