MECHANISMS OF ANTIBIOTIC RESISTANCE IN *NEISSERIA GONORRHOEAE* AND THE DEVELOPMENT OF LPXC INHIBITORS AS NOVEL THERAPEUTICS

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ABSTRACT

Shauna Marie Swanson: Mechanisms of antibiotic resistance in *N. gonorrhoeae* and the development of LpxC inhibitors as novel therapeutics
(Under the direction of Robert A. Nicholas)

*Neisseria gonorrhoeae* is a Gram-negative diplococcus that causes the sexually transmitted infection gonorrhea. Antibiotics are required to treat gonorrhea. Unfortunately, the gonococcus has developed resistance to all classes of antibiotics that have been used to treat gonorrhea. Antibiotics primarily diffuse through the outer membrane porin channels into the periplasm, but we have shown that they can also access the periplasm via the outer membrane secretin PilQ; mutation or deletion of PilQ increases resistance to penicillin by approximately threefold. In its mature form, PilQ exists as an SDS-resistant multimer. However, immature PilQ monomers are also present in the outer membrane. We initiated studies to determine if antibiotic permeation occurred through the mature secretin or via immature PilQ complexes. Our data indicate that immature, SDS-labile PilQ complexes form channels that allow antibiotics to traverse the outer membrane.

To help meet the growing need for new antibiotics for the treatment of gonorrhea, we have validated the enzyme LpxC as a drug target in *N. gonorrhoeae*. LpxC catalyzes the first committed step in the biosynthesis of lipid A, a major component of the outer membrane of Gram-negative bacteria. LpxC inhibitors are bactericidal to the gonococcus and retain activity against multi-drug resistant strains of gonorrhea. Two inhibitors were
tested *in vivo* using the murine model of gonorrhea, and one of these inhibitors was able to clear the infection in eight out of nine infected mice.

We also evaluated the capacity of *N. gonorrhoeae* to develop resistance to LpxC inhibitors. Spontaneously arising mutants displayed a 4- to 16-fold decrease in susceptibility to LpxC inhibitors, and in several of these mutants, mutation or deletion of the *pqIA* and *pqIB* genes was responsible for the resistance phenotype. The functions of PqiA and PqiB are unknown, but the loss of *pqIA* does not alter the MICs of other antibiotics and does not affect LpxC expression or growth rate. The results of the studies described within this dissertation further our understanding of antibiotic resistance mechanisms in *N. gonorrhoeae* and provide the basis for the development of LpxC inhibitors as potential new therapeutics for the treatment of gonorrhea.
To my family, especially my parents, for supporting me throughout college and graduate school. You did not always understand what I was working on, but you always believed in me.
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I’d also like to thank my friends and family for always being interested in hearing about whatever I was doing in graduate school. When you’re surrounded by people who have their PhD or are working on it, it is easy to forget what an accomplishment it truly is. Thank you for always encouraging and believing in me. Without you reminding me that there is life outside of graduate school, I might not have made it all the way through!
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CEACAM</td>
<td>carcinoembryonic antigen cell adhesion molecule</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>CMP-NANA</td>
<td>5'-cytidinemonophospho-N-acetyl neuramic acid</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor 3</td>
</tr>
<tr>
<td>DGI</td>
<td>disseminated gonococcal infection</td>
</tr>
<tr>
<td>DUS</td>
<td>DNA uptake sequence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESC</td>
<td>expanded-spectrum cephalosporins</td>
</tr>
<tr>
<td>GC</td>
<td>gonococcus</td>
</tr>
<tr>
<td>GGI</td>
<td>gonococcal genetic island</td>
</tr>
<tr>
<td>GISP</td>
<td>Gonococcal Isolate Surveillance Project</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPSG</td>
<td>heparin sulfate proteoglycan</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kdo</td>
<td>2-keto-3-deoxyoctulosonic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MSM</td>
<td>men who have sex with men</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MurNAc</td>
<td>N-acetylmuramic acid</td>
</tr>
<tr>
<td>PBP</td>
<td>penicillin-binding protein</td>
</tr>
<tr>
<td>PEA</td>
<td>phosphoethanolamine</td>
</tr>
<tr>
<td>PID</td>
<td>pelvic inflammatory disease</td>
</tr>
<tr>
<td>PG</td>
<td>peptidoglycan</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>Opa</td>
<td>opacity-associated proteins</td>
</tr>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>STI</td>
<td>sexually transmitted infection</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TFP</td>
<td>type four pili</td>
</tr>
<tr>
<td>T4SS</td>
<td>type IV secretion system</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>uridine diphosphate N-acetylglicosamine</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
Chapter 1. Introduction

1.1 Introduction

*Neisseria gonorrhoeae* is a Gram-negative diplococcus that causes the sexually transmitted infection (STI) gonorrhea. It is one of the two pathogenic members of the *Neisseriaceae* family; the other is *N. meningitidis*, the etiological agent of bacterial meningitis. Gonorrhea is the second-most prevalent reportable infectious disease in the United States (Division of STD Prevention 2012). Patients do not develop a protective immune response against gonorrhea, making antibiotic treatment necessary for curing the infection. However, antibiotic resistance in the gonococcus (GC) is a serious public health problem. In the US, gonorrhea is currently treated with the expanded-spectrum cephalosporin ceftriaxone in combination with either azithromycin or doxycycline (Centers for Disease Control and Prevention. 2012). Unfortunately, strains resistant to ceftriaxone have been isolated in the last several years (Unemo, Golparian et al. 2010, Ohnishi, Saika et al. 2011, Unemo, Golparian et al. 2012). This dissertation describes the interconnected mechanisms of antibiotic action and antibiotic resistance in *N. gonorrhoeae*, as well as the validation of a new potential drug target for the treatment of antibiotic-resistant gonorrhea.
1.2 Public health relevance

1.2.1 Epidemiology

The Centers for Disease Control and Prevention (CDC) received reports of 321,849 cases of gonorrhea in the United States in 2011, the most recent year for which data are available. This number represents a rate of 104.2 cases per 100,000 people. Alarmingly, the gonorrhea rate in the US increased in both 2010 and 2011 (Division of STD Prevention 2012). The increase occurred in all regions of the US and was most dramatic for the Northeast, but the overall rate remained the highest in the South (Figure 1.1). Because infections are often asymptomatic, especially in females, it is estimated that fewer than half of the gonorrhea cases that occur in the US are reported; thus, the actual burden is likely closer to 800,000 cases per year (Satterwhite, Torrone et al. 2013).

It is more difficult to estimate the world-wide burden of gonorrhea. Based on data collected in 2008, the World Health Organization (WHO) estimated that 106 million new gonococcal infections occur each year and that at any given time, 36.4 million people are infected with \textit{N. gonorrhoeae} (2012). These estimates are considerably higher (11% increase) than the same estimates made for 2005. Although the increase is partially driven by population growth, it represents a 21% increase in the global incidence of gonorrhea.
Figure 1.1 Geographical distribution of gonorrhea infections across the United States (adapted from the CDC Sexually Transmitted Diseases Surveillance Report (Division of STD Prevention 2012)).
1.2.2 Clinical manifestations

*N. gonorrhoeae* is an obligate human pathogen. It typically colonizes the genital tracts of men and women but can also infect other mucus membranes, including the anorectal canal, pharynx, and eye. Men typically develop symptoms including urethral discharge and a burning sensation while urinating within 3 to 7 days following exposure (Spence 1983). However, some men have less severe symptoms, and a small proportion of infected men may remain asymptomatic (Handsfield, Lipman et al. 1974, Spence 1983). Interestingly, heterosexual men that are co-infected with *N. gonorrhoeae* and *Chlamydia trachomatis* and men who have sex with men (MSM) and are infected with both gonorrhea and syphilis are more likely to be asymptomatic than men infected with *N. gonorrhoeae* alone (Bozicevic, Fenton et al. 2006). Rectal infections are common in MSM, and these infections are often asymptomatic (Miller 2006). Rectal infections can also be more difficult to detect by culturing, and infections are often missed if only the urogenital tract is sampled for nucleic acid amplification testing (van Liere, Hoebe et al. 2013).

Asymptomatic urogenital infections are more common in women, especially in cases where the infection has not yet ascended the genital tract. When symptoms are present, they include discharge and slight irritation (McCormack, Stumacher et al. 1977, Platt, Rice et al. 1983). Anorectal infections are also generally asymptomatic and most often occur secondary to endocervical infections due to the spread of the bacteria across the perineum (McCormack, Stumacher et al. 1977).

Approximately 10-20% of infected women who do not receive treatment for genital gonococcal infection progress to pelvic inflammatory disease (PID) (Haggerty, Gottlieb et al. 2010). As with uncomplicated gonococcal infections, PID due to gonorrhea can remain
asymptomatic, and these women continue to pose a public health threat as carriers of gonorrhea. When symptoms are present, they include abdominal pain, pelvic pain, and fever. If this condition remains untreated, it can result in ectopic pregnancy and even infertility due to damage to the fallopian tubes (Weström, Joesoef et al. 1992, Walker and Sweet 2011).

In both sexes, a small number (0.5 to 3%) of untreated gonorrhea cases progress to disseminated gonococcal infections (DGI), a bacteremia that can result in chronic joint infections, arthritis, or a skin rash (Britigan, Cohen et al. 1985, Bardin 2003). DGI is more common in women than men, likely because asymptomatic mucosal infections are more common in women and because menstruation is a risk factor for DGI (Holmes, Counts et al. 1971, Bleich, Sheffield et al. 2012). Similarly, DGI is more common among MSM than among heterosexual men because anorectal gonorrhea is more likely to be asymptomatic than urethral gonorrhea (Holmes, Counts et al. 1971). Once DGI is diagnosed, it can be treated with antibiotics (Miller 2006).

1.2.3 Gonorrhea and HIV co-infections

Asymptomatic gonorrhea infections are especially concerning in light of evidence that infection with *N. gonorrhoeae* enhances both the transmission and acquisition of human immunodeficiency virus (HIV) (Cohen 1998). Gonorrhea is one of the most frequently diagnosed bacterial STIs in people who have HIV (Page, Moore et al. 2008). Numerous epidemiological studies have described the association between HIV and gonorrhea (Zetola, Bernstein et al. 2009, Bernstein, Marcus et al. 2010). In addition to the overlapping risk factors for both of these diseases, several molecular mechanisms for
increased transmission and acquisition have been described. One of the first studies on the relationship between gonorrhea and HIV found that treating the gonococcal infection in co-infected patients reduced viral RNA levels in the semen (Cohen, Hoffman et al. 1997). Women infected with gonorrhea have greater numbers of endocervical CD4+ T-cells that can then be infected upon exposure to HIV (Levine, Pope et al. 1998), and infection with gonorrhea can enhance the transcription of HIV in T-cells infected with the virus, leading to increased viral expression and shedding (Chen 2003). Infection with gonorrhea also increases the susceptibility of immune cells to HIV. Ding et al. found the exposure of CD4+ T-cells to *N. gonorrhoeae* increased HIV infection of these cells in an TLR2-dependent manner (Ding, Rapista et al. 2010), and co-infection with gonorrhea enhances the infection of dendritic cells by HIV (Zhang, Li et al. 2005). These studies show that an individual with gonorrhea is at an increased risk of contracting gonorrhea, and an individual with both gonorrhea and HIV has an increased capacity to transmit HIV. Thus, treating gonococcal infections is one strategy to decrease the spread of HIV.

### 1.3 Structural components of the gonococcal cell wall

Like all Gram-negative bacteria, *N. gonorrhoeae* possesses a cell wall consisting of an inner (cytoplasmic) membrane, a thin layer of peptidoglycan, and an outer membrane. The outer membrane forms a permeability barrier and is composed of lipooligosaccharide, phospholipids, integral membrane proteins, and structures that are necessary for genetic transformation, adhesion to host cells, and the import and export of nutrients. These outer membrane components include the type four pili, Opa proteins, and porins.
1.3.1 Lipooligosaccharide

Gram-negative bacteria are characterized by the presence of lipopolysaccharide (LPS; also known as endotoxin) in the outer leaflet of the outer membrane. LPS is composed of lipid A, which anchors LPS to the membrane, a polysaccharide core, and O-antigen repeats that extend from the bacterial surface (Raetz and Whitfield 2002). Because *N. gonorrhoeae* has a shorter polysaccharide core and lacks the O-antigen repeats, the endotoxin of GC is called lipooligosaccharide (LOS) (Preston, Mandrell et al. 1996). Bacteria that produce LOS rather than LPS have cell surfaces that are more hydrophobic and are more likely to colonize the respiratory tract, genital tract, and other mucosal surfaces. In contrast, bacteria that produce LPS are more hydrophilic, and this property allows enteric bacteria to be more resistant to intestinal enzymes and bile (Preston, Mandrell et al. 1996).

*N. gonorrhoeae* lipid A consists of two glucosamine molecules, each with 3 acyl chains, bound to two Kdo (2-keto-3-deoxyoctulosonic acid) residues (Figure 1.2). This lipid A moiety is the basic structure of LOS required for cell viability and is the active component of endotoxin. Two heptose molecules are attached to one of the Kdo residues, and 3 variable oligosaccharide side chains can then be attached to the heptose molecules by a series of glycosyltransferases. The oligosaccharides added to the chain include glucose, galactose, GlcNAc, and GalNAc, but their exact composition and order depend on the presence of specific glycosyltransferase enzymes (Preston, Mandrell et al. 1996).

The *lgt* gene cluster in *N. gonorrhoeae* contains five genes that encode enzymes responsible for adding oligosaccharides to lipid A (Gotschlich 1994). The *lgt* genes display phase variation due to the presence of hypermutable poly-C and poly-G tracts. The lengths of these tracts can vary due to slip-strand mispairing errors during replication, resulting in
frame-shift mutations that turn the genes on or off, leading to subsequent antigenic variations in the sugars added to the LOS core (Danaher, Levin et al. 1995, Yang and Gotschlich 1996, Banerjee, Wang et al. 1998). Further variability in this region is conferred by the presence of multiple promoter regions in the lgtABCDE gene cluster. The presence of multiple promoters allows for slight variations in transcription that make it possible for a single cell to express multiple LOS variations simultaneously (Burch, Danaher et al. 1997, Braun and Stein 2004).

In addition to the varying expression of the glycosyltransferases, the structure of LOS can also be modified. The heptose II residue of LOS and the lipid A moiety itself can be decorated with phosphoethanolamine (PEA) (Cox, Wright et al. 2003, O’Connor, Piekarowicz et al. 2006). The presence of PEA affects the ability of the bacterium to resist host defenses such as serum killing, complement binding, and cationic antimicrobial peptides (Lewis, Choudhury et al. 2009, Balthazar, Gusa et al. 2011, Lewis, Shafer et al. 2012). A N. gonorrhoeae mutant that lacks the PEA transferase (LptA) (Cox, Wright et al. 2003) and is unable to add PEA modifications to lipid A was less fit compared to wild-type in both the murine and human male urethra models of gonococcal infection (Hobbs, Anderson et al. 2013).

Neisserial LOS can also be modified by sialic acid (Apicella, Mandrell et al. 1990, Mandrell, Lesse et al. 1990). This occurs when a bacterial sialyltransferase (Lst) located on the outer membrane (Shell, Chiles et al. 2002) adds a host-derived sialic acid precursor (5-cytidinemonophospho-N-acetylneuraminic acid, CMP-NANA) to the terminal galactose residue of LOS (Parsons, Andrade et al. 1989, Mandrell, Griffiss et al. 1993, Gilbert, Cunningham et al. 1997). This likely occurs after infection, as sialylation of LOS prior to
urethral infection or incubation with urethral epithelial cells *in vitro* reduces the infectivity of the gonococcus (Schneider, Schmidt et al. 1996, Harvey, Jennings et al. 2001).
Figure 1.2 Structure of Kdo₂-lipid A. Compared to the structure of *E. coli* Kdo₂-lipid A, the structure of gonococcal Kdo₂-lipid A differs in the location of one acyl chain.
1.3.2 Type IV pili

Type four pili (TFP) are phase-variable surface appendages in many Gram-negative bacteria that mediate multiple biological processes, including adhesion (Swanson 1973, Virji and Heckels 1984, Jonsson, Ilver et al. 1994), twitching motility (Swanson 1978, Henrichsen 1983), and DNA transformation (Sparling 1966) (Figure 1.3A). The presence or absence of pili can be observed by examining colony morphology under a dissecting microscope. Colonies that express pili are small, shiny, and rounded, whereas colonies containing non-piliated cells are large and appear dull and flat (Kellogg, Peacock et al. 1963). The pili can be rapidly extended and retracted in an ATP-dependent manner. A transposon mutagenesis screen in *N. meningitidis* identified fifteen proteins that are essential for the biogenesis of TFP (Carbonnelle, Hélaine et al. 2004). Figure 1.3B shows a model of type four pilus biogenesis and the locations of many of these proteins, and Table 1.1 lists pilus biogenesis proteins and their functions.

The pilus itself is a helical polymer that is primarily composed of subunits of the major pilin, PilE, with lesser amounts of the low-abundance minor pilins. The fibers are assembled in the periplasm, and assembly requires PilD, PilF, and PilG (Freitag, Seifert et al. 1995, Tonjum, Freitag et al. 1995, Wolfgang, van Putten et al. 2000). PilD is a pre-pilin peptidase responsible for cleaving the leader peptide from the pre-pilins (Freitag, Seifert et al. 1995), which promotes their assembly into filaments via interactions between their well-conserved hydrophobic N-terminal helices. This assembly leaves exposed the more variable C-terminal domains, which contribute to antigenic variation (Parge, Forest et al. 1995). There is some controversy regarding the exact location and function of the phase-variable protein PilC, but some reports show that it localizes to the tip of the pili where it
may stabilize the PilE polymers (Jonsson, Nyberg et al. 1991, Wolfgang, van Putten et al. 2000, Morand, Bille et al. 2004). There is also evidence that PilC serves as an adhesion factor for interactions between the gonococcus and human epithelial cells; however, this is also controversial and may apply only to specific tissue types (Nassif, Beretti et al. 1994, Rudel, Facius et al. 1995, Rudel, Scheurerpfug et al. 1995, Kirchner and Meyer 2005). PilC is also required for the natural competence of the organism (Rudel, Facius et al. 1995).

The polymerization (extension) and depolymerization (retraction) of the filaments requires the activity of the ATPases PilF and PilT, respectively (Freitag, Seifert et al. 1995, Wolfgang, Lauer et al. 1998, Forest, Satyshur et al. 2004). The assembled filaments extrude from the cell through the outer membrane secretin PilQ (Wolfgang, van Putten et al. 2000, Chen and Dubnau 2004). PilQ forms SDS-resistant multimers; the formation of these multimers requires the presence of the lipoprotein PilW (Collins, Davidsen et al. 2001, Carbonnelle, Hélaine et al. 2004, Szeto, Dessen et al. 2011). In *Pseudomonas aeruginosa*, a recently defined association of PilM, PilN, PilO, and PilP referred to as the “alignment complex” (Tammam, Sampaleanu et al. 2013) was shown to bridge the periplasm to link the inner membrane components of TFP assembly with the PilQ secretin. These proteins (PilMNOP) are all required for piliation in *N. gonorrhoeae*, and similar associations are suggested by the work presented in Chapter 2.

The pili display antigenic variability due to non-reciprocal recombination events, termed “gene conversion”, between the expressed pilE locus and the silent, incomplete pilS loci in which the pilS sequence remains unchanged (Haas 1986, Howell-Adams 2000). There are multiple pilS loci in most strains of gonorrhea, and each of these loci can contain up to 6 copies of a partial pilin gene (Haas and Meyer 1986, Segal, Hagblom et al. 1986,
Hamrick, Dempsey et al. 2001). These copies, or minicassettes, lack the N-terminal conserved region and contain increasing numbers of amino acid substitutions within the hypervariable C-terminal domain. Each minicassette is flanked by regions of repeats that match sequences found upstream and within pilE (Haas and Meyer 1986, Howell-Adams and Seifert 2000), and these sequences are important for recombination. Gene conversion occurs at a frequency of approximately $10^{-3}$ in vitro (Serkin and Seifert 1998), and variants appear rapidly following infection (Hamrick, Dempsey et al. 2001). It is possible that selective pressure during infection may increase the frequency of gene conversion to above that measured in vitro. Some recombination events can result in a deletion in the expression locus, and these deletions result in the phase variation from an “on” (piliated) to an “off” (nonpiliated) colony morphology (Segal, Billyard et al. 1985).
Figure 1.3 Type IV pili of *N. gonorrhoeae*. A. Scanning electron micrograph of piliated FA19 cells (magnification: 100,000×). B. Model showing the location of components involved in the biogenesis of TFP. The pilus is primarily composed of PilE subunits and the PilC adhesin and extrudes through the PilQ secretin in the outer membrane. The protein PilW is required for the stability of the secretin. The pilin subunits are processed by the pre-pilin peptidase PilD and polymerized through the activity of PilF. Pilus retraction is mediated by the PilT ATPase. The proteins PilM, PilN, PilO, and PilP are required for piliation, as discussed in Chapter 2 of this dissertation (Wolfgang, van Putten et al. 2000, Tammam, Sampaleanu et al. 2011).
Table 1.1 Proteins involved in type four pilus biogenesis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PilE</td>
<td>Pilin</td>
</tr>
<tr>
<td>PilF</td>
<td>ATPase, filament polymerization (extension)</td>
</tr>
<tr>
<td>PilT</td>
<td>ATPase, filament depolymerization (retraction)</td>
</tr>
<tr>
<td>PilD</td>
<td>Pre-pilin peptidase</td>
</tr>
<tr>
<td>PilG</td>
<td>Pilus assembly protein</td>
</tr>
<tr>
<td>PilX</td>
<td>Minor pilin</td>
</tr>
<tr>
<td>PilW</td>
<td>Stabilization of PilQ</td>
</tr>
<tr>
<td>PilM</td>
<td>Required for TFP biogenesis</td>
</tr>
<tr>
<td>PilN</td>
<td>Required for TFP biogenesis</td>
</tr>
<tr>
<td>PilO</td>
<td>Required for TFP biogenesis</td>
</tr>
<tr>
<td>PilP</td>
<td>Required for TFP biogenesis</td>
</tr>
<tr>
<td>PilQ</td>
<td>TFP secretin</td>
</tr>
<tr>
<td>PilC</td>
<td>Putative TFP adhesin</td>
</tr>
</tbody>
</table>
1.3.3 Opacity-associated proteins

The opacity-associated proteins (Opa) are antigenic- and phase-variable outer-membrane adhesins that are involved in the association of pathogenic *Neisseria* with host cells and contribute to the ability of the bacteria to evade the immune system. These proteins were named for the opaque appearance of Opa+ colonies on agar viewed under a dissecting microscope with a substage light supply (Swanson 1978). Gonococcal cells recovered from infected individuals are Opa+, even when the infecting inoculum contained only Opa− cells (James and Swanson 1978, Jerse, Cohen et al. 1994). *N. gonorrhoeae* strains typically encode 11 Opa proteins (Bhat, Gibbs et al. 1991), and a single cell may express any number of these proteins simultaneously. The Opa genes are constitutively transcribed, and phase variation occurs at the translational level. Phase variation is due to the presence of pentameric repeats (CTCTT) in the coding sequence (Stern, Brown et al. 1986); the number of these repeats varies due to slip-strand mispairing that can move the coding regions of the Opa genes in or out of frame (Murphy, Connell et al. 1989).

The structure of the Opa proteins has not been solved, but a two-dimensional model has been predicted based on protein sequences and epitope binding of monoclonal antibodies (Malorny, Morelli et al. 1998). This model predicts that eight transmembrane β strands form a barrel structure in the outer membrane. These transmembrane strands are connected by 4 extracellular loops (de Jonge, Bos et al. 2002). Loops 1, 2, and 3 contain variable regions. Loop 1 also contains some conserved sequences and is thus considered semi-variable, whereas loops 2 and 3 are considered hyper-variable. The fourth loop is well-conserved among Opa proteins (Malorny, Morelli et al. 1998).
The Opa proteins can be divided into two categories based on the host factors to which they bind. A small number of Opa proteins bind to heparin sulfate proteoglycans (HSPGs) (Chen, Belland et al. 1995, van Putten, Duensing et al. 1998). However, the majority of Opa proteins bind to members of the carcinoembryonic antigen cell adhesion molecule (CEACAM) family (Wang, Gray-Owen et al. 1998, Virji, Evans et al. 1999). These receptors are found on a variety of cell types and can influence the immune response to gonococcal infection (Liu, Feinen et al. 2011).

### 1.3.4 Peptidoglycan

Peptidoglycan (PG) is a polymer of carbohydrate and amino acids that is sandwiched between the inner and outer membranes of Gram-negative bacteria. This essential macromolecule confers structure and shape to the cell and protects it from osmotic stress. PG is composed of alternating N-acetylg glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) sugars linked with a β-1,4 glycosidic bond. In *N. gonorrhoeae*, these sugar chains average 80 to 100 disaccharides long. To each MurNAc residue a peptide chain composed of L-Ala-D-γ-Glu-m-DAP-D-Ala-D-Ala is attached (Hebeler and Young 1976). These peptide chains undergo transpeptidation to cross-link the glycan chains and form a sturdy meshwork structure. Transpeptidation is catalyzed by the penicillin-binding proteins (PBPs) (Ishino, Mitsui et al. 1980). These enzymes cleave the bond between the penultimate and terminal D-Ala residues and form a cross-link between the newly exposed terminal D-Ala and an m-DAP residue on a neighboring peptide chain (Rosenthal, Wright et al. 1980, Barbour 1981).
PG is constantly remodeled as bacterial cells grow and divide, and peptidoglycan fragments are released from the cell. This remodeling process requires the activity of endopeptidases and amidases that hydrolyze the peptide chains and lytic transglycosylases (LTs) that hydrolyze the glycan backbone (Johnson, Fisher et al. 2013). During remodeling, the LTs cleave the glycosidic bond between the GlcNAc and MurNAc residues of the glycan strand by catalyzing the formation of 1,6-anhydro bonds within the MurNAc residues (Rosenthal 1979, Chan, Hackett et al. 2012). Peptidoglycan fragments released during remodeling may contribute to the pathogenesis of gonorrhea. For example, when gonococcal PG monomers are applied to human fallopian tubes in organ culture, the ciliated cells are damaged and sloughed off of the mucosa, similar to what has been observed in patients suffering from PID (Melly, McGee et al. 1984).

1.3.5 Porin

Porins are abundant proteins that form water-filled channels through the outer membranes of Gram-negative bacteria to allow for the diffusion ions across the membrane (Young, Blake et al. 1983, Nikaido 1994). In addition to their general channel function, neisserial porins also interact with host cells and are involved in evading the immune system. Porins can insert into the host cell membrane during infection and arrest phagosome maturation, and they are involved in the invasion of host cells (Young, Blake et al. 1983, Lynch, Blake et al. 1984, Nikaido 1994, Rudel, Schmid et al. 1996, Mosleh 1998, van Putten, Duensing et al. 1998, Bauer, Rudel et al. 1999). Porins have a trimeric structure, with each monomer composed of a 16-strand β-barrel. Because each subunit produces a
membrane-spanning channel, the fully assembled porin contains three channels (Judd 1989, Nikaido 1994, Derrick, Urwin et al. 1999).

The gonococcal porin is encoded by the porB gene, and a given strain possesses only one of two porB alleles that encode either PorB_{1A} or PorB_{1B} (Knapp, Tam et al. 1984, Carbonetti, Simnad et al. 1988). The exact sequences of the two alleles can differ between strains, but depending on the strains compared, the alleles demonstrate 65 to 80% homology (Judd 1989). The PorB_{1A} proteins are slightly smaller than PorB_{1B} proteins, with apparent molecular masses of approximately 35 kDa compared to ~37 kDa for PorB_{1B} proteins (Gotschlich, Seiff et al. 1987). Some of the differences between the two PorB proteins and within each subclass have been exploited in various serotyping methods (Knapp, Tam et al. 1984, Sarafian and Knapp 1989).

Strains carrying PorB_{1A} are most often associated with disseminated gonococcal infection (Cannon, Buchanan et al. 1983). These strains are more invasive than PorB_{1B} strains (van Putten, Duensing et al. 1998) and are also more resistant to killing by human serum (Schoolnik, Buchanan et al. 1976, Carbonetti, Simnad et al. 1990). Two mechanisms by which PorB_{1A} interferes with complement binding have been described. It can bind C4b binding-protein, a component of the classical complement pathway, through interactions with the N-terminal surface-exposed loop (Ram, Cullinane et al. 2001). It can also resist complement attack by binding factor H, a regulatory protein in the alternative complement pathway, with its fifth surface-exposed loop (Ram, McQuillen et al. 1998). Together, these properties of PorB_{1A} may explain its increased ability to disseminate from the initial site of infection.
In contrast to the invasiveness of strains possessing PorB$_{1A}$, strains carrying PorB$_{1B}$ more often cause localized infections. This allele is also associated with increased antibiotic resistance. PorB$_{1B}$ strains demonstrate a lower baseline susceptibility to penicillin and erythromycin compared to strains with PorB$_{1A}$ (Woodford, Bindayna et al. 1989, Carbonetti, Simnad et al. 1990). However, PorB$_{1B}$ is able to develop mutations that can lead to resistance to penicillin, tetracycline, and other antimicrobials (Olesky, Hobbs et al. 2002).

1.4 Infection

1.4.1 Adhesion and invasion

Of the structural components described in the previous section, TFP, LOS, Opa, and porin are involved in mediating the attachment to and invasion of epithelial cells by gonococci. Pili mediate the initial attachment to host epithelial cells (Swanson 1973, McGee, Johnson et al. 1981, Merz, Rifembery et al. 1996, Mosleh, Boxberger et al. 1997), and PilC has been implicated as the adhesin component of the pilus (Rudel, Scheurerpflug et al. 1995). However, the identity of the host receptor for pilus-mediated bacterial attachment remains controversial. The host protein CD46 was demonstrated to be a receptor for PilC binding in ME180 cervical carcinoma cells (Källström, Liszewski et al. 1997, Källström, Blackmer Gill et al. 2001), but other studies have shown CD46-independent pilus binding (Tobiason and Seifert 2001, Kirchner, Heuer et al. 2005). In cervical epithelial cells, the host receptor for pilus-mediated binding has been identified as complement receptor 3 (CR3), and this interaction is also dependent on porin (Edwards, Brown et al. 2002). In some
tissues, including primary male urethral epithelial cells, the pilus has been shown to interact with the \( \alpha_1 \beta_1 \) and \( \alpha_2 \beta_1 \) integrins (Edwards and Apicella 2005).

Following the initial TFP-mediated contact with host cells, Opa proteins are required for tight binding to host cells. Gonococci express multiple Opa proteins, and these different Opa proteins bind to one of two types of mammalian receptors: carcinoembryonic antigen cell adhesion molecules (CEACAMs) or the heparin sulfate proteoglycans (HSPGs). Opa variants that bind to HSPGs are less common than those variants that bind CEACAMs. In some cell lines, Opa binding to HPSGs is sufficient for invasion (Chen, Belland et al. 1995); however, the majority of Opa proteins bind to CEACAMs (Dehio, Gray-Owen et al. 1998, Gray-Owen 2003). The CEACAM family receptors are differentially expressed by human tissues (Thompson, Grunert et al. 1991). Of the at least 12 CEACAM receptors, CEACAM1, CEACAM3, CEACAM5, and CEACAM6 are known to serve as receptors for the Opa proteins. Epithelial cells express CEACAM1, CEACAM5, and CEACAM6. Neutrophils express CEACAM3 and smaller amounts of CEACAM1 and CEACAM6. CEACAM6 is also found on monocytes, and Opa proteins also interact with CEACAM1 on T and B lymphocytes (Wang, Gray-Owen et al. 1998, Sadarangani, Pollard et al. 2011).

The interaction between Opa proteins and the CEACAM molecules on epithelial cells is sufficient to allow the cells to be engulfed, undergo transcytosis, and ultimately gain access to the submucosa (Wang, Gray-Owen et al. 1998, Gray-Owen 2003). The binding of gonococcal Opa proteins to CEACAM3 on neutrophils triggers a signaling cascade resulting in actin reorganization and the phagocytosis of the bacterium. The internalized bacteria are killed by a subsequent oxidative burst (Chen and Gotschlich 1996, Gray-Owen, Dehio et al. 1997, Sadarangani, Pollard et al. 2011). CEACAM1 is expressed by both T and B cells,
interactions between Opa proteins and the CEACAM1 receptor on these cells may result in immune suppression. The binding of Opa proteins to CEACAM1 can suppress the activation and proliferation of CD4+ T cells (Boulton and Gray-Owen 2002). In B cells, Opa binding can inhibit antibody production and induce cell death (Pantelic, Kim et al. 2005).

Using microscopy to observe interactions between the gonococcus and host cells, a number of labs have described the gross morphological changes in host epithelial cells during infection. Bacterial cells initially attach in clusters of 10 to 100 diplococci on the surface of the cells (Griffiss, Lammel et al. 1999), prompting the extension of the host cell microvilli towards the bacteria (Ward and Watt 1972, Shaw and Falkow 1988) and the formation of cortical plaques (Merz, Enns et al. 1999). Later in infection (6-18 h), the bacteria spread across the epithelial surface as individual diplococci, become tightly associated with the host membrane, and are subsequently engulfed by the host cells (McGee, Johnson et al. 1981, Shaw and Falkow 1988, Griffiss, Lammel et al. 1999). Following internalization, gonococci are observed in both the cytosol and within vacuoles. At approximately 40 hours post-infection, the bacteria egress from the epithelial cells and colonize the subepithelial tissues (McGee, Johnson et al. 1981, Apicella, Ketterer et al. 1996, Mosleh, Boxberger et al. 1997).

1.4.2 Bacterial defenses against the host immune response

As demonstrated by the fact that patients can be reinfected by N. gonorrhoeae, even by the same strain, the host immune response to gonococcal infections is not protective. In addition to its capacity to alter its surface components via phase and antigenic variation, N.
*gonorrhoeae* possesses multiple mechanisms to resist killing by the innate immune system and to suppress the adaptive immune response.

One way by which the innate immune system kills bacteria is through activation of the complement system. Activation of complement can lead to bacterial lysis, opsonization, and phagocytosis. However, several gonococcal surface structures are able to bind components of the complement system, preventing its activation and evading lysis and phagocytosis. Serum resistance in gonococci can be variable or stable. Variable serum resistance is mediated by the binding of factor H to sialylated LOS, and serum resistance can be restored to sensitive strains by adding 5'-cytidinemonophospho-N-acetyl neuramic acid (CMP-NANA), a sialyl residue donor, to the media (Parsons, Andrade et al. 1989, Ram, Sharma et al. 1998). Factor H is a cofactor for the cleavage of C3b to the inactive iC3b. When GC are bound to factor H, nearly all of the C3b that they are associated with becomes converted to iC3b, thus preventing complement-mediated killing. Stable serum resistance is mediated by the binding of complement components to porin. Loop 5 of PorB1A binds factor H, and loop 1 binds to C4b-binding protein (Ram, McQuillen et al. 1998, Ram, Cullinane et al. 2001). These interactions inhibit all three complement activation pathways.

Following infection with *N. gonorrhoeae*, neutrophils are recruited to the site of the infection. Neutrophils phagocytose bacteria and typically kill them via the production of reactive oxygen species (ROS). However, some gonococci are able to resist killing by neutrophils and are even able to replicate within the phagocytes (Simons, Nauseef et al. 2006, Criss, Katz et al. 2009, Johnson and Criss 2011). GC possess multiple mechanisms to resist oxidative stress. They produce superoxide dismutase (SOD), an enzyme that catalyzes the production of hydrogen peroxide (H₂O₂) from superoxide (O₂⁻) (Seib, Wu et
al. 2006); the hydrogen peroxide can then be neutralized by conversion to water and oxygen by catalase (KatA) and cytochrome c peroxidase (CCP) (Archibald and Duong 1986, Turner, Reid et al. 2003, Stohl, Criss et al. 2005). Superoxide and hydrogen peroxide can also be quenched by the manganese ion (Mn$^{2+}$), and gonococci import Mn$^{2+}$ into the bacterial cytoplasm via the MntABC transporter (Tseng, Srikhanta et al. 2001).

The ability of *N. gonorrhoeae* to vary its surface structures allows the gonococcus to evade antibody-mediated immune responses. There is a growing body of evidence that *N. gonorrhoeae* actively suppresses cell-mediated immunity as well. A study conducted using the mouse model of gonococcal infection showed that infection with *N. gonorrhoeae* increased the production of TGF-β, and this cytokine subsequently enhanced the Th17 response while inhibiting the differentiation and proliferation of Th1 and Th2 cells. These T-helper cells are involved in activating both CD8+ cytotoxic T-cells and antibody-producing plasma cells (Feinen, Jerse et al. 2010, Liu, Feinen et al. 2011, Liu, Islam et al. 2012). As described above, interactions between neisserial Opa proteins and CEACAMs expressed on activated CD4+ T cells also suppress the T-helper cell response (Boulton and Gray-Owen 2002), and Opa-CEACAM interactions on B cells can suppress the antibody response (Pantelic, Kim et al. 2005).

**1.4.3 Murine model of gonococcal infection**

Although humans are the only natural host for *N. gonorrhoeae*, a mouse model has been developed that allows for the investigation of GC pathogenesis in vivo. Early attempts to establish a long-term colonization of the female mouse genital tract with GC failed because colonization is dependent on the stage of the estrous cycle. Mice could be
colonized, but they then cleared the infection upon entering the post-ovulatory stages (Streeter and Corbeil 1981, Johnson, Tuffrey et al. 1989). To circumvent this issue, the current model of murine gonococcal infection, primarily developed by Ann Jerse’s group, uses estradiol treatment to make the genital tract of female BALB/c mice amenable to colonization (Taylor-Robinson, Furr et al. 1990, Jerse 1999). In this model, mice are injected with water-soluble estradiol on days -2, 0, and 2 of the infection and inoculated with *N. gonorrhoeae* on day 0. GC can be recovered on vaginal swabs for approximately 12 days post-infection until the infection is naturally cleared (Jerse 2011).

The development of the mouse model of gonorrhea made it possible to study the infection *in vivo* without using the human male urethral challenge mode. However, it does have some limitations. Many of the host proteins that the bacterium interacts with in its human host, including the pilus receptor CD46 and Opa-interacting proteins CEACAM1, 3, 5, and 6, are either not expressed by mice or have species-specific amino acid changes that prevent recognition by the bacterium. The murine complement proteins factor H and C4bp-binding protein also fail to bind to GC (Jerse 2011). Nevertheless, the murine model of gonorrhea has been used to study the immune response to GC and to evaluate the fitness cost or advantage of different strains of GC (Wu and Jerse 2006, Song, Condron et al. 2008, Warner, Shafer et al. 2008, Feinen, Jerse et al. 2010, Hobbs, Anderson et al. 2013).

In chapter 3 of this dissertation, we describe the use of the murine model of gonorrhea to evaluate the *in vivo* activity of a novel class of therapeutics against gonorrhea.
1.5 Genetic transformation

1.5.1 Natural competence

*N. gonorrhoeae* is one of the few naturally competent bacterial species (Johnsborg, Eldholm et al. 2007) (other naturally competent bacteria include *Streptococcus pneumoniae* and *Haemophilus influenzae*). The gonococcus is capable of acquiring DNA from its environment and recombining it into its chromosome, provided that the exogenous DNA contains a *Neisseria*-specific 10-bp uptake sequence and has regions of homology (generally >80%) with existing chromosomal DNA (Goodman and Scocca 1988, Elkins, Thomas et al. 1991). The ability of the gonococcus to take up and integrate exogenous DNA into its chromosome provides a means for the bacteria to undergo genetic variation and to become resistant to antibiotics and has aided the development of tools for the genetic manipulation of the organism. In contrast to the majority of the other naturally competent bacteria, *N. gonorrhoeae* remains competent for transformation throughout all phases of growth (Sparling 1966, Biswas, Sox et al. 1977).

Unlike many bacteria, DNA uptake in *N. gonorrhoeae* is sequence specific. Efficient DNA uptake by recipient cells occurs only if a specific DNA uptake sequence (DUS; 5’-GCCGTCTGAA-3’) is present in the free DNA (Graves, Biswas et al. 1982, Goodman and Scocca 1988, Elkins, Thomas et al. 1991). Some DUSs have an additional 2 bp at the 5’ end of the 10-bp DUS (AT), and the presence of this 12-bp DUS results in a transformation efficiency that is slightly higher than those obtained with the 10-bp DUS (Ambur, Frye et al. 2007). The 10- or 12-bp DUSs are distributed throughout the gonococcal chromosome and are also found in other *Neisseria* species. The DUS appears 1965 times in the 2.15 Mb genome of the strain FA1090 (approximately once every 1100 bp) (Davidsen 2004).
Although it occurs at reduced efficiency, some strains can also be transformed with DNA that lacks the DUS; however, the mechanism behind this non-specific uptake remains unclear (Duffin and Seifert 2010).

1.5.2 Sources of DNA

Because the gonococcus is an obligate human pathogen, the sources of DNA for transformation are other co-infecting strains of gonorrhea, the closely related pathogenic species, *N. meningitidis*, and the commensal species *N. lactamica, N. cinerea*, and *N. flavescens* (Hamilton and Dillard 2006). Notably, transformation with DNA from commensal *Neisseria* species contributes to the formation of an altered PBP allele that confers increased resistance to penicillin in *N. gonorrhoeae* (Spratt, Bowler et al. 1992) and mosaic PBP alleles that confer resistance to the expanded-spectrum cephalosporins, ceftriaxone and cefixime. DNA is released from these donor bacteria via autolysis or type IV secretion (Hamilton and Dillard 2006).

The type IV secretion system (T4SS) of *N. gonorrhoeae* is encoded on the gonococcal genetic island (GGI). The GGI is found in approximately 80% of gonococcal strains, and the strains that possess the GGI can secrete DNA into the extracellular environment via the T4SS (Dillard and Seifert 2001, Hamilton, Domínguez et al. 2005). DNA is secreted into the environment via the gonococcal T4SS and not directly into other cells, unlike other T4SSs that secrete DNA in a contact-dependent manner directly into recipient cells (Ramsey, Woodhams et al. 2011). Little is known about the regulation of DNA secretion via the T4SS, but co-culture experiments used to study the transfer of DNA showed that the majority of the DNA transfer occurs when the cells are in logarithmic *in vitro* growth (Dillard and...
Seifert 2001). This is in contrast with DNA donation that occurs due to the lysis and death of donor cells, which primarily occurs during the stationary phase of *in vitro* growth.

Autolysis occurs when cells are exposed to suboptimal growth conditions, including nutrient limitation (Hebeler and Young 1975). This process likely relies on the activity of amidases and LTs to breakdown the cell wall (Hebeler and Young 1975, Chan, Hackett et al. 2012). The DNA released due to the autolysis of the donor cells has been shown to be a viable source of DNA for transformation and can be taken up by recipient cells (Norlander, Davies et al. 1979).

1.5.3 DNA binding and uptake

The mechanism that mediates the initial binding of double-stranded DNA to the cell surface is unknown, but it occurs independently of the DUS and may involve Opa proteins and minor pilus proteins (Hill 2000, Aas, Løvold et al. 2002, Aas, Wolfgang et al. 2002). Once the DNA has made non-specific contact with the cell, the DUS binds to an unknown protein on the cell surface. The factors required for uptake of the DNA into the cell have been better characterized. Studies in the 1970s observed that piliated cells transformed much more readily than non-piliated gonococci (Biswas, Sox et al. 1977), and many of the components of the TFP have been shown to be essential for competence (Rudel, Facius et al. 1995, Wolfgang, Lauer et al. 1998, Zhao, Tobiason et al. 2005).

Although it has not been proven that DNA crosses the outer membrane of the gonococcus through the PilQ secretin, studies in other bacterial species suggest that this is the case. PilQ from *N. meningitidis* has been shown to bind DNA (Assalkhou, Balasingham et al. 2007), and the PilQ homolog of the thermophilic species *Thermus thermophilus* HB27
has been identified as a DNA transporter (Burkhardt, Vonck et al. 2011). Several proteins that are not involved in the biogenesis or function of the TFP have also been shown to be involved in competence after the DNA has crossed the outer membrane. ComP and PilV are pilin-like proteins that affect transformation efficiency and are important for specific DNA binding (Aas, Løvold et al. 2002). The lipoprotein ComL is located in the periplasm and may help traffic DNA through the peptidoglycan layer (Facius, Fussenegger et al. 1996, Fussenegger, Facius et al. 1996). The protein ComE is also located in the periplasm and binds DNA in a non-specific manner (Chen and Gotschlich 2001), but its role in competence has not yet been clearly defined. While the DNA is in the periplasm, some of the double-stranded DNA (dsDNA) is converted to single-stranded DNA (ssDNA), but the proteins involved in this process have not been identified (Biswas and Sparling 1981, Chaussee and Hill 1998). The protein ComA is predicted to be associated with the inner membrane where it may play a role in transporting DNA into the cytoplasm (Facius and Meyer 1993).

1.5.4 DNA integration

Once in the cytoplasm, ssDNA with sequence homology to a region of the chromosome can be integrated into the chromosome at that region via homologous recombination. This process requires the protein RecA, as RecA-deficient strains are not transformable (Koomey and Falkow 1987). N. gonorrhoeae possesses both the RecBCD and RecF pathways of recombination; both of these pathways are dependent on RecA (Koomey and Falkow 1987). However, RecF is dispensable for transformation, and strains lacking any one of the three components of the RecBCD pathway display an approximate 40-fold
reduction in transformation (Mehr and Seifert 1998). Therefore, there may be an additional, uncharacterized pathway involved in homologous recombination in gonococci.

1.6 Antibiotic resistance

1.6.1 History of antibiotic resistance

Because of the multitude of mechanisms that gonococci have developed to evade the host, antibiotic treatment is necessary to clear infections. However, *N. gonorrhoeae* has developed resistance to every antibiotic that has been used to treat gonorrhea over the last 70 years. Sulfonamides were the first antibiotic to be used to treat gonorrhea. The first reports of sulfonamide therapy for gonococcal infections appeared in 1937, but military reports from World War II show that treatment failures had become common by 1944 (Dunlop 1949, Kampmeier 1983). The development of resistance to the sulfa drugs in the 1940s foreshadowed the pattern of antibiotic implementation and the development of resistance a decade or two later that continues today.

Penicillin is perhaps the most “famous” antibiotic and was used to treat gonorrhea beginning in the mid-1940s. It was effective for over 4 decades, but decreased susceptibility to penicillin due to the acquisition of chromosomal mutations began to emerge in the late 1950s (Willcox 1970, Faruki, Kohmescher et al. 1985). The MICs of penicillin for gonococcal strains kept increasing until resistance (defined as an MIC ≥ 2 µg/ml) became widespread in the mid 1980s. During the time that gonococcal susceptibility to penicillin was decreasing, other treatment options had been developed and were used in lieu of penicillin. Antibiotics such as chloramphenicol, erythromycin, and
tetracycline were commonly used in the 1960s to the 1980s, until resistance again necessitated a switch to newly developed drugs (Figure 1.4). The second-generation fluoroquinolone ciprofloxacin was approved by the FDA in 1987 and was highly active against strains of *N. gonorrhoeae* that displayed resistance to other antibiotics. However, the emergence and spread of strains carrying mutations in DNA gyrase and DNA topoisomerase IV (*gyrA* and *parC*, respectively) during the mid-1990s resulted in the removal of fluoroquinolones from the recommended treatment guidelines in 2007 (Belland, Morrison et al. 1994, Centers for Disease Control and Prevention. 2007).

After removal of fluoroquinolones as recommended antibiotics for treatment of gonorrhea, the expanded spectrum cephalosporins (ESCs) were the only recommended antibiotics remaining that were effective against gonorrhea. These drugs, which include cefixime and ceftriaxone, have a similar mechanism of action to penicillin but remained effective against penicillin-resistant gonococci. Unfortunately, and predictably, mutations decreasing the susceptibility of *N. gonorrhoeae* to the ESCs have emerged over the last decade (Barry and Klausner 2009, Tapsall, Ndowa et al. 2009), and treatment failures with cefixime and ceftriaxone have been reported in Asia and Europe (Ison, Hussey et al. 2011, Ohnishi, Saika et al. 2011, Unemo, Golparian et al. 2011, Unemo, Golparian et al. 2012). In 2010, a strain of gonorrhea was isolated from the pharynx of a sex worker in Japan following treatment failure with ceftriaxone. This strain, designated H041, was found to have an MIC of 8 μg/mL for cefixime and 2-4 μg/mL for ceftriaxone (Ohnishi, Saika et al. 2011, Kirkcaldy, Kidd et al. 2013).

The Gonococcal Isolate Surveillance Project (GISP) has collected data and monitored trends in antimicrobial susceptibility collected throughout the US since 1986 (Schwarcz,
Zenilman et al. 1990). Although treatment failure with the ESCs has not been reported in the United States, strains with elevated MICs for cefixime and/or ceftriaxone have been observed. The GISP defines cefixime MICs ≥0.25 μg/mL and ceftriaxone MICs ≥0.125 μg/mL as elevated. Data collected between 2006 and 2012 show that the percentage of isolates with elevated MICs has increased for both of these ESCs. For cefixime, the percentage of GISP isolates with elevated MICs was 0.6% in 2006, reached 1.4% in 2010, and was 1.1% for the first six months of 2012. The increase in the percentage of strains with elevated cefixime MICs was much higher among the isolates from MSM (0.2% in 2006, 3.9% in 2010, and 2.9% in the first half of 2012) (Kirkcaldy, Kidd et al. 2013). These data led to the decision by the CDC to remove oral cephalosporins, such as cefixime, from the recommended treatment list in 2012 (Centers for Disease Control and Prevention. 2012).

The increase in the percentage of GISP isolates with elevated ceftriaxone MICs between 2006 and 2012 was less dramatic than that of cefixime. In 2006, 0.05% of the isolates had a ceftriaxone MIC ≥0.125 μg/mL; this number increased to 0.3% in 2009 and remained at 0.3% through the first six months of 2012. Of the isolates obtained from MSM from January to June of 2012, 0.9% displayed an elevated MIC for ceftriaxone (Kirkcaldy, Kidd et al. 2013). While strains with elevated MICs for ceftriaxone comprise only a small percentage of the GISP isolates analyzed, the increase in the prevalence of these strains is worrisome. The CDC has historically used a resistance prevalence of less than 5% to recommend an antibiotic for the treatment of gonorrhea (Moran and Levine 1995), and because ceftriaxone is currently a component of the recommended treatment protocol, increased selective pressure may speed up the development and spread of resistant isolates.
**Figure 1.4** Timeline of antibiotic resistance in *N. gonorrhoeae*. The dates below the line show the first year in which each drug was introduced for the treatment of gonorrhea. The text above the line shows the year in which resistance to the antibiotic develop or the year in which the antibiotic was no longer recommended to treat gonorrhea.
1.6.2 Plasmid-mediated antibiotic resistance

Antibiotic resistance in the gonococcus can be plasmid-mediated or chromosomally mediated, with the latter mode much more common. Plasmid-mediated resistance confers much higher levels of resistance than chromosomally mediated resistance determinants, which depend on the accumulation of mutations in endogenous genes that act together to increase resistance just over the breakpoint of the antibiotics. To date, plasmids that confer resistance to β-lactam antibiotics and tetracycline have been identified in the gonococcus. β-lactam antibiotics disrupt the peptidoglycan layer by binding to and inactivating the enzymes (PBPs) that are responsible for cross-linking peptidoglycan. The β-lactam bond of the antibiotic mimics the d-Ala-d-Ala peptide bond involved in cross-linking, and reacts with PBPs to form a stable acyl-enzyme complex. β-lactamases catalyze the hydrolysis of the β-lactam bond of penicillin and other β-lactam antibiotics, thereby inactivating the antibiotic and conferring high levels of resistance. Plasmids encoding a β-lactamases first appeared in gonococcal isolates from Asia and Africa in the 1970s (Ashford, Golash et al. 1976, Phillips 1976). The β-lactamase allele commonly found in these plasmids is active against penicillin, but little to no activity against the expanded-spectrum cephalosporins. Plasmid-mediated resistance never became common worldwide for reasons that remain unclear.

In contrast to the plasmids encoding a β-lactamase, plasmids encoding the TetM determinant that confers high-level resistance to tetracycline became much more widespread and contributed to the removal of tetracycline from the recommended treatment regimen for N. gonorrhoeae (Centers for Disease Control and Prevention. 1985). Tetracycline inhibits protein synthesis by binding to the 30S ribosomal subunit and
blocking interactions between the ribosome and aminoacyl-tRNA. The TetM protein disrupts this interaction and renders TetM-containing isolates resistant to tetracycline (Sanchez-Pescador, Brown et al. 1988). The tetM gene was first described in Streptococcus species and has been identified in microorganisms that colonize the genital mucosa, including Mycoplasma hominis, Ureaplasma urealyticum, and Gardnerella vaginalis. It is transferred between bacteria on a conjugative plasmid, and it can be transferred between gonococcal strains by both conjugation and transformation (Morse, Johnson et al. 1986).

1.6.3 Chromosomally mediated antibiotic resistance

Chromosomal mediators of β-lactam antibiotic resistance are acquired by the gonococcus in a stepwise manner through transformation of a sensitive strain (e.g., FA19) by DNA from an antibiotic-resistant donor strain (e.g., FA6140). Although each resistance allele on its own confers only a small increase in the MIC, the combination of these alleles results in a 100- to 400-fold increase in the MIC of penicillin or ESCs and leads to clinical resistance and treatment failure (Faruki and Sparling 1986, Zhao, Duncan et al. 2009).

The mechanism of resistance to penicillin is complex and multifaceted. The first resistance determinant that is transferred is an altered allele of the penA gene. This gene encodes penicillin-binding protein 2 (PBP2), the primary lethal target of penicillin. The PBP2 variants encoded by altered penA alleles contain mutations that decrease the rate of acylation by penicillin by approximately 16-fold and increase the MIC for penicillin by approximately 5-fold over that of the wild-type strain (Powell, Tomberg et al. 2009). The most commonly found PBP2 mutations include the insertion of an aspartate residue at position 345a and an additional 4-8 mutations in the C-terminus of the protein (Dowson,
Jephcott et al. 1989, Brannigan, Tirodimos et al. 1990). These mutations in gonococcal PBP2 likely originated from homologous recombination with the penA alleles of commensal Neisseria species (Spratt, Bowler et al. 1992). The penA alleles that confer resistance to the ESCs contain many more mutations (between 50-70 mutations), and these mosaic alleles were also generated via homologous recombination with penA alleles from commensal Neisseria species (Ameyama, Onodera et al. 2002).

The second resistance determinant acquired by penicillin-susceptible strains results in the overexpression of the MtrC-MtrD-MtrE multi-drug efflux pump. Unlike the penA determinant, which is specific for β-lactam antibiotics, the mtr determinant reduces susceptibility to a number of hydrophobic antibiotics and detergents (Maness and Sparling 1973). The mtr locus includes a repressor, mtrR, three genes (mtrC, mtrD, and mtrE) encoding the MtrC-MtrD-MtrE efflux pump, and an accessory gene (mtrF) involved in high level resistance to Triton X-100 and other detergents. The mtrR and mtrCDE genes are transcribed in opposite directions and are separated by their overlapping promoter regions. MtrR binds to this region and regulates transcription of itself as well as mtrCDE (Lucas, Balthazar et al. 1997). Overexpression of the MtrC-MtrD-MtrE efflux pump can result from mutations in the overlapping promoter region. Hagman and Shafer characterized a single-base-pair deletion located between the -10 and -35 regions of the mtrR promoter that also disrupts the -35 region of the mtrC promoter. This deletion blocks transcription of the mtrR gene and increases expression of mtrC by threefold, thereby decreasing susceptibility to its target antibiotics (Hagman, Pan et al. 1995, Hagman and Shafer 1995). This single-base-pair deletion is the most common mtr mutation, but other mutants that result in the overexpression of the efflux pump have also been isolated. These
include missense mutations in the $mtrR$ coding sequence, a dinucleotide insertion in the $mtrR$ promoter region, and the $mtr_{120}$ mutation in the MtrR binding site upstream of $mtrC$ that occurs in MS11 (Hagman and Shafer 1995, Zarantonelli, Borthagaray et al. 2001, Cousin, Roberts et al. 2004, Warner, Shafer et al. 2008).

The third resistance determinant, $penB$, encodes a mutated form of the porin protein PorB$_{1B}$ that contains amino acid substitutions in loop three of the protein. This loop folds into the β-barrel and forms part of the transmembrane channel of the porin; the substitutions encoded by $penB$ are thought to constrict the pore and decrease the influx of antibiotics (Gill, Simjee et al. 1998, Olesky, Hobbs et al. 2002). Surprisingly, the $penB$ mutations are phenotypically silent in the absence of an $mtr$ mutation. They have no effect on antibiotic susceptibility alone but appear to act synergistically with the efflux pump by a mechanism that is not yet understood (Veal, Nicholas et al. 2002, Olesky, Zhao et al. 2006).

Two other determinants that contribute to penicillin resistance have also been described. The first of these, $ponA$, encodes an altered form of PBP1 with a decreased rate of acylation for penicillin. This determinant does not increase resistance when transformed into a strain that has been transformed to express the first three determinants (e.g., FA19 $penA$ $mtrR$ $penB$), but when the $ponA$ determinant of a penicillin-resistant strain is replaced with the wild-type allele, resistance decreases 2-fold (Ropp, Hu et al. 2002). While $ponA$ is involved in high-level resistance to penicillin, it does not contribute to resistance to the ESCs (Zhao, Duncan et al. 2009). The second determinant, $pilQ2$, encodes mutations in the outer membrane TFP secretin, $pilQ$, and can also decrease penicillin susceptibility, provided that the first three determinants, $penA$, $mtr$, and $penB$, are present. The $pilQ2$ mutation, which has only been identified in vitro, encodes a missense mutation (E666K) in
PilQ that decreases the stability of the multimer, which was suggested to form a pore through which antibiotics could diffuse into the periplasm (Ropp, Hu et al. 2002, Zhao, Tobiason et al. 2005). In the second chapter of this dissertation, we describe other mutations in PilQ with a similar mechanism as the pilQ2 mutation, as well as mutations that occur upstream of pilQ that confer increased resistance to multiple classes of antibiotics via polar effects on pilQ expression.

1.7 LpxC inhibitors as a novel class of antibiotics

The emergence of multi-drug resistant bacterial pathogens has necessitated research into the development of inhibitors of novel drug targets. One avenue of research in Gram-negative bacteria has been the development of inhibitors of LpxC, an enzyme that is essential for the synthesis of lipid A. Chapter 3 of this dissertation describes our work to validate LpxC as a novel drug target in *N. gonorrhoeae*.

1.7.1 Lipid A biosynthesis

The synthesis of lipid A has been best characterized in *E. coli*. In *E. coli*, nine enzymes are involved in the synthesis of lipid A from the sugar nucleotide UDP-GlcNAc (uridine diphosphate N-acetylglucosamine) (Figure 1.5). The first seven of these enzymes are conserved in *N. gonorrhoeae* (Preston, Mandrell et al. 1996). Fatty acid acylation of UDP-GlcNAc by LpxA is the first step in the lipid A biosynthetic pathway, but it is thermodynamically unfavorable (Anderson, Bull et al. 1993). Thus, the reaction catalyzed by LpxC, the deacetylation of UDP-3-O-(acyl)-GlcNAc, is the first committed step of the pathway (Anderson, Robertson et al. 1988, Young, Silver et al. 1995, Sorensen, Lutkenhaus
et al. 1996). LpxC, and lipid A in general, are essential in nearly all Gram-negative bacteria
(Onishi, Pelak et al. 1996). LpxC is a zinc-dependent enzyme that has no sequence identity
with mammalian deacetylases, and it thus represents a promising target for the
development of novel antibiotics against Gram-negative pathogens (Jackman 2000,
Clements, Coignard et al. 2002) (discussed in Chapter 3).

In the third step of the pathway, LpxD adds a second acyl chain to the site that was
deacetylated by LpxC, forming UDP-2,3-diacylglucosamine (Kelly, Stachula et al. 1993).
LpxH then cleaves the pyrophosphate bond to remove UDP to generate the products lipid X
and UMP (Babinski 2002). Lipid X is linked with another UDP-2,3-diacylglucosamine
residue by the glycosyltransferase LpxB, forming a disaccharide and releasing another UMP
molecule (Radika and Raetz 1988). This disaccharide molecule is then phosphorylated by
the membrane protein LpxK to form the product known as lipid IV<sub>A</sub> (Ray and Raetz 1987).
The next two steps of lipid A synthesis are catalyzed by the enzyme KdtA (also known as
WaaA) (Clementz and Raetz 1991). This enzyme adds two Kdo (2-keto-3-deoxyoctulosonic
acid) residues from the sugar nucleotide CMP-Kdo onto one of the glucosamine residues of
lipid IV<sub>A</sub>. The Kdo then serves as the link between the membrane-tethered lipid A and the
surface exposed oligosaccharide.

The final two steps in lipid A synthesis add two more acyl chains to generate Kdo<sub>2</sub>-
lipid A. The locations of these two final acylations differ between <i>E. coli</i> and <i>N. gonorrhoeae</i>. In <i>E. coli</i>
and other Enterobacteriaceae species, the distal glucosamine residue receives
both of the final two acyl chains; however, in <i>Neisseria spp</i>, each glucosamine unit receives
a single additional acyl chain (Kulshin, Zähringer et al. 1992, Preston, Mandrell et al. 1996).
Once assembled, lipid A needs to make its way from the periplasmic face of the inner membrane to the outer leaflet of the outer membrane. The mechanisms involved in the export of lipid A are poorly understood but have been best characterized in *E. coli*. The essential ABC transporter MsbA is thought to be the “flippase” that flips the newly synthesized lipid A from the cytoplasm to the periplasmic surface of the cytoplasmic membrane. This function is supported by data showing that overexpression of MsbA can suppress the intracellular accumulation of lipid A in strains in which the lauroyltransferase LpxL has been mutated (Karow and Georgopoulos 1993, Polissi and Georgopoulos 1996, Zhou 1998). Studies in *N. meningitidis* have shown that MsbA is not essential, but *msbA* mutants do express much lower amounts of LOS. Thus, MsbA is not absolutely required for LOS export in *N. meningitidis* but does appear to be involved in the process. Notably, *msbA* from *N. meningitidis* can partially complement an *E. coli* *msbA* mutant, suggesting that the homologs have similar functions (Tefsen 2005). However, because lipid A is not essential in *N. meningitidis* but is required in *N. gonorrhoeae*, the requirement for MsbA may also differ between the two species.

The O-antigen oligosaccharide is thought to be flipped from the cytoplasmic face of the membrane to the periplasmic surface of the inner membrane by the enzyme Wzx (Feldman, Marolda et al. 1999). The oligosaccharide is then polymerized by the enzymes Wzy and Wzz, and the resulting polysaccharide is attached to lipid A by the ligase WaaL (Morona, van den Bosch et al. 1995). The periplasmic proteins LptA, YbrK, and LptB have been implicated in the transport of the fully assembled LPS to from the inner to the outer membrane, but the mechanism behind this trafficking remains entirely unknown (Sperandeo, Cescutti et al. 2006). Once LPS makes its way to the periplasmic surface of the
outer membrane, the outer membrane protein Imp and the lipoprotein RlpB are required for the transfer of LPS to the exterior surface of the cell (Braun and Silhavy 2002, Bos, Tefsen et al. 2004, Wu, McCandlish et al. 2006).
Figure 1.5 The lipid A biosynthesis pathway in *E. coli*. Of the nine enzymes shown here, the first seven are conserved in *N. gonorrhoeae*. 
1.7.2 History of LpxC inhibitors

The lpxC locus was first identified by screening smooth colony morphology mutants of a penicillin-resistant *E. coli* strain and was termed envA. The envA mutant grew slowly, produced filamentous cells, and exhibited increased susceptibility to a variety of antibiotics (Normark, Boman et al. 1969). The envA mutant produced approximately 30% less LPS compared to wild-type cells (Grundström, Normark et al. 1980), and envA was later shown to be essential in *E. coli* (Beall and Lutkenhaus 1987). Experiments showing that extracts from the envA mutant possessed less UDP-3-0-acyl-GlcNAc deacetylase activity and that the overproduction of envA resulted in increased deacetylase activity identified envA as the gene encoding UDP-3-0-acyl-GlcNAc deacetylase in the lipid A biosynthesis pathway (Young, Silver et al. 1995). This gene was then renamed lpxC.

The discovery that hydroxamate-containing compounds inhibited LpxC but analogs that contained a carboxylic acid rather than the hydroxamic acid did not provided the first evidence that LpxC might require a metal cofactor for activity (Onishi, Pelak et al. 1996). This hypothesis was further investigated in a study that showed that the activity of LpxC from *Pseudomonas aeruginosa* was inhibited by the addition of ethylenediaminetetraacetic acid (EDTA) to cell extracts and that deacetylase activity was restored by the addition of zinc to the extracts (Hyland, Eveland et al. 1997). Definitive proof that LpxC is a zinc metalloenzyme was obtained in a study that assayed the deacetylase activity of purified *E. coli* apo-LpxC incubated with varying concentrations of Zn$^{2+}$. This study also showed that metal chelators inhibited the enzymatic activity of the purified LpxC (Jackman, Raetz et al. 1999).
The structure of LpxC has been well-characterized by several groups. The tertiary structure of LpxC shows that the enzyme is composed of two domains that each consist of a 5-stranded β-sheet and 2 α-helices. The domains assemble in a sandwich with the helices on the interior, and residues on two of the helices interact with the zinc ion. The domains also have unique subdomains that together form the active site pocket at one end of the α/β sandwich. This active site contains a hydrophobic tunnel that accommodates the acyl chain of UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc (Coggins, Li et al. 2003, Whittington, Rusche et al. 2003, Coggins, McClerren et al. 2005).

The availability of structural information has facilitated the understanding of how LpxC interacts with its natural substrate and how this can be exploited by inhibitors (Figure 1.6). The first inhibitors of LpxC were identified by screening for compounds that blocked the incorporation of radiolabeled galactose in live E. coli cells. The screen identified the compound L-573,655, which contains a hydroxamic acid attached to a 2-phenyl-oxazoline ring, as an inhibitor of LPS biosynthesis. Multiple derivatives of this compound were synthesized in an attempt to improve the activity of the inhibitors, and the most potent compound, L-161,240, was obtained by the addition of multiple hydrophobic groups to the phenyl ring. This compound was tested in a mouse model of septicemia and was able to protect mice from a lethal dose of E. coli. However, L-161,240 showed little to no activity against other bacterial species (Onishi, Pelak et al. 1996).

To expand the spectrum of LpxC inhibitors, Jackman and colleagues designed a new class of LpxC inhibitors with a structure based on that of the native substrate of LpxC. These inhibitors contained a hydroxamate group and an acyl chain attached to a tetrahydropyran ring. The most potent of these inhibitors, TU-514 and TU-517, were
extremely active against purified LpxC \textit{in vitro}. However, they failed to inhibit the growth of 
\textit{E. coli} cells, suggesting that this class of compounds was not able to cross the cell wall
(Jackman 2000).

The compounds BB-78484 and BB-78485 were identified by screening a library of
metalloenzyme inhibitors for antibacterial activity against a strain of \textit{E. coli}; their target
was then confirmed to be LpxC. These sulfonamide compounds contained two hydrophobic
ring groups and a central hydroxamate, and they showed activity against several other
species of bacteria, including \textit{H. influenza}, \textit{Moraxella catarrhalis}, \textit{Serratia marcescens}, and
\textit{Burkholderia cepacia}, in addition to \textit{E. coli}. Neither compound was active against \textit{P. aeruginosa} (Clements, Coignard et al. 2002).

The LpxC inhibitor CHIR-090 was described by McClerran \textit{et al.} in 2005. It is an \textit{N}-
aroxyl-\textit{l}-threonine hydroxamic acid that binds slowly and irreversibly to LpxC. CHIR-090
was able to inhibit the growth of \textit{E. coli} and \textit{P. aeruginosa} and represented a major step in
the development of a broad-spectrum LpxC inhibitor (McClerrren, Endsley et al. 2005).
However, species-specific differences in the shape of the substrate-binding passage
prevented the somewhat bulky scaffold of CHIR-090 from potently inhibiting the growth of
other bacterial species (Lee, Liang et al. 2011).

LpxC inhibitors based on a narrower scaffold are a promising improvement on the
structure of CHIR-090. The first paper to describe LpxC inhibitors based on a linear
diacetylene scaffold was published in 2011 (Lee, Liang et al. 2011); the activity of these
compounds against \textit{N. gonorrhoeae} is described in detail in Chapter 3. The diacetylene
compound LPC-009 was shown to have greater activity than CHIR-090 against a number of
clinically relevant Gram-negative pathogens. This paper also presented the crystal
structures of LpxC orthologs from three different bacterial species in complex with LPC-009, revealing structural variations in the hydrophobic passage that binds the acyl chain of the physiologic substrate of LpxC. However, the flexibility of the diacetylene scaffold allowed LPC-009 to inhibit LpxC from various bacterial species despite differences in the conformation of the enzyme (Lee, Liang et al. 2011).
Figure 1.6. LpxC inhibitors. (A) LpxC inhibitors block the second step in the synthesis of lipid A. (B) Representative LpxC inhibitors developed by various companies and academic laboratories, including the compound LPC-067 that will be discussed in Chapter 3.
1.8 Introduction to the dissertation

As antibiotic-resistant strains of *N. gonorrhoeae* continue to emerge and spread worldwide, it is becoming increasingly important to understand the mechanisms that this pathogen uses to evade antibiotic killing. Individuals infected with gonorrhea require antibiotics to clear the infection, but we are running out of treatment options. The goals of my work were to determine the mechanism by which mutations in PilQ increase resistance to existing antibiotics and to validate LpxC as a target for new antibiotics.

1.8.1 Contribution of PilQ to antibiotic resistance

Mutation or deletion of the outer membrane secretin PilQ in a strain containing the first three resistance determinants (*penA, mtr*, and *penB*) results in an approximately threefold increase in the MIC of penicillin, indicating that antibiotics are able to travel through PilQ to gain access to the periplasm. I used a variety of genetic and biochemical techniques to determine whether the mature, SDS-resistant PilQ multimer or the immature, SDS-labile monomer contributed to antibiotic permeation. Our data support a model in which PilQ subunits associate in an immature complex that allows antibiotics to diffuse across the outer membrane.

1.8.2 Validation of gonococcal LpxC as a target for antibiotics

The enzyme LpxC catalyzes the first committed step in the biosynthesis of lipid A, a major component of the outer membrane of Gram-negative bacteria. Lipid A is essential for viability of nearly all Gram-negative bacteria, as are the enzymes that are involved in its biosynthesis. However, one of the exceptions is the close relative of *N. gonorrhoeae*, *N.*
\textit{meningitidis}, which can survive without lipid A. Therefore, upon beginning this project, I demonstrated that LpxC is essential in \textit{N gonorrhoeae}. Next, I demonstrated that LpxC is the primary target of the inhibitors. I also investigated the effects of existing resistance determinants on the susceptibility of \textit{N. gonorrhoeae} to LpxC inhibitors and showed that the inhibitors are effective against multi-drug resistant strains of gonorrhea. Working with our collaborators who developed a murine model of gonorrhea, we found that an LpxC inhibitor was able to clear a gonococcal infection in eight out of nine treated mice.

\textbf{1.8.3 Characterization of a mutant with reduced susceptibility to LpxC inhibitors}

Although sensitivity to LpxC inhibitors is not greatly reduced by existing resistance mechanisms, resistance to these inhibitors is certainly possible. Understanding the mechanisms that \textit{N. gonorrhoeae} might use to develop resistance and knowing the extent to which the mutations decreased sensitivity could be important factors in prolonging the therapeutic lifetime of LpxC inhibitors. Therefore, I isolated spontaneous mutants with reduced susceptibility to the inhibitor LPC-067 and identified the mutation responsible for the phenotype of one of these mutants.
Chapter 2. Diffusion of antibiotics through the PilQ secretin in *Neisseria gonorrhoeae* occurs through the immature, SDS-labile form

2.1 Overview

Antibiotic permeation across the outer membrane of *Neisseria gonorrhoeae* is a key parameter that determines the effectiveness of antibiotics for this pathogen. In strains harboring the *mtrR* and *penB* determinants, mutation or deletion of the PilQ secretin of type IV pili increases resistance to penicillin by ~3-fold, indicating a role for PilQ in antibiotic permeation. In this study, we examined spontaneously arising mutants with decreased susceptibility to penicillin. All of these mutants appeared to be non-piliated by colony morphology. One class of mutants had a phenotype indistinguishable from a previously characterized pilQ2 mutation that interfered with the formation of SDS-resistant multimers. A second class of mutants contained frame-shift mutations in the genes upstream of *pilQ* in the *pilmNOPQ* operon that increased resistance to similar levels as the pilQ2 mutation. In-frame deletions of these genes were constructed, but only the frame-shift mutations increased antibiotic resistance, suggesting that the mutations had polar effects on PilQ. Consistent with this result, titration of wild-type PilQ levels revealed that resistance correlated directly with the levels of PilQ expression. To determine which form

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1 This chapter was adapted from a manuscript that has been submitted for publication to the Journal of Bacteriology. Contributing authors include Sobhan Nandi, Joshua Tomberg, and Robert A. Nicholas (Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599). Present address of Sobhan Nandi: Synerca Pharmaceuticals, Inc., Chapel Hill, NC 27599).
of PilQ, monomer or multimer, was responsible for antibiotic permeation, the levels of the two PilQ forms were quantified; while multimer levels were only slightly decreased in frame-shift mutants, the levels of PilQ monomers were decreased to a much larger extent. Importantly, deletion of pilW, which is responsible for the maturation of PilQ into SDS-resistant multimers, had no effect on resistance, indicating that mature, SDS-resistant PilQ complexes do not serve as the route of entry of antibiotics into the periplasm. These data instead suggest that immature, SDS-labile complexes serve as secondary entry channels for antibiotics.
2.2 Introduction

*Neisseria gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhea, is one of the most prevalent STIs worldwide, with an estimated 106 million infections per year (World Health Organization. 2012). There is no vaccine against *N. gonorrhoeae*, and because patients do not develop immunological memory, there is a high rate of reinfection (Hedges, Mayo et al. 1999). Because of the lack of an adequate immune response, antibiotic therapy is the only option for curing gonorrhea infections, but antibiotic resistance has rendered many of the available therapeutics ineffective. Historically, gonorrhea has been treated with penicillin, tetracycline, and fluoroquinolones, but these antibiotics were discontinued because of widespread resistance (1987, 2007). The current CDC recommendation for the treatment of gonorrhea in the U.S. is dual therapy with ceftriaxone and azithromycin or doxycycline; however, strains resistant to each of these antibiotics have been reported (Unemo, Golparian et al. 2010, Ohnishi, Saika et al. 2011, 2012, Unemo, Golparian et al. 2012), eliciting concern that we are ushering in an era of untreatable gonorrhea (Unemo and Nicholas 2012).

The gonococcus develops resistance to penicillin either through the acquisition of a plasmid encoding a TEM-1-like β-lactamase or by acquiring multiple chromosomal mutations that incrementally increase resistance in a stepwise manner (Cannon and Sparling 1984). Because *N. gonorrhoeae* is naturally competent, chromosomal resistance alleles can be transferred from a penicillin-resistant strain to a susceptible strain by transformation and homologous recombination (Faruki and Sparling 1986). Four known resistance determinants (and a fifth unknown determinant) contribute to penicillin resistance; three of these determinants (*penA*, *mtr*, and *penB*) have been extensively
characterized and have well-documented effects on resistance, while the contributions of the remaining determinants (penA and the unknown determinant) are more complicated or have not been identified molecularly (Ropp, Hu et al. 2002). While each determinant by itself increases resistance by only 2- to 8-fold, the combination of all five determinants results in a 400-fold increase in resistance to penicillin and subsequent treatment failure (Zhao, Duncan et al. 2009).

The first resistance determinant acquired by a susceptible strain is the penA allele, which encodes altered forms of penicillin-binding protein 2 (PBP 2), the lethal target of penicillin, with a decreased rate of acylation by penicillin and ceftriaxone (Dowson, Jephcott et al. 1989, Brannigan, Tirodimos et al. 1990). The second resistance determinant, mtr, is encoded by any one of a collection of mutations located in the overlapping promoter regions of mtrR and mtrCDE or within the coding sequence of mtrR that increase the expression of the MtrC-MtrD-MtrE efflux pump (Pan and Spratt 1994, Hagman, Pan et al. 1995). mtr mutations confer only a two-fold increase in the MIC of penicillin, but are necessary for high-level penicillin resistance in clinical isolates (Veal, Nicholas et al. 2002). The third resistance determinant, penB, encodes mutations in the outer membrane porin, PorB_{1B}. These mutations decrease the influx of penicillin (and other antibiotics) through porin channels, thereby reducing the concentrations of the antibiotic in the periplasm (Gill, Simjee et al. 1998, Olesky, Hobbs et al. 2002, Olesky, Zhao et al. 2006). Surprisingly, penB mutations are phenotypically silent (i.e. they confer no resistance) unless there is a co-resident mtr mutation (Olesky, Zhao et al. 2006).

Given the importance of antibiotic resistance in gonococci, it is important that we understand all aspects of antibiotic action in this organism. To this end, we have described
a spontaneously occurring mutation in PilQ (pilQ2) that increases resistance to penicillin and other antibiotics by 2- to 3-fold in a strain (FA19 penA mtrR penB) harboring the first three resistance determinants (Ropp, Hu et al. 2002, Zhao, Tobiason et al. 2005). PilQ is a major component of the outer membrane and is essential for type IV pilus function, including twitching motility, uptake of extracellular DNA, and cell attachment and invasion (Newhall, Wilde et al. 1980, Tonjum and Koomey 1997, Helm, Barnhart et al. 2007). The pilus is composed of a polymer of pilin subunits that extends and retracts through a pore in the outer membrane formed by a multimeric PilQ complex (Wolfgang, van Putten et al. 2000). When gonococcal membranes are analyzed by SDS-PAGE and Western blotting, PilQ is found in two forms: a mature, SDS-resistant multimer that runs near the top of the running gel, and an immature, SDS-labile form that runs at the size of a monomer (Collins, Davidsen et al. 2001, Collins 2004). The mutation in the pilQ2 allele (E666K) prevents maturation of the complex such that all of the PilQ subunits migrate as a monomer on SDS-PAGE; this strain also exhibits a non-piliated colony morphology and a 50-fold decrease in transformation efficiency (Zhao, Tobiason et al. 2005). These data were consistent with a model in which the mature, SDS-resistant oligomer functions as a pore through which antibiotics and small molecules could diffuse into the periplasmic space, with the pilQ2 mutation preventing the formation of the mature oligomer and leading to an increase in antibiotic resistance.

In this study, we further investigated the influence of PilQ on antibiotic permeability. We isolated a number of spontaneously arising non-piliated mutants with reduced susceptibility to penicillin. Some of these mutants contained amino acid substitutions in the C-terminal portion of PilQ and had a phenotype identical to that of the
*pilQ2* mutant, i.e. loss of the SDS-resistant oligomer on SDS-PAGE. We also isolated resistant mutants with near-normal levels of PilQ oligomer but with frame-shift mutations in the genes upstream of *pilQ* in the *pilMNOPQ* operon. The effects of these frame-shift mutations on resistance were due to polar effects on PilQ, however, as in-frame deletions of the *pilMNOP* genes had no effect on penicillin resistance. Further examination of these mutants and others that allowed us to manipulate the levels of PilQ led us to develop a model in which PilQ subunits associate to form immature channels in the outer membrane, thus allowing antibiotics to enter the periplasm. In this model, the decrease in PilQ production in the frame-shift mutants results in the loss of immature PilQ oligomers, as most of the available subunits assemble into stable multimers that prevent antibiotics from entering the periplasm.
2.3 Materials and Methods

2.3.1 Bacterial strains.

*N. gonorrhoeae* strains FA19 (a penicillin-susceptible laboratory strain; MIC$_{\text{pen}}=0.01$ µg/ml) was kindly provided by Dr. Fred Sparling, University of North Carolina at Chapel Hill (Maness and Sparling 1973). PR100 (FA19 *penA mtrR penB*) and SZ3 (RM11.2 *penA mtrR penB*) have been previously described (Ropp, Hu et al. 2002, Zhao, Tobiason et al. 2005). RM11.2 (FA1090 *recA6*) has the *recA* gene under regulation of the *lac* operator/promoter (Seifert 1997). These strains and others used in this study are listed in Table 2.1.

We used SZ3 and PR100 interchangeably in this study. When each of the mutant genes described in this study were transformed into SZ3 and PR100, the resulting strains had nearly identical properties. Because the *pilE* allele can affect transformation efficiency, SZ3 was initially used in transformation efficiency experiments to prevent *recA*-dependent changes in *pilE* (Zhao, Tobiason et al. 2005), but PR100 was the parental strain in later experiments examining the complementation of *pilM*, *pilN*, *pilO* and *pilP* in-frame deletions. The only difference we observed between the two strains is that PR100 has a slightly higher MIC for penicillin (0.75 mg/ml) compared to SZ3 (0.5 mg/ml); introduction of the various mutations described herein resulted in the same fold increase in the MIC of penicillin whether the parental strain was PR100 or SZ3.
2.3.2 Bacterial media, growth conditions, and MIC determinations.

*N. gonorrhoeae* strains were grown at 37°C in a humidified 4% CO₂ incubator on GC medium base (GCB) agar plates fortified with supplements I and II (Kellogg, Peacock et al. 1963). *E. coli* strains were grown on Luria-Bertani plates or in 2×YT media as described (Ropp, Hu et al. 2002). For cloning in *E. coli*, genes were cloned into pUC18us (pUC18 containing the 10-bp gonococcal uptake sequence) or pGCC4, a complementation vector that places genes under control of the IPTG-inducible lac promoter/operator and allows for recombination into the silent intergenic region between lctP and aspC (Stohl, Brockman et al. 2003) in *N. gonorrhoeae*. Later experiments introducing an inducible, exogenous copy of pilQ into PR100 (described below) utilized pKH35, which is very similar to pGCC4, except that it has a more extensive polylinker and confers resistance to chloramphenicol instead of erythromycin. *E. coli* transformants were selected on either 100 mg/ml carbenicillin (pUC18us), 350 mg/ml erythromycin (pGCC4), or 34 µg/ml chloramphenicol. The plasmids were then used to transform gonococci using the protocol described below.

Minimum inhibitory concentrations (MICs) of antibiotics were determined by the spot method as described previously (Ropp, Hu et al. 2002). Briefly, cells were resuspended at a density of 1 × 10⁷ cells/ml and 5 µl (50,000 CFUs) were spotted onto GCB agar plates containing increasing concentrations of the indicated antibiotics. The MIC was defined as the lowest concentration at which no more than 5 colonies grew following incubation for 24 hr. MICs were determined for multiple transformants and represent the average ± S.E.M of at least three independent experiments.
2.3.3 Transformations and transformation efficiency assays.

Transformations were performed essentially as described previously. Because pilQ mutations and pilM, pilN, pilO, and pilP frame-shift mutations conferred resistance to penicillin, selection of these transformants was accomplished on penicillin plates. For pilM, pilN, pilO, and pilP in-frame constructs that did not confer increased penicillin resistance, the transformation mixture was plated on GCB plates with no selection, and colonies with a non-piliated morphology under the dissecting microscope were screened by colony PCR using gene-specific primers. All strains were verified by amplifying the appropriate regions by PCR with Pfu polymerase and sequencing the resulting DNA fragments. Transformants of pGCC4 plasmids into PR100 were selected on 6 mg/ml erythromycin, while transformants of pKH35 plasmids were selected on 3 µg/ml chloramphenicol. Because many of the constructs rendered the recipient strain non-transformable upon recombination, it was necessary to transform cells with the complementation vector first, followed by transformation of the mutant construct.

For quantification of transformation efficiency, piliated N. gonorrhoeae strains were passaged twice on GCB agar plates, resuspended in GCB + 10 mM MgCl₂ (GCB+) broth and diluted to $1 \times 10^8$ cells/ml. Twenty microliters of diluted cells and 100 ng of pSY6 DNA were added to 180 µl of GCB+ and incubated at 37°C with 5% CO₂ in a humid environment for 15 min. Following incubation, 1.8 ml GCB+ was added and the cells were incubated for a further 5 hours. Cells were then diluted with 10-fold serial dilutions in GCB+ broth, and 50 µl of the appropriate dilutions were plated in duplicate on GCB agar plates containing 1.5 µg/ml nalidixic acid. Isopropyl-b-D-thiogalactoside (IPTG) was added to GCB+ at 1 mM for transformations in SZ3 to induce expression of recA. To determine the total number of
colony-forming units (CFUs) in the transformation mixture, 50 μl of a 1 × 10^{-4} dilution was plated in duplicate on GCB agar plates. The transformation efficiency was calculated as the number of colony-forming units (cfu) on selective media/number of cfu in the transformation mixture.

2.3.4 Isolation of spontaneously arising penicillin-resistant clones.

To identify spontaneously arising mutations conferring resistance to penicillin, piliated PR100 or SZ3 were prepared as described for the transformation protocol, except that cells were plated directly (without addition of DNA) on GCB agar plates containing concentrations of penicillin just above their respective MICs (0.75 and 0.5 mg/ml, respectively). Spontaneously arising penicillin-resistant clones, all of which were non-piliated by colony morphology, were passaged onto fresh selective media for further analysis. A total of 615 clones were isolated and characterized.

All of the clones were analyzed by Western blotting with anti-PilQ antibody as described below. Those colonies that showed a similar phenotype as a pilQ2 mutant (i.e. loss of the SDS-resistant oligomer) were prepared for colony PCR with pilQ-specific primers. Briefly, cells were transferred to 25 μl water, the mixture boiled for 5 min, and 5 μl of the cleared lysate was amplified with either Pfu or Taq polymerase. The PCR products were sequenced directly at the UNC sequencing facility. For resistant clones that showed a PilQ phenotype similar to wild-type, the genes upstream of pilQ (pilM-pilP) were amplified and sequenced.
2.3.5 Scanning electron microscopy.

SZ3 and three spontaneously isolated mutants of SZ3 (G668S, insertion 631, and E712K) were grown to mid-log phase in GCB broth, and 500 μl of the suspension was pelleted via centrifugation. The cells were resuspended in 500 μl of a 3% glutaraldehyde, 0.15 M sodium phosphate buffer (pH 7.4), fixed at room temperature for 30 minutes, and stored at 4°C. The fixed cell suspension was then applied to 12 mm round poly-l-lysine coated coverslips and incubated for 2 hours at RT in a humid environment (BD BioCoat # 354085, BD Biosciences, San Jose, CA). After washing the coverslips three times with 0.15 M sodium phosphate buffer, pH 7.4, they were dehydrated with increasing concentrations of ethanol (30%, 50%, 75%, and 100%). The dehydrated coverslips were dried in a Samidri-795 critical point drier with carbon dioxide as the transitional solvent (Tousimis Research Corporation, Rockville, MD). The coverslips were mounted on 13 mm aluminum stubs and sputter coated with 10 nm of a gold-palladium alloy (60Au:40Pd, Hummer X sputter coater, Anatech, Ltd., Alexandria, VA). The bacterial cells were observed using a Zeiss Supra 25 FESEM operating at 5 kV, 5 mm working distance, and 10 μm aperture (Carl Zeiss SMT Inc., Peabody, MA).

2.3.6 Construction of pilM, pilN, pilO and pilP frame-shift and in-frame deletion mutants.

Genes were amplified from FA19, and the PCR products were cloned into pUC18us. Clai, Bcel and BsmI sites in pilM, pilN and pilP, respectively, were eliminated by digesting with the specified endonuclease, filling-in with Klenow fragment, and religating. For pilO, a silent Xbali site was introduced via Quik-change mutagenesis (Agilent Technologies, Santa
Clara, CA). All plasmids harboring frame-shift mutations in pilM, pilN, pilO and pilP were sequenced to confirm that the correct mutations were incorporated, and these plasmids were used to transform PR100 and SZ3 to decreased penicillin susceptibility. PCR amplification and digestion with the endonucleases listed above was used to confirm recombination of the mutant gene.

In-frame deletions in pilM, pilN, pilO, and pilP were created by amplifying 5’ and 3’ fragments of the gene of interest and cloning them into pUC18us. While the external primers contained different restriction sites at their 5’ ends, the internal primers contained a common restriction site that facilitated construction of the in-frame deletion. Each gene retained some 5’ and 3’ sequence, but approximately two-thirds of interior sequence was deleted. Specifically, the pilM in-frame deletion deleted codons 88-317 (bp 262 to 951), the pilN construct deleted codons 31-155 (bp 91 through 465), pilO was missing codons 26-170 (bp 76 through 510), and pilP was missing codons 31-144 (bp 91 to 432). Each deletion construct also contained ~300 bp of sequence both upstream and downstream of the deletion to facilitate successful recombination with chromosomal DNA. The in-frame deletion constructs were used to transform PR100, and because no selectable marker was transferred to the chromosome, non-piliated colonies were chosen for PCR screening to identify correct transformants. In-frame deletion clones were verified by sequencing and Western blotting.

2.3.7 pilW knock-out and overexpression.

An insertionally inactivated mutant of pilW was created by cloning pilW into pUC18us, digesting with BstXI and Hpal, filling in with T4 DNA polymerase, and ligating in a
kanamycin resistance cassette (from pUC4K). The kanamycin phosphotransferase gene was cloned in the same direction as the pilW gene. PR100 was transformed with the pUC18us-pilW::kan clone, and transformants were selected on GCB agar containing 50 µg/mL kanamycin. For overexpression of PilW, we utilized a plasmid (pUC18K-proAB-Q19e) containing the lacIg gene from pMJR200, the tac promoter/operator and multiple cloning site from pTTQ18, the erythromycin resistance gene, and flanking sequence from the gonococcal proAB operon to facilitate homologous transformation. pilW was cloned under the transcriptional control of the tac promoter, and the resulting plasmid was used to transform PR100 to erythromycin resistance (selected on 4 mg/ml erythromycin). To further increase PilW expression, pilW was cloned into pKH35. The different selectable markers allowed both expression constructs to be introduced into the same cell. Expression of PilW was induced by 0.1 to 10 mM IPTG.

2.3.8 Overexpression of PilQ.

Cloning of full-length pilQ in most vectors is toxic to E. coli. To avoid problems associated with expressing PilQ in E. coli cells while cloning, the 5’ and 3’ regions of pilQ were cloned separately. The 5’ end of pilQ, starting with a strong ribosome-binding site prior to the start ATG codon through base-pair 1180 was amplified and ligated into pKH35 as a Pac-Sacl fragment. This construct was transformed into N. gonorrhoeae strain FA19 and selected on chloramphenicol. The 3’ end of pilQ (base pairs 671-2196) were then amplified using a 5’ primer containing a HindIII site and a 3’ primer containing an XbaI site and an HA tag. The portion of the pKH35 vector that is downstream from the cloned gene was amplified using primers to add a 5’ XbaI site and a 3’ BamHI site, and this fragment was
cloned into the vector pUC18us along with the 3’ fragment of pilQ in a three-way ligation. The resulting plasmid therefore contained the 3’ end of pilQ with flanking sequences overlapping the pilQ sequence encoded in the first construct (overlap between base pairs 671 and 1180) and pKH35 vector sequence to mediate homologous recombination. To allow for selection of gonococcal transformants, a kanamycin resistance cassette was cloned into the XbaI site at the 3’ end of the pilQ sequence. This plasmid was then transformed into PR100 that had been previously transformed with the pKH plasmid containing the 5’ pilQ sequence. Because strain PR100 has reduced transformation efficiency, the entire IPTG-inducible pilQ locus was constructed in FA19, and genomic DNA was prepared from this strain and used to transform PR100 (transformations with genomic DNA occur at a higher frequency than transformations with plasmid DNA). The resulting strain, PR100 pKH35-pilQHA, therefore contained two copies of pilQ. Finally, this strain was then transformed with a construct that disrupts the endogenous copy of pilQ via the insertion of a spectinomycin resistance cassette. This strain, PR100 pKH35-pilQHA pilQ::Ω, contains only an IPTG-inducible copy of pilQHA.

2.3.9 Gel filtration.

Gel filtration was performed as previously described (Zhao, Tobiason et al. 2005). Briefly, FA19 cells were grown in liquid culture, and the cells were lysed with an Emulsiflex-C5 homogenizer (Avestin, Ottawa, Canada). The lysate was centrifuged at 100 000 g to isolate the membranes. PilQ proteins were extracted from the membranes via dounce-homogenization in 30 mM Tris, 1.6 mM NaCl, 2 mM EDTA, 2% SB-10, pH 8.0, and submitted to gel filtration on a Sephacryl S-500 column in 20 mM Tris, 500 mM NaCl, 1 mM
EDTA, 1.2% SB-10, pH 8. The eluted fractions were analyzed by Western blotting with PilQ antibody and quantified using QuantityOne software (Bio-Rad, Hercules, CA).

2.3.10 SDS-PAGE and Western blotting.

Cells were swabbed from GCB plates, resuspended in GCB+, and diluted to an OD$_{560}$ of 0.18 (~1 × 10$^8$ cells/ml). An aliquot (1 ml) of the diluted cells was centrifuged at 8,000 rpm for 3 minutes to pellet the cells, and 100-200 μl of SDS-PAGE loading buffer were added to each sample. The samples were heated in a boiling water bath for 5 minutes, and 5–10 μl were loaded onto SDS-polyacrylamide gels (8% for PilQ, 12% for all other proteins) and separated by electrophoresis. The proteins were then transferred onto nitrocellulose membranes overnight in 25 mM Tris, 192 mM glycine, 10% methanol and 0.02% SDS at 4 °C. The lower methanol concentration and presence of SDS appeared to increase the efficiency of transfer of the PilQ oligomer (Zhao, Tobiason et al. 2005). Following transfer, the membrane was rinsed in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) followed by blocking with 5% non fat dry milk in PBS-T for 1 hour at room temperature (RT), then probed with a 1:10,000 dilution of a rabbit PilM, PilN, PilO, PilP or PilQ polyclonal antibody for 2 hr at RT. Anti-PilP and PilQ antibodies were made by Charles E. Wilde III, Indiana University School of Medicine and obtained from Hank Seifert, Northwestern University. PilM, PilN, and PilO antibodies were produced by Covance (Princeton, NJ) from the purified recombinant proteins. The membrane was washed three times with PBS-T, and incubated for another hr with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ). After washing the membrane three times with PBS-T, the membrane was treated with
SuperSignal West Pico (Pierce Chemical, Rockford, IL) for 5 minutes at RT and expression of Pil protein bands were visualized by exposing the membrane to X-ray film (Kodak).

For quantitative Western blots, cell lysates were prepared as described above, and 15 μl of each sample were loaded on an 8% SDS-PAGE gel and run at 80V for approximately 150 minutes. Proteins were transferred to low fluorescence-polyvinylidene difluoride (LF-PVDF) membranes with a semi-dry transfer apparatus at 155 mA for 45 minutes in the presence of the transfer buffer described above. Membranes were blocked in PBS-T containing 5% non-fat dry milk and incubated with primary antibody overnight at 4 °C. Membranes were washed 3× in PBS-T, and incubated with an anti-rabbit Cy-3 conjugated secondary antibody (GE Healthcare, Piscataway, NJ) for 2 hours. Blots were washed twice with PBS-T, and once with PBS before imaging on a Typhoon 9400 imager (GE Healthcare). PilQ and PBP 1 bands were quantified using ImageQuant software.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>FA19</td>
<td>Penicillin-sensitive clinical isolate</td>
<td>29</td>
</tr>
<tr>
<td>PR100</td>
<td>FA19 $\text{penA mtrR penB}$</td>
<td>12</td>
</tr>
<tr>
<td>RM11.2</td>
<td>FA1090 containing $\text{recA}$ under IPTG regulation</td>
<td>30</td>
</tr>
<tr>
<td>SZ3</td>
<td>RM11.2 $\text{penA mtrR penB}$</td>
<td>22</td>
</tr>
<tr>
<td>PR100 $\text{pilQ2}$</td>
<td>PR100 containing a PilQ G666L mutation</td>
<td>22</td>
</tr>
<tr>
<td>PR100 $\text{pilW::kan}$</td>
<td>PR100 containing $\text{pilW}$ disrupted with a $\text{kan}^R$ cassette</td>
<td>This study</td>
</tr>
<tr>
<td>PR100 $\text{proAB-pilW pKH-pilW}$</td>
<td>PR100 containing two IPTG-inducible copies of $\text{pilW}$</td>
<td>This study</td>
</tr>
<tr>
<td>PR100 $\text{pKH35-pilQ}^{\text{HA}}$</td>
<td>PR100 containing an IPTG-inducible copy of $\text{pilQ}^{\text{HA}}$</td>
<td>This study</td>
</tr>
<tr>
<td>PR100 $\text{pKH35-pilQ}^{\text{HA}}$ $\text{pilQ::Ω}$</td>
<td>The above strain with a disrupted endogenous copy of $\text{pilQ}$</td>
<td>This study</td>
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2.4 Results

2.4.1 Phenotypic identification of spontaneously arising penicillin-resistant clones

We have shown previously that a spontaneously arising mutation in pilQ, pilQ-E666K (also referred to as pilQ2), increases resistance to penicillin and other antibiotics by 2- to 3-fold, provided that the strain also harbors the mtrR and penB resistant determinants (Zhao, Tobiason et al. 2005) that together decrease permeation through the outer membrane porin (Olesky, Zhao et al. 2006). The increase in resistance conferred by the pilQ2 mutation was nearly identical to that conferred by an insertionally inactivated allele. The pilQ2 mutation causes the PilQ secretin, which normally runs on SDS-PAGE as both a mature, SDS-resistant multimer and an immature, SDS-labile monomer, to migrate entirely as a 72 kDa SDS-labile monomer.

To examine in more detail how different mutations in pilQ regulate the formation and expression of SDS-resistant PilQ oligomers, we plated two intermediate-resistant strains, PR100 (FA19 penA mtrR penB; (Ropp, Hu et al. 2002)) and SZ3 (RM11.2 penA mtrR penB; (Zhao, Tobiason et al. 2005)) (Table 2.1), on GCB plates containing concentrations of penicillin slightly above the MIC of penicillin for the two strains (0.6 and 0.75 mg/ml for PR100 and SZ3, respectively). Spontaneously arising penicillin-resistant colonies, all of which had a non-piliated morphology under the dissecting microscope, were obtained with a frequency ranging from 3 x 10^-6 to 5 x 10^-6 for SZ3 and frequency of approximately 2.2 x 10^-6 for PR100. These colonies were analyzed by SDS-PAGE and Western blotting with an anti-PilQ antibody, revealing three distinct classes of mutants (a representative blot is shown in Figure 2.1A): i) Class 1 mutants had a phenotype identical to the pilQ2 mutants,
i.e. PilQ ran as an SDS-labile monomer and break-down products (Figure 2.1A, lanes 4, 5 and 13), ii) Class 2 mutants appeared to be similar to the parental strain (Figure 2.1A, lanes 3, 6-10), and iii) Class 3 mutants had either smaller fragments (i.e. unstable mutants) or a complete loss of pilQ (Figure 2.1A, lanes 11, 12 and 14). Of the 620 mutants analyzed, 14.9% were Class 1, 49.4% were Class 2, and 15.7% were Class 3.
Figure 2.1 Western blots for PilQ from spontaneous arising penicillin-resistant mutants of PR100. (A) Spontaneous mutations in PilQ were isolated by plating PR100 cells on GCB plates containing penicillin at concentrations just above the MIC. More than 650 non-piliated colonies with increased penicillin resistance were isolated. Western blots were performed on all 650 mutants, and three types of mutations were identified. A representative Western blot for PilQ is shown. (B) Thirteen different pilQ mutants were backcrossed into SZ3. A Western blot for PilQ from all 13 mutants is shown.
2.4.2 Characterization of class 1 mutants

Class 1 mutants had a phenotype identical to that of the pilQ2 mutant; therefore, we amplified and sequenced the pilQ gene from each of these mutants to identify the mutation(s) responsible for the loss of the SDS-resistant oligomer. Sequence analysis identified 13 distinct mutations (several were obtained multiple times) in PilQ that clustered within the C-terminal domain (aa 400-731) of PilQ: G441C, D472N, R483P, A550V, A585V, A585T, G617Y, DT619, a 7 amino acid insertion after I631, G668S, E709G, E712K, and P719T. We did not identify the previously described E666K mutation in pilQ2, although we did isolate a G668S mutation two amino acids distant. To ensure that other potential mutations in non-pilQ genes would not confound subsequent analyses, the mutant pilQ genes were backcrossed into SZ3 and clean mutants were selected for increased penicillin resistance and were verified by sequencing.

Western blots of lysates from the 13 backcrossed mutants (Figure 2.1B) revealed that PilQ from these strains migrated as SDS-labile monomers, although in four mutants, G688S, G441C, P719T, and D472N, the level of the SDS-resistant oligomer was markedly reduced but not eliminated (the levels varied based on the length of time and temperature of the heat treatment of the samples before loading the gel). To determine if the mutations interfered with the transport of PilQ to the outer membrane, we isolated outer membranes (Heckels 1977) from each of the 13 mutants and examined PilQ expression by Western blot. In each case, PilQ was present and showed the same banding pattern as in Figure 2.1B (data not shown). Therefore, these mutant PilQ variants, much like pilQ2, are transported to the outer membrane but are unable to mature into SDS-resistant multimers.
The effects of the different pilQ mutations on penicillin resistance and transformation efficiency were also determined. All of the mutant pilQ alleles increased the MIC of penicillin between 2.5- to 3-fold, the same fold increase as the previously identified pilQ2 mutation (Figure 2.2A). Increased MIC values were also observed for several other antibiotics, including ceftriaxone, vancomycin, and rifampicin (data not shown). Although each mutation conferred nearly identical increases in the MIC of penicillin, their effects on transformation competence were much more variable. Four of the mutations (G617W, I631-7aa insertion, R483P, and D619T) completely ablated transformation, five others (G441C, A550V, A585V, G668S, and P719T) had transformation efficiencies identical to the parental strain, and the remaining four mutations caused a disruption in transformation (~50- to 100-fold) similar in magnitude to that of the pilQ2 mutation (Figure 2.2B). These data reflect a lack of correlation between antibiotic influx and transformation efficiency.

Although the spontaneously isolated mutants exhibited a non-piliated colony morphology when observed under a dissecting microscope, the observation that many of the mutants remained competent for transformation led us to examine several of the mutants by SEM (Figure 2.2C). Three mutants with different transformation efficiencies were chosen: i) G668S, which exhibited a transformation efficiency equivalent to that of the parental strain SZ3, ii) the mutant with a 7 amino acid insertion at 631, which had a transformation efficiency below the limit of detection, and iii) E712K, which has a transformation efficiency similar to that of the pilQ2 mutant. Despite their non-piliated colony morphology, each of these three mutants expressed TFP, albeit in markedly reduced levels compared to the parental strain. Fewer cells expressed pili, and mutant cells that possessed pili had fewer pili than individual wild type cells. However, no correlation was
observed between the amount of pili produced by the mutants and their transformation efficiency.
**Figure 2.2** Phenotypes of spontaneously arising *pilQ* mutants. (A) The MIC of penicillin G was determined for the 13 strains carrying a distinct spontaneous mutation in *pilQ* as well as the parental strain (SZ3) and SZ3 *pilQ2*. (B) Transformation efficiencies of the 13 *pilQ* backcrossed mutants, SZ3, and SZ3 *pilQ2*. Each strain was transformed with a plasmid carrying a *gyrB* allele that confers resistance to nalidixic acid. SZ3 is a variant of strain RM11.2 that contains an inducible *recA* allele that allows for IPTG control of pilin antigenic variation. Each of the strains used in this experiment contained the same *pilE* sequence to minimize the influence of different PilE sequences on transformation efficiency. (C) Scanning electron microscopy was used to examine SZ3 and three spontaneous *pilQ* mutants.
2.4.3 Analysis of class 2 mutants

The class 2 mutants were interesting in that these strains appeared to have a PilQ phenotype identical to that of the parental strains. Because these strains were non-piliated by colony morphology, we suspected that the mutations either affected PilQ in a way that did not alter its migration on SDS-PAGE or that they mapped to a different gene in the pilus biosynthetic pathway. Sequencing of the pilQ from two of these strains showed a wild-type sequence. Because pilQ is the last gene of a large operon (pilMNOPQ), we next assessed whether mutations in the genes upstream of pilQ were responsible for the penicillin-resistant phenotype. Sequencing of the genes upstream of pilQ revealed that the pilM gene from both mutants contained a single base insertion after codon 19 that altered the reading frame of pilM. Analysis of additional class 2 mutants also revealed several mutants with frame-shift mutations in pilN.

To aid in subsequent analyses, we generated our own frame-shift mutations in pilM, pilN, pilO and pilP by digesting and filling-in a unique endogenous restriction site in plasmids harboring each of the genes, and then religating. These mutants were then used to transform SZ3 to increased penicillin resistance. All of the transformants were morphologically non-piliated as expected, and the pilM-engineered mutations appeared identical to the original frame-shift mutation that arose spontaneously. When these transformants were analyzed by Western blotting with an antibody against PilQ, the levels of the mature, SDS-resistant oligomer were very similar to the parental strain, whereas there was a marked decrease in the levels of the immature SDS-labile form and its breakdown products (Figure 2.3A). To examine whether the frame-shift mutations could be complemented, SZ3 was transformed with constructs that inserted an IPTG-inducible copy
of the wild-type gene into a silent region of the gonococcal genome, followed by transformation with the appropriate frame-shift \textit{pil} mutant (because the \textit{pil} mutants prevent subsequent transformation, this order of transformation was necessary). However, the expression of the intact gene with IPTG had no effect on penicillin resistance, suggesting that the effects of the frame-shift mutations were polar (data not shown).
Figure 2.3 Effects of frame-shift mutations. (A) After identifying spontaneously arising pilM and pilN frame-shift mutations in our screen, frame-shift mutations in pilM, pilN, and pilO were constructed in SZ3. These mutants were non-piliated and expressed similar levels of the SDS-resistant PilQ oligomer as the parental strain, but had decreased levels of PilQ monomer and break-down products. (B) The MICs of penicillin for the different frame-shift mutants transformed into PR100 was determined as described in Materials and Methods and compared to those of PR100 and PR100 harboring either pilQ2 or pilQ::kan.
2.4.4 Phenotypes of the in-frame *pilM, pilN, pilO* and *pilP* deletions

In-frame deletions in each of the four *pil* genes were constructed and used to transform SZ3 or PR100 to increased penicillin resistance, but no transformants could be selected. We then transformed PR100 with each of the constructs, and after plating on GCB, analyzing colonies with a non-piliated morphology for the presence of the deletion. When we determined the MICs of the transformants, none of the four in-frame deletions increased resistance above the levels of the parental strain (Figure 2.4A), which again suggested that the original frame-shift mutations that we isolated in the screen had a polar effect. As expected based on their non-piliated colony morphologies, the in-frame deletions of *pilM, pilN, pilO* and *pilP* completely ablated transformation, and unlike the frame-shift mutants, the loss of transformation in each mutant could be restored by expressing the corresponding wild-type gene (Figures 2.4B,C).

Polyclonal antibodies against purified PilM, PilN, and PilO were generated and used to determine the effects of the in-frame mutations on other Pil proteins. The *pilM* mutation had no noticeable effect on expression of any of the other proteins (Figure 2.5, lanes 3 and 4). In contrast, deletion of *pilN, pilO, or pilP* had profound effects on the stability of some or all of the other proteins. For example, deletion of PilN caused the loss of PilO, and deletion of PilO caused the loss of PilN (Figure 2.5, lanes 5-8), with levels of the other proteins unchanged. These data are consistent with recent reports that show that these two proteins form a complex, and thus we concluded that loss of one of the two proteins causes the misfolding, instability or degradation of the other. Interestingly, whereas deletion of PilN or PilO had no effect on the levels of PilP, deletion of PilP resulted in the loss of PilN.
and PilO (Figure 2.5, lanes 9 and 10). These data suggest that PilP acts to stabilize PilN and PilO, but that PilP is stable in the absence of PilN and PilO.

These results also suggested that resistance conferred by the frame-shift mutations was due to changes in translation of downstream genes. However, analysis of the frame-shift mutations with the different Pil antibodies showed the same banding pattern for PilM, PilN, PilO, and PilP as was observed with the in-frame deletions (Figure 2.5), suggesting that resistance increases were not due to changes in expression of these proteins. Moreover, in-frame deletions of these four Pil proteins had no effect on resistance levels, indicating that the loss of any of these proteins was not responsible for changes in antibiotic permeation. This result focused our attention back on PilQ, and to how the frame-shift mutations (e.g. pilM\textsuperscript{FS}) result in nearly the same level of resistance to penicillin as a complete deletion of the pilQ gene.
Figure 2.4 In-frame deletions of pilM, pilN, pilO and pilP affect transformation efficiency but not penicillin resistance. (A) In-frame deletions of the pilM, pilN, pilO and pilP genes were constructed and transformed into PR100, and the MIs of penicillin for each strain was compared to PR100 and PR100 pilQ2. (B) Deletion of pilM, pilN, pilO, or pilP in PR100 decreased the transformation efficiency to below the limit of detection, which could be complemented by expressing the deleted gene in trans. (C) Western blots of PilM, PilN, PilO, and PilP showing the wild-type strain, individual deletions, and the mutants complemented by expressing an IPTG-inducible allele of the deleted gene.
Figure 2.5 Effect of *pilM*, *pilN*, *pilO* and *pilP* deletions on the stability of the proteins encoded by other genes in the operon. Western blots showing the effect of deletion of *pilM*, *pilN*, *pilO*, or *pilP* on the levels of other proteins encoded by the *pilMNOPQ* operon relative to wild-type PR100 and the *pilQ2* mutant.
2.4.5 Effects of titration of PilQ levels on penicillin resistance

To determine whether levels of PilQ mediate gonococcal susceptibility to penicillin, we created a strain with an IPTG-inducible, HA-tagged PilQ inserted into the chromosome of PR100 and PR100 pilQ::Ω (PR100 in which the endogenous pilQ gene was insertionally inactivated) using the integration vector pKH35 to create PR100 pKH35-pilQHA and PR100 pKH35-pilQHA pilQ::Ω. The former strain has two copies of pilQ (one endogenous and one from the inducible pKH locus), whereas the latter strain has only the inducible pilQ gene. We then used these two strains and the parental strain PR100 to evaluate the effects of different levels of PilQ expression on susceptibility to penicillin.

The MIC of penicillin was determined for the three strains on GCB-pen plates containing increasing amounts of IPTG (Figure 2.6). As expected, the MIC of penicillin for PR100 did not change with the different concentrations of IPTG. For PR100 pKH35-pilQHA, the MIC for penicillin in the presence of 0.01 mM IPTG was identical to that of PR100, indicating a very low level of pilQ induction, but as the concentration of IPTG increased, the MIC decreased, strongly suggesting that the higher levels of PilQ increase permeation of penicillin into the periplasm, thereby increasing susceptibility to the antibiotic. In contrast, the MIC of penicillin for PR100 pKH35-pilQHA pilQ::Ω in the presence of 0.01 mM IPTG is similar to that observed in a strain that produces no PilQ, but as the concentration of IPTG was increased, the MIC decreased, and at 1.0 mM IPTG it was identical to that of PR100. These data strongly support our hypothesis that penicillin gains entry to the periplasm by diffusing through pores formed by PilQ.
Figure 2.6 Overexpression of PilQ increases susceptibility to penicillin. PR100 was transformed with a construct to insert a copy of pilQ under IPTG induction (PR100 pKH-pilQHA). The endogenous copy of pilQ was subsequently disrupted to create the strain PR100 pKH35-pilQHA pilQ::Ω. The MIC of penicillin for the IPTG-inducible PilQ strains was determined in the presence of three different concentrations of IPTG (0.01, 0.1, and 1.0 mM).
2.4.6 Analysis of PilQ levels in wild-type versus frame-shift mutants

There are three forms of PilQ that can be observed on a Western blot: 1) the mature, SDS-resistant multimer, 2) the immature, SDS-labile monomer, and 3) PilQ breakdown products. Theoretically, any (or all) of these forms could be responsible for allowing diffusion of antibiotics into the periplasm. One observation that was consistent over multiple blots was that the pilM frame-shift mutation resulted in decreased levels of all three forms of PilQ, but most notably affected levels of the monomer and breakdown products (Figure 2.3A). To examine the levels of the different PilQ forms in the parental strain versus the pilM frame-shift mutant, we quantified the amount of PilQ multimer and monomer present in lysates of wild-type and pilM mutant cells. We consistently found that the levels of PilQ monomer in the pilM frame-shift mutant were one third lower (38%) than those in the wild-type strain (Figure 2.7), whereas the decrease in the level of multimer in the frame-shift mutant was much less (14% decrease). These data suggested that the immature, SDS-labile form of PilQ (or its degradation products), and not the mature multimer, might be the entity responsible for forming the pores through which antibiotics diffuse into the periplasm.

To affect antibiotic flux, PilQ monomers would likely need to assemble into immature oligomers that are then disrupted by the presence of SDS. To investigate this possibility, PilQ proteins were detergent extracted from membranes of FA19 and chromatographed on Sephacryl S-500, and fractions were assessed for the levels of the different forms of PilQ. As reported previously, the SDS-resistant multimer eluted as a broad single peak centered at 1 MDa (Zhao, Tobiason et al. 2005). In contrast, the SDS-labile PilQ monomers eluted in two forms: a smaller shoulder of the main peak that co-
eluted at the same molecular mass as the SDS-resistant PilQ multimers, and the larger, broader peak that eluted with a molecular mass of ~350 kDa (Figure 2.8) (Zhao, Tobiason et al. 2005). These data suggest that a portion of the PilQ monomers observed following SDS-PAGE exist in the outer membrane as SDS-labile oligomers. These oligomers, like the SDS-resistant oligomer, could potentially allow diffusion of small molecules through their central pores.
Figure 2.7 The *pilM* frame-shift mutant decreases the levels of PilQ. (A) Western blot showing the effect of the *pilM* frame-shift mutation on the expression of PilQ multimers and monomers. (B) The anti-PilQ antibody was detected with a Cy-3-conjugated anti-rabbit secondary antibody using a Typhoon fluorescent imager. The levels of PilQ multimers and monomers were quantified using ImageQuant software.
Figure 2.8 Gel filtration of FA19 membranes. FA19 membranes were extracted with SB-10 and subjected to gel filtration on a Sephacryl S-500 column. The eluted fractions were assessed by SDS-PAGE and Western blotting for PilQ. The amounts of PilQ multimer and monomer were quantified using QuantityOne software. The arrows and molecular weights above the graph show the positions of protein standards.
2.4.7 Manipulation of PilW expression to regulate levels of the PilQ multimer

To further investigate the roles of the different forms of PilQ in antibiotic permeation, we manipulated levels of PilW, a protein required for the formation of the SDS-resistant PilQ multimer (Carbonnelle, Helaine et al. 2005, Trindade, Job et al. 2008, Koo, Tang et al. 2013). We reasoned that if the SDS-resistant multimer of PilQ was responsible for antibiotic influx, then the absence of the multimer would result in an increase in the MIC of penicillin to a level similar to that of the pilQ2 mutant. Therefore, we insertionally inactivated pilW with a kanamycin-resistance cassette, which resulted in complete conversion of the SDS-resistant PilQ multimer to the immature, monomeric form on SDS-PAGE (Figure 2.9A). FA19 pilW::kan cells demonstrated only a slight decrease in transformation efficiency (Figure 2.9C), demonstrating that an SDS-labile species of PilQ that runs as a monomer on SDS-PAGE can still form a pore through which the pilus can extrude. Consistent with our hypothesis that the pore is not formed by the SDS-resistant mature multimer, conversion of the multimeric PilQ to the monomeric form on SDS-PAGE had no effect on penicillin resistance (Figure 2.9B). These data suggest that the SDS-labile monomer is the species that forms pores in the outer membrane.

We next attempted to decrease the amount of the SDS-labile PilQ monomer by overexpressing PilW, which we predicted would convert more of the monomer to the mature form. Two additional inducible copies of pilW were inserted onto the chromosome while leaving the endogenous pilW gene intact, and Western blotting confirmed that PilW was indeed overexpressed (Figure 2.10A). However, there was no corresponding increase in the PilQ multimer, and we did not observe a decrease in the PilQ monomer or the proteolytic breakdown fragments compared to the PR100 parental strain (Figure 2.10A).
Moreover, overexpression of PilW had essentially no effect on the MIC of penicillin (Figure 2.10B). Taken together, these data are consistent with a model in which an overabundance of PilQ monomers, only some of which mature to the SDS-resistant multimer, loosely associate and mediate antibiotic influx into the cell. Thus, frame-shift mutations in *pilMNOP* exert their polar effects on penicillin resistance by decreasing the amount of PilQ monomers.
Figure 2.9 Insertional inactivation of pilW. (A) Fluorescent Western blot showing the impact of the pilW::kan mutation on the PilQ multimer:monomer ratio relative to wild-type cells, the pilQ2 mutant, and the pilM frame-shift mutant. (B) The disruption of pilW had no effect on the MIC of penicillin and (C) caused only a slight (~6 fold) decrease in the transformation efficiency.
Figure 2.10 Overexpression of PilW. Wild-type cells were transformed with a proAB-targeted expression construct (pUC18K-proAB-Q19e) containing pilW under control of the tac promoter. The resulting strain was also transformed with the plasmid pKH35-PilW, which introduced an additional copy of IPTG-inducible pilW. Thus, the resulting strain contained the native pilW gene as well as two IPTG-inducible copies in other regions of the chromosome. (A) Western blot showing the effect of expressing native PilW and IPTG-inducible PilW at varying concentrations of IPTG compared to wild-type cells, the pilQ2 mutant, the pilM frame-shift mutant, and the pilW::kan mutant. (B) The two IPTG-inducible copies of pilW had no effect on the MIC of penicillin.
2.5 Discussion

The contribution of PilQ to antibiotic susceptibility was first identified in a search for genes involved in high-level penicillin resistance. A spontaneously arising penicillin-resistant mutation, termed pilQ2, resulting from an amino acid change of Glu-666 to Lys, interfered with the oligomerization of PilQ, decreased transformation competency, and, in the presence of other resistance determinants, conferred a 2- to 3-fold increase in the MICs of penicillin and tetracycline (Zhao, Tobiason et al. 2005). In this study, we followed up on these initial observations by identifying additional spontaneously arising mutations that increase the MIC of penicillin. Surprisingly, in addition to expected mutations in pilQ, we identified mutations in the genes upstream of pilQ in the pilMNOPQ operon that resulted in a resistance phenotype similar to the pilQ2 mutant. The increased resistance of these mutants was due to polar effects on pilQ expression rather than the absence of these upstream genes. To verify that PilQ expression levels can alter sensitivity to penicillin, we created two strains expressing IPTG-inducible PilQ. The endogenous copy of pilQ was intact in one of these strains and inactivated in the other. We observed that as the induction level increased, the MIC decreased (Figure 2.6), suggesting that the overexpression of PilQ resulted in the formation of additional pores through which penicillin could access the periplasm.

We considered two models that were most likely to explain our results. The first model, which is perhaps the most straightforward and the one we favored at the start of these experiments, posits that antibiotics gain entry to the periplasm through mature PilQ oligomeric pores. This model is supported by the observation that the total amount of PilQ is decreased in the frameshift mutants, meaning that fewer pores exist that can allow the
antibiotics to permeate the outer membrane. However, the SDS-resistant oligomer was the
form of PilQ least affected by the frameshift mutations (14% decrease), and recent
evidence suggests that similar pores have mechanisms that allow them to close in vivo
(Burghout, van Boxtel et al. 2004, Collins 2004, Spagnuolo, Opalka et al. 2010). In addition,
deletion of pilW, the protein required for production of the stable, SDS-resistant PilQ
multimer (Carbonnelle, Helaine et al. 2005, Trindade, Job et al. 2008), did not affect
penicillin resistance. Finally, if SDS-resistant oligomers were responsible for antibiotic
influx, there should have been a linear relationship between the levels of oligomer and the
MIC; however, the levels of PilQ oligomer in strains containing a PilM frame-shift mutation
were decreased by only 14%, yet these strains had a nearly identical MIC as those with a
pilQ2 mutation or PilQ deletion (Figure 2.7 and 2.9 and data not shown). These data were
inconsistent with the model that the mature, SDS-resistant oligomer forms a pore for
antibiotics and other small molecules.

In contrast, our data are entirely consistent with the second model, in which
immature PilQ entities allow for the unregulated entry of antibiotics into the periplasm.
This model is supported by experiments showing that there is a much greater decrease in
the monomeric (on SDS-PAGE) PilQ in the frameshift mutants. This model also accounts for
the lack of a resistant phenotype in a strain lacking PilW; when pilW is disrupted, the SDS-
resistant PilQ multimer is completely absent and looks similar to the pilQ2 mutant by
Western blot but without a corresponding increase in antibiotic resistance. These
observations, in addition to gel filtration data of detergent extracts of gonococcal
membranes (Figure 2.8 and (Zhao, Tobiason et al. 2005)) demonstrating that a significant
portion of monomeric PilQ (by SDS-PAGE) elutes with a molecular mass identical to that of

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the SDS-resistant multimers, suggest that SDS-labile PilQ subunits associate in immature multimers that allow antibiotic diffusion into the periplasm.

It is important to distinguish the effects of mutations in PilQ, which both increase resistance and disrupt multimer formation, from the effects of frame-shift mutations in the upstream *pilMNOP* genes, which also increase resistance but have only minor effects on multimer formation. We surmise that resistance-conferring PilQ mutations prevent proper association of PilQ subunits (this is consistent with the observation that all mutations that affect the levels of PilQ multimer are located within the C-terminal beta-barrel domain thought to mediate subunit:subunit associations (Berry, Phelan et al. 2012)), and thus, in addition to preventing multimer formation, they would also decrease or prevent subunit association of immature PilQ subunits. In contrast, upstream mutations in *pilMNOP* decrease the concentration of PilQ monomers in the outer membrane, thereby decreasing permeation of antibiotics, but there are no mutations in PilQ that prevent or impair association; instead, the lower levels of total PilQ and especially of the SDS-labile monomeric form decrease the mass action association of PilQ monomers to form immature multimeric complexes that we predict serve as a route of entry of antibiotics.

For reasons not entirely clear, *N. gonorrhoeae* expresses an overabundance of PilQ subunits, only some of which become mature multimers. Our model predicts that there is a threshold level over which immature subunits begin to associate and form pores, which is consistent with the non-linearity observed between resistance and PilQ concentration. Thus, if a threshold level of PilQ monomers is required to promote formation of immature complexes by mass action, then decreasing PilQ expression by >30% overall could be sufficient to prevent association and increase resistance. Unfortunately, efforts to decrease
the levels of SDS-labile monomers by overexpressing the PilW protein involved in maturing PilQ complexes (Carbonnelle, Helaine et al. 2005, Trindade, Job et al. 2008), which would be a direct test of this model, were unsuccessful.

We cannot rule out the possibility that proteolytic fragments of immature PilQ subunits, which are observed in Western blots of both *N. gonorrhoeae* and *P. aeruginosa* membranes (Ayers, Sampaleanu et al. 2009), are the entities that form pores. Proteolysis of the N-terminal region of PilQ that leaves the C-terminal beta-barrel region intact could result in formation of pores lacking a “plug” that full-length, loosely associated complexes possess. However, the difficulties of preventing proteolysis of PilQ, particularly when oligomer formation is impeded or eliminated, precluded us from fully examining this possibility, and thus we cannot definitively distinguish whether full-length PilQ or its proteolytic fragments are responsible for antibiotic entry.

Thus far, PilQ mutants have not been found in clinical isolates; indeed, nearly all clinical isolates are piliated at the time of isolation (Kellogg, Peacock et al. 1963, Kellogg, Cohen et al. 1968). Whiley et al. examined the pilQ sequences of 63 *Neisseria gonorrhoeae* clinical isolates and found that mutations in PilQ did not contribute to decreased susceptibility to cefixime or ceftriaxone (Whiley, Jacobsson et al. 2010). Because all of the mutations that we identified in PilQ or upstream in PilM, PilN, PilO, or PilP that increase resistance also disrupted or decreased piliation, it appears likely that antibiotic resistant mutants that arise during the course of infection and antibiotic treatment would be unfit and competed out by other piliated strains. However, experimental infections of male volunteers using a non-piliated variant of F62 or an FA1090 pilE mutant showed that the pilus was not required for infection, although the symptoms were less severe in the non-
piliated infections (Hobbs, Sparling et al. 2011). Both of these strains possessed a normal copy of the pilQ gene, so it remains possible that PilQ is required for infection even in the absence of pili. Furthermore, the role of the pilus in disseminated gonococcal infections has not been examined, and it is possible that non-piliated antibiotic-resistant mutants could cause serious complications.

Although the pilMNOPQ mutations described in this paper may not be clinically relevant, our comparisons of the frame-shift and in-frame deletion mutants provide insight into the biogenesis of type four pili, a complex process that has not yet been entirely elucidated. A screen of N. meningitidis pilus-deficient mutants had previously identified pilMNOP as essential for pilus assembly (Carbonnelle, Helaine et al. 2005), and experiments performed in Pseudomonas aeruginosa have shown that the proteins encoded by these genes form an inner-membrane complex (Carbonnelle, Helaine et al. 2005, Ayers, Sampaleanu et al. 2009, Sampaleanu, Bonanno et al. 2009, Tammam, Sampaleanu et al. 2011, Tammam, Sampaleanu et al. 2013). The interactions between the proteins encoded by the pilMNOPQ operon were first shown in P. aeruginosa. Ayers et al. (Ayers, Sampaleanu et al. 2009) showed that in P. aeruginosa, mutation of PilN resulted in the loss of PilO expression, and vice versa, consistent with our results and others (Sampaleanu, Bonanno et al. 2009) that PilN and PilO from P. aeruginosa form heterodimers. Ayers et al. also showed that deletion of PilM resulted in the complete loss of PilN, PilO, and PilP expression, whereas deletion of PilN, PilO, and PilP had no effect on PilM levels. In contrast, we show here that in N. gonorrhoeae, deletion of PilM had no effect on PilN, PilO and PilP expression. Another difference between our results and those in P. aeruginosa is that deletion of either PilN or PilO in N. gonorrhoeae had no effect on PilP expression, whereas in P. aeruginosa,
deletion of PilP was reported to result in the loss of PilN and PilO. Moreover, deletion of any of the first four genes in the pilMNOPQ operon in *P. aeruginosa* decreased levels of PilQ (Ayers, Sampaleanu et al. 2009), whereas we see no difference in PilQ expression in strains with in-frame deletions of PilM, PilN, PilO or PilP. The differences between the two organisms is surprising, given that the gonococcal proteins are ~50% identical to their corresponding pseudomonal counterparts.

Despite these differences, our results are generally consistent with models for PilQ complexes proposed in both *P. aeruginosa* and *N. meningitidis*. This model has the cytoplasmic protein PilM interacting with the N-terminus of the inner membrane protein PilN, PilN interacting with PilO, and PilP interacting with PilN to form PilNOP heterotrimers (Ayers, Sampaleanu et al. 2009, Sampaleanu, Bonanno et al. 2009, Tammam, Sampaleanu et al. 2011, Georgiadou, Castagnini et al. 2012, Tammam, Sampaleanu et al. 2013). While relevant for pilus assembly, none of the studies on the complex formed by the first 4 proteins encoded by the pilMNOPQ operon explain how frame-shift mutations upstream of pilQ affect antibiotic susceptibility. Also to be considered is the phenotype of a previously described F595L mutation in pilQ (pilQ1), which confers increased antibiotic susceptibility. The pilQ1 mutation was isolated as a spontaneous suppressor mutation that allowed a strain of FA1090 with a deletion in the HpuA hemoglobin receptor protein to again utilize hemoglobin as the sole source of iron, likely by allowing free heme to diffuse into the cell (Chen, Tobiason et al. 2004). The different effects of these pilQ mutations on antibiotic susceptibility highlights the difficulty of using pilQ mutants to study the role that PilQ plays in membrane permeability.
Although the PilQ mutations isolated at the beginning of this study all exhibited increased penicillin resistance, the effects of the different mutations on transformation efficiency varied dramatically. For example, of the 13 class I mutations (i.e., those similar to the previously characterized pilQ2 mutant by Western blot), 9 had transformation efficiencies similar to those of the wild-type strain or the pilQ2 mutant, and 4 had transformation efficiencies that were below the limit of detection (Figure 2.2B). Thus, even though the 13 PilQ mutants appear identical by Western blot and MIC, the ability of their type IV pili to function properly is affected in different ways. Transformation competency depends on the ability of the TFP to extend and retract through the PilQ secretin, and therefore the PilQ in these 9 transformation-competent mutants must be able to form outer membrane pores even though SDS-resistant oligomers are not observed by SDS-PAGE. This further suggests that pores can be formed through interactions of PilQ complexes that are not fully mature.

In conclusion, this study identifies immature PilQ complexes, and not SDS-resistant PilQ multimer complexes, as the entity involved in forming pores in the outer membrane of *N. gonorrhoeae* and allowing permeation of antibiotics into the periplasm. Although not yet observed clinically, it remains possible that mutations that block permeation but have near normal piliation may arise in the future and provide additional resistance to antibiotics in strains that already possess mutations in PBP 2 and have decreased membrane permeability.
Chapter 3. Diacetylene LpxC inhibitors as novel antibiotics with activity against multi-drug-resistant *Neisseria gonorrhoeae*

3.1 Overview

*Neisseria gonorrhoeae* has developed resistance to nearly all classes of antibiotics that have been used to treat gonococcal infections, including the expanded-spectrum cephalosporins that are currently recommended for treatment, and there is an urgent need to develop new antibiotics against this pathogen. To this end, we have developed and optimized inhibitors of LpxC that display *in vitro* and *in vivo* activity against *N. gonorrhoeae*. LpxC (UDP-3-O-(acyl)-N-acetylglucosamine deacetylase) is the zinc metalloenzyme that catalyzes the first committed step in the biosynthesis of lipid A, the major component of the outer leaflet of the outer membrane of Gram-negative bacteria. Although LpxC is dispensable in the closely related species *N. meningitidis*, LpxC is essential in *N. gonorrhoeae*, and the inhibition of this enzyme is bactericidal. Furthermore, susceptibility to LpxC inhibitors is only slightly reduced by the presence of multiple resistance factors in cephalosporin-resistant isolates, and our LpxC inhibitors remain highly active against these MDR strains. Two inhibitors with potent activity *in vitro* were tested *in vivo* using the mouse model of gonococcal infection, and one of these inhibitors was able to clear the

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infection in all but one of the infected mice. The data presented herein validate LpxC as a drug target in *N. gonorrhoeae* and provide a promising start for the potential development of these compounds as novel antibiotics for the treatment of gonorrhea.
3.2 Introduction

*Neisseria gonorrhoeae* is the etiologic agent of the sexually transmitted infection (STI) gonorrhea, the second most common bacterial STI in the United States (Division of STD Prevention 2012). Gonorrhea is often asymptomatic in women, and when left untreated, it can lead to serious complications such as pelvic inflammatory disease, ectopic pregnancy, and infertility (Weström, Joesoef et al. 1992, Haggerty, Gottlieb et al. 2010, Walker and Sweet 2011). The immune system is unable to clear gonococcal infections, so antibiotic treatment is required. However, *N. gonorrhoeae* has developed resistance to all classes of antibiotics that have been used to treat infections. The current recommended treatment for gonorrhea in the US is the expanded-spectrum cephalosporin (ESC) ceftriaxone in combination with either azithromycin or doxycycline (Centers for Disease Control and Prevention. 2012), but strains resistant to ceftriaxone have recently been isolated (Unemo, Golparian et al. 2010, Ohnishi, Saika et al. 2011, Unemo, Golparian et al. 2012). Thus, there is an urgent need for new antibiotics to treat gonorrhea.

To help meet the growing need for new antibiotics, we have been working to develop inhibitors of the enzyme LpxC in the lipid A biosynthesis pathway as novel antibiotics against *N. gonorrhoeae*. Lipid A is the active component of endotoxin that causes septic shock during infections with Gram-negative bacteria (Raetz and Whitfield 2002). It is the major component of the outer leaflet of the outer membrane of Gram-negative bacteria and serves as the hydrophobic membrane anchor of lipooligosaccharide (LOS). LOS is similar to the lipopolysaccharide (LPS) produced by other Gram-negative bacteria but lacks the O antigen repeats found in LPS (Preston, Mandrell et al. 1996). Because lipid A is
conserved and essential in nearly all Gram-negative pathogens, the lipid A biosynthesis pathway represents a potential target for a new class of antibiotics.

The first step in lipid A biosynthesis is the thermodynamically unfavorable fatty acid acylation of UDP-\(N\)-acetylglucosamine (UDP-GlcNAc) by the enzyme LpxA (Anderson and Raetz 1987). The second step, in which LpxC removes the acetyl group from UDP-GlcNAc, is irreversible and is therefore the first committed step of the pathway (Figure 3.1). LpxC is a well-conserved zinc-dependent deacetylase (UDP-3-O-(acyl)-\(N\)-glucosamine deacetylase) that has no sequence identity with mammalian deacetylases (Young, Silver et al. 1995, Sorensen, Lutkenhaus et al. 1996, Barb and Zhou 2011). Following the reaction catalyzed by LpxC, eight additional enzymes are required for the synthesis of Kdo\(_2\) (2-keto-3-deoxyoctulosonic acid) lipid A, the minimal lipid A structure required for bacterial viability (Raetz and Whitfield 2002). The development of inhibitors of LpxC has been a goal of multiple research groups and companies over the past two decades, but previous efforts have not focused on optimizing LpxC inhibitors against \textit{N. gonorrhoeae}.

This paper describes the validation of LpxC as a novel drug target in \textit{N. gonorrhoeae}. We show that LpxC is essential in the gonococcus and is the primary target of our inhibitors. LpxC inhibitors are bactericidal against \textit{N. gonorrhoeae} and remain effective against recent ESC-resistant isolates. We have synthesized and tested nearly 200 compounds to optimize activity against \textit{N. gonorrhoeae}. Two of the compounds with the greatest activity \textit{in vitro} were tested in the mouse model of gonorrhea, and one of these compounds was able to clear the infection in all but one of the animals. These findings represent significant progress in the development of a new antibiotic for the treatment of MDR \textit{N. gonorrhoeae} infections.
Figure 3.1 LpxC inhibitors target the first committed step in the biosynthesis of lipid A. The first step in lipid A synthesis is catalyzed by LpxA and is thermodynamically unfavorable. Therefore, the reaction catalyzed by LpxC is the first committed step in this pathway. Eight additional steps are required to form Kdo₂-lipid A, the minimal structure required for bacterial viability. Compared to the structure of *E. coli* Kdo₂-lipid A, the structure of gonococcal Kdo₂-lipid A differs in the location of one acyl chain.
3.3 Materials and Methods

3.3.1 Bacterial strains

*Neisseria gonorrhoeae* strain FA19, a antibiotic-susceptible laboratory strain, was provided by Dr. Fred Sparling, University of North Carolina at Chapel Hill. FA1090 A25, which was obtained from Bill Shafer at Emory University, is an antibiotic-susceptible laboratory strain that was used in the mouse experiments. Strain 35/02 is a penicillin-resistant strain isolated in Sweden in 2002 that displays reduced susceptibility to ESCs (Lindberg, Fredlund et al. 2007). Two ESC-resistant resistant isolates, H041 and F89, were also used in this study. H041 was isolated from the pharynx of a sex worker in Japan in 2009 (Ohnishi, Saika et al. 2011), and F89 was isolated from the urethra of a man in France in 2010 (Unemo, Golparian et al. 2012). The former three strains were obtained from Magnus Unemo from Örebro University Hospital, Örebro, Sweden. The isogenic strains containing increasing numbers of resistance factors were created by transforming FA19 with PCR products of each of the resistance factors amplified from FA6140 (a penicillin-resistant strain) (Margaret Duncan and Rob Nicholas, unpublished data).

3.3.2 Bacterial growth conditions

*Neisseria gonorrhoeae* strains were maintained on GC medium base (GCB) agar plates containing supplements I and II in a humidified 37°C incubator with 4% CO₂. When necessary, cells were grown in flasks of GCB broth containing supplements I and II, supplement B, and 10 mM NaHCO₃ (GCB+) in a 37°C incubator with shaking (Kellogg, Peacock et al. 1963). *E. coli* strains were maintained on LB agar plates or cultured in LB
broth. *E. coli* cells transformed with the plasmids described in this paper were selected on 100 μg/ml carbenicillin (pUC18us), 50 μg/ml kanamycin (*lpxC* inactivation construct), or 34 μg/ml chloramphenicol (pKH35).

### 3.3.3 Antibiotic susceptibility testing

*LpxC* inhibitors were initially screened for activity using a disk diffusion assay. In this assay, GCB top agar (GCB broth with 0.7% agar) was melted, cooled in a 50°C water bath, and supplements I and I, supplement B, and 10 mM NaHCO$_3$ were added. Cells grown on GCB plates were resuspended in GCB broth to an OD$_{560}$ of 0.18 (approximately 1 x 10$^8$ cells per ml), and 100 μl of the diluted cells were mixed with 3 ml of the supplemented top agar. This suspension was poured on top of a GCB agar plate and allowed to solidify. The inhibitors were diluted in DMSO, and 5 μl of the inhibitor was spotted onto sterile 6 mm paper disks. The disks were then placed on the agar plates, and the zone of inhibition was measured 24 hours later.

The minimum inhibitory concentrations (MICs) of the *LpxC* inhibitors were determined by broth microdilution or agar dilution. For the broth microdilution MICs, cells were grown to mid-log phase in 25 ml cultures of GCB+ at 37°C with shaking. The cells were then diluted to an OD$_{560}$ of 0.02, and 100 μl of the diluted cells were transferred to 96-well plates containing 100 μl of two-fold dilutions of the inhibitor in 10% DMSO in duplicate. Thus, the starting density of cells was an OD$_{560}$ of 0.01. The plates were incubated at 37°C overnight on a microplate orbital shaker. The following day, the A$_{560}$ of the plates was read in a microplate spectrophotometer.
The agar dilution method of MIC determination has been previously described (Ropp, 2002). Briefly, cells maintained on GCB agar plates were resuspended in GCB+ and diluted to an OD$_{560}$ of 0.018. Five microliters of this dilution were spotted on GCB agar plates containing varying concentrations of the indicated inhibitor. The plates were incubated at 37°C for 24 hours, and the MIC was defined as the lowest concentration that prevented the growth of more than 5 colonies.

3.3.4 Bactericidal assay

To determine whether the LpxC inhibitors were bactericidal or bacteriostatic, FA19 cells were grown in GCB+ to mid-log phase (OD$_{560}$=0.6), and the culture was then split into three flasks. The cultures were treated with vehicle (1% DMSO), 4× the MIC of LPC-011 (8 μg/ml), or cefixime (0.12 μg/ml) as a bactericidal positive control. The OD$_{560}$ of these cultures was determined every hour for four hours, and aliquots of the cultures were diluted and spread on agar plates for CFU determination every two hours.

3.3.5 Transformation of N. gonorrhoeae

Piliated FA19 cells maintained on GCB agar were resuspended in GCB+ containing 10 mM MgCl$_2$ and diluted to an OD$_{560}$ of 0.18. Aliquots of the diluted cells (900 μl) were then mixed with 100 μl of 1× SSC buffer containing 1 to 2 μg of plasmid DNA. The cells were then incubated with the DNA for 5 h at 37°C in a humidified incubator containing 5% CO$_2$. At the end of the incubation period, varying amounts of the cells were spread on GCB agar plates containing the appropriate antibiotics.
### 3.3.6 Knockout and complementation of NgLpxC

Two plasmids were constructed to test the essentiality of *lpxC* in *N. gonorrhoeae*. The plasmid pUC18us-NgLpxC-E(kanR) was constructed by cloning the *lpxC* coding sequence into the vector pUC18us and then digesting with *Eco*RI, which cuts in the middle of *lpxC*, and inserting a kanamycin cassette. This construct was designed to inactivate *lpxC* by inserting the kanamycin resistance cassette into the endogenous *lpxC* gene via homologous recombination. The second plasmid, pUC18us-NgLpxC-B(kanR) was constructed similarly, except that the kanamycin resistance cassette was inserted at a *Bst*BI site 8 bp downstream of the *lpxC* stop codon; this plasmid was designed to serve as a transformation control for the knockout plasmid. Gonococcal cells transformed with these plasmids were selected on 50 μg/ml kanamycin.

The plasmid pKH35-NgLpxC was constructed by cloning the *lpxC* sequence under the control of the *lac* promoter in the vector pKH35. This plasmid also contains the coding sequences of the genes *lctP* and *aspC*, which serve as recombination sites so that the gene of interest, the *lacI* gene, and the chloramphenicol acetyltransferase gene, *catG*, can be inserted onto the chromosome. Gonococcal transformants containing this plasmid were selected on 0.6 μg/ml chloramphenicol. Because *lpxC* is essential in *N. gonorrhoeae*, this complementation plasmid was transformed into FA19 prior to inactivating the endogenous copy of *lpxC* in the presence of 0.01 mM IPTG.

### 3.3.7 Construction of strains expressing RlLpxC and EcLpxC

Strains expressing LpxC from either *E. coli* or *Rhizobium leguminosarum* were constructed by cloning the coding sequence of *lpxC* from these organisms into the vector
pKH35. The resulting plasmids were then transformed into FA19 (selected on 0.6 μg/ml chloramphenicol), and the gonococcal *lpxC* was inactivated using the knockout construct described above in the presence of 0.01 mM IPTG.

### 3.3.8 In vivo efficacy testing of LpxC inhibitors

Thirty-five female BALB/c mice (6-8 weeks old, National Cancer Institute) were treated with 5 mg slow-release 17-β estradiol tablets beginning two days prior to infection (Jerse 1999). Because estradiol treatment results in the overgrowth of the commensal vaginal flora, the mice were also given antibiotics. On days -2 through +1, the mice were giving streptomycin sulfate (2.4 mg) and vancomycin hydrochloride (0.6 mg) via intraperitoneal (IP) injection twice daily, and trimethoprim sulfate (0.4 g/L) was provided in the drinking water from day -2 through the duration of the study. From day +2 through the end of the study, streptomycin sulfate (5 g/L) was also added to the drinking water.

The mice were inoculated vaginally with a dose of 1.76 x 10^6 CFUs of *N. gonorrhoeae* strain FA1090 in PBS on day 0, and treatment was initiated on day +2. The LpxC inhibitors LPC-169 and LPC-174 were solubilized in 40% (2-hydroxypropyl)-β-cyclodextrin (HBC) at a concentration of 6.8 mg/ml and were delivered via IP injection at a dose of 40 mg/kg (in a 0.1 ml volume) twice daily on days +2 through +6 for a total of 10 doses. A single dose of ceftriaxone (15 mg/kg in H2O; 0.1 ml) delivered via IP injection was used as a positive control. The vehicle control was 0.1 ml of 40% HBC administered via IP injection twice daily on days +2 through +6 (Figure 3.9).

Colonization was assessed daily throughout the study beginning on day +1 by swabbing the vagina and plating samples on GC-VCNTS (GC agar supplemented with
vancomycin, colistin, nystatin, and trimethoprim sulfate). Mice that were culture-negative for four consecutive days were considered to have cleared the infection. At the end of the study (day +9), the mice were euthanized in a CO₂ gas chamber. All animal protocols were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee.
3.4 Results

3.4.1 LpxC inhibitors are active against \textit{N. gonorrhoeae}

Preliminary disk diffusion and MIC studies showed that LpxC inhibitors had activity against \textit{N. gonorrhoeae}. Figure 3.2A shows the results of one set of disk diffusions in which the inhibitors LPC-040 and LPC-013 were tested for their ability to inhibit the growth of strains FA19 and 35/02. The MICs of LPC-040 and LPC-013 were then determined, and these compounds had MICs that were between 2 and 6 \( \mu \text{g/mL} \). Subsequent inhibitors based on the same scaffold were synthesized, and these showed even better activity, with LPC-091 having an MIC of 0.003 \( \mu \text{g/mL} \) for FA19 and 0.156 \( \mu \text{g/mL} \) for 35/02 (Figure 3.2B). The structures of these compounds are shown in Figure 3.2C.

To determine whether the inhibitors were bactericidal or bacteriostatic against \textit{N. gonorrhoeae}, FA19 cells were grown in liquid culture to mid-log phase and treated with vehicle or 4\( \times \) the MIC of LPC-011. Cells treated with 4\( \times \) the MIC of cefixime served as a positive control. As shown in Figure 3.3A, the cells treated with the LpxC inhibitor lysed rapidly, and very few viable cells remained after four hours (Figure 3.3B). These data demonstrate that LPC-011, and presumably all LpxC inhibitors, are bactericidal against \textit{N. gonorrhoeae}.
Figure 3.2 Screening and optimization of LpxC inhibitors. (A) Bacterial disk diffusion assay. Tested compounds include: (1) DMSO (negative control), (2) 2 µg LpxC inhibitor, (3) 20 µg LpxC inhibitor, and (4) 100 ng cefixime. (B) MICs of several LpxC inhibitors. (C) Structures of five diacetylene LpxC inhibitors.
Figure 3.3 LpxC inhibitors are bactericidal for *N. gonorrhoeae*. (A) OD$_{560}$ of FA19 grown in GCB broth + supplements in the presence of DMSO (vehicle), cefixime (0.12 mg/ml; 4X MIC), or LPC-011 (8 mg/ml; 4X MIC). (B) The CFU/ml were determined at 0, 2 and 4 hours after compound addition by plating serial dilutions of the cell suspensions.
3.4.2 LpxC is essential in *N. gonorrhoeae*

Although LpxC is essential in nearly all Gram-negative bacteria, it is dispensable in the closely related bacterium *N. meningitidis*. To determine if LpxC was essential in *N. gonorrhoeae*, we constructed two plasmids for use in a transformation assay (Figure 3.4). These plasmids each contained the *lpxC* gene and a kan$^R$ cassette, but the location of the kan$^R$ cassette varies between the two plasmids. In the *lpxC* inactivation construct pUC18us-NgLpxC-E(kan$^r$), the resistance marker was inserted at an endogenous EcoRI site in the middle of the *lpxC* coding sequence (Figure 3.4A). The transformation control construct pUC18us-NgLpxC-B(kan$^r$) contains the intact *lpxC* coding sequence with the resistance marker inserted at an engineered BstB1 site 8 bp downstream of the stop codon (Figure 3.4B). When wild-type FA19 cells were transformed with the linearized plasmids, only the transformation control construct yielded kan$^R$ transformants. The absence of transformants with the inactivation construct but not the transformation control construct indicates that *lpxC* is essential in *N. gonorrhoeae*.

To further explore the requirement for *lpxC*, FA19 cells were first transformed with the complementation construct pKH35-NgLpxC. Transformation with this plasmid introduced a second, IPTG-inducible copy of *lpxC* between the *lctP* and *aspC* genes on the gonococcal chromosome via homologous recombination (Figure 3.4C). We then repeated the transformation assay using the inactivation and transformation control plasmids in the presence and absence of 0.01 mM IPTG. The control construct yielded kan$^R$ colonies with and without IPTG, but *lpxC* knock-out transformants were obtained only when IPTG was present (i.e., only when the second copy of *lpxC* was expressed). These data provided further evidence that *lpxC* is essential in *N. gonorrhoeae*. 
Figure 3.4 LpxC is essential in *N. gonorrhoeae*. Strain FA19 was transformed with a construct to inactivate *lpxC* (A), but inactivation of *lpxC* was achieved only when the expression of a second, complementing copy of *lpxC* (C) was induced with IPTG. As a transformation control, we were able to transform a construct containing a kanamycin resistance cassette immediately downstream of the native *lpxC* (B) both in the presence and absence of the complementing copy of *lpxC*.
3.4.3 LpxC is the primary target of LpxC inhibitors in *N. gonorrhoeae*

A strategy similar to the one used to show that LpxC is essential in the gonococcus was employed to verify that it is the primary target of the LpxC inhibitors. The *lpxC* genes from *E. coli* (inhibited by diacetylene compounds with high potency) and *R. leguminosarum* (inhibited by diacetylene compounds with low potency) were cloned into the complementation vector pKH35. The resulting plasmids were then used to transform FA19. Transformation was verified by PCR amplification and sequencing. The native copy of *lpxC* was then inactivated by transformation with the knock-out construct in the presence of IPTG, and the MIC of LPC-067 was determined for the strains expressing LpxC from different species.

To determine the amount of IPTG to use to induce the expression of LpxC, the endogenous copy of LpxC was HA-tagged. An inducible copy of NgLpxC was also tagged, and the expression from the *lac* promoter in the presence of varying amounts of IPTG was compared to the expression from the native promoter by Western blotting. We found that induction with 0.01 mM IPTG resulted in an expression level equivalent to that of the native promoter (Figure 3.5).

The strain expressing EcLpxC demonstrated a 15-fold decrease in the LPC-067 MIC, and the strain expressing RILpxC displayed a 2.5-fold increase in the MIC (Figure 3.6). These data are consistent with previous studies showing that *E. coli* and *R. leguminosarum* display different degrees of susceptibility to diacetylene LpxC inhibitors and provide evidence that LpxC is the target of these inhibitors in *N. gonorrhoeae.*
**Figure 3.5** IPTG-inducible expression of LpxC. The expression level of LpxC-HA was determined using a strain of FA19 in which the endogenous copy of LpxC had been HA-tagged (FA19 NgLpxC-HA; last four lanes on the right). The expression of an IPTG-inducible copy of LpxC-HA by a strain in which the endogenous copy of LpxC had been disrupted (FA19 pKH-NgLpxC-HA lpxC::kan) was assessed following growth in media containing different amounts of IPTG (0, 0.01, and 0.1 mM). The expression at 0.01 mM IPTG was equivalent to the expression of LpxC under its native promoter.
**Figure 3.6** LpxC is the primary target of LpxC inhibitors in *N. gonorrhoeae*. The MICs of LPC-067 were determined for FA19 strains expressing the LpxC orthologues from *N. gonorrhoeae*, *E. coli*, or *Rhizobium leguminosarum* in the presence of 0.01 mM IPTG.
3.4.4 Effect of existing resistance factors on susceptibility to LpxC inhibitors

A set of step-wise isogenic strains expressing different resistance factors was used to determine the influence of existing resistance factors on the susceptibility of *N. gonorrhoeae* to LpxC inhibitors. These strains were created by transforming resistance factors from the penicillin-resistant strain FA6140 to the sensitive strain FA19 (Margaret Duncan, unpublished). The first determinant, *penA*, encodes a mutated copy of penicillin-binding protein 2 (PBP2). As expected, this mutation had no effect on the MIC of the inhibitor LPC-067. In contrast, the addition of the *mtrR* mutation that results in the overexpression of the MtrCDE efflux pump increased the MIC of LPC-067 by approximately 4-fold over that for the parental strain FA19. The increase in MIC with the *mtrR* mutation strongly suggests that LpxC inhibitors are substrates of the MtrC-MtrD-MtrE efflux pump (Figure 3.7).

No further increase in the MIC was observed following the addition of the *penB* mutation that encodes a mutated form of the outer membrane porin, PorB1B. This suggests that either the LpxC inhibitors do not rely on porin to gain entry to the cells or that they diffuse through the mutant porin at the same rate as wild-type porin. The final resistance determinant tested, *ponA*, encodes a mutated copy of penicillin binding protein 1 (PBP1). As expected, the addition of *ponA* to the strain containing the other 3 mutations did not increase the MIC. Thus, the overexpression of the MtrC-MtrD-MtrE efflux pump is the only transformable resistance determinant that contributes both to high-level resistance to the expanded-spectrum cephalosporins and to decreased susceptibility to LpxC inhibitors.
Figure 3.7 Effect of known resistance factors on susceptibility to LpxC inhibitors. The MICs of LPC-067 for FA19 expressing known resistance factors from the penicillin-resistant strain FA6140 were determined.
3.4.5 LpxC inhibitors are active against ESC-resistant strains of *N. gonorrhoeae*

In the last several years, strains resistant to the ESCs have been isolated and verified. Few options exist for treating these cefixime- and/or ceftriaxone-resistant strains. We tested the activity our diacetylene LpxC inhibitors against multiple strains of gonorrhea, including two of these recent isolates, F89 and H041. As shown in Figure 3.8, LPC-174 inhibits the growth of both isolates. The MIC of LPC-174 for H041 is 8-fold greater than that for FA19, and this is partially due to the presence of the *mtrR* mutation in H041. However, the MIC of F89 is only slightly higher than that of FA19 (Figure 3.8), and it contains the *mtrR* mutation. H041 and F89 have similar ceftriaxone and cefixime MIC values, and the reason for the increased sensitivity of F89 to LpxC inhibitors is unknown. We hypothesize that there is an unknown resistance factor present in some antibiotic-resistant strains that confers increased resistance to bactericidal drugs (Margaret Duncan and Rob Nicholas, unpublished). This factor may be present in H041 but not F89.
Figure 3.8. Activity of LpxC inhibitors against MDR strains of *N. gonorrhoeae*. The activity of LpxC inhibitors was assessed against multiple strains of gonorrhea, including isolates (F89 and H041) that are resistant to the expanded-spectrum cephalosporins.
3.4.6 LpxC inhibitors display in vivo activity against *N. gonorrhoeae*

Thirty-five mice were inoculated with strain FA1090 A25 on day 0. Colonization was assessed prior to initiating treatment, and mice that were culture negative were excluded from the study. The final numbers of mice in each treatment group were as follows: LPC-169, seven mice; LPC-174, nine mice; ceftriaxone, 7 mice; and 40% HBC (vehicle), 8 mice. Treatment was initiated on the afternoon of the second day following inoculation, and colonization was assessed each day (Figure 3.9).

Figure 3.10A shows the percentage of mice in each group colonized with *N. gonorrhoeae* throughout the study period. The majority of the mice (87.5%) of the mice that received the vehicle control (40% HBC) remained colonized for the duration of the study. Ceftriaxone was used as the positive control, and 100% of the mice that received a single dose of ceftriaxone had cleared the infection by the following day (day +3). Treatment with LPC-174 was significantly better than the vehicle control, and all but one mouse cleared the infection within four days post treatment (89% clearance). However, treatment with LPC-169 did not improve clearance compared to the vehicle control. At the end of the study, 71% of the mice treated with LPC-169 remained colonized. The average number of gonococci recovered for each group is shown in Figure 3.10B. Notably, the colonization level on days +8 and +9 in the group treated with LPC-174 is due to a single mouse. The other 8 mice in the LPC-174 group cleared the infection by day +6.
Figure 3.9 Protocol for the \textit{in vivo} efficacy testing of LpxC inhibitors. Two days prior to infection, mice were treated with 17-β estradiol and antibiotics. The mice were inoculated with $1.76 \times 10^6$ CFUs of strain FA1090. Treatment was initiated on the afternoon of day two following infection. Colonization was monitored for 7 days following the initiation of treatment.
Figure 3.10 *In vivo* efficacy of LpxC inhibitors. The LpxC inhibitors LPC-169 and LPC-174 were tested for their ability to reduce the duration of colonization of mice infected with *N. gonorrhoeae*. (A) Colonization of all groups over the 9-day infection period. The arrow denotes the time at which treatment with LPC-169, LPC-174, ceftriaxone, or 40% HBC was initiated. (B) Mean log_{10} CFU/ml of *N. gonorrhoeae* recovered from each group of mice over 9 days. The arrow marks the time at which treatment was initiated, and the dashed line marks the limit of detection (20 CFUs).
3.5 Discussion

Our data demonstrate that LpxC is a promising drug target in *N. gonorrhoeae* and that our compounds are effective inhibitors of gonococcal growth both *in vitro* and *in vivo*. Significant progress was made in optimizing inhibitors that had been originally designed to inhibit EcLpxC. Whereas the first inhibitors had MICs that were above 1 mg/mL, subsequent inhibitors displayed MICs that were nearly 1000-fold lower than our starting lead compound. Furthermore, the fact that a strain of gonorrhea that expressed only EcLpxC had an MIC of LPC-067 that was approximately 100-fold lower than the same strain expressing NgLpxC indicates that the compounds are readily able to gain access to the enzyme and suggests that, with further optimization, our compounds will be able to inhibit NgLpxC at much lower concentrations. These future modifications will likely be enhanced by ongoing work in the Zhou lab to obtain the crystal structure of *Neisseria* LpxC bound to a diacetylene LpxC inhibitor.

In light of the recently isolated cephalosporin-resistant strains of gonorrhea, it is particularly important that these LpxC inhibitors maintain activity against strains possessing multiple resistance factors. Although the MIC of the inhibitor LPC-174 for the ESC-resistant strain H041 is 8-fold greater than that of the antibiotic-sensitive strain FA19, this is quite negligible compared to the approximately 400-fold increase in the MIC of ceftriaxone for H041 compared to FA19. The experiments using isogenic strains expressing increasing numbers of resistance factors show that much of the difference in the MICs of the inhibitors for H041 and FA19 can be attributed to the presence of the *mtrR* mutation that results in the overexpression of the MtrC-MtrD-MtrE efflux pump. Previous work in our lab has suggested the presence of an unknown resistance factor that confers increased
resistance to bactericidal antibiotics (Margaret Duncan, unpublished). This factor is present in 35/02 and may also be responsible for the decreased sensitivity of strain H041. The absence of this factor from strain F89 could explain the relative sensitivity of this ESC-resistant isolate to LpxC inhibitors.

The data showing that LPC-174 is active in the murine model of gonorrhea demonstrate that this inhibitor is able to travel through host tissues to gain access to the bacteria at the site of infection. Although it would be ideal to identify an inhibitor that is able to clear the infection with a single dose, this study provides important proof-of-principle data that diacetylene LpxC inhibitors are effective in vivo. The half-life of LPC-174 is approximately one hour (data not shown), and increasing the half-life of future compounds would likely improve the in vivo efficacy. Furthermore, the activity of the compounds is affected by binding to human serum albumin (HSA). The MIC of the inhibitors increases 10- to 25-fold in the presence of 2% HSA (data not shown), and this sequestration of free drug affects the activity of the compounds in vivo. Lastly, LPC-174 is a racemic mixture, with only one of the two enantiomers showing activity, and thus may be even more effective than the numbers indicate.

It was somewhat surprising that LPC-169 was not active in vivo despite displaying similar activity to LPC-174 in vitro, but we may be able to use this information to identify and avoid modifications to the inhibitor that are disadvantageous in vivo. Efficacy studies with the ESC-resistant strain F89 will be initiated soon, and optimization of the inhibitors to improve their activity both in vitro and in vivo while increasing their half-life and reducing binding to HSA is ongoing.
Chapter 4. Deletion of *pqiAB* decreases the susceptibility of *N. gonorrhoeae* to LpxC inhibitors

4.1 Overview

*Neisseria gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhea, has developed resistance to nearly all antibiotics that have been used to treat infections, and resistance to currently recommended antibiotics has already emerged. We are investigating inhibitors of LpxC, the enzyme that catalyzes the first committed step in lipid A biosynthesis, as a novel class of antibiotics against *N. gonorrhoeae*. LpxC is essential in *N. gonorrhoeae*, and LpxC inhibitors are bactericidal for this bacterium. These inhibitors are effective against recently isolated cephalosporin-resistant strains.

Because LpxC is a novel target, no specific resistance mechanisms to LpxC inhibitors exist in the gonococcus. However, we have isolated spontaneous mutants *in vitro* that display decreased susceptibility to LpxC inhibitors. R7, a mutant with an 8- to 16-fold increase in the MIC, was chosen for further characterization. We found that deletion of two genes, *pqiA* and *pqiB*, was responsible for the increased resistance, and expressing these genes in the R7 mutant in *trans* restores sensitivity to LpxC inhibitors. Although both *pqiA* and *pqiB* are required for susceptibility to LpxC inhibitors, the mechanism of resistance due to the loss of these genes is unclear. The loss of *pqiAB* is not a general antibiotic resistance

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mechanism, as susceptibility to other drugs is not affected. Furthermore, the loss of pqiAB does not alter *in vitro* growth or LpxC expression. Deletion of pqiAB in a strain of gonorrhea that expresses LpxC from *E. coli* in place of gonococcal LpxC still increases the MIC of LpxC inhibitors, suggesting that the resistance mechanism is distinct from LpxC itself. We hope that gaining an understanding of potential resistance mechanisms will extend the therapeutic lifetime of this new class of antibiotics.
4.2 Introduction

*Neisseria gonorrhoeae* is the causative agent of the sexually transmitted infection gonorrhea. Infected individuals require treatment with antibiotics to clear the infection; however, the gonococcus has developed resistance to nearly all antibiotics that have been used to treat infections. Ceftriaxone is currently the only recommended antibiotic for monotherapy in the United States (the current recommended regimen is dual therapy with ceftriaxone and azithromycin), but isolates that are resistant to this drug have recently emerged (Unemo, Golparian et al. 2010, Ohnishii, Saika et al. 2011, Centers for Disease Control and Prevention. 2012, Unemo, Golparian et al. 2012).

We are investigating inhibitors of LpxC (UDP-3-O-(acyl)-N-acetylglucosamine deacetylase), the zinc-dependent enzyme that catalyzes the first committed step in lipid A biosynthesis (Anderson and Raetz 1987), as a novel class of antibiotics against *N. gonorrhoeae*. Lipid A is a unique saccharolipid that is a major component of the outer surface of the outer membrane of Gram-negative bacteria. It is the component of endotoxin that can cause septic shock during infections with Gram-negative bacteria (Raetz 1993, Raetz and Whitfield 2002). The biosynthesis of lipid A has been best characterized in *E. coli*. Of the nine enzymes required for the synthesis of lipid A in *E. coli*, the first seven are conserved in *N. gonorrhoeae* (Preston, Mandrell et al. 1996).

The enzyme LpxC is a deacetylase that catalyzes the first committed step in the pathway (Anderson, Robertson et al. 1988, Young, Silver et al. 1995, Sorensen, Lutkenhaus et al. 1996). LpxC has no homology with mammalian deacetylases and is a promising target for the development of a new class of antibiotics (Jackman 2000, Clements, Coignard et al. 2002). As described in Chapter 3, LpxC is essential in *N. gonorrhoeae*, and LpxC inhibitors
are bactericidal for this bacterium. These inhibitors are effective against recently isolated cephalosporin-resistant strains and are active \textit{in vivo} in the mouse model of gonorrhea.

However, despite the promising \textit{in vitro} and \textit{in vivo} activity of the LpxC inhibitors against a range of Gram-negative bacteria, mutants with resistance to LpxC inhibitors have been described previously in \textit{Escherichia coli} and \textit{Pseudomonas aeruginosa} (Clements, Coignard et al. 2002, Caughlan, Jones et al. 2011, Zeng, Zhao et al. 2013). Thus, it is possible that the gonococcus might also be able to develop resistance to this class of drugs should it be used in the clinic. Gaining an early understanding of potential bacterial resistance mechanisms to these compounds could provide valuable information as to the best clinical use of LpxC inhibitors and could inform guidelines to preserve the therapeutic lifetime of this new class of antibiotics.

This paper describes the isolation of \textit{N. gonorrhoeae} mutants with reduced susceptibility to LpxC inhibitors and the identification of a locus that is required for sensitivity to the inhibitors. The mechanism responsible for the approximately 10-fold increase in the MIC of LpxC inhibitors of the R7 mutant remains unknown, but it is specific for LpxC inhibitors and is not due to alterations in the structure or expression of the target enzyme.
4.3 Materials and Methods

4.3.1 Bacterial strains and growth conditions

*N. gonorrhoeae* strain FA19 (provided by Dr. Fred Sparling, University of North Carolina at Chapel Hill) was used for strain construction throughout this study (Maness and Sparling 1973). R7 is a spontaneously arising mutant of FA19.

*N. gonorrhoeae* strains were cultured on GC medium base (GCB) agar plates containing supplements I and II and were grown in a 37°C incubator with 4% CO₂ (Kellogg, Peacock et al. 1963). The cells were also grown in GCB broth containing supplements I and II and 10 mM NaHCO₃ in a 37°C incubator with gentle shaking. The *E. coli* strains used for cloning in this study were grown on LB agar plates or in LB broth. *E. coli* strains transformed with the constructs described in this paper were selected on 50 μg/ml kanamycin (knock-out constructs), 20 μg/ml chloramphenicol (pKH35), or 100 μg/ml carbenicillin (pUC18us).

4.3.2 Isolation of spontaneous mutants

Spontaneous mutants of FA19 with reduced susceptibility were obtained by growing FA19 to mid-log phase (OD₅₆₀ ~0.5-0.6) in liquid culture and then plating 100 μl of the cells on media containing 4× the MIC of the inhibitor LPC-067. To calculate the spontaneous mutation frequency, cells were also diluted and plated on GCB agar without selection.
4.3.3 Susceptibility testing

The MICs of LpxC inhibitors for the strains described in this study were determined using the agar dilution method, as previously described (Ropp, Hu et al. 2002). Briefly, cells grown on GCB agar plates were suspended in GCB broth and diluted to an OD$_{560}$ of 0.018. Plates containing varying concentrations of the indicated inhibitor were prepared, and five microliters of the cell suspension were spotted on each plate. The plates were incubated 37°C for 24 hours. The MIC was defined as the lowest concentration of inhibitor that prevented the growth of more than five colonies.

4.3.4 Transformation of *N. gonorrhoeae*

Piliated FA19 cells were swabbed from GCB agar and resuspended in GCB+ containing 10 mM MgCl$_2$. The cells were diluted to an OD$_{560}$ of 0.18, and 900 µl of these cells were mixed with 100 µl of 1× SSC buffer containing approximately 1 µg of DNA. This mixture was incubated for 5 h at 37°C in a humidified incubator containing 5% CO$_2$. At the end of the transformation period, cells were plated on GCB agar plates containing the appropriate antibiotics.

4.3.5 Identification of the R7 mutation

To identify the mutation responsible for the phenotype of the R7 mutant, genomic DNA was prepared from R7 and digested with the enzyme *DraI*. After confirming that the digested DNA retained the ability to transform FA19 to the same level of resistance as the R7 mutant, the digests were electrophoresed, and 11 fragments ranging in size from 1 kb to greater than 10 kb were purified from the gel. The ability of each of these fragments to
transform FA19 to resistance was assessed. After identifying the Dral fragments that produced transformants with elevated MICs, the gel-purified fragments were treated with alkaline phosphatase and cloned into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The TOPO reaction was transformed into TOP10 cells and selected on LB plates containing kanamycin. Over 400 colonies were isolated and transferred to individual wells of 96-well plates containing 150 µl LB broth. The plates were incubated overnight to allow for cell growth, 75 µl of 50% glycerol was added the following day, and the plates were stored at -80°C.

To isolate the plasmid containing the mutation, 10 µl of cells from each well in a single row of the 96-well plates were pooled and transferred to culture tubes. Three milliliters of LB were added, and the cells were grown overnight. Plasmid DNA was then prepared from these cultures and used to transform strain FA19. After identifying the pool that yielded transformants, the colonies in the individual wells within that pool were cultured overnight in three milliliters of LB. Plasmid DNA was isolated from these individual strains and again used to transform strain FA19. The plasmid that yielded colonies that were able to grow on 0.3 µg/mL LPC-091 was then sequenced using the vector primers included with the TOPO cloning kit.

### 4.3.6 Complementation of the R7 mutant

The genes pqiA and pqiB were cloned into the complementation vector pKH35 both individually and together. This vector places the gene(s) of interest under the control of the lac promoter so that the cloned DNA can be expressed by IPTG induction. This plasmid also
contains the genes *lctP* and *aspC* to serve as recombination sites to insert the cloned DNA and the chloramphenicol acetyltransferase gene *catG* into the gonococcal chromosome. *N. gonorrhoeae* transformants containing this plasmid were selected on 0.6 μg/ml chloramphenicol and confirmed by PCR and sequencing.

### 4.3.7 Gene disruption and knock-out

The genes *pqIA* and *pqIB* were simultaneously deleted from strain FA19 using overlap-extension PCR to amplify the 5′ end of *pqIA* and the 3′ end of *pqIB* with a *DraI* site at the junction of the two genes. This PCR fragment was cloned into the vector pUC18us, and a kanamycin resistance cassette was inserted at the *DraI* site of the resulting plasmid. Plasmid DNA was prepared, sequence confirmed, and used to transform *N. gonorrhoeae*. A similar strategy was employed to construct the Δfrag1-4 constructs shown in Figure 4.4. The genes *pqIA* and *pqIB* were also individually inactivated in FA19. Each gene was amplified with primers that added a 5′*HindIII* site and a 3′*XbaI* site and cloned into pUC18us. After confirming that the genes had been inserted into pUC18us, a kanamycin resistance cassette was inserted into endogenous restriction sites in the genes (*MluI* for *pqIA* and *ClaI* for *pqIB*). The resulting constructs were used to inactivate *pqIA* and *pqIB* individually in FA19.
4.4 Results

4.4.1 Isolation and characterization of spontaneously arising mutants

We isolated mutants with reduced susceptibility to LpxC inhibitors by growing FA19 cells in liquid culture and then plating the cells on media containing 4× the MIC of the inhibitor LPC-067. Mutants were isolated at a frequency of approximately $1.1 \times 10^{-6}$. The MICs of LPC-091 for these strains were determined and ranged from 4- to 16-fold higher than the parental strain, as shown in Figure 4.1. Based on previous studies in *E. coli* and *P. aeruginosa* (Clements, Coignard et al. 2002, Caughlan, Jones et al. 2011, Zeng, Zhao et al. 2013), several genes were considered as potential candidates for the sites of the mutations. These genes, *fabG*, *fabZ*, *lpxA*, *lpxC*, and *mtrR* and their promoter regions, were sequenced in mutants R1, R2, R4, and R7, but no mutations were identified.

The R7 mutant was chosen for further characterization. To determine if its decreased susceptibility to LpxC inhibitors was due to a growth defect, its growth in GCB broth was assessed in comparison to the parental strain FA19. As shown in Figure 4.2A, no growth defect was observed. The expression of LpxC in the R7 mutant was also evaluated using a strain in which the endogenous copy of LpxC was HA-tagged (described in Chapter 3), but no differences in LpxC expression were observed between R7 and the wild-type strain FA19 (Figure 4.2B). We also determined the MICs of a variety of other antibiotics for R7 and the other spontaneous mutants but found no differences in the MICs of these antibiotics for the mutant strains compared with the parental strain (data not shown). Therefore, we concluded that the mechanism responsible for the decreased susceptibility to LpxC inhibitors was not a general resistance mechanism.
Genomic DNA prepared from the R7 mutant was used to transform a strain of gonorrhea that expresses LpxC from *E. coli* (EcLpxC) rather than gonococcal LpxC. The MIC of the R7 EcLpxC strain was dramatically elevated compared to the MIC of FA19 EcLpxC, indicating that the mechanism of reduced susceptibility is distinct from the LpxC enzyme itself (Figure 4.2C).
Figure 4.1 Spontaneous mutants with reduced susceptibility to LpxC inhibitors. Mutants with reduced susceptibility to LpxC inhibitors were isolated and their phenotypes were confirmed by MIC assays.
Figure 4.2 Phenotype of the R7 mutant. (A) The growth of the R7 mutant was assessed in comparison with the parental strain FA19 by monitoring the OD$_{600}$ of the two strains during growth in GCB broth containing supplements I, II, and B and sodium bicarbonate. This experiment was done three times, and a representative growth curve is shown. (B) The endogenous copy of LpxC was HA-tagged in the R7 mutant to assess the effect of the mutation on expression of LpxC via Western blotting for the HA tag. (C) The R7 mutant displays an elevated LPC-091 MIC even when LpxC from *E. coli* (EcLpxC) is expressed in place of gonococcal LpxC.
4.4.2 Identification of the R7 mutation

To determine the site of the mutation in the R7 mutant, genomic DNA was prepared from R7 and digested with the enzyme Dral. FA19 was transformed with the digested DNA to confirm that the digestion did not disrupt the site of the R7 mutation. Then, the digested DNA was electrophoresed, and fragments of DNA ranging in size from 1 kb to greater than 10 kb were purified from the gel (Figure 4.3A). The ability of each of these fractions to transform FA19 and yield colonies with reduced susceptibility to LPC-091 was evaluated. We found that transformation with fraction 6, which contained DNA ranging in size from 3 to 4 kb, produced the most colonies (Figure 4.3B). DNA fragments contained in this fraction were subsequently cloned into the TOPO-blunt vector, and approximately 250 TOPO clones were screened for their capacity to transform strain FA19. The plasmid that yielded the greatest number of transformants was sequenced and was found to contain DNA that flanked a large deleted region. This was confirmed by PCR amplification of the corresponding region in the R7 mutant. We found that R7 contained a large deletion that resulted in the truncation of two genes and deletion of nine others (Table 4.1 and Figure 4.4).

To identify which of the eleven truncated or deleted genes were responsible for the phenotype of the R7 mutant, smaller regions containing two to four genes were deleted from strain FA19 (Figure 4.4). Deletion of the two genes at the 5’ end of the large deletion in R7, pqiA and pqiB, in strain FA19 resulted in a strain (FA19 ΔpqiAB) with an LPC-091 MIC similar to that of the R7 mutant (Figure 4.5). The deletion of any of the other genes missing in R7 did not alter the MIC compared to the parental strain FA19.
Figure 4.3 Isolation of the Dral DNA fragment containing the mutation. (A) Genomic DNA prepared from the R7 mutant was digested with the enzyme Dral, and the resulting DNA fragments were electrophoresed on a 1% gel. Fragments of the indicated sizes were extracted from the gel and purified. (B) FA19 was transformed with the Dral fragments indicated in panel A, and the number of transformants isolated on 0.3 μg/mL LPC-091 was recorded.
### Table 4.1 Genes that were truncated or deleted in R7.

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Name</th>
<th>Length</th>
<th>Predicted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGO-1311*</td>
<td>aprT</td>
<td>600</td>
<td>Adenine phosphoribosyltransferase</td>
</tr>
<tr>
<td>NGO-1312</td>
<td></td>
<td>810</td>
<td>Hydrolase</td>
</tr>
<tr>
<td>NGO-1313</td>
<td></td>
<td>111</td>
<td>Neisseria-specific protein</td>
</tr>
<tr>
<td>NGO-1314</td>
<td></td>
<td>1356</td>
<td>Pro tease, undefined</td>
</tr>
<tr>
<td>NGO-1315</td>
<td></td>
<td>474</td>
<td>Neisseria-specific protein</td>
</tr>
<tr>
<td>NGO-1316</td>
<td>tbpA</td>
<td>1053</td>
<td>Transferrin binding protein A</td>
</tr>
<tr>
<td>NGO-1316.1</td>
<td>hmbR</td>
<td>852</td>
<td>Transferrin binding protein A</td>
</tr>
<tr>
<td>NGO-1317</td>
<td>tpn</td>
<td>651</td>
<td>IS1016 transposase</td>
</tr>
<tr>
<td>NGO-1318</td>
<td>hemO</td>
<td>711</td>
<td>Heme oxygenase/iron starvation protein</td>
</tr>
<tr>
<td>NGO-1319</td>
<td>pqiA</td>
<td>1302</td>
<td>Paraquot-inducible protein A</td>
</tr>
<tr>
<td>NGO-1320*</td>
<td>pqiB</td>
<td>1659</td>
<td>Paraquot-inducible protein B</td>
</tr>
</tbody>
</table>

* indicates truncated genes
**Figure 4.4** Map of the region deleted in R7. Four smaller fragments were deleted to identify the genes that were responsible for the decreased susceptibility of the R7 mutant to LpxC inhibitors (gene maps modified from the STDGEN Database (Los Alamos National Laboratory 2003)).
4.4.3 Function of PqiA and PqiB

Because *pqiA* and *pqiB* were first identified in *E. coli* as part of the *soxRS* regulon that is induced upon exposure to paraquat (Koh and Roe 1995), we hypothesized that these genes may play a role in mediating the response to oxidative stress despite the lack of SoxRS orthologs in *N. gonorrhoeae*. However, the R7 mutant showed no difference in sensitivity to H$_2$O$_2$ or to paraquat compared to the parental strain FA19 (data not shown). No other function has been described or predicted for these proteins. PqiA is a 434 amino acid protein with a predicted molecular weight of 47854 and eight predicted transmembrane domains. PqiB is a 553 amino acid protein with a predicted molecular weight of 60470, one transmembrane domain, and a coil-coil domain. Neither protein has a predicted signal sequence. Orthologs of PqiA and PqiB are found throughout a wide range of Gram-negative bacteria with varying degrees of sequence identity, but they are absent from Gram-positive bacteria (Table 2). Interestingly, lipid A is also only found in Gram-negative bacteria.
Table 4.2 Amino acid sequence identity of PqiA and PqiB. Tables showing the amino acid sequence identity of gonococcal PqiA (A) and PqiB (B) with PqiA and PqiB from other Gram-negative bacteria.

### A  PqiA

<table>
<thead>
<tr>
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<th>Pae</th>
<th>Vch</th>
<th>Hin</th>
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<td>29</td>
<td>17*</td>
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</tr>
<tr>
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<td>21*</td>
<td>40</td>
<td>25</td>
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<tr>
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<td>38</td>
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<tr>
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<td></td>
<td>20*</td>
<td>16*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vch</td>
<td>- - -</td>
<td></td>
<td></td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hin</td>
<td>- - -</td>
<td></td>
<td></td>
<td></td>
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### B  PqiB

<table>
<thead>
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<th>Pae</th>
<th>Vch</th>
<th>Hin</th>
</tr>
</thead>
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<td>35</td>
<td>23</td>
<td>37</td>
<td>14#</td>
</tr>
<tr>
<td>Eco</td>
<td>- - -</td>
<td>82</td>
<td>22</td>
<td>43#</td>
<td>13#</td>
<td></td>
</tr>
<tr>
<td>Kpn</td>
<td>- - -</td>
<td>23</td>
<td>43</td>
<td>11#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pae</td>
<td>- - -</td>
<td>23</td>
<td>19#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vch</td>
<td>- - -</td>
<td></td>
<td>8#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hin</td>
<td>- - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


*Pae* PqiA contains a large deletion; *Hin* PqiB contains a large insertion.
4.4.4 Requirement of both \textit{pqiA} and \textit{pqiB} for sensitivity to LpxC inhibitors

The genes \textit{pqiA} and \textit{pqiB} were disrupted simultaneously or individually in FA19 to determine if the loss of one gene was sufficient to increase the MIC of the LpxC inhibitor LPC-091. We found that deletion of both genes resulted in the largest increase in the MIC, and deletion of either gene individually resulted in an intermediate phenotype. Conversely, we also evaluated the ability of these genes together and alone to restore sensitivity to the R7 mutant. Complementation with \textit{pqiAB} together restored the MIC of the mutant to the same level as the wild type strain FA19. However, complementation with either \textit{pqiA} or \textit{pqiB} alone did not alter the MIC of R7 (Figure 4.5). Therefore, we concluded that both genes are required for sensitivity to LpxC inhibitors.
Both *pqiA* and *pqiB* are required for susceptibility to LpxC inhibitors. The bars to the left of the dashed line are the MICs of strains in which *pqiA* and *pqiB* have been deleted separately or simultaneously from strain FA19. The bars to the right of the dashed line show the MICs of strains in which *pqiA* and *pqiB* have been express separately or simultaneously in strain R7.
4.4.5 Complementation of other spontaneous mutants with *pqiAB*

To determine if mutations in *pqiAB* were responsible for the phenotypes of the R1, R2, and R4 mutants, the construct pKH35-*pqiAB* was transformed into these strains. Expression of *pqiAB* was able to restore sensitivity to LpxC inhibitors in the R2 and R4 mutants but not R1 (Figure 4.6). This indicates that the gonococcus can develop multiple mechanisms of resistance to LpxC inhibitors. Sequence analysis of *pqiAB* from R1, R2, and R4 confirmed the results of the complementation experiment. No mutations were detected in the *pqiAB* coding sequence or promoter region of R1. However, a single base pair substitution in *pqiB* in R2 that results in an amino acid change to a premature stop codon (W340stop) was identified, and a frame-shift mutation in *pqiA* was found in R4 (data not shown).
Figure 4.6 Expression of *pqiA* and *pqiB* in other mutants. The expression of *pqiA* and *pqiB* restores sensitivity to the R2 and R4 mutants but not to R1.
4.5 Discussion

LpxC is a promising target for new antibiotics with activity against *N. gonorrhoeae*, including recently isolated cephalosporin-resistant strains. No current antibiotics target the lipid A biosynthesis pathway, even though it is essential in nearly all Gram-negative bacteria. Notably, LpxC is dispensable in the closely related strain *N. meningitidis*, but we have shown that it is essential in *N. gonorrhoeae* (Chapter 3). LpxC is a zinc-metalloenzyme with little homology to mammalian deacetylases, and the development of inhibitors of this enzyme has been the focus of multiple groups over the last two decades (Onishi, Pelak et al. 1996, Clements, Coignard et al. 2002, Mdluli, Witte et al. 2006, Barb, McClerren et al. 2007, Bodewits, Raetz et al. 2010, Lee, Liang et al. 2011).

Our lab has shown that the LpxC inhibitor LPC-174 has activity against gonorrhea *in vivo* using the mouse model of gonococcal infection (Chapter 3), and we believe that this class of drugs represents a promising new potential option for the treatment of MDR gonorrhea. However, despite the excellent *in vitro* and *in vivo* activity of LpxC inhibitors, past experience tells us that it would only a matter of time before the gonococcus developed resistance to these drugs should they be used in the clinic.

Mechanisms of resistance to LpxC inhibitors have been previously studied in *E. coli* and *P. aeruginosa*. These mechanisms have included mutations in the fatty acid biosynthesis pathway, overexpression of efflux pumps, overexpression of LpxC, and mutation of LpxC. Mutations in *fabZ* in *E. coli* and *fabG* in *P. aeruginosa* result in decreased susceptibility to LpxC inhibitors. FabZ and LpxA share a common substrate, R-3-hydroxymyristoyl-ACP. Therefore, mutations in the fatty acid biosynthesis pathway results in the accumulation of substrate for LpxA and increased lipid A synthesis (Clements,
A similar phenomenon was observed in *P. aeruginosa* with mutations that altered the expression or activity of the fatty acid biosynthesis gene *fabG* (Caughlan, Jones et al. 2011). Both of these studies also isolated mutants with mutations in LpxC. It is somewhat surprising that of the four mutants (R1, R2, R4, and R7) isolated in this study, no mutations in *fabG*, *fabZ*, or *lpxC* were identified. The isolation and characterization of additional spontaneous mutants may yield one of these previously characterized mechanisms. A more recent study in *E. coli* also identified mutations in *fabZ* as conferring increased resistance to LpxC inhibitors (Zeng, Zhao et al. 2013) as well as mutations in the tRNA synthase ThrS that conferred an additional increase in resistance to LpxC inhibitors. This mutation impaired protein synthesis and resulted in >200-fold increase in resistance to LpxC inhibitors. Moreover, these two mutations were independent, i.e. the same fold decrease in susceptibility was observed no matter what order the mutations were introduced.

Additional studies are needed to characterize the mechanism responsible for the reduced susceptibility conferred by the loss of PqiA and PqiB. The observation that the MICs of other antibiotics are not altered by the loss of PqiAB strongly suggests that the mechanism conferring the increased resistance is specific for LpxC inhibitors. Because these proteins are both predicted to be membrane bound, it is possible that their presence enhances transport of the inhibitors into the cytoplasm of *N. gonorrhoeae*. The development of a fluorescently tagged or radioactive LpxC inhibitor would allow us to assess the ability of the inhibitors to access the cytoplasm of the *pqiAB* mutant and would be a valuable tool in continued efforts to identify the resistance mechanism.
Other future studies should include attempts to isolate and characterize mutants with even further reduced susceptibility to LpxC inhibitors. Because the mutation responsible for the resistance phenotype of the R1 mutant does not occur within the \textit{pqiAB} locus, it may be possible to transform the R7 mutant with DNA from the R1 mutant to isolate a strain with greater resistance to LpxC inhibitors. Furthermore, because many circulating strains of antibiotic-resistant gonorrhea contain the \textit{mtrR} mutation that results in the overexpression of the MtrCDE efflux pump, which confers a 4-fold increase in the MIC of LpxC inhibitors (Chapter 3), the simultaneous effect of the \textit{mtrR} mutation and the loss of \textit{pqiAB} should be evaluated. Because R7 confers an approximately 10-fold increase in the MIC, the presence of both of these mutations may result in a 40-fold increase relative to the wild type strain FA19.

In conclusion, although LpxC inhibitors have potent bactericidal activity against MDR \textit{N. gonorrhoeae} isolates \textit{in vitro} and display activity \textit{in vivo} using the mouse model of gonorrhea, history tells us that no antibiotic is immune to the development of resistance mechanisms. Because of the dire need for new antibiotics, it will be important to extend the therapeutic lifetime of any new classes of drugs that can be developed. Studying potential resistance mechanisms in the lab before they occur in nature may provide information that could help set guidelines for the use of these drugs in the clinic.
Chapter 5. Discussion and future directions

5.1 Summary and significance

The threat of untreatable gonorrhea is a very real public health issue. In the fall of 2013, the CDC released a report summarizing antibiotic resistance threats in the US. For the first time, it prioritized bacteria according to the threat that antibiotic-resistant strains of these species pose to public health. Drug-resistant *N. gonorrhoeae* was assigned to the highest threat level, “urgent”, along with *Clostridium difficile* and the carbapenem-resistant Enterobacteriaceae (Centers for Disease Control and Prevention. 2013). To help control antibiotic-resistant bacteria, the CDC has recommended the following four actions:

1) prevent infections and the spread of antibiotic resistant bacteria,
2) analyze the spread of resistant bacteria,
3) improve the use of existing antibiotics, and
4) develop new antibiotics and diagnostics.

Accomplishing each of these goals will require a multifaceted approach and collaboration between clinicians and other healthcare workers, basic scientists, and members of the biotech industry. This dissertation describes work that makes significant contributions to several of these recommended actions.

The work reported in Chapter 2 characterizes a mechanism of resistance that can result from mutations that decrease the expression of the PilQ secretin. Understanding current resistance mechanisms is an important component of tracking antibiotic-resistant
infections. Furthermore, observations made while characterizing the contribution of PilQ to antibiotic permeation suggest that the proteins encoded by the genes upstream of pilQ form a periplasmic complex. This complex has been previously identified in P. aeruginosa, and although our data are similar, we have identified several species-specific differences regarding the interactions between these proteins.

Chapter 3 describes our progress in developing LpxC inhibitors, a new class of antibiotics with a novel target that inhibits the growth of antibiotic-resistant strains of N. gonorrhoeae. LpxC is an exciting drug target because it is essential for the production of lipid A, the active component of endotoxin and the membrane-bound tether of lipooligosaccharide. LOS (or LPS in other species) forms a formidable barrier that antibiotics must cross to inhibit the growth of Gram-negative bacteria.

The work detailed in Chapter 4 builds upon that described in Chapter 3. I identified a potential mechanism that N. gonorrhoeae could utilize to decrease susceptibility to LpxC inhibitors, and this data could be useful if they become an approved class of antibiotics. Understanding how resistance to LpxC inhibitors may develop could improve the usage of these drugs by informing guidelines regarding dosage and combination therapy recommendations based on the degree of resistance conferred by individual mutations.

N. gonorrhoeae has demonstrated an incredible capacity to develop resistance to every class of drugs that have been used in the treatment of gonorrhea. The recent emergence of cephalosporin-resistant gonorrhea has added a new level of urgency to attempts to develop novel antibiotics that are effective against MDR strains. The work described in this dissertation and the future studies that will build upon this research will
hopefully contribute to our ability to continue to control gonococcal infections with antibiotic therapy.

5.2 Pathogenic potential of pilQ mutants

Our lab previously identified a mutation in PilQ (Glu-666 to Lys; designated pilQ2) that increased antibiotic resistance, disrupted piliation, and decreased transformation competence. When lysates of this mutant were subjected to SDS-PAGE and Western blotting for PilQ, we found that the pilQ2 mutation interfered with the production of the SDS-resistant multimeric form of PilQ that in wild-type strains of *N. gonorrhoeae* migrates to the interface of the stacking and resolving gels in SDS-PAGE (Zhao, Tobiason et al. 2005). In contrast to the phenotype of pilQ2, an additional pilQ mutant referred to as pilQ1 demonstrated increased susceptibility to antimicrobials and showed no effects on formation of the SDS-resistant multimer. However, similar to the pilQ2 mutant, the pilQ1 mutant also displayed decreased piliation and had reduced transformation efficiency (Chen, Tobiason et al. 2004). The work described in Chapter two of this dissertation was initiated to better understand how PilQ contributes to antibiotic resistance.

Mutations in or upstream of PilQ have not been described in clinical isolates (Whiley, Jacobsson et al. 2010); however, non-piliated strains remain infective but are less inflammatory (Hobbs, Sparling et al. 2011). We used SEM to examine several spontaneous mutants that exhibited increased resistance and a non-piliated colony morphology and observed that these mutants expressed pili, albeit at reduced numbers and with fewer interacting bundles of pili from different cells (Figure 2.2C). Electron microscopy with immunogold labeling of the pilQ2 mutant showed that some of the mutant cells had very
small projections that were labeled with the anti-pilin antibody (Zhao, Tobiason et al. 2005), but these projections were much smaller than those described in Chapter 2. The differences in piliation observed between the spontaneous mutants isolated in Chapter 2 and the pilQ2 mutant may be due to the different techniques used to visualize the cells and pili. In Chapter 2, we used SEM without any labeling to observe the cells; Zhao et al. used an anti-pilin antibody and immunogold labeling to detect the pili with transmission electron microscopy (TEM). However, it is unlikely that the immunogold labeling method would fail to detect pili such as those shown in Figure 2.2C if they were in fact present on the surface of the pilQ2 mutant.

Much like the pilQ2 mutant, the spontaneous mutants described in Chapter 2 all showed elevated penicillin MICs compared to the parental strain. In contrast, these mutants displayed a broad range of transformation efficiencies. The transformation efficiency assay is used as an indicator of pilus function, and the large variations in transformation efficiency suggests that pilus function and antibiotic permeation through the PilQ oligomer are independent of each other. Therefore, it remains possible that mutations could develop that alter the structure or expression of PilQ in ways that would render gonococci refractory to antibiotic treatment without eliminating pilus function.

PilQ, and type IV pili in general, are well conserved in a number of Gram-negative pathogens, many of which also display concerning levels of antibiotic resistance. Due to the high degree of similarity between the TFP systems of different bacteria, mutations in PilQ may increase antibiotic resistance in other species as well. In contrast to the lack of gonococcal clinical isolates with mutations in PilQ, a strain of P. aeruginosa isolated from a patient with cystic fibrosis (CF) was found to have a 281-bp deletion in pilQ, while another
strain isolated from a different CF patient had a mutation in \textit{pilF}, the pseudomonal homolog of gonococcal \textit{pilW} (Chang, Klockgether et al. 2007, Klockgether, Miethke et al. 2013). These mutants are both non-piliated (they do not produce extracellular pilin); however, the \textit{pilQ} mutant retains motility due to the expression of flagella and the upregulation of the type IVb pilus and CupE fimbriae.

Antimicrobial susceptibility testing was not performed for these mutants, but the authors did find that the \textit{pilQ} mutant was resistant to killing by leukocytes and was able to grow within PMNs (Klockgether, Miethke et al. 2013). This study shows that in the context of a chronic infection, such as is found in the CF lung, pseudomonal PilQ is dispensable. The infectivity of this strain is unknown, but CF patients can remain colonized with the same strain of \textit{P. aeruginosa} for many years, and the loss of a functional PilQ may provide a fitness benefit during chronic infection. It is also unknown if mutations in pseudomonal \textit{pilQ} can increase the MICs of antibiotics; any mutation that renders the treatment of chronic \textit{P. aeruginosa} infections in CF patients more difficult could have clinically relevant consequences.

\textbf{5.3 LpxC overexpression}

In Chapter 3, we showed that LpxC inhibitors are active against \textit{N. gonorrhoeae}. To demonstrate that gonococcal LpxC is the primary target of LpxC inhibitors, we created two strains that expressed IPTG-inducible LpxC from either \textit{E. coli} (EcLpxC) or \textit{R. leguminosarum} (RlLpxC). The endogenous copy of LpxC (NgLpxC) was then disrupted so that the foreign LpxC was the only copy of LpxC expressed. EcLpxC is potently inhibited by diacetylene LpxC inhibitors, and therefore a corresponding decrease in the MIC of the
inhibitor was observed for the strain expressing this species of LpxC. In contrast, RlLpxC is poorly inhibited by diacetylene LpxC inhibitors, and we observed an increase in the MIC when RlLpxC was expressed in *N. gonorrhoeae* (Figures 3.4 and 3.6) (Barb, Jiang et al. 2007, Barb, McClerren et al. 2007).

To further demonstrate that LpxC was the target of our compounds, we expressed NgLpxC (with and without an HA tag) from an IPTG-inducible promoter and determined the MIC of an LpxC inhibitor at different levels of induction. Although the expression of the HA-tagged NgLpxC increased with increasing levels of IPTG (Figure 3.5), no corresponding increase in the MIC of the LpxC inhibitor was observed (data not shown). This result was surprising because we expected that increasing the amount of target (LpxC) would result in an increase in the amount of inhibitor required to kill the cells. However, it is possible we might be better able to observe a target-dependent effect if we instead focused on under-expressing LpxC and detecting increased susceptibility to LxpC inhibitors.

A recent paper described the development and use of mathematical models to explain why target overexpression does not always increase drug resistance (Palmer and Kishony 2014). The authors examined the effect of overexpressing the targets of seven different antibiotics in *E. coli*. They found that resistance due to overexpression occurred for three of the drugs tested, and that this phenomenon corresponded to drugs that inhibit a single target (trimethoprim, triclosan, and coumermycin). However, resistance due to target overexpression does not occur when the drug sequesters an essential metabolite (such as the binding of sulfamethoxazole to pteridine diphosphate). Furthermore, target overexpression can also increase sensitivity to drugs when the target/drug complex is
responsible for the toxicity of the drug, such as for the interaction of ciprofloxacin with DNA gyrase.

Because diacetylene LpxC inhibitors occupy the hydrophobic substrate-binding passage of the enzyme that is occupied by the natural substrate acylated UDP-GlcNAc (Lee, Liang et al. 2011), this mechanism of action would suggest that target overexpression should result in antibiotic resistance (Palmer and Kishony 2014). There are several possible explanations for our observation that the overexpression of NgLpxC does not alter the MIC. One possibility is that interactions between LpxC and the enzymes preceding or following it in the lipid A biosynthetic pathway (LpxA and LpxD, respectively) are required for the progression of the pathway, and that these interactions are blocked by the LpxC/inhibitor complex. For example, if interactions between LpxC and LpxD are required for the binding of LpxD to its substrate, then inhibitor-bound LpxC could sequester LpxD from interacting with substrate-bound LpxC molecules even when LpxC is overexpressed.

A perhaps more likely possibility is that when LpxC is overexpressed, the amount of free Zn\(^{2+}\) in the cell is not sufficient to bind to the LpxC apoenzymes. The zinc ion is critical for the recognition of fatty acids by LpxC and for the catalytic activity of the enzyme (Hernick and Fierke 2006). Therefore, even though the total amount of LpxC was overexpressed in our system, the increase in active LpxC may have been negligible. The hypothesis that Zn\(^{2+}\) is limiting when LpxC is overexpressed could be tested by assessing the MIC of LpxC inhibitors following LpxC overexpression in media supplemented with additional zinc (perhaps as ZnSO\(_4\)). It may also be necessary to increase the expression of the MntABC metal transport system that is responsible transporting both manganese and zinc into gonococci (Lim, Jones et al. 2008).
5.4 LpxC stability and regulation

As an alternative to using LpxC overexpression to test the requirement of LpxC for the killing activity of LpxC inhibitors, we also attempted a strategy of growing cells that express an IPTG-inducible copy of NgLpxC-HA but have an inactivated endogenous copy of *lpxC*. Cells were grown to mid-log phase in liquid media containing IPTG, and the cells were then pelleted, washed with media, resuspended in broth lacking IPTG, and split into two flasks. One flask was treated with $4 \times$ the MIC of the LpxC inhibitor LPC-091, and the other flask was left untreated. Aliquots were removed each hour to monitor the OD$_{560}$. Surprisingly, no decrease in cell density was observed, even in the flask that was treated with the LpxC inhibitor. After four hours of incubation, cells were removed from each flask and used to prepare samples for SDS-PAGE and Western blotting. The resulting Western blot showed that HA-tagged LpxC was still present in the cells that had been treated with inhibitor and in the untreated cells.

There are several possible explanations for these observations. The first is that LpxC inhibitors are only active against cells that are growing. Gonococci are very sensitive to changes in their growth conditions, and washing and resuspending the cells may have been sufficient to switch the cells to stationary phase even if LpxC induction had continued. This is the most straight-forward explanation for the observations made regarding the untreated cells. The continued density of the cells treated with the LpxC inhibitor could be due to a similar phenomenon. If the cells were no longer actively growing, then treatment with the inhibitor could be expected to have no effect on cell density.
The detection of HA-tagged LpxC at the end of the four-hour incubation in absence of IPTG suggests that gonococcal LpxC is quite stable and has a low turn-over rate. It also suggests that the binding of inhibitor to the enzyme does not alter protein stability, as presumably the majority of LpxC present in the cells in the treated flask was bound to inhibitor. Little is known about the regulation of LpxC at the protein level in *N. gonorrhoeae*, and it is possible that the presence of the HA-tag subverts normal protein turnover. However, the observations described above regarding the continued growth of cells after the removal of IPTG from the media were also observed for cells that expressed a non-HA-tagged copy of NgLpxC. Therefore, it is unlikely that the HA-tag is responsible for the persistence of the enzyme even in the absence of induction.

In *E. coli*, LpxC levels are regulated by the protease FtsH, and both the lack of LpxC and the overexpression of LpxC are lethal (Ogura, Inoue et al. 1999). Degradation of LpxC by FtsH requires the presence of a C-terminal degradation signal (Führer, Langklotz et al. 2006, Führer, Müller et al. 2007). Although LpxC is well-conserved in Gram-negative bacteria, the C-termini are quite divergent. The C-terminal degradation signal is present in enterobacteria, but is absent in many other bacteria, including the betaproteobacteria (e.g., *N. gonorrhoeae*) (Langklotz, Schakermann et al. 2011). LpxC is regulated by the Lon protease in at least some of the alphaproteobacteria (Langklotz, Schakermann et al. 2011); this protease is present in gonococci, but its potential role in regulating LpxC levels has not been explored.
5.5 Broad vs. narrow spectrum antibiotics

Antibiotic research has traditionally focused on the development of drugs with a broad spectrum of activity, e.g., tetracycline and other antibiotics with activity against a wide variety of pathogens. From a financial standpoint, devoting funding to the development of a broad-spectrum antibiotic has a higher potential return on investment than devoting the same resources to a novel narrow-spectrum drug. There are also clinical advantages associated with broad-spectrum antibiotics. When a clinician diagnoses a bacterial infection, treatment can be initiated prior to determining the identity of the infecting organism. However, the major drawbacks of broad-spectrum antibiotics include the impact of the drugs on the natural flora, which can lead to complications such as C. difficile-associated diarrhea, and the risk of antibiotic resistance acquisition and spread (Carbon and Isturiz 2002, Spellberg, Powers et al. 2004).

The targeted use of narrow-spectrum antibiotics may prevent or slow the development of antibiotic resistance, but it will be necessary to develop/improve rapid pathogen diagnostic methods before narrow-spectrum drug development becomes a priority. It would be difficult to initiate clinical trials to evaluate the effectiveness of narrow-spectrum drugs unless the infecting pathogen could be quickly identified. Therefore, the development of narrow-spectrum drugs against aggressive pathogens that cause life-threatening diseases may be more difficult than the development of broad-spectrum antibiotics.

*N. gonorrhoeae* is perhaps an ideal pathogen for targeting with narrow-spectrum drugs. It rarely causes life-threatening disease, and rapid testing methods are being developed (Benzaken, Galban et al. 2006, Vickerman, Watts et al. 2006, Samarawickrama,
Cheserem et al. 2014). The diacetylene LpxC inhibitors described in this work are effective against many Gram-negative pathogens, but we have focused on optimizing them against *N. gonorrhoeae*. There is an urgent need for new antibiotics effective against ESC-resistant gonorrhea, and we hope that this novel class of inhibitors will meet the clinical and regulatory requirements to help fill that need.

**5.6 Future directions**

The LpxC inhibitor LPC-174 was tested in the murine model of gonococcal infection and was able to clear the infection in 8/9 infected animals. While the data obtained from the mouse studies demonstrate that diacetylene LpxC inhibitors are active *in vivo*, the inhibitors need to be further optimized to reduce sequestration of free drug by proteins in the serum and to improve the stability and half-life of the drugs. We also plan to test the activity of LPC-174 or a similar compound against a recent isolate, F89, that is resistant to cefixime and ceftriaxone. Evaluating the *in vivo* activity against F89 is an important next step in demonstrating the potential of LpxC inhibitors to be used in the treatment of ESC-resistant gonococcal infections.

Disrupting lipid A synthesis may reduce the severity of septic shock, and the use of LpxC inhibitors in combination therapy may restore the activity of other antibiotics that have previously been considered obsolete by increasing membrane permeability. Exploring potential synergistic interactions between LpxC inhibitors and other classes of drugs is an important future direction for this project. Even in the absence of drug synergy, combination therapy should be considered to decrease the potential of resistance developing to both antibiotics.
Because very few new antibiotics are being developed, it is critical that we safeguard the use of any new antibiotics to ensure that they are used to their full potential. The presence of PqiA and PqiB is required for maximum sensitivity to LpxC inhibitors in *N. gonorrhoeae*, but the mechanism behind this requirement remains unknown. Because PqiA and PqiB are only found in Gram-negative bacteria, they may play a yet-undescribed role in lipid A biosynthesis or transport. Alternatively, because they are both predicted to be membrane proteins, their presence may increase the ability of the LpxC inhibitors to access the cytoplasm. Characterization of the structure of lipid A by mass spectrometry and the development of LpxC inhibitors with radioactive or fluorescent tags would allow for both of these possibilities to be evaluated.
REFERENCES


