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Abstract

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Examination of *Neisseria gonorrhoeae* Opacity Protein Expression During
Experimental Murine Genital Tract Infection

Amy N. Simms, Doctor of Philosophy, 2005

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The opacity (Opa) proteins of *Neisseria gonorrhoeae* are a family of phase-variable outer membrane proteins that bind to host cells. Phase variable expression occurs via a reversible frameshift mechanism within each *opa* gene. Opa protein expression is selected for, or induced during experimental genital tract infection of female mice, similar to that which was reported in male volunteers. Using a genetically marked strain of FA1090 to follow recovery of a specific population of Opa variants during murine infection, here we showed that selection of a pre-existing population of Opa-positive gonococci present in the inoculum was responsible for the reisolation of mainly Opa-positive variants early during infection. We conclude that the preferential

recovery of Opa-positive gonococci observed early during murine infection is due to selection of a pre-existing population of Opa-positive variants caused by factors other than binding to human CEACAM receptors. In long-term infection of mice, a cyclical pattern of Opa protein expression was observed in which a decreased recovery of Opa-positive variants followed early selection for Opa protein expression; reemergence of Opa-positive gonococci occurred later in infection. To examine rates of phase variation in a *N. gonorrhoeae* background under physiologically relevant conditions, we engineered a translational *opaB::phoA* fusion and introduced it into the chromosome of *N. gonorrhoeae* strain FA1090 to generate strain ANS100. No change was seen in the frequency or rate of *opaB::phoA* phase variation in all in vitro conditions tested. We next examined whether increased phase variation of the *opaB::phoA* fusion occurred during murine infection. No significant change occurred in the frequency of “on” variants among vaginal isolates when mice were inoculated with predominantly “off” variants; however, a marked increase in the recovery of “off” variants was observed following inoculation with predominantly “on” variants. The inability to show differences in *opa* gene phase variation under different conditions in vitro leads us to conclude at this time that induction of *opa* gene phase variation may be spontaneous and random. However, the in vivo studies suggest that increased *opa* gene phase variation may occur under conditions that we have not yet been able to mimic in vitro.

To my parents, for everything

**Examination of *Neisseria gonorrhoeae* Opacity Protein Expression
During Experimental Murine Genital Tract Infection**

By

Amy Nicole Simms

Dissertation submitted to the Faculty of the
Department of Microbiology and Immunology Graduate Program of the
Uniformed Services University of the Health Sciences
F. Edward Hebert School of Medicine
in partial fulfillment of the
requirements for the degree of
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Chapter 1: Introduction

Overview of *Neisseria gonorrhoeae* infections and public health impact

Neisseria gonorrhoeae is the causative agent of the sexually transmitted disease gonorrhea. Gonorrhea is a very common infectious disease in the United States, second only in reported cases to *Chlamydia trachomatis*. In 2000, 358,995 cases of gonorrhea were reported to the Centers for Disease Control and Prevention (1). However, this number is considered to represent only half of the actual cases; an estimated 650,000 cases of gonorrhea occur in the United States every year (32). The reported incidence of gonorrhea in the United States is the highest of any industrialized country, with the highest percentage of cases in young men and women between 15 and 24 years of age (1).

The most common types of infections caused by *N. gonorrhoeae* are uncomplicated lower urogenital tract infections in both men and women. The most frequent site of infection is the endocervix of females and the urethra of males (93); however, the female urethra is also frequently infected. Rectal and pharyngeal infections in both genders are also common. Many mucosal gonococcal infections are asymptomatic (93), which is important in that untreated gonococcal infection can lead to serious local and systemic complications. The most common complication of gonococcal urethritis in men is epididymitis, which can lead to sterility if untreated (12). In women, ascension of gonococci to the upper reproductive tract from the endocervix causes pelvic inflammatory disease (PID). PID is any combination of upper genital tract syndromes

including endometritis, salpingitis, tubo-ovarian abscess, and pelvic peritonitis (214). PID, which occurs in 10-20% of women with endocervical infection (93), is the mostcommon and most important complication of gonorrhea in terms of public health because of its acute symptoms and long-term sequelae, such as internal abscesses and chronic pelvic pain. PID can also damage the fallopian tubes to cause involuntary fertility and increased risk of ectopic pregnancy. Disseminated gonococcal infection (DGI) is also a serious complication of gonococcal infection of both genders. DGI occurs in 0.5-3% of patients with untreated mucosal infection (93). DGI occurs as a result of gonococcal bacteremia and manifests most commonly as acute arthritis, joint pain, and, or dermatitis. Lastly, a rare complication of DGI is gonococcal endocarditis, which occurs in 1 to 3% of patients with DGI (92, 130).

Adaptation and Survival of the Gonococcus in the Genital Tract

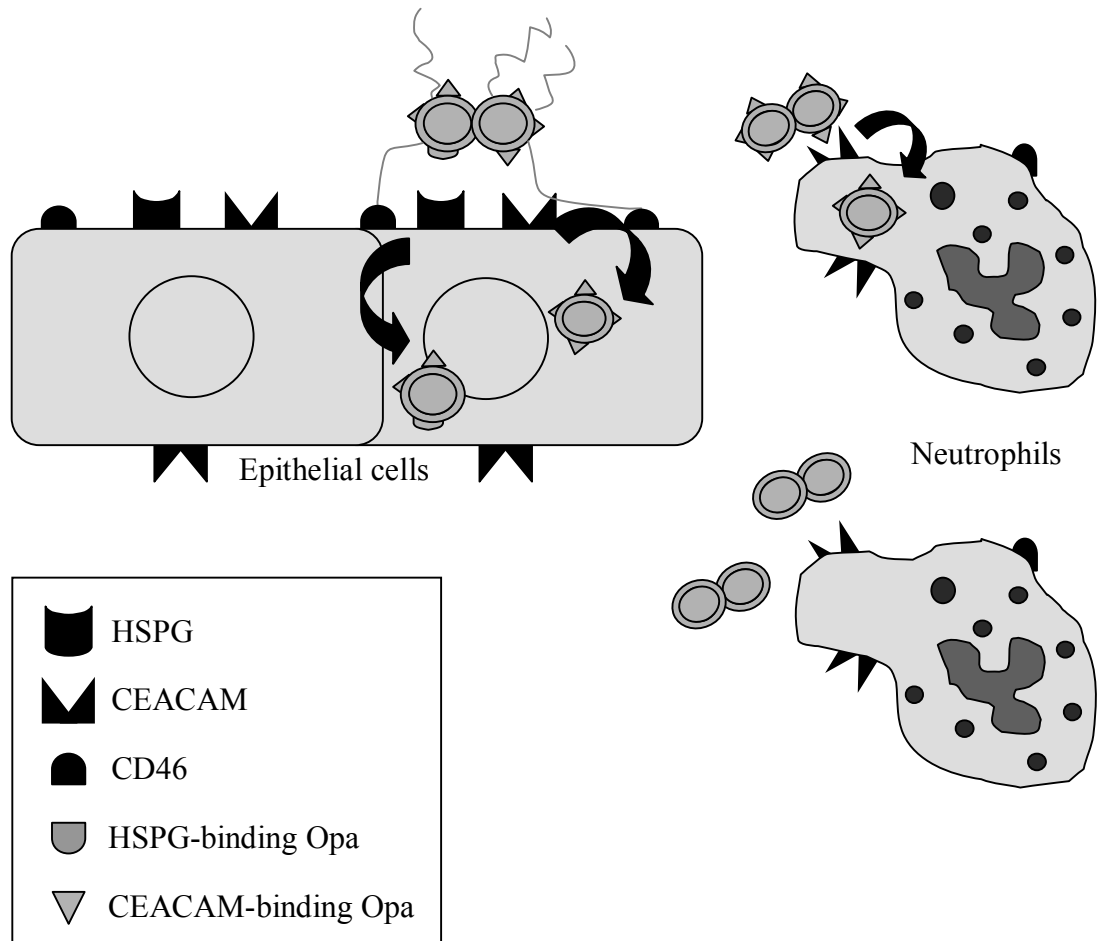
N. gonorrhoeae is a human-specific pathogen with no outside reservoir. In order to establish and maintain infection, the gonococcus must adapt to the host environment. Attachment of the gonococcus to the mucosal epithelium is mediated by several phase-variable surface structures, including colonization pili, lipooligosaccharide (LOS), and the opacity (Opa) proteins. This attachment allows the gonococcus to persist despite the constant shedding of mucous in the lower genital tract of females and the force of urine expulsion in the urethra. The initial adherence of the gonococcus to the mucosal surface is hypothesized to occur via binding of colonization pili to epithelial cells. The putative pilus receptor on the host epithelium is the human membrane cofactor protein (CD46) (112). After this initial interaction is formed, the gonococcus then binds more intimately

to the epithelium either by LOS-mediated binding to the asialoglycoprotein receptor (ASGP-R) (80) or via adherence of Opa proteins to specific receptors (21, 35, 38, 72, 205, 208) (Figure 1). This intimate attachment can lead to invasion of epithelial cells, possibly followed by transcytosis into the subcellular space (210).

The gonococcus also possesses many factors that allow it to survive in the presence of mediators of the host innate defense. Two well characterized active efflux pump systems exist in *N. gonorrhoeae*, which protect the gonococcus from the toxic effects of hydrophobic antimicrobial compounds in the rectum (75, 179, 218) and the female lower genital tract (104). The concentration of free iron on mucosal surfaces is very low due to iron sequestration by host glycoproteins. The gonococcus persists in the absence of free iron by expressing specific receptors for host transferrin, lactoferrin, and hemoglobin (17, 34, 46). One hallmark of symptomatic gonococcal infection is a strong inflammatory response. Gonococci can evade complement-mediated defenses by down-regulating complement activation (158). Gonococci can also survive in the presence of phagocytes by the use of enzymes that neutralize toxic oxygen radicals (6, 81, 108, 203) or repair oxidatively damaged proteins (183).

N. gonorrhoeae also can persist in the presence of an adaptive immune response as evidenced by the fact that many individuals are often re-infected by gonococci of the same serovar or strain despite the presence of specific anti-gonococcal antibodies (171). One mechanism the gonococcus uses to survive in the presence of a specific antibody response is the induction of blocking antibodies that reduce the effectiveness of antibodies to gonococcal porin. Porin is a highly immunogenic outer membrane protein and anti-porin antibodies are bactericidal (87, 186). However, porin is physically

Figure 1. Schematic of Opa-mediated adherence to and invasion of epithelial cells and non-opsonic uptake by neutrophils. The two classes of Opa protein receptors, heparin sulfate proteoglycan molecules (HSPG) and carcinoembryonic antigen cell adhesion molecules (CEACAM), are indicated. Initial attachment of the gonococcus to the epithelium is thought to be mediated by attachment of pili to its putative receptor, CD46 (112). Two neutrophils are depicted; in one, Opa-mediated binding to the CEACAM receptor leads to non-opsonic uptake of the gonococci, in the other, Opa-negative gonococci are not taken up by the neutrophils, which may be a mechanism the gonococcus uses to survive in the presence of neutrophils.



associated with a protein called reduction-modifiable protein (Rmp) (20, 135, 151) and with LOS molecules (90). Blocking antibodies, directed against Rmp (59, 110, 134, 164, 165, 166) or LOS (59), hinder the binding of anti-porin antibodies to their target, thus negating an efficacious response. Molecular mimicry is also utilized by the gonococcus to establish and maintain infection. One example of this mimicry is the use of host 5-cytidine-monophospho-N-acetylneuraminic acid CMP-NANA by gonococcal sialyltransferase to sialylate LOS molecules. Sialylation of the LOS, which occurs at the terminal galactose of the lactoneoseries LOS structure (73, 128, 155), increases gonococcal serum resistance, as well as resistance to porin-specific bactericidal antibodies [reviewed in (185)]. Gonococci also produce a secretory IgA1 protease, which degrades IgA1 molecules and the intracellular lysosome/ late endosome marker LAMP1 (84, 125). Cleavage of LAMP1 contributes to the survival of the gonococcus in phagocytes and epithelial cells (125). However, a gonococcal strain without this specific virulence factor was not attenuated in infection of the human male urethra (106). Finally, membrane blebbing, which creates a decoy, and invasion into cells are other ways the gonococcus can avoid specific antibody responses [reviewed in (42, 190)].

The opacity (Opa) Proteins of the *Neisseriae*

Differences in colony photo-opacity in *N. gonorrhoeae* are caused by the expression of a family of outer membrane proteins called the Opa proteins. LOS also plays a role in colony photo-opacity via its interactions with Opa proteins to cause intragonococcal adhesion (19). The Opa proteins were originally referred to as P.II proteins in *N. gonorrhoeae* and class 5 proteins in *N. meningitidis*. Opa proteins range

from 25 to 30 kD in mass and the individual proteins are encoded by separate, unlinked chromosomal genes; *N. gonorrhoeae* strains possess 8 to 11 *opa* genes; the repertoires of *N. meningitidis* and other commensal *Neisseria* spp. are limited in comparison, with only 3 to 4 *opa* alleles present in these species (3, 91, 141, 200, 217). Individual *Neisseria* strains carry different sets of *opa* alleles and the complete Opa protein repertoire has only been fully defined for two gonococcal strains (14, 44). Three regions within each *opa* gene are responsible for the differences in molecular mass and antigenicity: the semivariable region (SV) and two hypervariable regions (HV₁ and HV₂) (Figure 2). These regions form surface-exposed loops in the mature Opa protein (Figure 3) as predicted by two-dimensional models (127) and antibody binding studies (50, 127). A fourth loop is also surface exposed. This fourth loop appears to be highly conserved among all Opa proteins (127).

Phase variation of Opa protein expression

By the use of cloned *opa* genes expressed in *E. coli*, it was determined that phase variation of Opa protein expression occurs by a frameshift mechanism that involves insertion or deletion of one or more copies of a pentameric DNA sequence (CTCTT) (11, 146). This repeated region is found within the signal sequence-encoding region of each gene (191). Also, these studies indicated that *opa* gene phase variation is RecA-independent (11, 146). Unlike pilus antigenic variation, which is the direct result of sequence changes in the pilin structural genes, antigenic variation of Opa phenotype is the result of phase variation of individual *opa* genes.

The mechanism of *opa* gene phase variation has been shown to be consistent with

Figure 2. Diagram of an *opa* gene. The three regions of variability (SV, semivariable, HV₁, and HV₂, hypervariable regions 1 and 2) that define different *opa* alleles are shown in black. The signal-peptide coding region, which contains a number of pentameric CTCTT repeats and is responsible for *opa* gene phase variation, is indicated by the striped box.

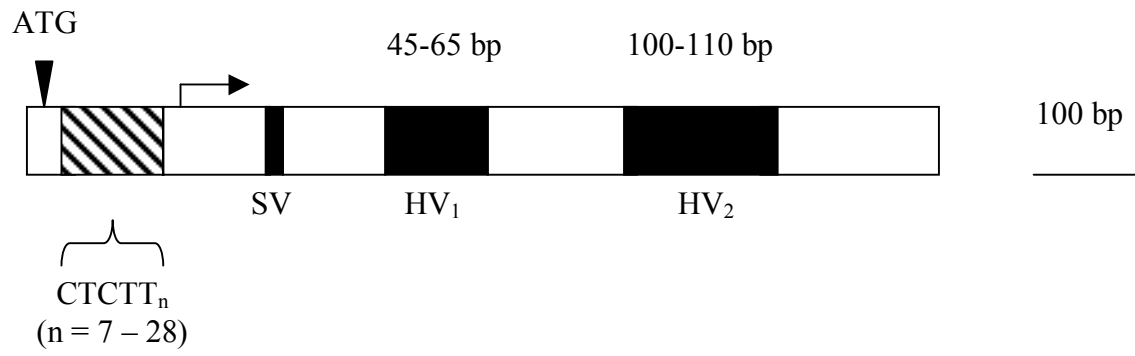
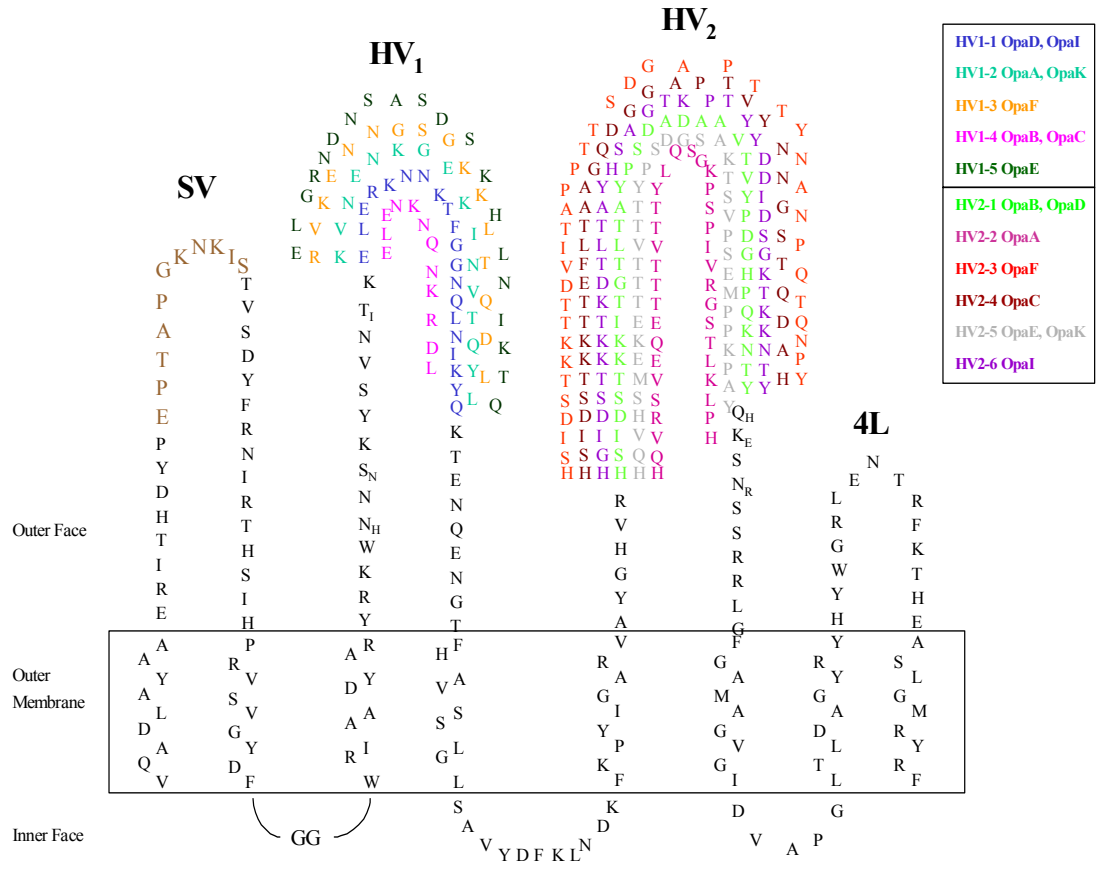


Figure 3. Two dimensional model of the predicted amino acid sequence of the Opa proteins of *N. gonorrhoeae* strain FA1090, based on the model proposed by Malorny et al (127). Four surface-exposed loops are present: the semi-variable (SV) loop, hypervariable loops 1 & 2 (HV₁, HV₂), and a conserved 4th loop (4L). Amino acids that are conserved among all of the Opa proteins of strain FA1090 are present in black text, variable sequences are indicated by different colored text.



that of slipped strand mispairing (SSM) (11, 146, 191). In SSM, mutational events occur during DNA replication as a result of local denaturation of a DNA duplex followed by mispairing of bases within the tandem repeat region (123). High frequency deletions or insertions occur only in the repeat region, involve an integral number of repeats, and are strongly biased toward the loss or gain of one repeat (122). Also, the frequency of frameshifting increases with the length of the repeat region (122). In order for misalignment of the repeat region to occur, SSM requires transient formation of single-stranded DNA. Interestingly, the pentameric repeat region of the *opa* genes, as well as the A-rich region that flanks the repeated region, was shown to adopt an unusual triple-stranded H-form or H-DNA conformation (8). This triple stranded region is characterized by tandem, direct mirror repeats with a purine/ pyrimidine strand bias and is highly dependent on supercoiling (139, 213). H-DNA, by the features of its structure, presents a single-stranded region that is highly sensitive to single strand-specific nuclease (96, 97, 109, 213). In the H-DNA conformation present in the repeat region of the *opa* genes, the purine strand (AG) is displaced from the structure (Figure 4), as was shown by increased protection against S1 nuclease by an oligonucleotide complementary to the purine strand (8).

Despite evidence that *opa* gene phase variation occurs via SSM, the rate of *opa* gene phase variation has not been well studied in its native gonococcal background. The rate of change of colony opacity phenotype of various gonococcal strains as viewed under a stereomicroscope was determined to be $\sim 10^{-3}$ per cell per generation (131). Variations in temperature, pH, oxygen concentration, piliation, and the addition of excess DNA or DNase, did not significantly influence this rate. Unfortunately, the results from

Figure 4. Proposed H-DNA structure of the pentameric repeat region of the *opa* genes, adapted from Belland (8). A box surrounds residues within the proposed triple-stranded region. Hoogsteen base pairs between acceptor purines and uncharged or protonated pyrimidines are indicated by the minus and plus signs respectively (97). The arrow indicates the direction of transcription of the *opa* gene.

this study are compromised by the fact that some Opa proteins do not confer detectable opacity to a colony. Therefore, the actual rate of *opa* gene phase variation in the gonococcus may in fact be much higher. Phase variation of recombinant *opa* genes cloned in *E. coli* was measured to occur at a rate of $\sim 10^{-3}$ per cell per generation (11, 146). Studies in *E. coli* also suggested that promoter strength might be important in *opa* gene phase variation. In a study using transcriptional *opa::lacZ* fusions and translational *opa::phoA* fusions in an *E. coli* background, Belland et al. (10) determined a correlation between expression rates from specific *opa* gene promoters and the phase variation rates of the corresponding *opa* gene. In contrast to what others reported (191), this study also indicated that *opa* gene transcription may not be constitutive. However, similar experiments have not been performed in *N. gonorrhoeae* and, therefore, it is not known whether specific conditions in the host environment drive *opa* gene phase variation during infection or whether this is a random event.

The role of Opa protein expression in vivo: Clinical Studies

Early clinical studies indicated that gonococci express Opa proteins during infection. Surveys by James and Swanson (99, 100) of gonococci isolated from 102 males and 176 females revealed that urethral isolates from men were predominantly opaque, and in general, endocervical isolates were more transparent. However, endocervical isolates varied in opacity levels and trypsin-sensitivity depending on the phase of the menstrual cycle. Recovery of transparent, trypsin-resistant gonococci was markedly increased near or at menstruation, while opaque, trypsin-sensitive gonococci were predominantly recovered from cervical isolates cultured around ovulation (99, 100).

These patterns were seen in both women who were taking oral contraceptives and those who were not. However, less fluctuation in colony opacity phenotype and trypsin-sensitivity was observed among gonococci isolated from women using oral contraception. These authors also had the opportunity to study isolates from three women infected with the same strain of *N. gonorrhoeae* due to an outbreak of β -lactamase-producing *N. gonorrhoeae* in Salt Lake City, UT. Sequential cervical cultures, collected 7 to 10 days apart due to treatment failure, from all three women indicated that gonococci isolated nearest to menstruation lack proteins expressed by gonococci isolated nearest to midcycle (99). From these observations, it was hypothesized that phase variation of gonococcal Opa proteins may serve to evade hostile factors present in the genital tract. It was also hypothesized that production of Opa proteins may render the gonococcus sensitive to proteases present in menstrual blood (99, 100). Subsequent research on the sensitivity of Opa protein variants to progesterone indicated that opaque variants were more susceptible to the actions of progesterone than transparent variants from the same strain (170). Taken together, these early observations suggested that hormonally-driven factors or progesterone itself might play a selective role against Opa protein expression in women.

Gonococcal Opa protein expression also appears to differ depending on the body site of infection. Rectal and pharyngeal isolates from both men and women were predominantly opaque (100), as compared to corresponding genital isolates. Also, a comparison of paired samples of urethral and cervical isolates from women infected by a single strain of penicillin-resistant *N. gonorrhoeae* in Durham, NC in 1983, showed that different Opa variants were recovered from these two infection sites in four of the seven

women studied (174). In addition, significantly more transparent gonococci were recovered from upper reproductive tract compared to the lower genital tract in a study of paired endocervical isolates and fallopian tube isolates collected by laparoscopy from seven women with acute gonococcal salpingitis (55). All of the endocervical isolates expressed one or more Opa protein(s), while none of the fallopian tube isolates expressed Opa proteins as determined by SDS-PAGE. In summary, data from clinical studies have revealed that gonococcal Opa protein expression appears to be hormonally regulated in women, preferentially expressed during infection of the male urethra, and differs depending upon site of the infection.

Opa Protein Function and Receptor Identification

Clinical studies suggest an important role in Opa protein expression in gonococcal infection; however, the specific function of these proteins has not yet been elucidated. Early in vitro analysis using tissue culture cell lines (13, 120, 126, 192), primary buccal cells (121), and fallopian tube tissue (53) indicated that Opa proteins were involved in gonococcal attachment to and invasion of epithelial cells. In these studies, Opa-positive gonococci adhered to and invaded cells more efficiently and in greater numbers than their Opa-negative counterparts. In addition, it was shown that antibodies specific to the Opa proteins of a particular strain blocked epithelial cells adherence and invasion of the corresponding Opa variant(s) (192). Opa-mediated invasion of epithelial cells occurred in the absence of pili (126) and rearrangement of the actin cytoskeleton (13, 126) was essential. Studies using primary human leukocytes determined that gonococci that expressed certain Opa proteins were phagocytosed in the absence of serum (138, 161,

168), and that induction of both neutrophil and monocyte chemiluminescence occurred upon uptake (161). As shown previously for various epithelial cell lines, antibodies specific to Opa proteins also blocked these interactions with phagocytes (60, 162). Finally, conclusive evidence that Opa proteins mediate adherence to, and invasion of epithelial cells, as well as non-opsonic uptake by neutrophils, was provided by attachment and invasion studies in which the individual *opa* genes were expressed in *E. coli* (9, 180).

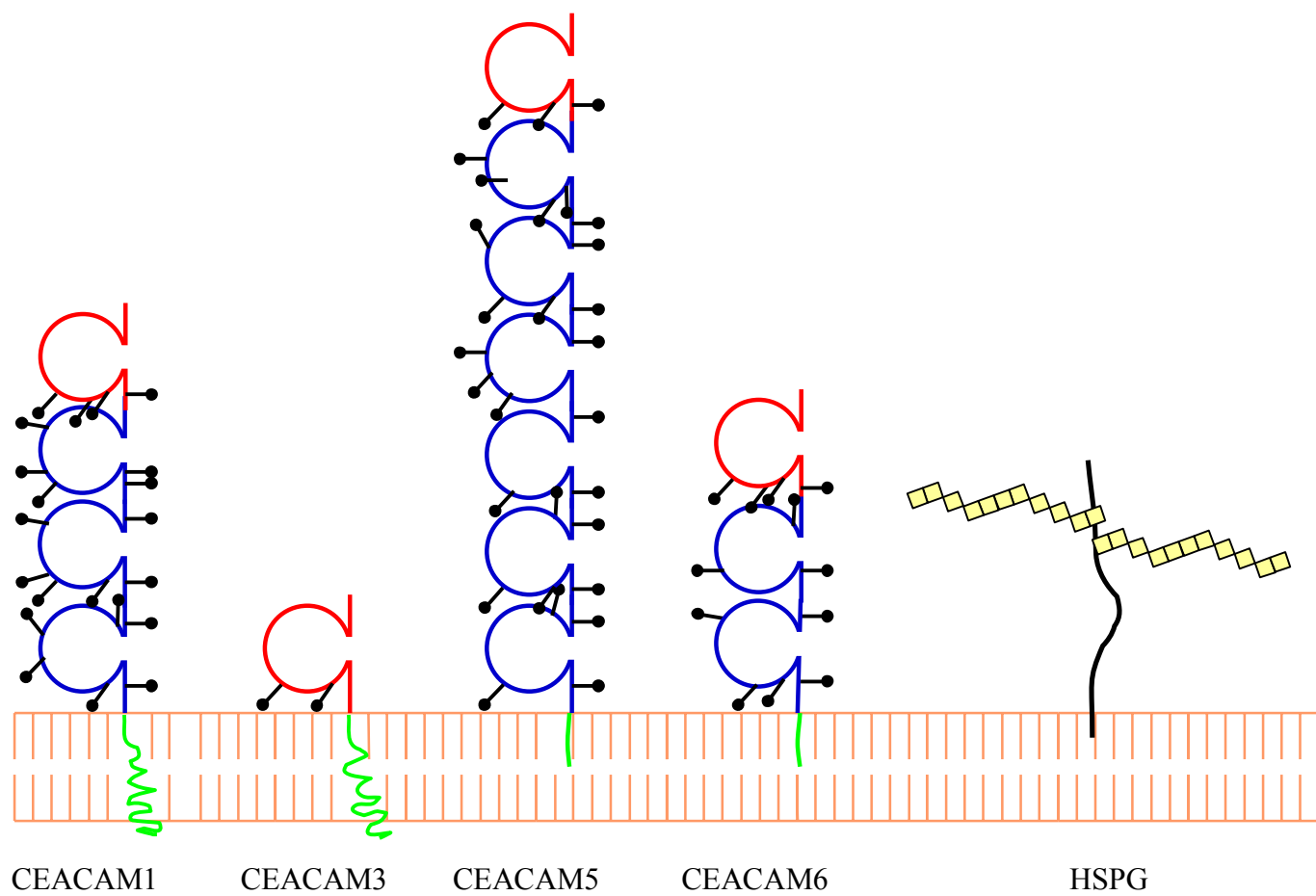
More specific analysis of the Opa proteins of different gonococcal (13, 120, 126, 180, 207, 211) and meningococcal strains (207) revealed that different Opa variants possessed different tissue tropisms in vitro. The Opa proteins of *N. gonorrhoeae* strain MS11 illustrate an example of Opa-mediated tropism in *N. gonorrhoeae*. It was discovered that only one Opa variant from this strain was able to effectively invade Chang conjunctiva epithelial cells (126). Additionally, all of the other Opa proteins of this strain mediated uptake by neutrophils (120). This apparent paradox was resolved by the identification of two classes of Opa protein receptors, syndecan-like heparin sulfate proteoglycans (HSPG) and members of the carcinoembryonic antigen cell adhesion molecule family (CEACAM) (21, 35, 38, 72, 205, 208). The CEACAM molecules are members of the immunoglobulin (Ig) superfamily and are divided into two major subgroups: the CEA subgroup and the pregnancy specific glycoprotein subgroup [reviewed in (76)]. Of the 12 genes present in the CEA subgroup, only seven members are expressed. The Opa proteins of the *Neisseriae* only bind four CEACAM molecules, namely CEACAM1, 3, 5, and 6. Also, within the CEACAM family, differences exist in the expression of the various molecules on different cell types and tissues. CEACAM1,

5, and 6 are present in a broad range of host tissues, while CEACAM3 is only expressed on mature neutrophils [reviewed in (76)]. The structure of the CEACAM molecules consists of an IgV-like N-terminal domain, which consists of 108 amino acids, and a variable number of or no IgC-like domains, of which two types are present, type A (93 amino acids) or type B (85 amino acids) (Figure 5) (216). The CEACAM molecules are membrane-linked, either via a GPI-linkage of the carboxy terminus domain (CEACAM5, 6) or as transmembrane proteins (CEACAM1, 3) (199). The cytoplasmic domain of both CEACAM1 and CEACAM3 contains modified immunoreceptor tyrosine based activation/ inhibition motifs (ITAM/ ITIM), and within these motifs are two tyrosine residues that may be phosphorylated (98, 147).

HSPG molecules consist of a protein core and one or more covalently attached glycosaminoglycan (GAG) chains (Figure 5) (116). HSPG molecules are anchored to the membrane of cells by either a transmembrane intracellular domain (syndecans) or via a glycosylphosphatidylinositol (GPI) link (glypicans) [reviewed in (202)]. In contrast to the CEACAM molecules, HSPG molecules are present at the epithelial cell surface in nearly all mammalian tissues, while CEACAM molecules are present on a variety of human cells, including epithelial cells and neutrophils (94).

Much work has been done to characterize the receptor-ligand interactions and subsequent events for Opa-mediated uptake via HSPG or CEACAM molecules. The importance of HSPG molecules in Opa-dependent invasion of epithelial cells was first observed using chemical modification and enzymatic removal of the proteoglycans on Chang conjunctiva cells (205). These alterations abolished Opa-dependent invasion. Also, cells deficient in proteoglycan synthesis were resistant to gonococcal invasion.

Figure 5. Models of the CEACAM molecules and the syndecan-like HSPG molecule, adapted from Hammarstrom (76) and Tumova et al (202). The IgV-like N domains of the CEACAM molecules are shown in red, while the IgC-like domains are in blue. A straight green line indicates the GPI-linkage of the CEACAM5 and 6 molecules to the cell membrane. CEACAM1 and 3 contain long cytoplasmic domains, as indicated by wavy green lines. Potential glycosylation sites are shown as lollipops. The syndecan-like HSPG molecule contains a short cytoplasmic domain. The HSPG molecule shown here contains two short heparin sulfate chains attached to the HSPG core protein.



Heparin affinity chromatography and binding assays indicated that one Opa protein of *N. gonorrhoeae* strain MS11, Opa₃₀ (or OpaA) mediated invasion of these cells, as well as human endometrial and corneal cell lines via binding of HSPG molecules (205). These results were further supported by research from another group, in which invasion of other non-human cell lines by the same Opa variant was blocked by addition of exogenous heparin or heparin sulfate to the culture (35). Adherence of HSPG-specific Opa variants causes a transient accumulation of actin surrounding the attachment point, which leads to uptake of the bacteria (71). This process involves activation of the acidic sphingomyelinase signaling pathway in Chang conjunctiva epithelial cells and primary fibroblasts (70). Serum factors, specifically vitronectin and fibronectin, are needed for HSPG-dependent invasion in certain other epithelial cell lines, and act to bridge the Opa-proteoglycan complex and host cell β -integrin receptors (56, 57, 67, 204). Interestingly, only one Opa protein from each of the three strains for which the Opa protein receptors have been characterized can utilize HSPG as a receptor for invasion of epithelial cells (35, 205). Binding of these Opa proteins to HSPG molecules is dependent on conformation. Structure function analysis of an HSPG-binding Opa protein revealed that while deletion of the SV or HV₂ region decreased receptor recognition, the HV₁ region was absolutely necessary for binding and internalization of gonococci via binding of HSPG (69).

The identification of CEACAM molecules as receptors for the majority of the Opa proteins was first observed via transfection of COS (African green monkey kidney) cells with a human cDNA library (208). In this study, co-precipitation and receptor ligand assays identified one particular CEACAM variant, CEACAM1, as a receptor for

Opa-dependent attachment and invasion. Infection of human epithelial cell lines and neutrophils led to the identification of other CEACAM molecules (CEACAM3, 5, and 6) as receptors for the Opa proteins (21, 37, 38, 72, 209). The addition of monoclonal antibodies to either the CEACAM molecules or to specific Opa proteins blocked attachment and invasion of Opa protein-expressing gonococci (37, 38, 209). It was also shown that addition of heparin or heparin sulfate to these cultures did not block CEACAM-mediated uptake of specific Opa variants by these cells (21, 38). This result indicated that Opa-mediated invasion by this pathway was not dependent on recognition by the HSPG molecules.

Binding of Opa proteins to the CEACAM receptors is dependent on the conformation of both the CEACAM molecules and the Opa proteins. Analysis of CEACAM receptor family binding by gonococcal or meningococcal Opa proteins indicated that the two HV loops of the Opa protein are critical for receptor binding (24, 49, 50, 69). Specific combinations of the HV regions conferred tropism for different receptors. In contrast, the SV loop was completely dispensable for CEACAM binding (24). In studies concerning the structure of the CEACAM molecules, it was observed that the majority of the CEACAM molecules are recognized by multiple Opa proteins via their homologous N-terminal domains (209). Glycosylation of the CEACAM molecule does not appear to be important (25); it was later shown that Opa protein binding occurs on the non-glycosylated face of the CEACAM molecule (23). Three residues in the CEACAM5 molecule (serine 32, glycine 41, and phenylalanine 29) appear to be critical for maximum receptor activity, based on homologue scanning mutagenesis (23). Interestingly, Ser₃₂ and Gly₄₁ are conserved in all four of the Opa-binding CEACAM

molecules, whereas the Phe₂₉ is present in all except CEACAM6. In a similar study, in which several predicted exposed amino acids were mutated, two other amino acids, tyrosine 34 and isoleucine 91, which are conserved in all the Opa-binding CEACAM molecules, were identified as essential for Opa protein binding (207). Ser₃₂ was also identified as important, but not critical for Opa protein binding to the CEACAM1 molecule. The results by these two groups are perplexing in that the same conserved amino acids were not identified in both analyses. A possible explanation for these conflicting results may reside in differences in the techniques applied and also may be due to differences in the structure of the individual CEACAM molecules.

Opa proteins and the Immune Response

In order for the gonococcus to establish and maintain infection in the genital tract, the organism must overcome many non-specific factors of the immune response, such as complement fixation, phagocyte surveillance, low pH, antimicrobial peptides, and gonadal steroids. Several lines of evidence suggest that phase variable expression of the Opa proteins contributes to immune evasion in various ways, including conferring resistance to complement-mediated killing and progesterone, immune suppression, decreased uptake by neutrophils, as well as evasion of Opa-specific antibodies. The Opa proteins also allow the gonococcus to intimately attach to epithelial cells, which may provide a way to avoid the constant sloughing of the mucous, as well as withstand the force of urine expulsion in the urethra. It is likely that complement components are present in the male urethra as part of the serum exudate during inflammation, and complement proteins were detected on gonococci isolated from infected women by

endocervical lavage (136). Therefore, the bactericidal action of complement is another innate host factor that the gonococcus must escape in the genital tract. In studies completed in vitro, Opa-positive gonococci were more resistant to the bactericidal action of normal human serum (NHS) than their Opa-negative counterparts (22, 39). Some Opa variants of the gonococcus also bound heparin, and in doing so, were protected from killing by normal human serum (39). Both of these studies indicate that Opa protein expression may contribute to survival of the gonococcus through evasion of host innate defenses. However, the molecular mechanism of enhanced serum resistance of Opa-positive gonococci has not been fully elucidated.

A hallmark feature of gonococcal infection is the presence of a purulent exudate containing neutrophils with intracellular gonococci (93). Gonococci have been shown to interact intimately with neutrophils, monocytes, and macrophages in blood and tissue (78, 152). As described earlier, certain Opa proteins mediate nonopsonic uptake of the gonococcus by neutrophils and monocytes via binding of the CEACAM3 receptor (9, 63, 120, 121). Opa-negative gonococci and some Opa-positive variants do not associate with, and consequently, are not killed by neutrophils or monocytes in the absence of serum (9, 60, 63, 118, 120, 148). Opa protein-mediated binding of CEACAM3 increases activity of the Src family kinases Hck and Lyn (184), which triggers phosphorylation of the ITAM sequence in the cytoplasmic domain of CEACAM3 (36, 83, 132) and stimulation of the small GTPase Rac (15, 172). As a result, transient rearrangement of the actin cytoskeleton occurs (15, 132), which eventually results in uptake of the bacteria. CEACAM3 is expressed only on mature or stimulated neutrophils (37, 62). Opa-mediated uptake via CEACAM3 in neutrophils is detrimental to the gonococcus, in that

gonococci engulfed via this mechanism trigger the respiratory burst and are killed (172). Therefore, phase variation of Opa expression may play an important role in generating Opa variants that can avoid phagocyte killing before opsonins are present. However, it has also been suggested that Opa-mediated ligation of the CEACAM3 receptor enhances the death rate of neutrophils (36), which possibly contributes to overall survival of *N. gonorrhoeae* in the presence of a strong inflammatory response. Interestingly, the interaction of Opa-positive gonococci with neutrophils does not appear to stimulate the release of reactive oxygen intermediates into the extracellular milieu (149). This result may possibly explain the persistence of extracellular gonococci in the presence of high concentrations of neutrophils.

In contrast to other bacterial pathogens, repeated infection with *N. gonorrhoeae* is very common, even in the presence of specific antibodies, and can occur with the same strain as that which caused the previous infection (171). Investigations of the immune response to *N. gonorrhoeae* during and following natural infection indicate that local and systemic antibody responses are not strong compared to the level of cross-reacting antibodies present in a control population (88, 89). These studies used whole gonococci as the target in their screening assay. Others used radioimmunoprecipitation with patient sera and surface-labeled gonococci, followed by SDS-PAGE, to detect antibodies to gonococcal surface antigens (219). These authors (219) detected Opa-specific antibodies in patients with gonococcal genital tract infection. Interestingly, in this study of sexual contacts infected with the same strain, serum from one woman did not react with any of the Opa variants isolated from her urethra or cervix or from the urethra of her male partner. However, her sera did react with Opa proteins expressed by gonococci isolated

from the cervix of another female contact of the male (219). One possible explanation of this result is that phase variation of the Opa proteins occurred in the different patients, indicating that phase variation of Opa protein expression may facilitate immune evasion. The generation of Opa specific antibodies during natural infection is also supported by a reported correlation between Opa-specific antibody and a reduced risk of gonococcal salpingitis (156). This study of commercial sex workers in Nairobi, Kenya, suggests that exposure to multiple Opa types might increase the likelihood that antibodies specific to a more conserved region of the Opa proteins would be produced.

Some new and exciting data demonstrate that Opa-mediated binding to CEACAM1 on CD4⁺ T cells can down-regulate the activation and proliferation of these cells (26). This result is intriguing in light of the recent discovery that neisserial infection induces CEACAM1 expression in both epithelial and primary endothelial cells via an NF- κ B-dependent signaling pathway (143, 144). However, it is not known to what extent these in vitro experiments reflect events that occur in infected patients or whether there are a significant number of CD4⁺ lymphocytes present in the genital lumen for an overall immunosuppressive event to occur. There is scant evidence to indicate that aggregated lymphoid follicles similar to the Peyer's patches of the small intestine are present in the urogenital tract (58, 198, 215). In addition, it remains to be seen whether other Opa proteins from different gonococcal strains, which also bind CEACAM1, also cause this immunosuppressive event in vitro. However, this finding has led some to hypothesize that Opa-mediated immunosuppression increases susceptibility to repeated infection or to concomitant infection with other pathogens (26).

Opa Protein Expression in Experimental Infection Models of Gonorrhea

A challenge of examining Opa protein expression in vivo is the limited number of infection models. Experimental urethral infection of male volunteers is highly relevant to gonococcal pathogenesis in natural infection of the male genital tract. No similar model exists for female infection because of the high risk of complications that can arise from localized gonococcal infection in women. In the male volunteer model of experimental urethritis, subjects are inoculated intraurethrally with a suspension of gonococci by the use of a pediatric catheter (41, 194). Infected subjects develop urethritis that is characterized by a purulent exudate within one to five days (40). Antibiotic treatment is initiated upon the development of signs of infection or at the end of the trial (41, 195). A variety of gonococcal virulence factors have been studied during experimental infection of male volunteers. Gonococcal mutants deficient in production of pilin (40), RecA (40), or IgA1 protease (106) were not attenuated in this model. Interestingly, a strain unable to produce the transferrin or lactoferrin receptor was strongly attenuated in male volunteers as compared to the wild-type control (47); expression of the lactoferrin receptor in the absence of the transferrin receptor was sufficient for infection (5). These results suggest transferrin or lactoferrin are adequate sources of iron for gonococci during urethral infection of men. Phase variation of surface molecules has also been examined during urethral infection of male volunteers. Pilin antigenic variation (178, 195) and LOS (173) phase variation occur early during infection of male volunteers. The kinetics of Opa protein expression was also examined in the male infection model as discussed below (102, 194).

To examine the importance of Opa proteins during urethral infection, two independent laboratories infected male volunteers with suspensions consisting of predominantly Opa-negative variants and analyzed the output gonococci (urine or urethral discharge) for Opa protein expression (102, 194). In one study in which *N. gonorrhoeae* strain MS11 was used, three men were inoculated with >98% Opa-negative gonococci. In all 3 infected subjects, 95% of urine cultures collected within 24-50 hours post inoculation contained a majority of Opa-positive gonococci (194). One Opa variant, OpaC, predominated in all urine cultures. However, in a semen sample collected at 50 hours post-inoculation from one volunteer, high numbers of gonococci that expressed 3 different Opa variants were isolated. Opa protein expression of *N. gonorrhoeae* strain FA1090 was also examined in urethral infection of male volunteers (102). In this study, 15 men were inoculated with a predominantly Opa-negative population (78-100%). Nine of these men became infected, with subsequent urine cultures containing an increased percentage of Opa-positive gonococci compared to that of the inoculum. The majority (>90%) of gonococci reisolated in six of these men were Opa-positive (102). In contrast to the study with strain MS11, no specific Opa variant was preferentially recovered from all infected subjects. In addition, a large percentage of reisolated gonococci expressed multiple Opa proteins in conjunction with the original Opa protein expressed as infection progressed. This result indicates phase variation of *opa* genes most likely occurred in these men over the course of infection. Due to the ethical constraints placed on human studies, long-term genital infection of men could not be examined. The authors concluded that selection and, or, induction of Opa protein expression occurred early during urethral infection; however, no selective forces were identified. Knowledge of

Opa protein expression in the endocervix is less developed due to the ethical issues that prohibit the infection of female volunteers.

Estradiol-treated mice have been used as a surrogate host for *N. gonorrhoeae* to study gonococcal adaptation to the female lower genital tract due to the high numbers of physiological and histological similarities between genital tract of women and female mice (101, 103, 104). Estrogen treatment of mice leads to maintenance of an estrus-like state (197), which is the stage of the reproductive cycle that is permissive to gonococcal colonization in normal cycling mice (27, 48, 115). In this model, high numbers of gonococci are recovered from the lower genital tract of mice following intravaginal inoculation for an average of twelve days in a fourteen-day period (and for as long as forty days) (101, 103, 104). Quantitative cultures evaluated over time indicate that replication of gonococci occurs in vivo. Inflammation occurs in infected mice and gonococci are seen within neutrophils (101). In contrast to the human male infection model, the transferrin and lactoferrin receptors of the gonococcus are not critical in this infection model (103). The hemoglobin receptor is also not critical for murine infection (103). These results suggest that other sources of usable iron must be present in the lower genital tract, which are not present in the male urethra. Finally, non-piliated gonococci are frequently recovered from the genital tract of infected mice, indicating that gonococcal pili may not play a role in murine infection (101). Loss of piliation during murine infection may be due to the absence of the putative pilus receptor, CD46 (112), in mice (142).

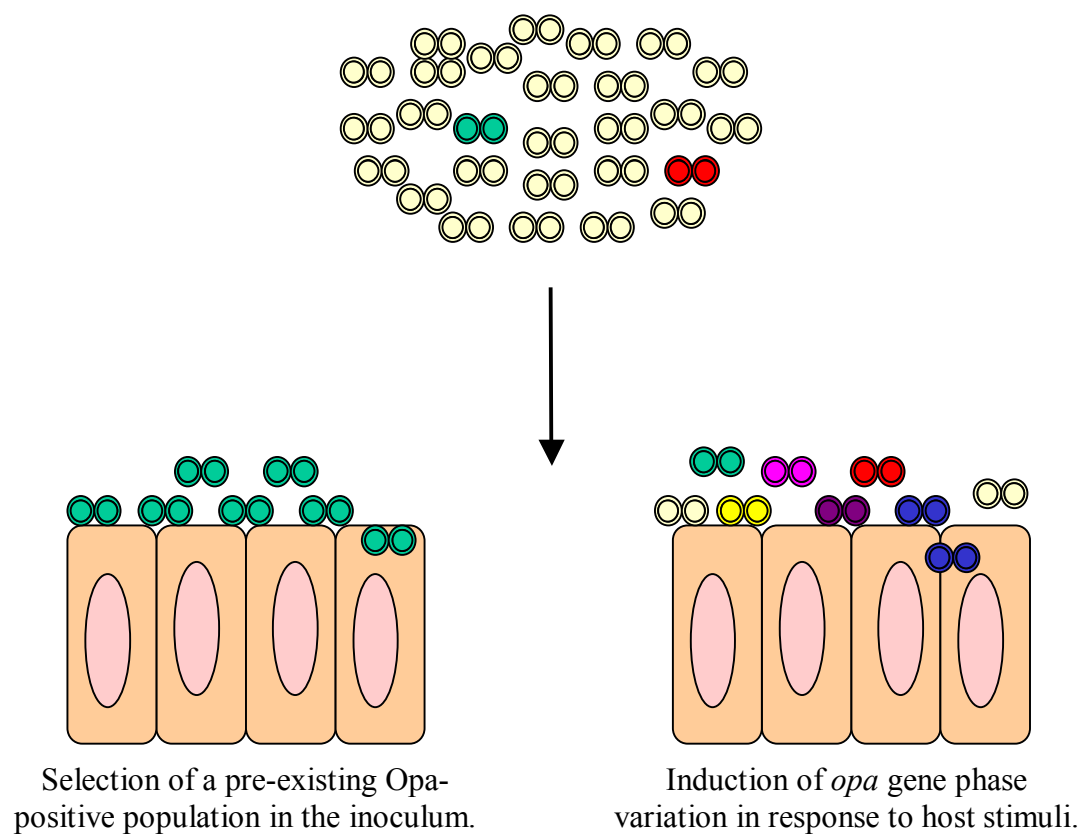
Opa protein expression was also examined during experimental genital tract infection of female mice. Similar to results seen in the human male infection model,

primarily Opa-positive gonococci were recovered early in infection after intravaginal inoculation of mice with a predominantly Opa-negative population (101). Similar to male volunteer studies, it was not determined whether the recovery of Opa-positive gonococci was due to selection of a pre-existing population of Opa-positive gonococci or to induction of *opa* gene phase variation in vivo. HSPG molecules are expressed on almost all mammalian epithelium and have been identified on the vaginal epithelium of mice (85). However, only one homolog of the human CEACAM molecules (CEACAM1) is present in mice (77) and the two molecules are not highly related. Additional factors that may select for certain Opa phenotypes, such as progesterone, complement proteins, and other components of the host innate immune response are present in the mouse genital tract.

Specific Aims of this Dissertation

The overall goal of this thesis was to test if selection and, or induction of Opa protein expression is responsible for the predominance of Opa-positive variants recovered during experimental infection of female mice (Figure 6). Two hypotheses were examined. The first hypothesis was that selection of a pre-existing Opa-positive population in the inoculum is responsible for the reisolation of mainly Opa-positive variants. The second hypothesis was that induction of *opa* gene phase variation by host stimuli contributes to Opa protein expression in vivo. Two specific aims were designed to test these hypotheses. The first specific aim was to determine whether selection or induction of Opa protein expression occurred early in the murine genital tract by using a genetically marked Opa variant of *N. gonorrhoeae* FA1090. The results of this study are

Figure 6. Diagram depicting the events that occur following infection of experimental hosts with a predominantly Opa-negative population of gonococci (101, 102, 194). Two possible events are illustrated, selection of a pre-existing Opa-positive population or induction of *opa* gene phase variation in vivo. Opa-negative gonococci are light yellow, whereas different Opa-positive variants are shown in various colors.



presented in Chapter 2. The second specific aim was to explore the possibility of increased *opa* gene phase variation in vivo by the use of a chromosomally-encoded translational *opaB::phoA* fusion in *N. gonorrhoeae* FA1090. The *opaB::phoA* phase variation rate was examined in vivo and in vitro under conditions likely to be present in the host. The results of this second specific aim are presented in Chapter 3.

Chapter 2: Selection for *Neisseria gonorrhoeae* Opacity Protein Expression during Experimental Murine Infection in the Absence of Human CEACAM Receptors

Introduction

The opacity (Opa) proteins of *N. gonorrhoeae* are a family of phase variable outer membrane proteins that mediate intimate attachment to, and invasion of cultured epithelial and endothelial cells, as well as non-opsonic uptake by neutrophils [reviewed in reference (52)]. Expression of different Opa proteins affects the photo-opacity of a colony, with colony phenotypes ranging from transparent to deeply opaque. Opa proteins range from 25 to 30 kDa in molecular mass and are encoded on separate unlinked chromosomal genes. A single gonococcus can express 8 to 11 antigenically distinct Opa proteins, each the product of its own structural gene, whereas only 3 to 4 *opa* genes are present in *N. meningitidis* (3, 91, 141, 217) and commensal *Neisseria* sp. (200). The *opa* gene repertoire of different neisserial strains is distinct. Phase variable expression of Opa proteins is a high-frequency event (131), which occurs at a rate of 10^{-3} /cell/generation via insertion or deletion of one or more copies of a pentameric DNA sequence within the signal sequence-encoding region of each gene (11, 146, 191). The resultant frameshift leads to expression of a full-length protein or proteins that are prematurely terminated during translation. A single gonococcus can express no Opa proteins, one Opa protein, or multiple Opa proteins simultaneously.

Opa proteins mediate attachment to and, or invasion of many different cultured cell lines, including cervical, endometrial, endothelial, and Chang human conjunctiva

cells (13, 120, 126, 180, 207, 211). Some Opa proteins also mediate uptake by phagocytes in the absence of opsonization (9, 21, 38, 63, 120, 180). Differences in the binding specificity of different Opa proteins within a strain can be explained by the identification of two separate classes of Opa protein receptors, the carcinoembryonic antigen cell adhesion molecule family (CEACAM) and heparin sulfate proteoglycan molecules (HSPG) (21, 38, 72, 205, 208). Members of the CEACAM receptor family are present on a variety of cells, including human epithelial and endothelial cells and professional phagocytes. Within the CEACAM family, differences exist in the expression of the various CEACAM molecules on different cell types and tissues. HSPG molecules are expressed on the majority of epithelial cells in mammalian tissue (94). The majority of Opa proteins from strains in which the Opa protein receptor specificity has been identified bind to members of the CEACAM family, specifically CEACAM1, 3, 5, and 6. Only one Opa protein from each of these strains also utilizes HSPG as a receptor for attachment and invasion of epithelial cells (35, 205).

Analyses of urethral isolates from naturally and experimentally infected men suggest that Opa protein expression is important in establishment and, or maintenance of urethral infection. A majority of urethral isolates from men with natural infection expressed one or more Opa proteins (100), and in male volunteer studies, Opa-positive gonococci were recovered following intra-urethral inoculation with a predominantly Opa-negative population of gonococci (102, 194). These data strongly suggest selection and, or, induction of Opa protein expression occurs early during infection of the male urethra. No selective forces have been definitively identified, however, and due to the ethical

constraints placed on studies with humans, Opa protein expression during long-term urethral infection of men was not examined.

Estradiol-treated mice have been used as a surrogate host for *N. gonorrhoeae* to study gonococcal adaptation to the female lower genital tract (101, 103, 104). We previously reported that expression of Opa proteins appeared to be selected for in this model (101), even though the murine CEACAM1 molecule is not highly related to its human counterparts (77). To better characterize expression of these phase variable proteins during experimental murine infection, here we report the kinetics of the recovery of Opa-positive variants from estradiol-treated mice over time following inoculation with defined mixtures of Opa-negative and Opa-positive gonococci. Furthermore, to examine if selection of a pre-existing population of Opa-positive variants is responsible for the increased recovery of gonococci expressing Opa proteins from the mouse lower genital tract, we constructed a genetically marked strain of FA1090 to follow a specific population of gonococci throughout the course of infection.

Materials and Methods

Bacterial strains *N. gonorrhoeae* strain FA1090 was initially isolated from a female patient with disseminated gonococcal infection (41). Opa protein expression of this strain during gonococcal urethritis was characterized in male volunteers (102). The Neisserial Insertional Complementation System (NICS, a generous gift of H.S. Seifert, Northwestern University) was implemented to create a chloramphenicol resistant (Cm^R) gonococcal strain (FA1090Cm^R). This system allows one to introduce markers into a chromosomal locus with no known function located between the *aspC* and *lctP* genes.

NICS vector pGCC5 contains a transposon-encoded chloramphenicol acetyltransferase (*cat*) gene in this intragenic region (137). Plasmid pGCC5 was digested with ClaI to release a 5.2 kB fragment containing the *aspC*, *lctP* genes and the Cm resistance marker. After gel purification, this fragment was introduced into an Opa-negative, pilated variant of *N. gonorrhoeae* strain FA1090 by spot transformation (74), and transformants were selected on GC agar (101) containing 0.5 µg/ml chloramphenicol (Cm). Polymerase chain reaction (PCR) amplification using primers IcatB (5' TTAAGGGCACCAATAACTGC) and IcatC (5' AACTGGTAGGTATGGAAGATCTCTAGA), which are located within the *cat* gene, was used to confirm the presence of the *cat* gene in the Cm^R transformants, and a stable transformant (FA1090Cm^R) was selected for further characterization. In order to confirm that integration of the *cat* gene occurred in the desired region, primers F1451A (5' CATCCGTTCTGCTCTATACCC) and R1451B (5' GTCCCATTTCTTCAAAGTCGG) were designed, which correspond to the nucleotide sequences immediately adjacent to the transposon insertion in pGCC5. As predicted, PCR analysis of wild-type FA1090 gonococcal DNA with F1451A and R1451B led to production of a 400 bp fragment; in contrast, a 1.6 kB fragment was amplified from FA1090Cm^R genomic DNA (data not shown), which is consistent with the insertion of the transposon-encoded *cat* gene. Restriction enzymes, taq polymerase, and Deep Vent polymerase were from New England Biolabs (Beverly, MA). Standard recombinant DNA methods, including restriction enzyme digestions and plasmid purification, were performed in accordance to manufacturer's instructions. PCR amplifications were completed in a Thermolyne Amplitron II Thermal Cycler (Barnstead/ Thermolyne Corp., Dubuque, IA). Reactions

consisted of 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1 % Triton X-100, 0.25 mM of each dNTP, 200 ng of each primer, 0.5 U of taq Polymerase, and 200 ng of template. PCR amplification with both the IcatB and IcatC and F1451 and F1451B primers was completed by cycling 25 times at 94°C for 1 min, 59°C for 1 min, 72°C for 2 min, followed by a 4°C indefinite hold. Plasmid DNA was prepared with the Qiagen Plasmid Midi Kit and QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Purification of PCR products and restriction enzyme fragments were performed with the QIAquick gel extraction kit (Qiagen). Chromosomal DNA was isolated via the cetyltrimethyl-ammonium bromide (CTAB) protocol (111).

Preparation of Bacterial Inocula Opa variants used for each experiment were pre-screened for Opa protein expression as follows. Two days before inoculation of mice, the frozen stock culture of the Opa variant to be tested was subcultured to GC agar. Twenty-four hours later, suspensions of non-piliated individual colonies that exhibited a photo-opacity consistent with expression of the desired Opa protein when viewed under a stereomicroscope were dotted onto multiple nitrocellulose filters (Schleicher & Schuell, Keene, MA), and tested by immunoblot with Opa-specific sera. Aliquots of each suspension were also inoculated onto GC agar plates with Kellogg's supplements at 37°C in an atmosphere of 5% CO₂ as described previously (102). After 20-21 hours of incubation, isolated colonies on plates inoculated with colonies that were confirmed to express the desired Opa phenotype were suspended in sterile phosphate buffered saline. Bacterial suspensions were filtered through 1.2 µm filters to remove clumped gonococci as described (101) and adjusted to an A₆₀₀ of 0.08 [10⁸ colony forming units (CFU)/ml].

Aliquots were mixed to obtain the desired ratio of Opa variants. Mice were then inoculated vaginally with 20 μ l of the mixed suspension; aliquots of the inoculum were serially diluted and cultured on GC agar to determine the dose. The percentage of each Opa variant present in the inoculum was determined by colony suspension immunoblot as described below. In experiments in which mice were inoculated with mixtures of wild-type gonococci and FA1090Cm^R, the inoculum was also plated on GC agar with Cm selection.

Mouse infection protocol Female BALB/c mice (6-8 weeks old, National Cancer Institute) were treated with 17 β -estradiol and antibiotics to induce susceptibility to *N. gonorrhoeae* as described (101, 104). Two days after initiation of estradiol treatment, mice were inoculated intravaginally with bacterial suspensions containing primarily non-piliated *N. gonorrhoeae* FA1090 of the desired Opa phenotypes (Opa-negative, OpaI, or OpaB variants). In all experiments, vaginal mucus was collected daily and quantitatively cultured on GC-VCNTS agar for *N. gonorrhoeae* as described (104). GC-VCNTS plates were examined after 20-24 hours of incubation and the number of CFU recovered was determined. Individual colonies were analyzed from both the inoculum plates (96 colonies, limit of detection: 1%) and primary vaginal cultures (36 colonies, limit of detection: <3%) by colony suspension immunoblots to determine the percentage of each Opa variant within the input and output populations as described below. In experiments using mixtures of FA1090Cm^R and FA1090, colonies were subcultured onto GC agar with Cm to determine Cm^R phenotype. In these experiments, selection was defined as three times that which was present in the inoculum. Animal experiments were conducted

in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol that was approved by the University's Institutional Animal Care and Use Committee.

Production of Opa-specific antisera Strain FA1090 possesses 11 different *opa* genes, which collectively encode 8 antigenically distinct Opa proteins (A, B, C, D, E, K, F, and I) (44, 54). When viewed under a stereomicroscope, Opa-negative, OpaB, OpaE, and OpaK variants of this strain produce transparent colonies. OpaC and OpaF produce slightly opaque colonies, and OpaA, OpaD, and OpaI produce deeply opaque colonies (102). Two surface-exposed hypervariable regions (HV₁ and HV₂) of each *opa* gene are responsible for the observed differences in molecular weight and antigenicity of individual Opa proteins. The Opa phenotype of individual colonies was determined by colony suspension immunoblot with HV₂-specific polyclonal rabbit sera or monoclonal antibodies (Mabs) specific for Opa proteins of strain FA1090. HV₂-specific polyclonal rabbit antisera was produced by immunization with peptides specific to unique sequences within the HV₂ regions of Opa proteins A, B/D, C, F, and I (Table 1). OpaB and OpaD variants can be distinguished by a difference in electrophoretic mobility on SDS-PAGE (Figure 7A), as well as by colony photo-opacity. Peptide synthesis and conjugation, immunizations, and affinity-purification of the rabbit antisera were performed by Bethyl Labs (Montgomery, TX). Antibody specificity was confirmed by ELISA against the corresponding peptide. Optimal titers for colony suspension immunoblots and Western blots were determined empirically. The titers of the Opa-specific rabbit antisera for

Table 1. Rabbit antisera specific for the HV₂ regions of the Opa proteins of *N. gonorrhoeae* FA1090

Serum	Specificity	Peptide sequence ^a
Rb-A	OpaA	YLQSGKPSPIVRGSTL
Rb-BD ^b	OpaB/D	AYPSDADAADVTV
Rb-C	OpaC	TTEFLTAAGQDGGA
Rb-F	OpaF	VITAPPTTSDGA
Rb-I	OpaI	HSAGTKPTYDDIDSGKTKK
Rb-AB4L	OpaA, B, C, D, F, K, I ^c	HYWGRLNTRFKTHE

^a Peptide sequence corresponds to unique regions within the HV₂ loop of each Opa protein (44).

^b OpaB and OpaD proteins share the same HV₂ sequence.

^c AB4L does not recognize the OpaE protein well. mAb H164 was used to determine OpaE/K variants (102).

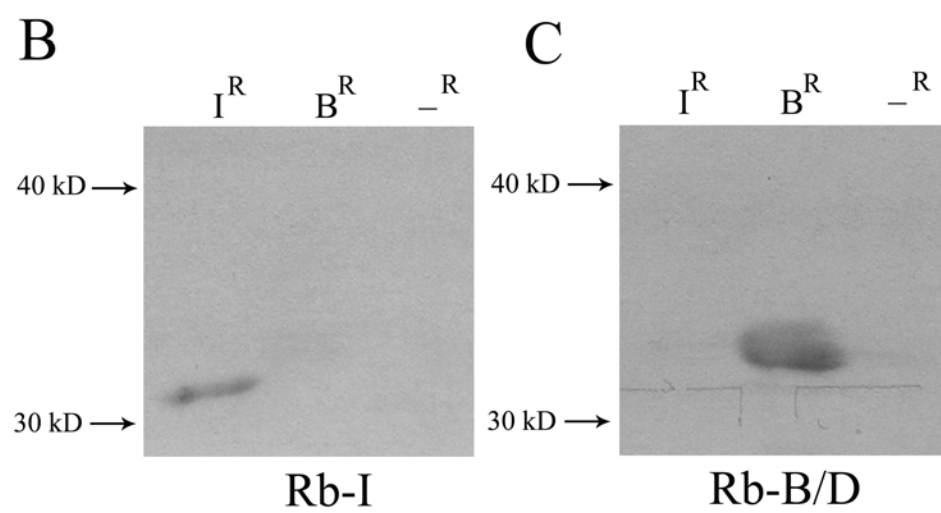
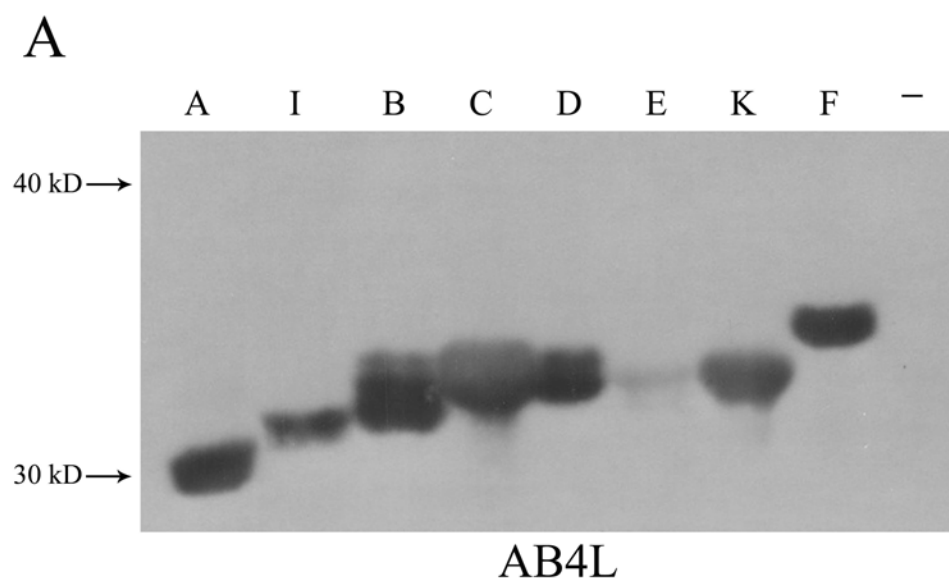
colony suspension immunoblots were Rb-A and Rb-B/D: 1:10,000, Rb-C: 1:5,000, Rb-F: 1:4,000, and Rb-I: 1:9,000, and for western blots Rb-A and Rb-B/D: 1:150,000, Rb-C: 1:45,000, Rb-F: 1:30,000, and Rb-I: 1:20,000. Mab H164 (a gift from Janne Cannon, University of North Carolina, Chapel Hill) was used to detect OpaE and OpaK variants by colony immunoblotting and western blot analysis (102). Mab H164 binds both the OpaE and OpaK proteins of strain FA1090, which share the same HV₂ region and differ only slightly in electrophoretic mobility on SDS-PAGE (7, 18). OpaE and OpaK variants were not distinguished in this study; therefore, H164-binding variants are referred to as OpaE/K variants.

Determination of Opa phenotype The predominant Opa phenotype within a colony (i.e. primary vaginal isolates, inoculum isolates) was determined by colony suspension immunoblots as previously described (101). Briefly, suspensions of individual colonies were inoculated onto multiple nitrocellulose membranes (Schleicher & Schuell). Each filter was probed with one of the Opa-specific polyclonal rabbit antisera or mAb H164. Bound antibody was detected with goat anti-mouse IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP, 1:50,000, Sigma Chemical Company, St. Louis, MO), followed by addition of enhanced chemiluminescence substrate (Amersham Biosciences, Piscataway, NJ). The remaining colony suspensions were frozen for further confirmation by Western blot if needed. If a colony suspension bound more than one Opa-specific serum, the frozen stock was cultured and several colonies were tested by colony suspension immunoblot to distinguish between variants that expressed more than one Opa protein simultaneously from those that were mixtures of single-expressing

variants. If a colony suspension failed to bind any of the Opa-specific antisera, western blotting with the broadly cross-reactive anti-Opa MAb 4B12 (2) (a gift of M. S. Blake, CBER, FDA) or polyclonal rabbit serum AB4L was performed to confirm the Opa-negative phenotype. AB4L was generated against a peptide that corresponds to the fourth surface exposed Opa protein loop (Table 1). This loop is highly conserved among the neisserial Opa proteins, with the only difference among the Opa proteins of strain FA1090 being the presence of an asparagine residue instead of a tyrosine in the OpaB, OpaC, OpaD, and OpaF proteins. Analysis of the AB4L antiserum by Western blot determined that AB4L, like mAb 4B12 (102), recognizes all known Opa proteins of strain FA1090 with the exception of OpaE (Figure 7A). For Western blot analysis of whole cell lysates, bacteria were harvested from one in vitro passage on GCB agar, suspended in 2x Laemmli loading buffer (Sigma), and boiled at 100°C for 10 minutes. Outer membrane proteins were prepared via the lithium acetate method as described (18, 86). Samples were run on 11% SDS-PAGE gels as described (102). Separated proteins were transferred electrophoretically to PVDF membranes (Biorad, Hercules, CA) and probed with Opa-specific antisera. Bound antibody was detected with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma) as described for colony suspension immunoblots.

Indirect fluorescent-antibody staining (IFA) Detection of OpaI and OpaB variants in vaginal smears from infected mice was performed by a sequential staining procedure using Rb-I or Rb-B/D polyclonal antisera as the primary antibody. Vaginal mucus from infected mice was smeared in duplicate onto teflon printed IFA slides (Electron

Figure 7. Opa proteins of *N. gonorrhoeae* strain FA1090 and the predominant Opa protein phenotype of FA1090Cm^R stocks used in this study. Outer membrane proteins of all of the Opa variants of strain FA1090, as well as OpaI (I^R), OpaB (B^R), and Opa-negative (-^R) variants of strain FA1090Cm^R, were prepared by lithium acetate extraction, subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with Opa-specific polyclonal rabbit antisera. (A) Western blot analysis of all Opa proteins of strain FA1090 with a polyclonal rabbit antiserum specific to the fourth conserved loop of the Opa proteins (AB4L). (B) Western blot analysis of OpaI, OpaB, and Opa-negative variants of strain FA1090Cm^R with polyclonal rabbit antiserum specific to OpaI (Rb-I). (C) Duplicate Western blot probed with antiserum specific to OpaB/D (Rb-B/D).



Microscopy Sciences, Hatfield, PA) (103). Slides were fixed in 100% methanol at -20°C . Slides were blocked for 1 hour at room temperature in PBS containing 1% immunoglobulin-free bovine serum albumin (Sigma) to block non-specific binding. Samples were then incubated with Rb-I (1:4000) or Rb-B/D (1:4000) for 1 hour at room temperature. Goat anti-rabbit immunoglobulin G (IgG, Alexa 488 conjugate, 1:500, Molecular Probes, Eugene, OR) was used as the first secondary antibody. Slides were then incubated with a second primary antibody, a polyclonal rabbit serum raised against heat killed *N. gonorrhoeae* strain FA1090 (anti-GC, 1:3000) for 1 hour at room temperature. Goat anti-rabbit IgG conjugated to Texas Red (Sigma) was used as a second secondary antibody (1:800). All antisera were diluted in PBS containing 1% immunoglobulin-free bovine serum albumin. Slides were rinsed three times with PBS containing 0.05% Tween 20 after incubation with each primary and secondary antibody. Evans Blue (Sigma) was used to counterstain vaginal epithelial cells by diluting a 0.5% (wt/vol) stock 1:30,000 in the final secondary antibody solution. Slides were examined with an Olympus BX60 system microscope with a BX-FLA reflected light fluorescence attachment and Olympus U-M41001, U-M41002, and U-N51006 filters. All images were obtained with a SPOT RT CCD digital camera (Diagnostic Instrument, Inc., Sterling Heights, MI). All images were obtained at an original magnification of 40x.

Statistical analysis The frequency of recovery of Cm^{R} vaginal isolates between experimental groups was analyzed using repeated measures analysis of variance (ANOVA). A mixed model approach (SAS PROC MIXED, version 8.0) was used to

accommodate animals for which data were collected on fewer than three days. The main effect of group was evaluated using Tukey's adjustment for multiple comparisons.

Results

Kinetics of Opa protein expression over time Previous data from our laboratory showed that expression of Opa proteins is selected for and, or, induced in the lower genital tract of female mice. In these experiments, high percentages of Opa-positive gonococci were recovered from 4 of 8 mice within 4 days post-inoculation, following inoculation with a predominantly Opa-negative population of *N. gonorrhoeae* (101). Different Opa variants predominated in different mice. To further characterize this initial description of Opa protein expression during murine infection, as well as examine the kinetics of Opa protein expression later in infection, mice were inoculated with defined mixtures of OpaI-expressing and Opa-negative gonococci. OpaI variants were initially chosen because OpaI variants are easily identified on GC plates due to their very dark colony opacity phenotype and OpaI-expressing gonococci were frequently represented among murine vaginal isolates in our previous report (101). In two experiments in which a total of 10 mice were inoculated with bacterial suspensions consisting primarily of Opa-negative (62-72%) and OpaI variants (28-38%), three distinct phases of Opa protein expression were observed. An apparent selection of OpaI-expressing gonococci was seen between days 1 and 3 post-inoculation in 9 of 10 (90%) infected mice (Table 2). We refer to this phase as the early phase of infection and consider it similar to that which we reported previously (101). A mid-phase of infection, characterized by a decrease in the recovery of Opa-positive gonococci, was observed between days 3 and 7 post-inoculation

Table 2. Three Phases of Opa protein expression during genital tract infection of female BALB/c mice, following inoculation with a mixture of OpaI and Opa-negative gonococci.

Number of mice showing phase/ total number of mice per experiment (percentage)			
	Early Phase ^a	Mid-phase ^b	Late phase ^c
Experiment I	4/4 (100%)	3/4 (75%)	1/1 (100%) ^d
Experiment II	5/6 (83%)	3/6 (50%)	1/2 (50%) ^e
Total	9/10 (90%)	6/10 (60%)	2/3 (67%)

^a A majority of Opa-positive gonococci recovered.

^b Loss in the recovery of Opa-positive variants.

^c Re-emergence of Opa-positive variants.

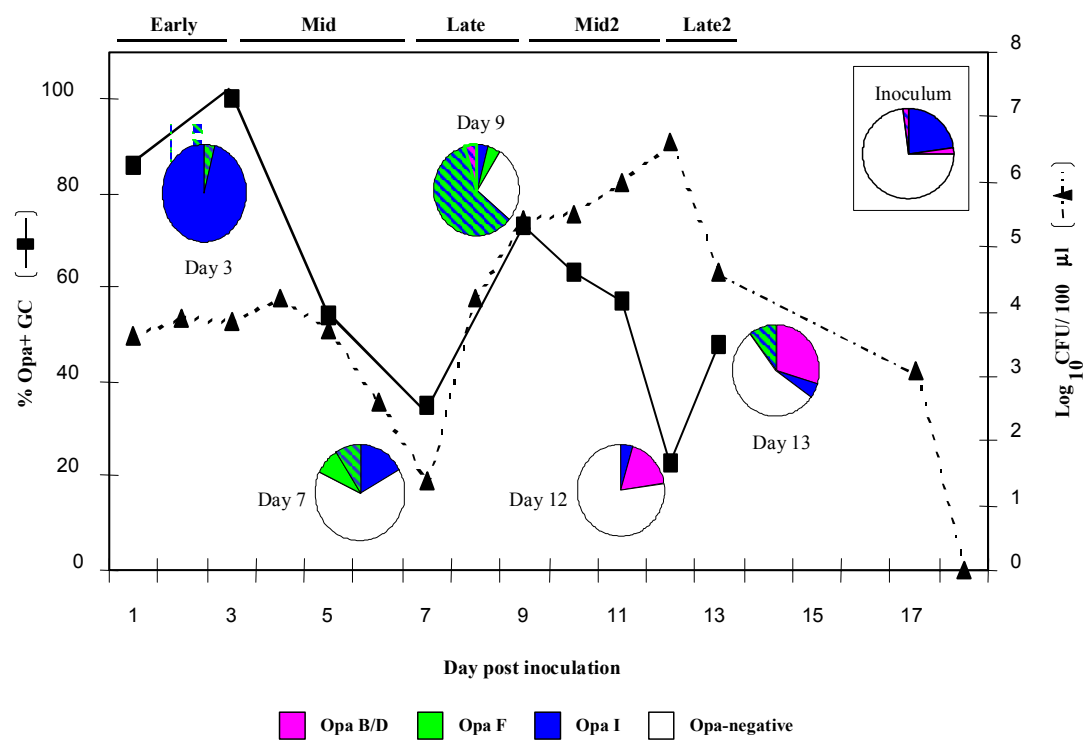
^d Only one mouse in this experiment was infected for longer than 8 days.

^e Only two mice in this experiment were infected for longer than 8 days.

in 6 of 9 mice (67%) that displayed the early phase. In these mice, the reduced recovery of Opa-positive variants paralleled a decrease in overall recovery of *N. gonorrhoeae*. Interestingly, the mid-phase was followed by a re-emergence of OpaI variants, which also coincided with an increase in overall recovery of gonococci in two of three mice in which infection progressed for longer than eight days. This stage was termed the late phase of infection. No dramatic change in Opa phenotype was seen over time when an Opa-negative variant of strain FA1090 was serially cultured without single colony passage in vitro (102). Also, as reported previously in mice and male volunteers (101, 102), gonococci that expressed multiple Opa proteins simultaneously were recovered at all phases of infection, with the highest percentages in the two later phases. Gonococci that express multiple Opa proteins are rare during in vitro passage and this phenotype was rapidly lost upon subculture (Simms and Jerse, unpublished).

An example of the distribution of Opa phenotypes over time in one mouse that displayed all three phases of infection is shown in Figure 8. This mouse remained colonized with gonococci for 17 days, at which point the experiment was ended. Variants that expressed multiple Opa proteins were recovered from this mouse at high percentages during the late phase, with some gonococci expressing up to three Opa proteins simultaneously. After the late phase, another decrease in the number of Opa-positive variants recovered was seen on day 12 post-inoculation, followed by a slight increase in Opa-positive isolates on day 13. These latter two changes in Opa phenotype are reminiscent of both the mid- and late phases characterized above and are tentatively referred to as mid2 and late2. The patterns shown in mice infected for longer than 8 days

Figure 8. Kinetics of Opa protein expression in female mice following inoculation with a mixture of predominantly OpaI and Opa-negative gonococci. The phase of Opa protein expression, as defined in Table 1, is indicated above the graph. Results from one representative mouse that was infected for 17 days are shown here. The percentage of Opa-positive gonococci recovered and the overall gonococcal recovery are shown on the y axes. Pie graphs are utilized to illustrate the percentage of each Opa variant recovered per time point. The composition of the input inoculum was 28% OpaI and 72% Opa-negative, with other Opa variants at <1%. Gonococci that expressed more than one Opa protein simultaneously are represented by stripes.

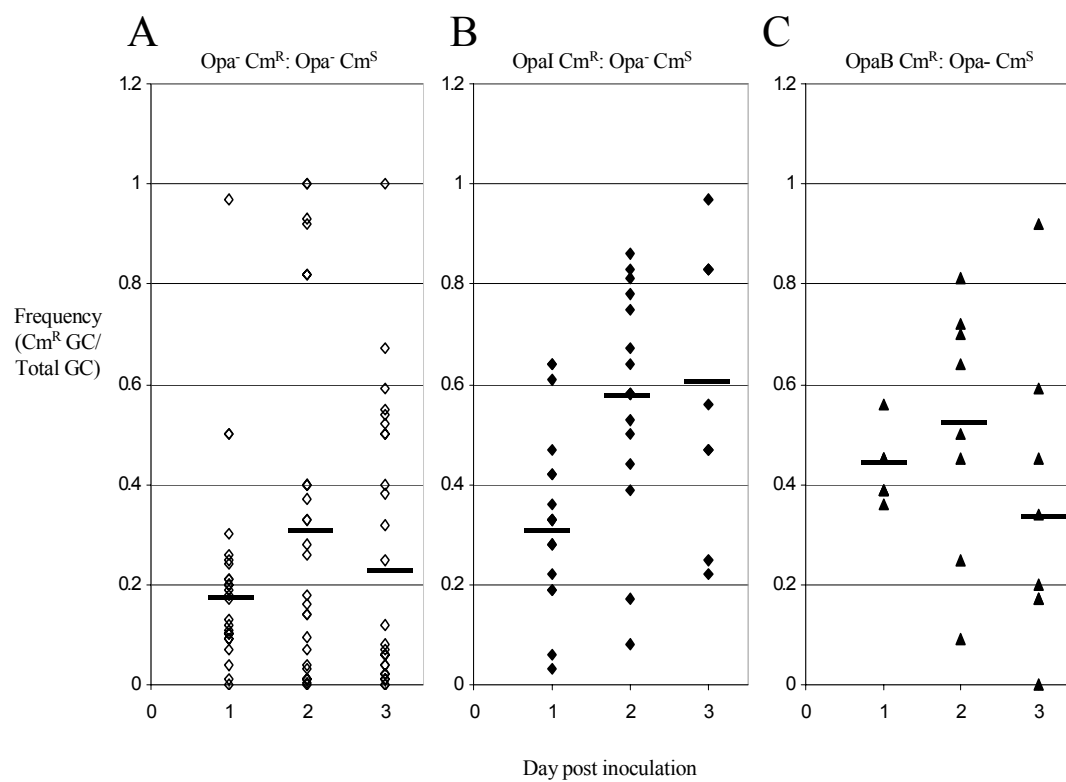


suggest Opa protein expression during genital tract infection in female mice may have a cyclical pattern.

Pre-existing populations of Opa-positive variants are selected for in vivo. We hypothesized that selection of a pre-existing population of OpaI variants in the inoculum used in the experiments described above was responsible for the reisolation of mainly OpaI-expressing gonococci in the early phase of infection. An alternative hypothesis is that the increased recovery of OpaI variants was due to an increase in Opa protein expression via induction of increased *opa* gene phase variation in vivo. In order to distinguish between these two possibilities, we constructed a chloramphenicol-resistant (Cm^R) strain of *N. gonorrhoeae* FA1090 (FA1090Cm^R) as described in Materials and Methods to provide a means of following specific populations of gonococci over time. OpaI, OpaB, and Opa-negative variants of FA1090Cm^R were identified by colony suspension immunoblotting, and non-piliated stocks of each were frozen for use in subsequent experiments. Nonpiliated gonococci were used to lessen the likelihood of genetic transfer of the *cat* gene to wild-type gonococci during infection. The predominant Opa phenotype of each of these frozen stock cultures was confirmed by Western Blot analysis with Opa-specific antisera (Figure 7).

FA1090Cm^R did not have a different growth rate compared to the wild-type strain when cultured independently and together in supplemented GC broth (data not shown). We then examined if strain FA1090Cm^R had an advantage compared to the wild-type parent strain (Cm^S) in vivo, which was independent of Opa phenotype. In four different experiments, groups of 5-9 mice (total n = 30) were inoculated with mixtures consisting

Figure 9. Frequency of Cm^{R} gonococci recovered from mice inoculated with mixtures of Opa-negative variants of FA1090 and Opa-negative, OpaI, or OpaB variants of FA1090 Cm^{R} . (A) Mice inoculated with a mixture of both Cm^{S} and Cm^{R} Opa-negative variants (Control group, 4 experiments, $n=5-9$ mice/experiment, total $n=30$). The range of the input inocula ratios in these experiments was 11-19% Opa-negative, Cm^{R} and 68-73% Opa-negative, Cm^{S} gonococci. (B) Mice inoculated with mixtures of OpaI, Cm^{R} and Opa-negative, Cm^{S} variants (3 experiments, $n=5-6$ mice/experiment, total $n=16$). The range of the input inocula ratios in these experiments was 8-18% OpaI, Cm^{R} and 72-79% Opa-negative, Cm^{S} gonococci. (C) Mice inoculated with a mixture of OpaB, Cm^{R} and Opa-negative, Cm^{S} variants ($n=8$). The input inoculum ratio in this experiment was 11% OpaB, Cm^{R} and 81% Opa-negative, Cm^{S} gonococci. A significant difference in the frequency of Cm^{R} gonococci recovered was detected in mice inoculated with wild-type gonococci (Cm^{S}) spiked with Cm^{R} Opa-positive variants (OpaI or OpaB) compared to mice inoculated with Opa-negative variants of both FA1090 and FA1090 Cm^{R} , as determined by repeated ANOVA analysis (OpaI, Cm^{R} $P < 0.001$; OpaB, Cm^{R} $P < 0.008$).



of 11-19% Opa-negative, Cm^R gonococci and 68-73% Opa-negative, wild type (Cm^S) gonococci. The percentage of Opa-positive variants was $\leq 10\%$, with the majority Cm^S as expected. No significant increase in the mean frequency of Cm^R gonococci among vaginal isolates was observed compared to that of the inoculum (Figure 9A). We conclude from these experiments that FA1090Cm^R had no inherent growth or survival advantage independent of Opa protein expression in vivo and therefore, would be a suitable tool for following a specific population of gonococci during the early phases of murine infection.

We next predicted that if selection of Opa-positive variants occurred early in infection, inoculation of mice with a defined ratio of Opa-positive, Cm^R gonococci and Opa-negative, Cm^S gonococci would result in the reisolation of the Opa-positive, Cm^R population. Groups of mice were first inoculated with suspensions of primarily Opa-negative, Cm^S gonococci spiked with smaller numbers of OpaI-expressing, Cm^R gonococci. The percentage of OpaI, Cm^R gonococci in the inocula of these three experiments ranged from 8-18%. In these experiments, a significantly higher frequency of Cm^R gonococci occurred among vaginal isolates within two days post-inoculation when compared to mice inoculated with Opa-negative, Cm^R and Opa-negative, Cm^S gonococci (Figure 9B, $P < 0.001$). An analysis of both Opa and Cm^R phenotypes showed that the vast majority of Cm^R gonococci expressed OpaI at each time point in each of three different experiments. Using the criteria by which selection was defined (greater or equal to three times the percentage found in the inoculum), selection for OpaI, Cm^R gonococci occurred in 12 of 16 (75%) mice (Table 3). The results of a representative experiment are shown in Figure 10.

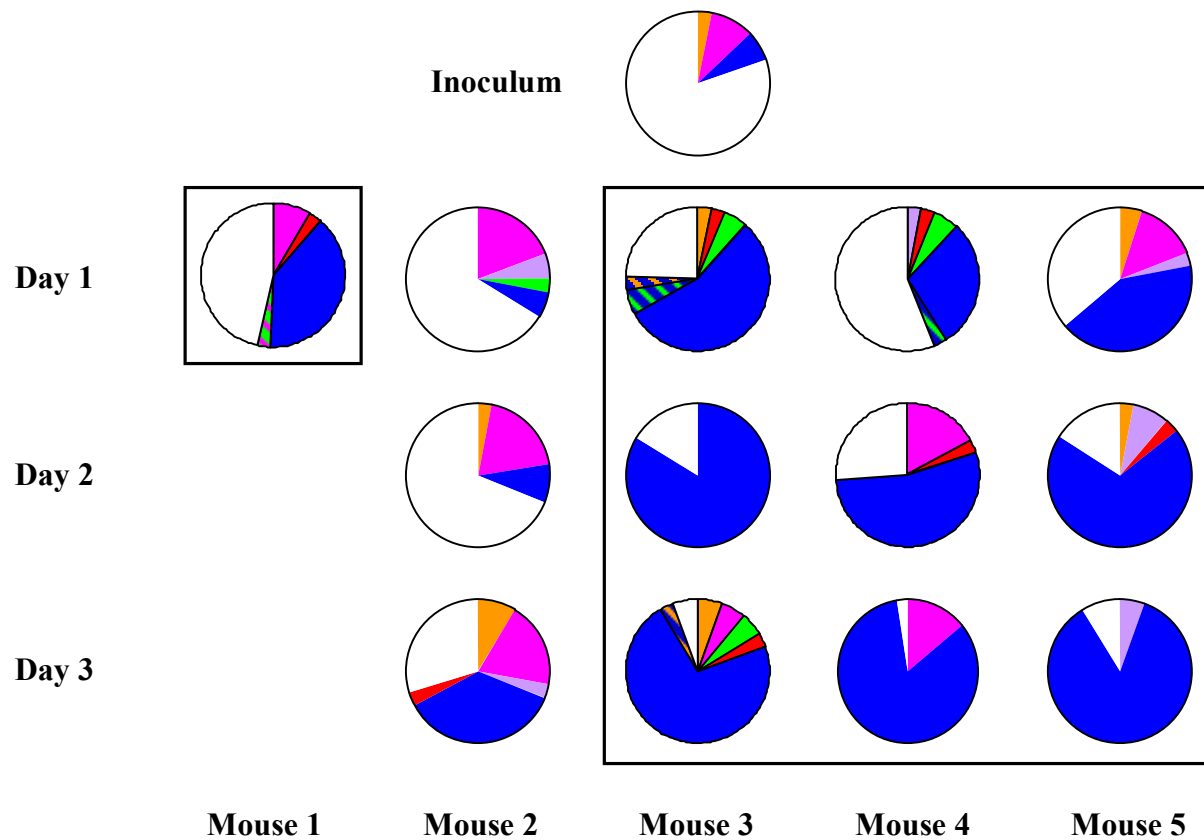
Table 3. Selection experiments using mixtures of OpaI, Cm^R and Opa-negative, Cm^S variants^a.

Number of mice showing selection of OpaI, Cm ^R variants / Total number of mice in experiment (%)	
Experiment #	Selection of OpaI, Cm ^R GC ^b
I	5/6 (83%)
II	3/5 (60%)
III	4/5 (80%)

^a The predominant phenotypes in the inocula were OpaI, Cm^R (range: 8-18%) and Opa-negative, Cm^S(range: 72-79%).

^b Selection was defined as three times or greater than that which was present in the inoculum.

Figure 10. Opa phenotype of vaginal isolates from mice inoculated with a mixture of OpaI, Cm^R and Opa-negative, Cm^S gonococci. Selection was defined as a threefold increase in the percentage of OpaI variants over that which was present in the inoculum. In this experiment, OpaI-expressing gonococci were selected for in 4 of 5 mice, of which the vast majority (90-100%) were Cm^R. Gonococci were only recovered from Mouse 1 on day 1. Time points at which selection of OpaI, Cm^R gonococci was observed are enclosed in black boxes. In this experiment, infection was continued for longer than three days and in 3 of the 4 mice in this experiment in which selection of OpaI, Cm^R gonococci was observed early, evidence of a mid-phase of Opa protein expression (a decrease in the recovery of Opa-positive variants) was seen by day 5 post-infection (data not shown). All Rb-B/D-positive and H164-positive OpaE/K variants in the OpaI mixture experiments were not analyzed by western blotting, therefore, they are referred to as OpaB/D and OpaE/K.



■ OpaA
 ■ OpaB/D
 ■ OpaC
 ■ OpaE/K
 ■ OpaF
 ■ OpaI
 ■ Opa-negative

As discussed, the receptor repertoire of the Opa proteins of the pathogenic *Neisseria* consists of members of the human CEACAM family, as well as HSPGs (21, 38, 72, 205, 208). Similar to the existence of only one Opa protein in strain MS11 that binds to HSPG (35, 205), the OpaI protein of *N. gonorrhoeae* strain FA1090 was reported to be the only Opa protein of this strain that binds to HSPG; all other Opa proteins from this strain only bind CEACAM receptors (N. B. Guyer, M. C. Piriou, M. M. Hobbs, and J. G. Cannon, Abstr. 101st Gen. Meet. Amer. Soc. Microbiol., abstr. D-135, 2001). Unlike HSPG, which is present in the murine genital tract (85), it is important to note that the murine CEACAM1 molecule is not closely related to its human CEACAM counterpart (77). Therefore, to examine whether selection of Opa-positive gonococci occurs when mice are infected with an Opa protein that only binds CEACAM receptors, an experiment was designed to follow the recovery of OpaB variants in vivo. Eight mice were infected with a mixture of predominantly Opa-negative, Cm^S (81%) and OpaB, Cm^R (11%) gonococci. As seen with the OpaI, Cm^R variant, a significant increase in the frequency of Cm^R reisolates occurred early in infection, as compared to mice inoculated with Opa-negative, Cm^R and Opa-negative, Cm^S gonococci (Figure 9C, $p < 0.008$). Using our criteria for selection (recovery of OpaB, Cm^R variants at greater than or equal to three times the percentage present in the inoculum), selection for OpaB, Cm^R gonococci was seen in all eight infected mice (Table 4). The outcome of this experiment suggests that selection of Opa protein expression in the murine genital tract is not dependent on the presence of human CEACAM receptors.

Reciprocal experiments were performed, in which mice were infected with bacterial suspensions containing predominantly Opa-negative, Cm^R gonococci (71-78%)

Table 4. Selection experiments using mixtures of OpaB and Opa-negative variants of FA1090Cm^R and FA1090 (wt, Cm^S).

Number of mice showing selection of pre-existing OpaB population/ Total number of mice in experiment (%)	
Inoculum ^{a,b}	Selection ^c of OpaB variants
OpaB, Cm ^R : Opa-negative, Cm ^S	8/8 (100%)
OpaB, Cm ^S : Opa-negative, Cm ^R	2/7 (29%)

^a The predominant phenotypes present in the inocula of the OpaB, Cm^R experiment were 11% OpaB, Cm^R and 81 % Opa-negative, Cm^S.

^b The predominant phenotypes present in the inocula of the OpaB, Cm^S experiment were 12% OpaB, Cm^S and 78% Opa-negative, Cm^R.

^c Selection was defined as three times or greater that which was present in the inoculum.

and smaller numbers of OpaI, Cm^S (15-24%) or OpaB, Cm^S (12%) gonococci. The recovery of OpaI-expressing gonococci was not as dramatic in these OpaI mixture experiments. Selection for Opa-positive gonococci occurred in two of three experiments (Table 5). In these two experiments, the percentage of mice in which selection occurred was 13% and 60%. However, in all mice in which OpaI variants predominated, those OpaI-expressing gonococci were Cm^S. These results are consistent with selection of a pre-existing population when selection for Opa protein expression occurred. None of the other mice had high percentages of Opa-positive gonococci among their vaginal isolates. In reciprocal experiments designed to test the selection of OpaB variants, selection of OpaB, Cm^S gonococci occurred in only two of seven infected mice (Table 4; Figure 11, mice 9 and 15). Interestingly, a predominance of OpaE/K variants was isolated from the other five infected mice. The majority of these isolates were also Cm^R. The percentage of OpaE/K Cm^R gonococci in the inoculum (7%) was similar to that of the OpaB, Cm^S (11%) population. Taken together, these data indicate that regardless of Cm^R phenotype, Opa-positive gonococci present in the inoculum were selected for in vivo. However, given the fact that the reciprocal experiments were not as selective, it is possible that FA1090Cm^R may have a slight advantage in vivo that was not detected in the early in vivo control studies (Figure 9A). Alternatively, another phase variable factor present in either population may have influenced the gonococcal recovery in the different experiments.

Both OpaI and OpaB variants are cell-associated in vaginal smears. Gonococci expressing Opa proteins are prone to aggregation, particularly highly opaque colony

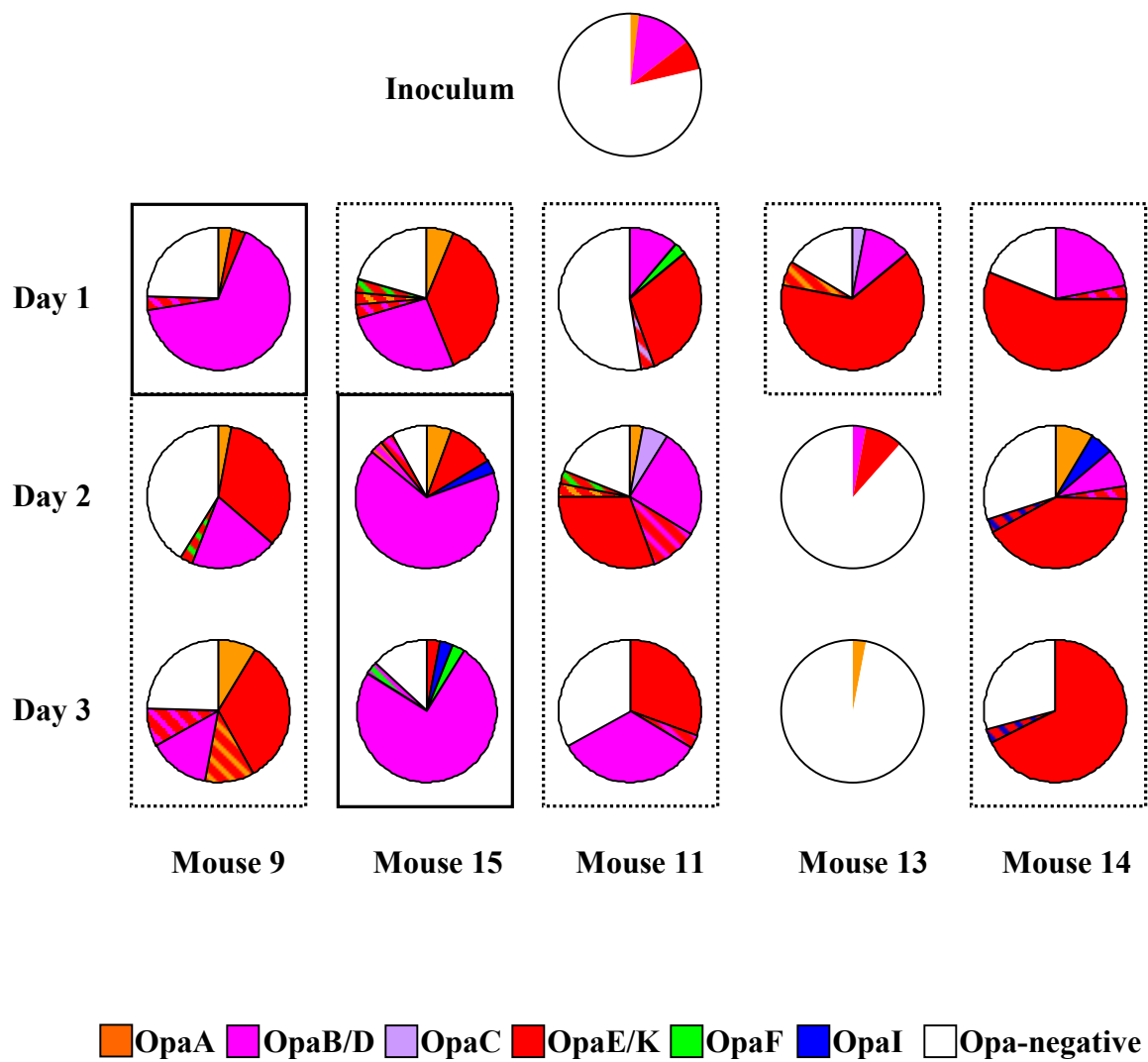
Table 5. Selection experiments using mixtures of OpaI, Cm^S and Opa-negative, Cm^R variants.

Number of mice showing selection of OpaI, Cm ^S variants / Total number of mice in experiment (%)	
OpaI, Cm ^S : Opa-negative, Cm ^R inoculum ^a	
Experiment #	Selection of OpaI, Cm ^S GC ^b
I	0/7 (0%)
II	1/8 (13%)
III	3/5 (60%)

^a The predominant phenotypes present in the inocula were OpaI, Cm^S (range: 15-24%) and Opa-negative, Cm^R (range: 71-74%).

^b Selection was defined as three times or greater that which was present in the inoculum.

Figure 11. Opa phenotype of vaginal isolates from mice inoculated with a mixture of OpaB, Cm^S gonococci and Opa-negative, Cm^R gonococci. Boxes highlight those time points at which selection of OpaB, Cm^S gonococci (solid lines) or OpaE/K, Cm^R gonococci (dashed lines) occurred. 2 of 7 mice in this experiment (Mice 9 and 15) showed selection of OpaB, Cm^S gonococci. Selection of a population of OpaE/K, Cm^R gonococci occurred in the remaining 5 mice; pie graphs of 3 of these 5 mice (Mice 11, 13, and 14) are shown.

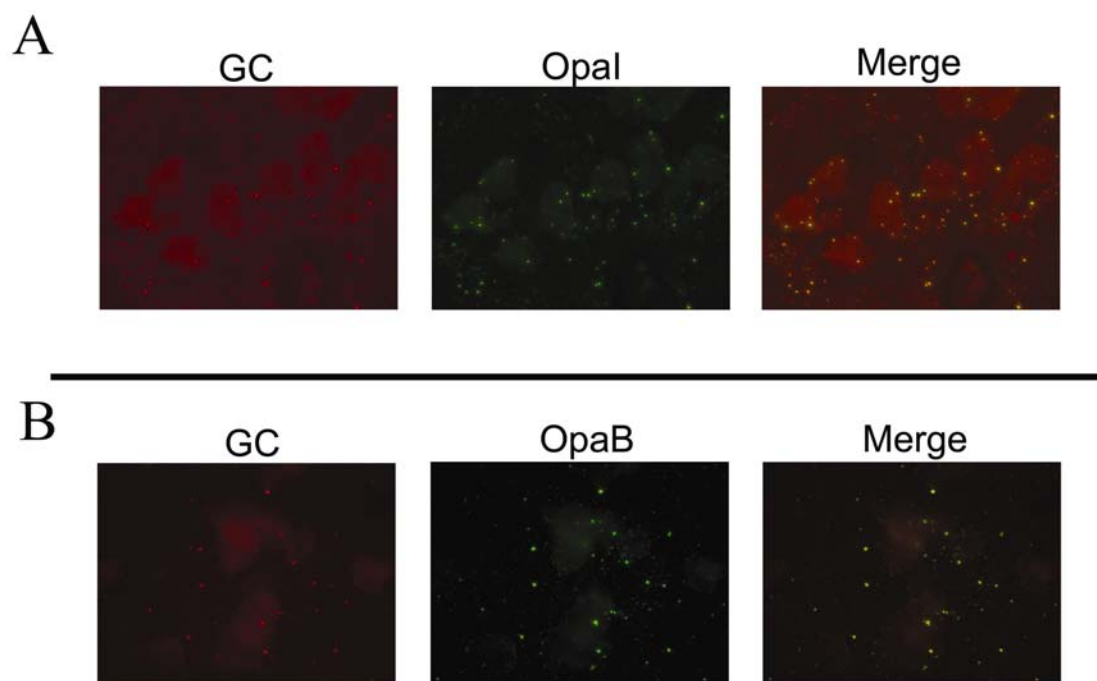


variants such as the OpaI variants of strain FA1090. This phenomenon can skew quantitative culture results. Therefore, the Opa phenotype of gonococci in vaginal smears from infected mice was analyzed using IFA to confirm immunoblot results by another method, as well as to determine if gonococci were cell associated. The smears were stained via a sequential IFA technique with Opa-specific polyclonal sera (Rb-I or Rb-B/D) and a polyclonal rabbit antiserum raised by our laboratory against whole gonococci (Rb-GC) (102). IFA results were consistent with the immunoblot results in that large numbers of gonococci that were reactive with the OpaI or OpaB/D antiserum were observed in mice from which a majority of OpaI or OpaB/D variants were recovered respectively. Furthermore, both OpaI and OpaB variants were associated with epithelial cells during infection (Figure 12). We often saw gonococci that did not react with the OpaI or OpaB/D antiserum attached to cells (data not shown). It is not known if these GC were Opa-negative or if they represented small numbers of gonococci that expressed other Opa proteins. At this time, we do not know the receptor-ligand interaction responsible for adherence of gonococci to mouse vaginal epithelial cells. Mice do not express the putative gonococcal pilus receptor, CD46 (112), and as discussed, it is unlikely that the gonococcal Opa proteins can exploit mouse CEACAM molecules as receptors.

Discussion

Earlier studies on Opa protein expression during experimental urethral infection in male volunteers and genital tract infection in female mice suggested Opa protein

Figure 12. Association of OpaB and OpaI variants with murine vaginal cells. IFA of vaginal smears of mice infected with a mixture of OpaI, Cm^R and Opa-negative, Cm^S gonococci (top panels) or OpaB, Cm^R and Opa-negative, Cm^S gonococci (bottom panels). Smears were stained with polyclonal rabbit antisera raised against the OpaI (A) or OpaB/D (B) proteins of strain FA1090 (green), as well as a polyclonal rabbit serum specific for whole gonococci (red). Results from one mouse in each group are shown.



expression was selected for, and or, induced in vivo (101, 102, 194). In order to examine the reason for the increased isolation of Opa-positive variants early during murine infection, here we engineered a derivative of strain FA1090 that carries an antibiotic resistance marker. This strain, FA1090Cm^R, allowed us to follow one specific population during the first three days within the murine lower genital tract. We showed that two Opa proteins (OpaB and OpaI) of strain FA1090 were selected for in vivo. An additional Opa variant (Opa E/K) was also recovered in high numbers in one experiment. No difference was observed in the degree of association of these Opa variants with epithelial cells in vaginal smears. From these studies, we conclude that selection of a pre-existing population of Opa variants is responsible for the recovery of predominantly Opa-positive populations of gonococci in vivo.

In light of the role played by Opa proteins in adherence and invasion of human cells, a simple explanation for these observations might be selection for Opa protein expression due to a colonization advantage. However, as discussed, the major family of Opa protein receptors, the CEACAM family, is not expressed in normal mice. Only one homolog of the human CEACAM molecules (CEACAM1) has been identified in mice (77), and the two molecules do not appear to be highly related. Structural analysis of the human CEACAM1 molecule by Virji et al (207) revealed two critical amino acids for Opa binding, tyrosine 34 and isoleucine 91. Other amino acids that enhanced Opa binding to the CEACAM molecule were glutamine 44 and 89, serine 32, and valine 39 (207). Sequence alignment of the human CEACAM1 molecule and mouse CEACAM1 (77) revealed that tyrosine 34 was conserved between the two genes, but the other critical

and accessory amino acids were not (data not shown). Therefore, it is unlikely that the neisserial Opa proteins can utilize murine CEACAM1 as a cellular receptor.

Some Opa proteins bind HSPG molecules (35, 205). HSPG molecules are expressed on almost all mammalian epithelium and have been identified on the vaginal epithelium of mice (85). Serum factors, specifically vitronectin, enhance invasion via the HSPG binding pathway (56, 67). The CEACAM receptor repertoire of the Opa proteins of strain FA1090 was recently reported and only the OpaI protein of this strain binds to HSPG molecules (N. B. Guyer, M. C. Piriou, M. M. Hobbs, and J. G. Cannon, Abstr. 101st Gen. Meet. Amer. Soc. Microbiol., abstr. D-135, 2001). This presented a paradox, in that we frequently recover other Opa variants of this strain from the genital tract of mice (101). Here, experiments using a CEACAM-binding Opa protein (OpaB) suggested that this Opa variant was also selected for in vivo, much like the HSPG-binding Opa protein, OpaI. We conclude that unless there are other Opa receptors not yet identified in mice Opa-mediated colonization is not responsible for selection of Opa-positive gonococci in mice. Interestingly, it was reported that some members of the CEACAM family, specifically CEACAM1, 5, 6, and 8, are not present on primary male urethral cells, and that CEACAM3 is detected on only a small population of these cells (79). It therefore seems unlikely that the adherence function of the Opa proteins is the selective force behind Opa protein expression during male urethritis. Such a selective pressure cannot be ruled out, however, since studies using a CEACAM1-specific Opa variant of strain MS11 indicated that the CEACAM1 molecule is upregulated in primary human ovarian surface epithelial cells via activation of nuclear transcription factor $\kappa\beta$ (143). It

remains to be seen if upregulation of CEACAM1 occurs in primary male urethral cells during infection.

It is important to note that neither the human challenge model nor experimental infection in female mice fully mimics *N. gonorrhoeae* infection in women. While some human cell lines of female genital origin, such as ME180 (cervical carcinoma) and NC9 (cervical) cells, surface-express CEACAM molecules (196), it was also reported that CEACAM molecules are not always expressed by all genital epithelial cells. Specifically, neither Hec-1-B (endometrial) nor HeLa (endocervical) cells expressed CEACAM molecules (196). Also, in one clinical study, it was shown the CEACAM-5 molecule (CEA) was not always present on primary cervical tissue from all subjects (140). The recent development of transgenic female mice that express many of the human CEACAM receptor molecules (33) may be a useful tool to further elucidate the importance of CEACAM receptor binding in selection of Opa-positive gonococci in vivo.

An alternative hypothesis to explain the apparent advantage displayed by Opa-positive gonococci early during murine infection is that Opa-negative gonococci may be more sensitive to innate host defenses. In order to persist in the lower genital tract of females, the gonococcus must evade many nonspecific physiological and immunological factors, such as complement-mediated bacteriolysis and opsonization, low pH, antimicrobial peptides, inhibitory commensal flora, and gonadal steroids. Bos et al (22) presented evidence that complement may play a role in selection of Opa protein expression in vivo. In this study, Opa-positive gonococci of strain MS11 were more resistant to the bactericidal action of normal human serum (NHS) than were their Opa-negative counterparts. Some Opa variants of the gonococcus can also bind heparin (35),

which was shown to protect Opa-positive gonococci from killing by NHS (39). The mechanism of Opa-mediated serum resistance is not yet known. However, these studies indicate that Opa protein expression may contribute to survival of the gonococcus by increasing resistance to complement-mediated bacteriolysis. Complement components exude onto mucosal surfaces during inflammation, and complement proteins were detected on the surface of gonococci isolated from infected women by endocervical lavage (136). Complement components have also been detected in mouse uterine luminal fluid during the high-estrogen phase of the estrus cycle (105). Exogenous estradiol administration stimulates the synthesis of C3 in the endometrium of both ovariectomized and normal cycling mice (124). Therefore, we can surmise that C3 was present in the genital tract of our mice at the time of infection. Unfortunately, strain FA1090 is inherently resistant to the bactericidal action of NHS, which complicates studying the relative serum resistance of different FA1090 Opa variants *in vitro*.

Another interesting finding in this study was that the predominant Opa phenotype among vaginal isolates appeared to fluctuate between Opa-positive and Opa-negative in several mice for which Opa protein expression was examined for greater than eight days. We defined three phases of Opa protein expression *in vivo*: an early phase in which selection of Opa-positive gonococci occurred, a mid phase characterized by a decrease in the percentage of Opa-positive variants recovered, and a late phase, in which a re-emergence of Opa-positive gonococci was observed. Interestingly, during these three phases, the changes in Opa protein expression corresponded with fluctuations in total number, with Opa-positive variants associated with higher bacterial counts. This observation is intriguing when one considers clinical observations regarding culture rate

in women with gonorrhea with respect to the menstrual cycle. In 1947, Koch (119) noted a link between windows of culture negativity in female patients with gonorrhea and the phase of the patients' menstrual cycle. In four hospitalized patients, positive gonococcal cultures were obtained during the proliferative or high estrogen phase of the menstrual cycle. Endocervical cultures from these same patients taken during the luteal or high progesterone phase of the menstrual cycle were consistently negative. Similar observations were made in other clinical studies (99, 107, 133).

Hormonally-driven changes in pH, commensal flora, progesterone, complement, and immunological factors may challenge survival of the gonococcus during the menstrual cycle. Also, hormonally-driven changes in expression of cellular host receptors could lead to gonococcal invasion of epithelial cells. While it is not known which factors control the association between hormonal state and culture positivity in women with gonorrhea, evidence indicates phase variation of the Opa proteins may help the gonococcus evade host factors. This hypothesis is based on work by James and Swanson in which endocervical isolates from women in the luteal or high progesterone phase of the cycle were generally transparent; opaque variants were often recovered in the proliferative phase (99, 100). Transparent colonies, which occurred most frequently during the week of menstruation and least frequently at mid-cycle were trypsin-resistant, whereas the opaque variants were trypsin-sensitive (99). Others reported that opaque gonococci are more resistant to progesterone in vitro (170), although we have been unable to reproduce this result using defined Opa variants of strain FA1090 (Simms and Jerse, unpublished). Also, as discussed, complement, which is produced more in the high estrogen phase, may select for Opa-positive gonococci (22).

Consistent with our results, which suggest a cyclical variation in Opa protein expression in genital infection of female mice, Kita et al reported changes in Opa phenotype with respect to the estrous cycle in SLC:ddY mice (114). These mice were not treated with estradiol. Although the mice in the experiments described in our study were treated with β -estradiol, the pituitary and ovaries remained intact, thus allowing for hormonal influences, including progesterone secretion. Therefore, it is possible that hormonally-driven host factors in estradiol-treated mice may have contributed to the decreased isolation of Opa-positive gonococci at the mid phase of infection, either by selection against Opa-positive gonococci or through an increase in the phase variation rate of the *opa* genes, leading to recovery of primarily Opa-negative gonococci. An alternative explanation of the decrease in recovery of Opa-positive variants during the mid-phase of infection is the possibility of Opa-mediated invasion of the gonococcus into the host epithelium. If this occurred, the likelihood of recovering intracellular gonococci by vaginal swabs would perhaps be reduced.

In summary, earlier reports regarding Opa protein expression in experimental infection of male volunteers and 17β -estradiol-treated mice supported a role for Opa proteins in establishment of gonococcal genital tract infection. The advantage Opa protein expression confers in these two infection models is not yet clear. Many host factors may select for or against Opa protein expression; whether they act independently or in concert is not known. However, in light of the host restrictions between humans and mice, adherence to CEACAM receptors is unlikely to be responsible for the selection of Opa-positive gonococci in the murine genital tract.

Chapter 3: Examination of *opa* gene phase variation in vitro and in murine infection using a chromosomally-encoded *opaB::phoA* fusion expressed in *Neisseria gonorrhoeae*

Introduction

Neisseria gonorrhoeae is the etiological agent of gonorrhea. It is a human specific pathogen with no environmental reservoir. In contrast to most other bacterial pathogens, reinfection with *N. gonorrhoeae* is very common and can occur with the same strain (171). The gonococcus encodes many factors that allow it to establish infection and evade host factors. One mechanism the gonococcus uses for this purpose is antigenic and phase variation of surface molecules. The opacity (Opa) proteins are a family of phase variable outer membrane proteins that mediate attachment and invasion of epithelial cells (13, 120, 126, 180, 207, 211), non-opsonic uptake by neutrophils (9, 21, 37, 63, 120), and evasion of innate host defenses (22, 39, 99, 100). Individual Opa proteins are encoded by separate unlinked chromosomal genes; *N. gonorrhoeae* strains possess between 8 to 11 different *opa* genes, whereas *N. meningitidis* strains possess 3 to 4 different *opa* genes. *opa* gene phase variation is *recA* independent (11, 146) and occurs by a reversible frameshift mechanism that involves insertion or deletion of one or more copies of a pentameric DNA sequence (CTCTT) found within the signal sequence-encoding region (11, 146, 191). Transcription of the *opa* genes is constitutive (191); therefore, detection of phase variation is usually determined at the level of translation. Individual *opa* genes phase vary independently of each other, and as a result, gonococci can express no Opa proteins, a single Opa protein, or multiple Opa proteins

simultaneously. Differences in colony photo-opacity of *N. gonorrhoeae* are caused by the expression of different Opa proteins.

The mechanism of *opa* gene phase variation is consistent with that of slipped strand mispairing (SSM) (11, 146, 191). In SSM, mutational events occur during DNA replication as a result of local denaturation of a DNA duplex followed by mispairing of bases within the tandem repeat region (123). High frequency deletions or insertions occur only in the repeat region, involve an integral number of repeats, and are strongly biased toward the loss or gain of one repeat (122). Also, the frequency of frameshifting increases with the length of the repeat region (122). In order for misalignment of the repeat region to occur, SSM requires transient formation of single-stranded DNA. Interestingly, the pentameric repeat region of the *opa* genes was shown to adopt an unusual triple-stranded H-DNA conformation (8), which by the features of its structure, presents a single-stranded region that is highly sensitive to single-strand-specific nuclease.

Phase variation of recombinant *opa* genes occurs at a high frequency (10^{-3} / cell/ generation) when expressed on single copy plasmids in *Escherichia coli* (11, 146). Despite the evidence that *opa* gene phase variation occurs via SSM, the rate of *opa* gene phase variation has not been well studied in its native gonococcal background. The rate of change of colony opacity phenotype of various gonococcal strains was examined previously by Mayer (131) and determined to be $\sim 10^{-3}$ per cell per generation. Variations in temperature, pH, oxygen concentration, piliation, and the addition of excess DNA or DNase, did not significantly influence this rate. Unfortunately, the results from this study are compromised by the fact that some Opa proteins do not confer detectable opacity to a

colony. Therefore, the actual rate of *opa* gene phase variation in the gonococcus may be higher. Promoter strength may also be important in *opa* gene phase variation. In a study using transcriptional *opa::lacZ* fusions and translational *opa::phoA* fusions expressed in *E. coli*, Belland et al. (10) established a correlation between transcription levels from different *opa* promoters and the phase variation rates of the corresponding *opa* genes. These workers also presented evidence that *opa* gene transcription may not be constitutive.

Studies using experimental infection models have indicated that Opa protein expression is selected and, or induced in vivo (101, 102, 194). Inoculation of male volunteers with populations of predominantly Opa-negative gonococci led to the recovery of primarily Opa-positive variants early in infection. We previously reported a similar result in female mice (101), and have recently shown that selection of Opa-positive gonococci is responsible for the recovery of Opa-positive variants at early time points post-inoculation (Simms and Jerse, in preparation/ Chapter 2). High numbers of gonococci that expressed multiple Opa proteins simultaneously were recovered from both the urethras of male volunteers (102) and the lower genital tract of mice (101). Although selection of small populations of spontaneously generated multiple expressers may be responsible for this observation, it is possible that induction of *opa* gene phase variation, followed by selection for variants that express several Opa proteins occurs in vivo.

The factors that select for Opa-positive gonococci remain unidentified and the effect of host environment on the rates of *opa* gene phase variation have not been examined. To examine the rate of phase variation of the *opa* genes in the gonococcus, here we constructed a chromosomal *opaB::phoA* fusion in *N. gonorrhoeae* strain

FA1090. The *opaB* gene was chosen because OpaB variants of this strain were frequently recovered from the lower genital tracts of both male volunteers and female mice (101, 102). The effect of environmental conditions designed to mimic those of the urogenital tract on the phase variation rate of the *opaB::phoA* fusion were examined. In addition, we measured the frequency of *opaB::phoA* phase variants among vaginal isolates during experimental genital tract infection of female mice.

Materials and Methods

Bacterial strains and plasmids All bacterial strains and plasmids used in this study are listed in Table 6. *Neisseria* spp. were grown on GCB agar containing Kellogg's supplements (113) and incubated in 5% CO₂ at 37°C. *E. coli* strains were grown in Luria-Bertani (LB) broth or on agar at 37°C. Antibiotics were added at the following concentrations for *E. coli*: chloramphenicol (Cm), 25 µg/ml; erythromycin (Erm), 250 µg/ml; kanamycin (Km), 50 µg/ml. For *N. gonorrhoeae*, the antibiotic concentrations were: Erm, 2 µg/ml and streptomycin (Sm), 100 µg/ml. All antibiotics were obtained from Sigma Chemical Company, St. Louis, MO. GCB agar with VCNTS selection (vancomycin, colistin, nystatin, and trimethoprim sulfate and streptomycin) was used to isolate gonococci from the murine genital tract as described (101, 103, 104).

Recombinant DNA methods Standard recombinant DNA methods, including restriction enzyme digestions, ligation reactions, agarose gel electrophoresis, and chemical transformations, were performed in accordance to manufacturer's instructions. *E. coli*

Table 6. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source
<i>Neisseria</i> sp.		
FA1090	<i>N. gonorrhoeae</i> , wild-type, porin type 1B, Sm ^R	(41)
ANS100	FA1090 with an <i>opaB::phoA</i> fusion inserted in the genome between <i>lctP</i> and <i>aspC</i> , Erm ^R	This study
RF4738	<i>N. cinerea</i> , commensal strain	ATCC
F6865	<i>N. lactamica</i> , commensal strain	ATCC
<i>E. coli</i>		
Top10	Host strain for pCR-Blunt cloning vector	Invitrogen
DH5 α MCR	Host strain for plasmids	Invitrogen
S17-1 (λ pir)	Host strain used for conjugation	(181)
Plasmids		
pCR-Blunt	pCR-Blunt cloning vector	Invitrogen
pANS-1	pCR-Blunt carrying the <i>opaB</i> gene in MCS	This study
pANS-2	pCR-Blunt carrying the <i>opaB::phoA</i> fusion in MCS	This study
pAM400	Derivative of pUT/mini-Tn5 <i>phoA</i> , Cm ^R	A.D. O'Brien
pGCC2	NICS integration vector, Erm ^R	H.S. Seifert
pANS-3	<i>opaB::phoA</i> fusion in SalI site of pGCC2	This study

DH5 α were transformed by electroporation using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Restriction enzymes, T4 DNA ligase, and Deep Vent polymerase were purchased from New England Biolabs (Beverly, MA). All PCR amplifications were completed in a Thermolyne Amplitron II Thermal Cycler (Barnstead/ Thermolyne Corp., Dubuque, IA). Reactions consisted of 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1 % Triton X-100, 0.25 mM of each dNTP, 200 ng of each primer, 3 U of Deep Vent Polymerase, and 200 ng of template. Nucleotide sequencing and primer synthesis (Table 7) were performed by the Uniformed Services University Biomedical Instrumentation Center. To determine the number of CTCTT repeats present in the signal peptide coding region of "on" and "off" variants of ANS100, a 1.2 kB fragment containing the *opaB::phoA* fusion was PCR amplified from lysates prepared directly from primary colony isolates using the F3-fus and R3-fus primers. The amplification was completed by cycling 30 times at 94°C for 1 min, 58°C for 1 min, 72°C for 4 min, followed by a 4°C indefinite hold. The resultant PCR product was gel purified and sequenced directly with F1-*opaB* as the sequencing primer. The pCR-Blunt cloning system was purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's instructions. Plasmid DNA was prepared with the Qiagen Plasmid Midi Kit and QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Gel extractions of PCR products and restriction enzyme digests were performed with the QIAquick gel extraction kit (Qiagen). Chromosomal DNA was isolated via the cetyltrimethyl-ammonium bromide (CTAB) protocol (111).

Table 7. Oligonucleotides used in this study.

F1-opaB	5' GATTGCGGTATTGTCGGGAAT 3'
R1-opaB	5' GGTGCTTCATCACCTTAGGGAAC 3'
F2-phoA	5' GCTCTGCGTGATTCTCTTAGC 3'
R2-phoA	5' GTACGTTGCTTTCGGTCCTAG 3'
F3-fus	5' <u>GTCGACT</u> GCTTCATCACCTTAGGGAAC 3'
R3-fus	5' <u>GTCGACCGT</u> GATCTGCCATTAAGTCTG 3'
F4-lctP	5' GCGTCTTACCAAACGCTGTA 3'
R4-lctP	5' AAGAAAATCATTGCCGCGAC 3'

Underlined sequences indicate those primers where a *SalI* site was incorporated.

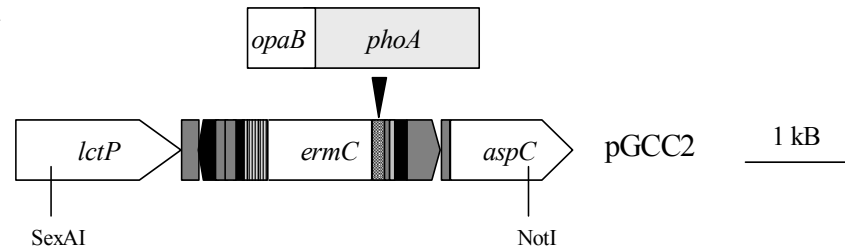
Strain construction The *opaB* structural gene of *N. gonorrhoeae* FA1090 and 170 bp of upstream DNA, which contains the -10 and -35 promoter elements (43), was PCR amplified using primers F1-*opaB* and R1-*opaB* and Deep Vent polymerase. PCR conditions were as described above, except the extension time was performed for 4 min. The resultant 1.1 kB fragment was ligated into the cloning vector pCR-Blunt and transformed into competent *E. coli* Top10 cells. Km^R transformants were screened via colony suspension immunoblot with rabbit anti-serum raised against the OpaB protein of strain FA1090 (Simms and Jerse, manuscript in preparation/ Chapter 2). One OpaB-positive clone was named pANS-1 and used to create the *opaB::phoA* fusion. A promoterless *phoA* gene that lacks the signal sequence region was obtained from plasmid pAM400 (kindly provided by Alison O'Brien), which is a derivative of the suicide vector pUT/mini-Tn5*phoA*Km^R (51), in which the Km^R gene within the *phoA* cassette was replaced with a chloramphenicol acetyltransferase gene (*cat*) (175). pAM400 was introduced into *E. coli* Top10 (pANS-1) via conjugation. Briefly, *E. coli* donor [*E. coli* S17-1 λ pir (pAM400)] and recipient [*E. coli* Top10 (pANS-1)] strains were grown overnight in static LB broth cultures with either chloramphenicol or kanamycin respectively. Overnight cultures were diluted 1:20 into fresh LB broth with antibiotics and grown statically for 3 hours. A total of 5×10^6 recipient and 2×10^6 donor cells were mixed and placed onto a nitrocellulose filter (Millipore, Billerica, MA; 0.22 μ m) on an LB agar plate. After four hours of incubation at 37°C, the bacteria were suspended in a small amount of LB broth. This suspension was serially diluted and cultured on LB agar containing kanamycin or chloramphenicol, or both antibiotics, and incubated at 37°C for 24 hours. All Km^R, Cm^R transconjugates were suspended together in LB broth with

antibiotics, grown to mid-log-phase, and the plasmids were extracted. The bulk plasmid prep was electroporated into *E. coli* DH5 α MCR. Plasmid DNA from a Km^R, Cm^R transformant that exhibited the blue colony color phenotype on media containing 5-bromo-4-chloro-3-indolyl phosphate (XP, 40 μ g/ml, Sigma Chemical Company) substrate was extracted and sequenced to determine the location of the fusion junction. The fusion was in-frame with the translational start codon of *opaB* and located around 100 bp downstream of the signal peptide-coding region (CTCTT repeat region).

The Neisserial Insertional Complementation System (NICS, a gift of H.S. Seifert, Northwestern University) was implemented to insert the *opaB::phoA* fusion into a non-essential locus of the genome of *N. gonorrhoeae* strain FA1090. NICS plasmid pGCC2 contains a fragment of the gonococcal chromosome in which a mini-transposon was inserted into an intragenic region of the chromosome between the *lctP* and *aspC* genes from which there is no detectable transcription (137). Within this transposon are an erythromycin resistance gene (*ermC*) and a multi-cloning site. The *opaB::phoA* fusion was amplified using primers F3-fus and R3-fus as described above. The amplified fusion was cloned into pCR-Blunt to create pANS-2. pANS-2 was digested with Sall to release the 1.8 kB fragment that contains the fusion. The 1.8 kB fragment was then ligated into the Sall site of pGCC2 to obtain pANS-3. pANS-3 was transformed into *E. coli* DH5 α via electroporation. Resultant Erm^R transformants were screened for blue colony color phenotype on LB agar supplemented with XP and via restriction enzyme digest of the plasmids with Sall. pANS-3 was digested with restriction enzymes SexAI and NotI to release the 6.1 kB fragment that contains the fusion (Figure 13A). Broth transformation of this fragment into *N. gonorrhoeae* FA1090 was performed as described (177) and

Figure 13. Schematic of the *opaB::phoA* fusion, as well as the sequence of “on” and “off” variants of strain ANS100. (A) The *opaB::phoA* fusion was inserted into a unique *Sall* site within the intergenic region between the *aspC* and *lctP* genes, creating strain ANS100. (B) Nucleotide sequence of the *opaB::phoA* fusion from colonies of both “on” (top) and “off” (bottom) variants of ANS100. Colonies examined by PCR were also passaged to GC media, and expression of the fusion was confirmed by XP colony lifts. The region containing the translational start site of the *opaB* gene and fusion junction with *phoA* is shown. The predicted amino acid sequence is indicated above each DNA sequence. The *phoA* sequence, including the 5’ end of the original mini-tn5*phoA* transposon, is underlined. The arrow indicates the beginning of the mature alkaline phosphatase protein.

A



B

In frame: 12 repeats

M N P A R K K P S L L F S S L L F
 ATG AAT CCA GCC CGC AAA AAA CCT TCT CTT CTC TTC TCT TCT CTT CTC TTC
 S S L L F S S L L F S S A A Q A A
 TCT TCT CTT CTC TTC TCT TCT CTT CTC TTC TCT TCG GCA GCG CAG GCG GCA
 S E G N G R G P Y V Q A D L A Y
 AGT GAA GGC AAT GGC CGC GGC CCG TAT GTG CAG GCG GAT TTA GCC TAC
 A A E R I T H D Y P E P D S Y T
 GCC GCC GAA CGC ATT ACC CAC GAT TAT CCG GAA CCT GAC TCT TAT ACA
 Q V A S W T E P F P F C P V L E
CAA GTA GCG TCC TGG ACG GAA CCT TTC CCG TTT TGC CCT GTT CTG GAA

Out of frame: 11 repeats

M N P A R K K P S L L F S S L L F
 ATG AAT CCA GCC CGC AAA AAA CCT TCT CTT CTC TTC TCT TCT CTT CTC TTC
 S S L L F S S L L F G S A G G K STOP
 TCT TCT CTT CTC TTC TCT TCT CTT CTC TTC GGC AGC GCA GGC GGC AAG TGA

 AGG CAA TGG CCG CGG CCC GTA TGT GCA GGC GGA TTT AGC CTA CGC CGC

 CGA ACG CAT TAC CCA CGA TTA TCC GGA ACC TGA CTC TTA TAC ACA AGT

AGC GTC CTG GAC GGA ACC TTT CCC GTT TTG CCC TGT TCT GGA A

transformants were selected on GCB agar containing erythromycin. The desired strain was identified by the presence of blue colonies on GCB agar containing XP, and by PCR with primers F2-phoA and R2-phoA to look for the predicted 750 bp fragment. One colony, which met the above criteria, was named ANS100 and chosen for further analysis.

Southern blot analysis Chromosomal and plasmid DNA were digested to completion with SmlI and probed by Southern blot analysis with digoxigenin-labeled (DIG) probes that correspond to unique sequences within the *lctP* and *phoA* genes. Probes were created and labeled by the PCR DIG Probe Synthesis Kit and the use of primers F4-lctP and R4-lctP, and primers F2-phoA and R2-phoA, as described by the manufacturer (Roche Applied Science, Indianapolis, IN). Prehybridization and hybridization steps were performed at 68°C in DIG Easy Hyb buffer (Roche Applied Science). The DIG Luminescent Detection Kit (Roche Applied Science) was used for detection of DIG-labeled bands.

Colony Suspension Immunoblots Colony suspension immunoblots were performed as previously described (102). Briefly, suspensions of individual colonies from *E.coli* DH5 α MCR carrying either pANS-1 or pANS-2 were spotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH), air-dried, and probed with a polyclonal rabbit serum specific for both the OpaB and OpaD proteins of *N. gonorrhoeae* strain FA1090 (RbB/D; Simms and Jerse, in preparation, Chapter 2). Bound antibody was detected by probing with goat anti-rabbit IgG bound to horseradish peroxidase (Sigma

Chemical Company), followed by enhanced chemiluminescence substrate (Amersham Biosciences, Piscataway, NJ).

SDS-PAGE and Western blotting Bacteria were harvested from one in vitro passage on solid GCB or LB agar plates with the appropriate antibiotic selection, suspended in 2x Laemmli loading buffer (Sigma Chemical Company) and boiled at 100°C for 10 minutes. Purified bacterial alkaline phosphatase (250 U, Invitrogen) was also suspended in 2x Laemmli loading buffer and boiled as above. Samples were run on 11% SDS-PAGE gels as described (102). Separated proteins were transferred electrophoretically to PVDF membranes (Biorad) and probed with anti-alkaline phosphatase (*E. coli*) polyclonal rabbit serum (1:50,000, Rockland Immunochemicals, Gilbertsville, PA). Bound antibody was detected by probing with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:50,000, Sigma Chemical Company), followed by enhanced chemiluminescence substrate (Amersham Biosciences).

XP Colony lift analysis Due to the sensitivity of *N. gonorrhoeae* to the XP substrate (31), a colony lift assay was used to examine the frequency of “on” (blue) and “off” (white) variants of ANS100. Gonococci were lifted from GCB agar plates onto nitrocellulose membranes (VWR, West Chester, PA). Membranes were then placed on filter paper that had been soaked in XP substrate (40 µg/ml) and the reaction was allowed to proceed for 1 hour at room temperature. Membranes were air-dried at room temperature and the numbers of blue and white colonies were counted. Between 1000-1500 colonies were counted for each time point and condition tested. The frequency of

phase variation was calculated by dividing the number of blue or white colonies by the total number of colonies present. The rate of phase variation was calculated by dividing the frequency of phase variation by the number of bacterial generations.

In vitro culture conditions for examining *opa* gene phase variation rate The frequency and rate of phase variation under standard growth conditions was determined as follows. Briefly, non-piliated, predominantly “on” or “off” variants of strain ANS100 were grown to late-log phase for 14-16 hours in supplemented GC broth (GC broth with Kellogg’s supplement I (113), 10 μ M ferric nitrate, and 5 mM sodium bicarbonate) as described (104). 50 ml of supplemented GC broth was inoculated with gonococci to a concentration of 10^7 CFU/ ml and incubated at 37°C with aeration. The absorbency of each culture at 600 nm was measured over time, and samples that corresponded to the early, mid-, and late-logarithmic phase were serially diluted and cultured on GCB agar.

The effects of iron limitation, pH, and the presence of the gonadal hormone progesterone on the frequency and rate of *opaB::phoA* phase variation were also examined using liquid culture conditions. For all test conditions, non-piliated, predominantly “on” or “off” variants of strain ANS100 were grown to late-log-phase as described above and used as starter cultures as follows. Aliquots of these starter cultures were centrifuged briefly and resuspended in fresh GC broth without supplements. The A_{600} of the bacterial suspension was determined to estimate the bacterial concentration and 10^6 CFU of the resuspended bacteria were inoculated into 50 ml of GC broth as follows. For pH experiments, supplemented GC broth of pH 5.8, 6.5, and 7.2 was tested. The pH of each broth was adjusted using 1M HCl. For the iron limitation experiments, 2

50 ml aliquots of GC broth with Kellogg's supplement I (113) and 5 mM sodium bicarbonate were added to both the iron-repleted and iron-depleted cultures. Ferric nitrate (10 μ M) was also present in the iron-repleted culture. The chelator deferoxamine mesylate (DFM, 150 μ M, Sigma Chemical Company) was added to the iron-depleted culture after one generation of growth to ensure limitation of iron availability. The effect of progesterone was examined in supplemented GC broth containing 50 μ l of a 10 mg/ml working stock of progesterone, which had been dissolved in DMSO (Sigma Chemical Company, 10 μ g/ml). GC broth containing 50 μ l of DMSO without progesterone was inoculated for comparison. For all test conditions, cultures were incubated with aeration and only late log phase cultures were sampled to assess the rate of *opaB::phoA* phase variation, as described above. XP lifts on colonies isolated from the test conditions were performed after 24 hours of growth as described above.

N. gonorrhoeae can grow anaerobically if nitrite is provided as a terminal electron acceptor (117). The effect of low oxygen concentration on *opaB::phoA* phase variation was examined using solid agar conditions. Briefly, GC broth suspensions containing 10^7 CFU/ml of non-piliated, predominantly “on” or “off” variants of strain ANS100 were prepared, and sterile cotton swabs soaked in this suspension were spread over the surface of GCB agar plates. Sterile disks soaked in 20% (w/v) sodium nitrite (Sigma Chemical Company) were added to the middle of each plate (95). A disk soaked in sterile water was used as a negative control. Plates were incubated in an anaerobic jar with an AnaeroGen paper sachet (Oxoid Inc., Ogdensburg, NY) at 37°C for 24 hours. Aerobic cultures were also performed for comparison by serially diluting and plating the initial gonococcal suspension on GCB agar followed by incubation at 37°C in 5% CO₂ for 16

hours to allow only slight growth. Gonococci grown both anaerobically and aerobically were suspended in GC broth, serially diluted, and plated onto GCB agar, followed by incubation at 37°C, 5% CO₂. After 24 hours of incubation, XP colony lifts were performed as described above to detect the frequency of *opaB::phoA* phase variation.

Effect of natural transformation on *opa* gene phase variation The effect of excess extracellular DNA on *opaB::phoA* phase variation was performed via broth transformation as described (4). Briefly, predominantly “on” or “off” variants of strain ANS100 that expressed pili, which is needed for natural competence (16, 169, 177, 220), were suspended in GC broth with 10 mM MgCl₂ to a concentration of 4 x 10⁷ CFU/ ml. One microgram of chromosomal DNA from *N. gonorrhoeae* FA1090, *N. cinerea* RF4738, or *N. lactamica* F6865 suspended in 100 µl of 1X SSC (sodium chloride-sodium citrate solution, Roche Applied Science) was added to 900 µl of the ANS100 suspension and incubated at 37°C. Bacterial suspensions to which no DNA was added were also tested. After 30 minutes of incubation, each suspension was added to 4 ml of pre-warmed supplemented GC broth containing sodium bicarbonate and incubated for five hours at 37°C with aeration. After this incubation, the suspensions were serially diluted, cultured on GCB agar, and XP lifts were performed as described above to detect the frequency of *opaB::phoA* phase variation.

Experimental Murine Genital Tract Infection Female BALB/c mice (6-8 weeks old, National Cancer Institute) were treated with 17β-estradiol and antibiotics as described to induce susceptibility to gonococcal infection (101). Two days after initiation of estrogen

treatment, mice were inoculated intravaginally with a suspension of non-piliated ANS100, consisting primarily of either “on” or “off” variants in sterile PBS. Isolated growth from agar plates was suspended in sterile PBS and filtered through 1.2 μm filters to remove clumped gonococci as described (101). Filtered suspensions were adjusted to an A_{600} of 0.08 and mice were inoculated intravaginally with 20 μl of suspension (10^6 gonococci per mouse). The inoculum was serially diluted and cultured on GCB agar to determine the dose. The frequency of “on” and “off” variants present in the inoculum and among vaginal isolates collected on days 1, 3, 5, 7, and 9 post-inoculation was determined by XP colony lifts as described above. Vaginal mucus was collected daily and quantitatively cultured for *N. gonorrhoeae* as described (101, 103, 104). GC-VCNTS plates were examined after 20-24 hours of incubation and the number of CFU recovered was determined. Animal experiments were conducted in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol that was approved by the University’s Institutional Animal Care and Use Committee.

Results

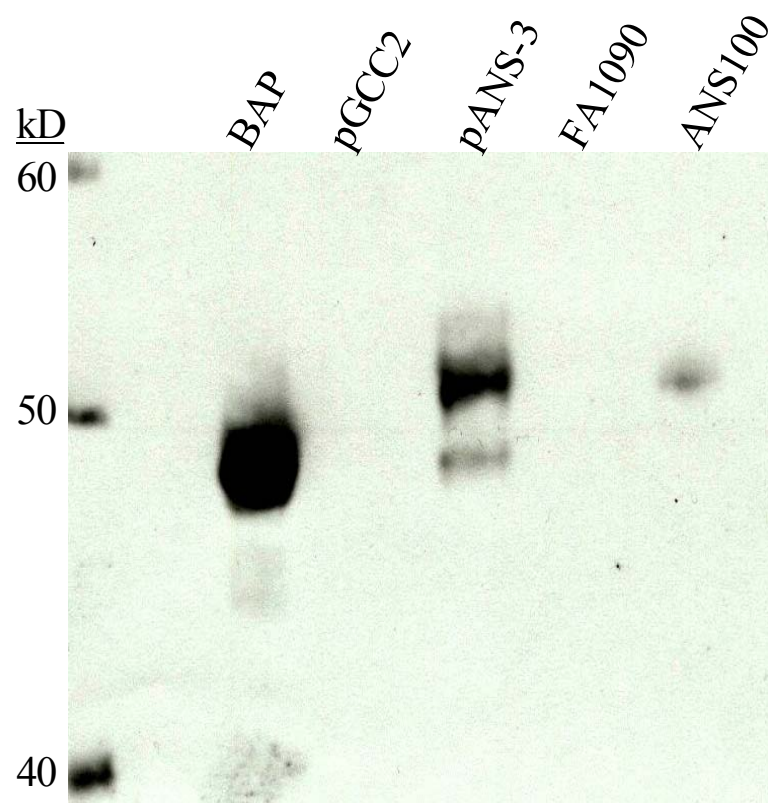
Construction and characterization of an *opaB::phoA* fusion in *N. gonorrhoeae*

FA1090. An *opaB::phoA* fusion was created and inserted into a non-essential locus of the genome of *N. gonorrhoeae* strain FA1090 as described in Materials and Methods (Figure 13A). This resultant recombinant strain was named ANS100. “On” and “off” variants of ANS100 were isolated via XP colony lift analysis and sequenced to determine the number of CTCTT repeats present in the signal peptide-coding region. “On” variants of ANS100 contained 12 CTCTT repeats, which is consistent with an in-frame configuration. “Off” variants of this fusion strain contained 11 CTCTT repeats, which results in a premature stop codon before the fusion junction (Figure 13B).

To determine if the *opaB::phoA* fusion in strain ANS100 was able to undergo phase variation, Western blot analysis with anti-alkaline phosphatase antibody was performed (Figure 14). Whole cell lysates from *E. coli* DH5 α carrying the pGCC2 NICS vector and from *N. gonorrhoeae* FA1090 did not contain any proteins that reacted with the antibody. A protein that bound to the anti-alkaline phosphatase antibody was detected in lysates of *E. coli* DH5 α containing the pANS-3 plasmid and from *N. gonorrhoeae* strain ANS100. The apparent molecular weight of the *opaB::phoA* fusion protein in both pANS-3 and strain ANS100 was ca. 53 kD (predicted size: 54 kD); the molecular weight of the bacterial alkaline phosphatase control was ca. 49.5 kD in size (predicted size: 50 kD).

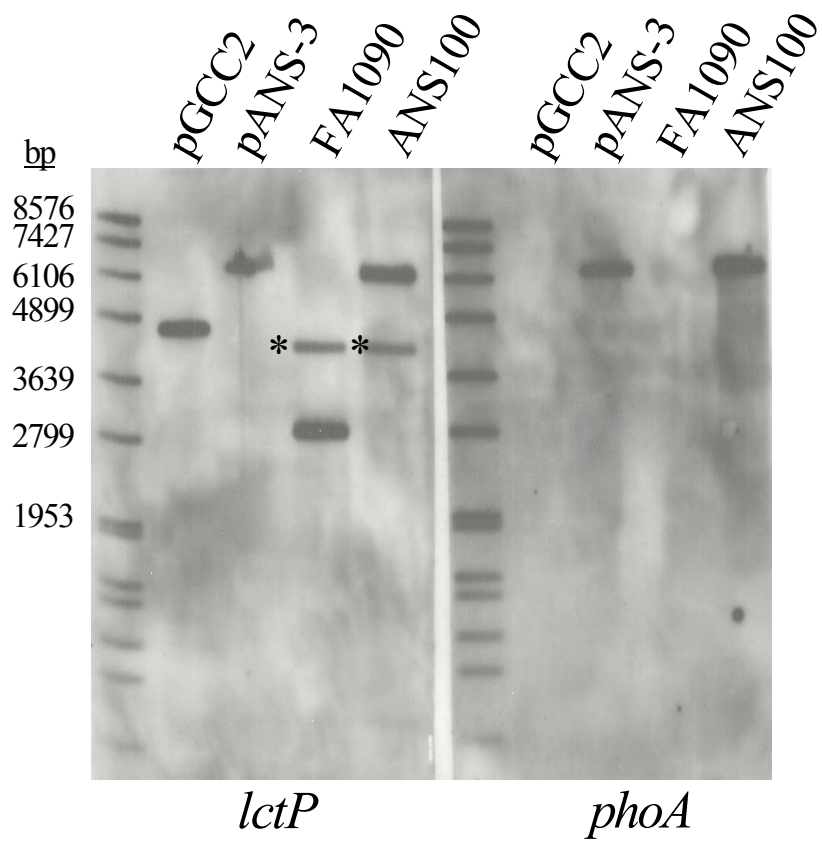
Southern blot analysis was performed to confirm integration of the *opaB::phoA* fusion into the gonococcal chromosome between the *lctP* and *aspC* alleles. SmlI cuts both *aspC* and *lctP*. An analysis of the genome sequence of strain FA1090 indicated that

Figure 14. The *opaB::phoA* fusion undergoes phase variation. Western blot analysis of whole cell lysates of bacterial alkaline phosphatase (BAP), *E. coli* DH5 α MCR carrying either the pGCC2 vector or pANS-3, *N. gonorrhoeae* strain FA1090, and *N. gonorrhoeae* strain ANS100 probed with an anti-alkaline phosphatase antibody is shown. The predicted sizes of the bacterial alkaline phosphatase and the *opaB::phoA* fusion protein are 50 and 54 kD, respectively. Measurement of the migration of the anti-alkaline phosphatase reactive bands relative to the molecular weight standards (MagicMark Western Protein Standard, Invitrogen) indicates that the bacterial alkaline phosphatase is ca. 49.5 kD and the *opaB::phoA* fusion protein in both pANS-3 and strain ANS100 is ca. 53 kD.



no SmlI sites were present in the intergenic region between *aspC* and *lctP*, which we confirmed by restriction enzyme digest (data not shown). Plasmids pGCC2 and pANS-3 and chromosomal DNA from *N. gonorrhoeae* strain FA1090 and ANS100 were digested to completion with restriction enzyme SmlI and hybridized with oligonucleotide probes for the *lctP* and *phoA* genes as described in Materials and Methods. The *phoA* gene was only detected in those samples in which the fusion was present (pANS-3 and ANS100) (Figure 15). The size of the *phoA* band in these two digests was identical in size (ca. 6.3 kB). Hybridization with the *lctP* probe indicated that recombination of the *opaB::phoA* fusion occurred in the desired region of the genome (Figure 15). In the pGCC2 digest, an *lctP*-reactive band of around 4.5 kB was present. A shift to 6.3 kB was seen in the *lctP*-reactive band in the pANS-3 digest as predicted by insertion of the 1.8 kB *opaB::phoA* fusion into the MCS of pGCC2. An identical band was seen in the digest of strain ANS100, indicating that the *opaB::phoA* fusion present in this strain was located in the expected region. Finally, a 2.8 kB band was seen in the digest of strain FA1090, as predicted by analysis of the FA1090 genome sequence. Interestingly, an unexpected *lctP*-reactive band was present between 3.6 and 4.9 kB in both gonococcal digests. Hybridization of these digests with a probe to the gonococcal *kat* gene showed a single band, which based on the restriction enzyme map of the gonococcal *kat* gene, indicated that this band was not the product of an incomplete digest. Furthermore, hybridization of an SmlI digest of *E. coli* DH5 α with the *lctP* probe indicated that this band is not *lctP*-specific, but appears to have been produced during probe synthesis by amplification of a gene present in *E. coli* DH5 α MCR, which is also present in *N. gonorrhoeae* strain FA1090 (data not shown).

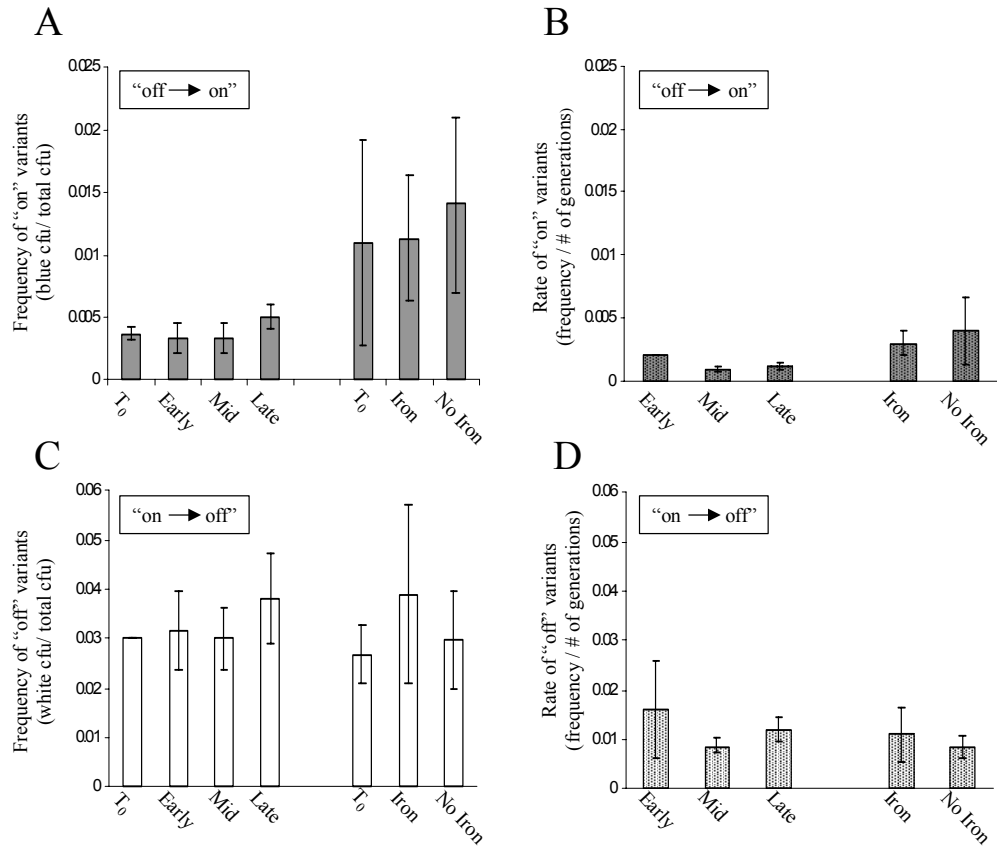
Figure 15. Location of the SexAI/NotI fragment containing the *opaB::phoA* fusion in the gonococcal chromosome of strain ANS100. Five nanograms of SmlI digests of pGCC2 and pANS-3, as well as five micrograms of SmlI-digested chromosomal DNA from *N. gonorrhoeae* strain FA1090 and strain ANS100 were electrophoresed on a 0.7% agarose gel, followed by transfer to Nytran membrane and Southern blot analysis with probes to the *lctP* (left panel) and *phoA* (right panel) genes. The apparent molecular weights of the *lctP*-reactive bands were as follows: pGCC2: 4.3 kB; pANS-3 and ANS100: 6.1 kB; and FA1090: 2.8 kB. The *phoA*-reactive bands in the pANS-3 and ANS100 digests were ca. 6.1 kB. The asterisks indicate an unexpected *lctP*-reactive band present in both gonococcal digests as described in the text.



The frequency and rate of *opaB::phoA* phase variation in vitro. Strain ANS100 was constructed to allow us to examine the rate of *opa* gene phase variation at the level of a single copy in the gonococcal chromosome. The rate of *opaB::phoA* phase variation under standard broth conditions was determined to be approximately 10^{-3} per cell per generation, which confirms earlier results with cloned *opa* genes in *E. coli* (11, 146). There was no significant difference in the growth rate of strain ANS100 in vitro as compared to the wild-type strain FA1090 (data not shown), and the frequency and rate of *opaB::phoA* phase variation during standard growth conditions did not significantly vary between growth phases, regardless of whether ANS100 “on” or “off” variants were used to inoculate the cultures (Figure 16). Finally, there was no significant difference in the phase variation frequency or rate between the “on to off” or “off to on” cultures of strain ANS100.

Transcriptional factors, not yet defined, may contribute to Opa protein expression during infection as suggested by evidence that differences in promoter strength correlate with rates of *opa* gene phase variation (10). It is therefore conceivable that stimuli within the host may drive phase variation of certain *opa* genes rather than *opa* genes being turned on or off randomly. The significance of this is that induction of *opa* gene phase variation by host stimuli may contribute to Opa protein expression in vivo. Host factors that have been hypothesized to play a role in Opa protein expression include iron concentration (176), pH variation (119), the gonadal hormone progesterone (99, 100, 170), and oxygen tension (64, 163). Accordingly, in order to examine the effects of these various conditions on *opa* gene phase variation frequency, predominantly “on” or “off” variants of ANS100 were cultured under different conditions. The frequency or

Figure 16. In vitro analysis of phase variation of strain ANS100 during standard and iron limited conditions. The frequency (A) and rate (B) of “on” variants recovered after inoculation of GC broth with predominantly “off” variants of ANS100. The frequency (C) and rate (D) of “off” variants recovered after inoculation of GC broth with predominantly “on” variants of ANS100. Early-, mid-, and late-log-phase gonococci cultured during standard GC conditions were analyzed. Late-log-phase gonococci were cultured from iron-replete and iron-deplete cultures. Data shown are representative of three separate experiments. The graphs illustrate the average frequency or rate with the standard deviation indicated.



rate of ANS100 phase variants did not change when grown in GC broth at pH values that correspond to that of endocervical mucous at different stages of the menstrual cycle (5.8, 6.5, and 7.2) (119, 182). Similarly, the frequency of ANS100 phase variants also did not change when grown on solid agar plates under lower oxygen tensions when compared to the standard aerobically grown controls (data not shown). Iron is limited on mucosal surfaces (212) and the phase variation frequency or rate in gonococci cultured in the absence of iron also did not differ compared to that of gonococci grown in iron-replete broth (Figure 16). No change was seen in the phase variation frequency or rate of the *opaB::phoA* fusion in the presence of the hormone progesterone (data not shown).

A major source of genetic diversity in the *Neisseriae* stems from the natural competence of these organisms. This process is the principal mode of DNA exchange in *Neisseria* species (188) and efficient natural transformation is reliant on expression of pili (16, 169) and the presence of a 10 bp uptake sequence in donor neisserial DNA (61, 68). Recently, it was shown that the presence of excess extracellular DNA increases the phase variation rate of the *hmbR* gene of *N. meningitidis* in a transformation-dependent manner (4). Similar to the *opa* genes, the *hmbR* gene also phase varies by means of SSM (167). Therefore, experiments were designed to investigate whether DNA from commensal *Neisseria* species of the female lower genital tract influenced phase variation of the *opaB::phoA* fusion. Piliated “on” or “off” variants of strain ANS100 were incubated with excess amounts of neisserial DNA as described in Materials and Methods. No change was seen in the frequency of phase variants or the rate when genomic DNA from wild type strain FA1090 and two commensal *Neisseria* strains, *N. lactamica* and *N. cinerea*, was added to the culture (data not shown).

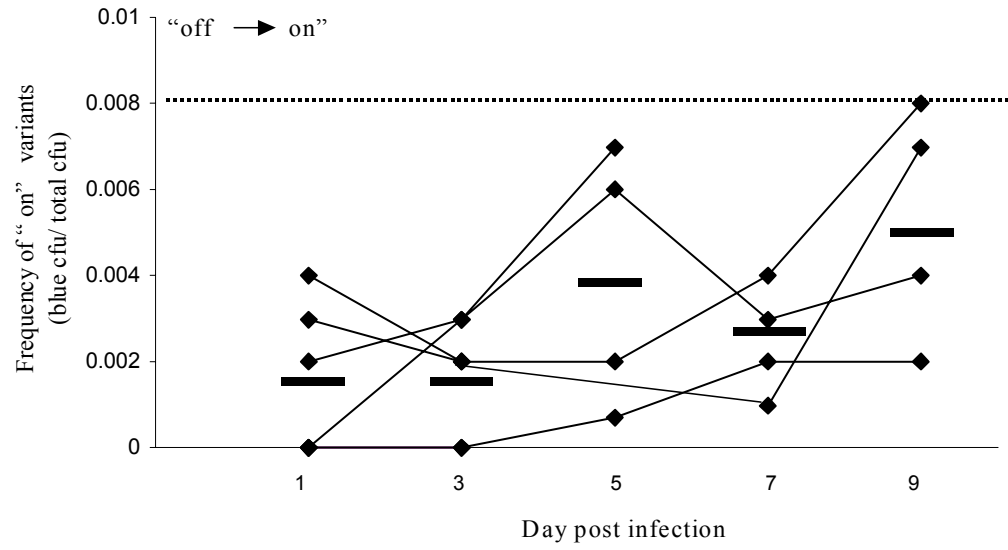
The frequency of *opaB::phoA* phase variation during murine genital tract infection.

Several lines of evidence suggest Opa phenotype may be important in gonococcal infection and that anatomical site, gender differences, and hormonal state may influence Opa protein expression (55, 99, 100). As described in experimental infection of male volunteers (102, 194) and female mice (101), selection of Opa-positive gonococci appears to occur in the genital tract following inoculation with a predominantly Opa-negative population; however, induction of *opa* gene phase variation may also contribute to the recovery of primarily Opa-positive variants. In order to examine if induction of *opa* gene phase variation by host stimuli contributes to Opa protein expression in vivo, estradiol-treated mice were inoculated with suspensions of nonpilulated strain ANS100 that consisted predominantly of *opaB::phoA* “off” variants. The frequency of *opaB::phoA* “off” variants in murine vaginal isolates recovered was determined. In two separate experiments, 6 mice (3 per experiment) were infected with a population of predominantly “off” variants of strain ANS100 (frequency of “on” variant: 0.008). The mean duration of recovery of gonococci in these two experiments was 9 days (range: 8-9 days). Based on XP colony lift analysis, no significant increase in the frequency of recovery of “on” variants of strain ANS100 was seen over the course of infection in any mouse, with “on” frequency values ranging from <0.001 to 0.008 (Figure 17A).

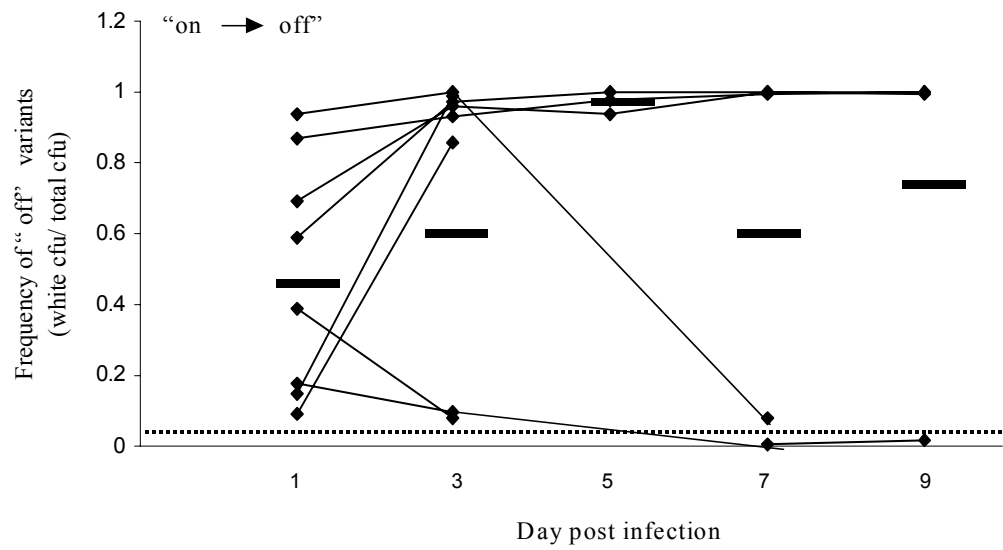
The reverse experiment was performed twice, in which a total of 8 mice (3 and 5 mice per experiment) were infected with primarily ANS100 “on” variants. The frequency of “on” variants in both inocula was 0.98 or 980 colonies out of 1000. The mean duration of recovery of gonococci in these two experiments was 7.7 days (range: 3-9

Figure 17. Frequency of recovery of “on” or “off” variants of strain ANS100 during murine lower genital tract infection, following inoculation of mice with predominantly “off” or “on” variants. Black diamonds represent the frequency of (A) “on” variants recovered and (B) “off” variants recovered from each mouse during experimental infection. The frequency of “on” variants in the predominantly “off” inocula (A) was 0.008, while the frequency of “off” variants in the predominantly “on” inocula (B) was 0.02, as shown by the dotted lines on the graphs. The data shown in each graph are cumulative of two separate experiments. For all experiments, between 1000 and 1500 CFU were counted to determine the frequency for each data point. Thick horizontal bars indicate the average frequency of ANS100 phase variants recovered for that particular day.

A



B



days). Interestingly, in the majority of the mice infected (6 of 8), large increases (25-50 fold) were seen in the recovery of “off” variants, as based on XP colony lift analysis. The mean frequency of “off” variants recovered in these two experiments was considerably increased on each day examined as compared to the inocula levels, ranging from 0.49 to 0.97 (Figure 17B).

Discussion

In this study, we constructed a chromosomally-encoded translational *phoA* fusion in the *opaB* gene of *N. gonorrhoeae* strain FA1090. Two identical copies of the *opaB* gene are present in the genome of *N. gonorrhoeae* strain FA1090 (44, 54). The design of the fusion was such that changes in *opa* gene phase variation could occur independently of any selection for or against Opa protein function by introducing the *opaB::phoA* fusion into a non-essential locus of the genome. In this way, neither *opaB* gene was disrupted. The *opaB::phoA* fusion exhibited phase variation, as evidenced by phase variable alkaline phosphatase activity. The rate of *opaB::phoA* phase variation occurred at a rate similar to that which was reported previously for cloned *opa* genes in *E. coli* (11, 146). No environmental condition was identified that altered the phase variation rate of the *opaB::phoA* fusion.

Historically, the focus on *opa* gene expression has been on phase variation and not transcriptional regulation. However, it was recently reported that the gonococcal Fur protein regulates the *opa* genes of strain FA1090 (176). Using electrophoretic mobility shift assays, these authors showed that the gonococcal Fur protein bound to promoter fragments from all of the *opa* genes. A putative Fur binding sequence of 21 bp, with

61% identity to the gonococcal Fur consensus binding sequence, was identified 6 bp downstream of the -10 promoter region of the *opa* genes (Figure 18). It was not determined if this putative regulation of the *opa* genes by Fur actually occurs and if the outcome is positive or negative. In our studies with strain ANS100, the phase variation rate of the fusion did not change following chelation of iron in the growth media. Therefore, it appears unlikely that Fur-mediated regulation of the *opaB* gene in strain FA1090 contributes to phase variation of the OpaB protein.

In both in vitro and in vivo experiments with strain ANS100, we observed that the frequency and, or rate of “on to off” *opaB::phoA* phase variation was higher than the “off to on”. The difference in vivo was more dramatic than that observed in vitro. The frequency of phase variants did not change significantly when mice were infected with primarily “off” variants of strain ANS100. Interestingly, when mice were infected with predominantly “on” variants of strain ANS100, the majority of isolates recovered were *opaB::phoA* “off”. One explanation for the apparent preference for “off” variants concerns the number of CTCTT repeats present in the “off” and “on” conformations. As determined by nucleotide sequencing, the “on” conformation of the *opaB::phoA* fusion contained 12 repeats. The loss or gain of one repeat during DNA replication would lead to an “off” conformation. In contrast, the chance of recovering an “on” variant from an “off” variant is lower, with only the addition of one repeat to the 11 repeat “off” conformation leading to an “on” variant. The loss of one repeat from this 11 repeat region would not cause an in-frame conformation. A similar scenario would occur for “off” fusions that contain 13 repeats; the addition of one repeat would lead to an “off” conformation, whereas loss of one repeat leads to an “on” variant. Therefore, consistent

Figure 18. ClustalW multiple-sequence alignment of the promoter regions of the *opa* genes of *N. gonorrhoeae* strain FA1090. The –35 region (10, 43) in all of the *opa* gene promoters of strain FA1090 are indicated by blue boxes. The –10 region (10, 43) in the promoters of the *opaA*, *opaB1*, *opaB2*, *opaC*, *opaD*, *opaF*, and *opaK* genes is surrounded by a green box. Both the *opaE* and *opaI* genes lack a discernable –35 region. The sequence of the gonococcal consensus Fur box (65) is indicated in red, and aligned with the corresponding putative Fur binding sequence in the *opa* genes, as determined by Sebastian et al (176). Nucleotides in the *opa* gene that are identical to the consensus Fur box are also red.

with what we observed in vitro, the rate of “on to off” phase variation in ANS100 is predicted to occur twice as frequently than “off to on”. The dramatic change in frequency of “on” to “off” variants in mice suggests other factors may be involved in vivo. One possible explanation for the marked loss of *opaB::phoA* expression in vivo is that expression of the *phoA* gene product is selected against in the mouse genital tract. The *opaB::phoA* fusion junction is present in the first surface-exposed loop of the Opa protein, thus it is likely that the fusion protein is expressed on the outside of the bacterium. Another possibility is that increased transcription of *opaB* occurs in vivo, leading to increased numbers of PhoA molecules in the membrane, which might be detrimental to survival of the fusion strain. Due to the limitations that may be inherent to our reporter system, we choose not to make strong conclusions about “on to off” phase variation of ANS100 in mice at this time.

N. gonorrhoeae strain MS11 preferentially expresses certain Opa variants in vitro and also during experimental infection of 3 male volunteers (194). Belland et al (10) suggested that this observation might be due to the promoter strength of these specific variants with reasoning that relaxation of strands during transcription may increase frequency of triple helix formation, which is the basis of SSM. To test this hypothesis, these investigators constructed transcriptional *lacZ* and translational *phoA* gene fusions with the *opa* genes that were frequently expressed by strain MS11 (*opaA*, *opaB*, and *opaC* genes). The fusions were expressed on single-copy plasmids in *E. coli*. A correlation was seen between the promoter strength of the *opa* genes as measured by LacZ activity and their corresponding phase variation rates as measured by PhoA expression from the translational fusions. Expression of the *opaA::lacZ* fusion was the

highest followed by that of the *opaB::lacZ* and *opaC::lacZ* fusions. The phase variation rate of the *opaA::phoA* translational fusion was also the highest followed again by the *opaB::phoA* and *opaC::phoA* fusions. The authors concluded that the promoter strengths of the *opaA* and *opaC* genes in *N. gonorrhoeae* strain MS11 were responsible for the preferential recovery of those variants in both in vitro and in vivo passage.

In contrast to the volunteer study with strain MS11, volunteer experiments using strain FA1090 did not show a preferential recovery of one Opa type or small subset of Opa types from all subjects. It is important to note here that the nomenclature of the Opa proteins from strain MS11 is not the same as that for the Opa proteins from strain FA1090. Six of 8 different Opa proteins were highly represented in urethral isolates taken from the 9 men who became infected, and the predominant Opa proteins expressed varied between patients (102). Interestingly, sequence analysis of the *opa* promoters of strain FA1090 indicates that the majority of the *opa* genes share the same promoter sequence (Figure 18) (176). The *opaB* promoter sequence is identical to that of the *opaA*, *opaC*, *opaD*, *opaK*, and *opaF* genes of strain FA1090. Both copies of the FA1090 *opaB* gene share the same promoter sequence as the promoter sequence of the *opaB* gene of strain MS11, which was previously shown to be of intermediate strength (10). Also, the *opaI* and *opaE* genes of strain FA1090 share the same promoter sequence as the *opaC* gene of MS11. Belland et al showed that this MS11 *opaC* promoter had the least amount of transcription in vitro (10). This result is interesting in light of the fact that FA1090 OpaI variants are frequently recovered from both the male urethra and the genital tract of female mice (101, 102). Finally, the *opaH* promoter of strain FA1090 is identical to that of the MS11 *opaA* promoter sequence. The *opaH* and *opaF* structural genes in strain

FA1090 are identical and, therefore, are duplicate alleles (44, 54). Variants that reacted with mAbs specific to the HV₂ loop of OpaF and OpaH were frequently recovered from the male urethra during experimental infection (102). However, in similar experiments, we did not often recover OpaF/H variants from the genital tract of female mice (101). In summary, the correlation between predicted promoter strength, based on homology to known promoters in strain MS11, and expression in vivo does not appear to hold true in strain FA1090.

In conclusion, *opa* gene phase variation in response to the environmental conditions examined in vitro appears to be spontaneous and random. In our mouse infection experiments, no change in the frequency of phase variation from “off to on” was detected. However, in experiments examining the frequency of phase variation from “on to off”, the dramatic recovery of “off” variants is suggestive of increased SSM due to in vivo conditions that we have not yet been able to mimic in vitro. However, further studies are needed to determine the possible effect(s) of the particular reporter gene used.

Chapter 4: Discussion

Summary

The major results of this dissertation research address the specific aims of the project as defined in the introduction. These findings were as follows. First, a Cm^R strain of *N. gonorrhoeae* FA1090 was constructed to determine whether selection or induction of Opa protein expression occurred early in experimental murine infection. Using mixtures of defined Opa variants of FA1090Cm^R and wild type FA1090, it was determined that selection of a pre-existing population of Opa-positive gonococci occurred within 3 days following infection of the lower genital tract of mice, rather than induction of Opa protein expression in the Opa-negative population. Second, a translational *opaB::phoA* fusion was constructed and inserted into a non-essential locus of the genome of *N. gonorrhoeae* strain FA1090 to create strain ANS100. Consistent with the previous estimates by others, based on changes in colony photo-opacity and ectopic expression of *opa* genes in *E. coli* (11, 131, 146), the rate of *opa* gene phase variation in this *N. gonorrhoeae* background was determined to be $\sim 10^{-3}$ / cell/ generation during standard growth conditions. *opa* gene phase variation was also examined under conditions designed to mimic the host environment. No conditions that affected the frequency or rate of *opa* gene phase variation were identified. A slightly faster rate from “on” to “off” occurred in these experiments, which might be explained by the increased likelihood of recovering an “off” variant from an “on” variant following a SSM event. Infection of mice with primarily “off” variants of ANS100 did not reveal evidence of increased *opa* gene phase variation in vivo. In similar experiments using “on” variants

of ANS100, high numbers of “off” variants were recovered. Conclusions concerning these events may be premature due to concerns about the particular reporter gene used. However, these data are intriguing in that they suggest that in the absence of selection, *opa* genes are turned off. An additional discovery in this thesis was the identification of three distinct phases of Opa protein expression during long-term colonization of female mice. The pattern of Opa protein expression over 9 days suggested that the early selection event is followed by a cyclic pattern of Opa protein expression, in which Opa-negative variants were primarily recovered at the mid-phase of infection, followed by a re-emergence of Opa-positive gonococci in the late phase.

Opa-positive gonococci appear to have a survival advantage early in infection.

Our results show that Opa-positive gonococci are preferentially recovered after infection of mice with mixtures of Opa-negative and Opa-positive variants. This result was observed when either OpaI or OpaB variants were used to spike a mostly Opa-negative inoculum. The Opa repertoire of *N. gonorrhoeae* strain FA1090 contains 11 genes, which encode 8 different Opa proteins (OpaA, OpaB, OpaC, OpaD, OpaE, OpaF, OpaK, and OpaI) (44). As described earlier, two classes of cellular Opa protein receptors have been identified, HSPG and human CEACAM molecules (21, 35, 38, 72, 205, 208).

The Opa protein receptor repertoire of *N. gonorrhoeae* strain MS11 has been well studied. Only the OpaA protein is able to utilize HSPG as a receptor for attachment and invasion (35, 205). The other Opa proteins of this strain bind to different CEACAM molecules and some of these Opa proteins mediate invasion of epithelial cells and neutrophils via these receptors (21, 37, 38, 72). Unfortunately, we cannot easily use

strain MS11 for Opa work, due to the fact that antibodies specific to the different Opa variants of this strain are not available. As described previously, the receptor repertoire of the Opa proteins of strain FA1090 was also analyzed (N. B. Guyer, M. C. Piriou, M. M. Hobbs, and J. G. Cannon, Abstr. 101st Gen. Meet. Amer. Soc. Microbiol., abstr. D-135, 2001) and OpaI-expressing gonococci were the only FA1090 Opa variants that bound HSPG. Binding of OpaI variants to HSPG molecules in the human male urethra and the lower genital tract of mice (85) may explain why OpaI variants were often recovered from both the male urethra and the genital tract of female mice.

One caveat of the receptor identification of *N. gonorrhoeae* strain FA1090 is that this report has not been peer-reviewed at this time. Therefore, the receptor repertoire of *N. gonorrhoeae* strain FA1090 needs to be further elucidated. Infection of wild-type Chinese hamster ovary (CHO) cells and isogenic mutants with deficiencies in HSPG biosynthesis was used to identify the HSPG-binding Opa protein of *N. gonorrhoeae* strain MS11 (35, 205). Therefore, infection of these cells with defined Opa variants of strain FA1090 might allow us to clearly state whether the OpaI protein binds and utilizes HSPG as its receptor, as well as whether any of the other Opa proteins of this strain share this activity. Addition of heparin or heparan sulfate to these adherence assays, receptor binding assays, and immunoprecipitations with purified Opa proteins should also be completed to confirm that HSPG binding is responsible for OpaI-mediated attachment and invasion. The identification of the CEACAM molecules, CEACAM1, 3, 5, and 6 as receptors for different Opa proteins of strain MS11 was performed using transfected HeLa cells that expressed the different CEACAMs (21, 37, 38, 72). These cells were infected with defined Opa variants of this strain and bound and intracellular gonococci

were enumerated. Repetition of these experiments using the different Opa variants of strain FA1090 should yield information concerning the full CEACAM receptor repertoire of the Opa proteins of this strain. Furthermore, immunoprecipitation and receptor overlay assays (209) using purified Opa proteins and CEACAM molecules should further contribute to the determination of the full extent of CEACAM receptor binding by the Opa proteins of strain FA1090. In conclusion, these important experiments would allow us to determine the receptor specificity of the Opa proteins of strain FA1090, as well as strengthen our conclusions concerning adherence as an unlikely selective force in our mouse studies.

Our experiments using a non-HPSG-binding Opa protein (OpaB) suggested that this Opa variant was also selected during murine infection. Further research is needed to establish if the Opa proteins of *N. gonorrhoeae* can bind to and utilize the mouse CEACAM1 molecule as a receptor, but based on structure function studies with human CEACAM1 (207) and comparison with the nucleic acid structure of the murine CEACAM1 molecule (77), it is unlikely. Therefore, our data indicate that Opa-mediated binding to the human CEACAM molecules may not be as critical in establishment and maintenance of infection as suggested by tissue culture models. One piece of evidence to support this idea is the report that CEACAM molecules are not always constitutively expressed by the genital epithelial cells used in typical cell culture models (196). It was also reported in a clinical study that the CEACAM-5 molecule (CEA) was not present on cervical tissue from all women sampled (140). Furthermore, it has been reported that some members of the CEACAM family, specifically CEACAM1, 5, 6, and 8, are not present on primary male urethral cells, and that CEACAM3 is detected on only a small

population of these cells (79). Collectively, from these data, it could be argued that Opa-mediated adherence to epithelial cells may not be vital for survival in the genital tract. There is much redundancy present in the gonococcus in terms of colonization factors; for example, expression of pili and LOS may be sufficient to enable the bacteria to establish an infection. The development of transgenic mice expressing all of the CEACAM molecules identified as Opa protein receptors (CEACAM1, 3, 5, 6) would allow further delineation of the importance of CEACAM molecules in Opa protein expression in the genital tract.

It is possible that another Opa receptor, distinct from those already reported, may be expressed on human urethral epithelial cells, as well as in the mouse genital tract. However, if another Opa receptor is not present in the mouse genital tract, it is likely that selection of Opa protein expression is due to the effects of innate host defenses. One such host factor that can be easily examined in the mouse system is the complement cascade. Complement components are present in the vaginal lumen of 17- β -estradiol treated mice, based on studies that show exogenous estradiol administration stimulated the synthesis of C3 in the uteri of both ovariectomized and normal cycling mice (124). The gonococcus possesses two well-studied virulence factors that allow it to avoid the bactericidal actions of complement, namely LOS and porin. Serum sensitive strains of *N. gonorrhoeae* evade killing by normal human serum (NHS) via sialylation of LOS molecules using host 5-cytidine-monophospho-N-acetylneuraminic acid (CMP-NANA) in concert with its own sialyltransferase (66, 129, 150, 155, 206). This CMP-NANA-dependent form of serum resistance is termed unstable serum resistance, due to the fact that this serum resistance is rapidly lost upon in vitro subculture. The mechanism of this

CMP-NANA-dependent serum resistance involves binding of factor H to the sialic acid on the LOS (160). Binding of factor H to the gonococcal surface downregulates the alternative pathway, which causes increased surface deposition of C3bi and blocks the complement cascade at the level of C3 (153, 154). Alternatively, some porin molecules mediate stable serum resistance. Gonococcal porins are the most abundant proteins in the outer membrane, with two main isoforms identified, PorIA and PorIB (30, 189). PorIA strains are often associated with disseminated infections, while PorIB strains are usually associated with urogenital tract infections and PID (29, 30, 189). Ram et al showed that PorIA isolates resist complement-mediated killing by directly binding factor H to the surface exposed 5th loop of PorIA (159). Interestingly, these researchers also showed that the complement regulator C4b-binding protein (C4bp) binds to PorIA or PorIB molecules of serum resistant strains, which leads to down-regulation of the classical complement pathway, and thus provides an additional mechanism for the gonococcus to stably evade complement-mediated bacteriolysis (157).

It was also shown that Opa-positive gonococci are more resistant to the bactericidal action of NHS in vitro than their Opa-negative counterparts (22). Therefore, to examine if complement selectively killed Opa-negative gonococci, which would lead to the increased recovery of Opa-positive variants observed in our selection experiments, genetically defined complement-deficient mice or mice treated with cobra venom factor (CVF) could be utilized. CVF is an anionic protein purified from *Naja naja* venom that depletes both C3 and C5 of the complement cascade (201). CVF-treatment of mice is preferential over the use of knockout mice in that CVF is inexpensive and the actions of CVF deplete the entire complement cascade. If complement selects against Opa-negative

variants, we would predict that infection of CVF-treated mice with defined mixtures of Opa-positive and Opa-negative gonococci would result in recovery of the same ratio of Opa phenotypes as in the inoculum, whereas in the untreated group, selection of Opa-positive gonococci would occur. These experiments could also be completed in knockout mice deficient in production of specific complement components to delineate the specific factors that are responsible for the selection events. Finally, other host factors, including protegrins, inflammation, and interactions with commensal flora, may effect the survival of different Opa variants in the genital tract of mice; whether they act independently or in concert is not known.

An interesting observation in this research, as well as in earlier experiments performed with male volunteers and female mice, is that almost all the Opa variants of *N. gonorrhoeae* strain FA1090 are recovered in vivo. No specific Opa variant was predominantly recovered from the nine men who became infected in experiments in which these volunteers were inoculated intraurethrally with a predominantly Opa-negative population of strain FA1090 (102). However, certain Opa variants, OpaB, OpaF, OpaE, OpaK, and OpaI, were preferentially recovered from different subjects. Also, relatively few OpaA, OpaC, and OpaD variants were recovered from any of these men. Furthermore, large numbers of gonococci expressing multiple Opa proteins simultaneously were reisolated from the last culture (range: days 2-6 post-inoculation) of a majority of these men. An unpublished study in which human volunteers were infected with predominantly OpaA variants showed that OpaA expression was maintained throughout the course of infection in these subjects (Jerse, unpublished). This result suggests that OpaA expression is not detrimental in the human male urethra.

Similar to Opa protein expression in male volunteers, Jerse reported that infection of mice with a predominantly Opa-negative population resulted in the recovery of Opa-positive variants, and different Opa variants predominated in vaginal isolates recovered from different mice on days 4 and 8 post-inoculation (101). A single Opa variant, OpaI, was highly represented among vaginal isolates from 2 of 3 mice; in the other mouse, OpaB variants were preferentially recovered. Similar to the male urethral isolates, small numbers of OpaA and OpaD variants were recovered from these mice. No OpaC variants were detected; the limit of detection in these experiments was 5%. In the experiments reported here to test selection, the Opa variant used to spike a primarily Opa-negative inoculum (OpaI, OpaB) was predominantly recovered from the majority of mice infected. However, OpaE/K variants were also highly represented in vaginal isolates from mice in one experiment. Also, small numbers (range: 1-20%) of all the Opa variants, including OpaC, were recovered from different mice at different times in all these experiments.

The explanation of the differences in Opa variants recovered in vivo might be in the number of alleles of each *opa* gene in the *N. gonorrhoeae* genome, as well as in possible selection advantages conferred by expression of different Opa variants. *N. gonorrhoeae* strain FA1090 has two functional copies of both the *opaB* and *opaF* genes (44). These gene duplications could explain why both OpaB and OpaF variants were preferentially recovered from male volunteers, as well as why OpaB variants were highly represented in gonococci reisolated from the murine genital tract. An intriguing direction for this research is to examine if the other Opa variants of strain FA1090 (OpaA, OpaC, OpaD, OpaE, OpaK, and OpaF) are selected for when mice are infected with mixtures containing primarily Opa-negative gonococci spiked with smaller numbers of the other

Opa variants. It is possible that OpaB and OpaI variants have a stronger advantage over the other Opa variants of strain FA1090 in mediating the selection seen early in infection. Competition experiments using mixtures of OpaI and OpaB variants to infect mice would allow us to determine if either of these Opa variants possesses a selective advantage over the other *in vivo*.

Transcriptional regulation of the *opa* genes

Transcriptional regulation of the *opa* genes is an understudied area of research and is complicated by conflicting data from different laboratories. It was initially reported that *opa* gene transcription is constitutive (145, 191). In these studies, the authors showed that bulk mRNA purified from both Opa-negative and Opa-positive variants reacted with oligonucleotide probes specific to a conserved sequence in the CTCTT repeat region, as well as with probes reactive to the HV₁ region from two different *opa* genes. However, others presented evidence that Opa protein expression may be regulated at the level of transcription (10). It is possible that the half-life of the untranslated *opa* mRNA might be the reason for the discrepancies in *opa* gene transcription between these studies. In the study by Belland et al (10), Northern blotting analysis of RNA with probes specific to unique regions within the HV₂ sequences of the *opaA*, *opaB*, and *opaC* genes of *N. gonorrhoeae* strain MS11 indicated that full-length mRNA from a specific *opa* gene was found primarily in variants that expressed the corresponding Opa protein. However, weaker signals were detected for mRNA from all Opa-positive and Opa-negative variants examined with the different HV₂ probes, indicating that *opa* gene transcription may in fact be partially constitutive.

Sequence analysis revealed three different promoter sequences in the *opa* genes of *N. gonorrhoeae* strain MS11; the *opaA* gene contains one sequence, the *opaB*, *D*, *E*, *F*, *G*, *H*, *I*, and *K* genes contain another, and the *opaC* and *J* genes contain the third (10). It is not yet known whether *opa* gene transcription or phase variation is responsive to environmental signals. One possible exception is the presence of a putative Fur box in the *opa* genes (176), however, it has not yet been determined if iron influences *opa* gene expression. Belland et al (10) constructed transcriptional *lacZ* fusions in the *opaA*, *opaB*, and *opaC* genes of strain MS11 on plasmids and the expression levels of these three fusions in *E. coli* differed significantly when bacteria were grown under standard conditions. Interestingly, these transcription initiation rates correlated with the phase variation rates of translational *phoA* fusions with the same *opa* genes expressed in *E. coli*. Therefore, the authors concluded that promoter differences in the *opa* genes result in significantly different levels of expression and that these differences correspond to rates of *opa* phase variation, possibly due to positive supercoiling ahead of the transcription complex. Further research needs to be completed in different strains of *N. gonorrhoeae* to determine if these observations regarding the basal level of *opa* gene transcription are significant and if transcription levels always correspond with phase variation rates.

Induction of *opa* gene phase variation is not influenced by host factors.

Our studies using strain ANS100 indicated that the rate of *opa* gene phase variation does not change when exposed to environmental factors designed to mimic those in the host. Iron starvation, pH variation, progesterone, and oxygen tension did not affect the frequency or rate of phase variation of “on” or “off” variants of strain ANS100.

In addition, exposure to extracellular neisserial DNA did not influence the rate of phase variation of the *opaB::phoA* fusion, as was reported for phase variation of the hemoglobin receptor in *N. meningitidis* (4). Our results are similar to the reported rate of change in gonococcal colony opacity phenotype in the presence of various environmental factors. Mayer (131) determined that the presence of 194 different compounds or mixtures of compounds did not affect the rate of change of colony opacity phenotype of *N. gonorrhoeae* strain F62. As mentioned earlier, a caveat of this study is that not all Opa proteins convey a distinct colony photo-opacity. In our mouse infection experiments, no change in the frequency of phase variation from “off to on” was detected. However, in experiments examining the frequency of phase variation from “on to off”, the dramatic recovery of “off” variants is suggestive of increased SSM due to in vivo conditions that we have not yet been able to mimic in vitro.

Further experiments should be completed to strengthen these results. The studies by Belland et al (10) and a comparison between the promoter sequences of strains MS11 and FA1090 (Figure 18) indicate that the FA1090 *opaB* promoter is of intermediate strength, due to its identity with the promoter sequence of the *opaB* gene of strain MS11 (10). The *opaB* gene was chosen in our studies because of the presence of two *opaB* alleles in the genome (44, 54), as well as the fact that its promoter is identical to that present in the majority of the functional *opa* genes of strain FA1090 (6 of 10, Figure 18). To fully determine the scope of *opa* gene phase variation in *N. gonorrhoeae*, translational fusions could be constructed in all the *opa* genes present in strain FA1090. If these constructs were made and introduced into the genome of strain FA1090, a panel of 11 strains, each carrying an *opa* translational fusion, would be produced. The rate of *opa*

gene phase variation of each of these strains would then be tested in vitro and in vivo as with strain ANS100. Next, the construction of isogenic strains containing transcriptional fusions between promoter regions in the corresponding *opa* genes and the *lacZ* gene would be completed. Comparison of the transcription initiation rates of the *opa::lacZ* fusions and the phase variation rates of the *opa* translational fusions would allow us to examine if promoter strength is a factor in *opa* gene expression in *N. gonorrhoeae*.

An interesting result in our mouse experiments using primarily “on” variants of strain ANS100 was that predominantly “off” variants were recovered from the majority of mice, possibly due to increased phase variation without selection. However, when mice were infected with “off” variants of this strain, no significant change in the frequency of phase variation occurred. A concern that arose from these conflicting results involves the choice of *phoA* as the reporter gene in the *opaB* fusion. The product of the XP substrate has been reported to be toxic to *N. gonorrhoeae* (31). We did not isolate any environmental condition that influenced the rate of *opaB::phoA* phase variation in vitro. However, if some factor in the mouse genital tract causes *phoA* expression to be toxic for the gonococcus, it is possible that increased phase variation does occur in vivo, but would not be seen in the “off to on” phenotype as this would lead to increased amounts of alkaline phosphatase in the membrane of the gonococcus. A gonococcal strain that constitutively expresses *phoA* could be constructed and examined in single and mixed infection experiments with the wild type strain in mice to determine if *phoA* expression is detrimental to the gonococcus in vivo. Alternatively, this potential problem could be alleviated by the use of another reporter gene in the fusion constructs.

Opa protein expression in the genital tract of female mice is cyclical.

Using mixtures consisting of primarily Opa-negative gonococci spiked with a smaller population of OpaI variants, we propose that three phases of Opa protein expression occur during experimental infection of the murine genital tract. The first phase was characterized by selection of a pre-existing population of Opa-positive gonococci present in the inoculum (OpaI). This phase lasted for 1 to 3 days post-inoculation. Following this selection for Opa protein expression, a decrease in the recovery of Opa-positive variants occurred in 6 of 9 mice. This second stage was termed the mid-phase of infection and it occurred between days 3 and 7 post-inoculation. Notably, loss of Opa-positive variants coincided with an overall decrease in gonococcal recovery. In two of three mice in which infection progressed for longer than eight days, a third phase of Opa protein expression was identified, which we called the late phase. In the late phase of infection, a re-emergence of Opa-positive gonococci was observed. Gonococci that expressed multiple Opa proteins simultaneously were identified in all phases, with the highest percentages occurring in the later two phases. The description of these phases was initially based on 2 experiments using mixtures of OpaI and Opa-negative gonococci. The first phase of infection, selection of Opa-positive gonococci present in the inoculum, was reproduced in later experiments. However, a sufficient amount of mice did not maintain infection long enough to firmly establish the proposed last two phases of Opa protein expression in the murine genital tract. Evidence of a mid-phase of infection did occur in one experiment in which mice were infected with a population consisting of predominantly Opa-negative, Cm^S gonococci mixed with a smaller percentage of OpaI, Cm^R variants. In 3 of the 4 mice in this experiment in which

selection of OpaI, Cm^R gonococci was observed early, evidence of a mid-phase of Opa protein expression was seen by day 5 post-infection (Simms and Jerse, in preparation/Chapter 2). These experiments should be repeated to strengthen the statistical validation of our results.

The occurrence of different phases indicates a cyclical variation in Opa protein expression in genital infection of female mice. Similar observations on Opa protein expression were reported during genital tract infection of SLC:ddY mice; these mice were not treated with estradiol (114). Also, using endocervical isolates recovered from women with natural infection, correlations have been made between the luteal or high progesterone phase of the menstrual cycle and the isolation of transparent colonies; opaque variants were often recovered in the proliferative or high estrogen phase (99, 100). The factors responsible for each of the observed phases of Opa protein expression *in vivo* are not known. As discussed earlier, the selection of a pre-existing OpaI population in the inoculum may be due to adherence of OpaI-expressing variants to HSPG molecules present on the murine genital epithelium or to enhanced resistance of OpaI-expressing gonococci to host factors, such as complement-mediated bacteriolysis.

Several possibilities exist to explain the mid-phase of Opa protein expression *in vivo*. It is feasible that the decrease in Opa-positive variants recovered in the mid-phase is due to invasion of OpaI-expressing gonococci into host cells. This is consistent with the decrease in total gonococcal recovery. In a histochemical study of infected murine vaginal and endocervical tissue, no intracellular gonococci were observed (Song and Jerse, unpublished). However, tissue from the genital tract of mice infected with OpaI-expressing gonococci has not been examined. Further studies to examine Opa-mediated

invasion in the mouse genital tract are hindered by the lack of murine genital tract tissue culture cells. Experiments using histological staining of tissue from the genital tract of mice in the mid-phase would reveal whether invasion occurred and if this invasion was Opa-mediated.

Hormonally driven factors may also contribute to the decrease in OpaI variants recovered during the mid-phase. This hypothesis is supported by the second mid-phase seen in Figure 10 (Chapter 2). The estrous cycle of female mice consists of four stages: proestrus, estrus, metestrus, and diestrus, and lasts for 4 to 5 days (187). Similar to the menstrual cycle in humans, fluctuations in the gonadal hormones result in changes in vaginal pH, histology, mucus secretion, and commensal flora load (27, 45). Even though the mice in the experiments described in our study were treated with 17- β -estradiol, the pituitary and ovaries remained intact, thus allowing for hormonal influences, including those affected by progesterone secretion. Hormone secretion also influences synthesis of complement components. Estradiol administration stimulates the synthesis of C3 in mice and rats, whereas simultaneous or delayed administration of progesterone inhibits the synthesis of this protein (28, 82, 193). Also, the synthesis of C3 is maximal in the high estrogen phase of the estrus cycle, estrus, and diminishes in metestrus and diestrus, where the concentration of progesterone is at its highest (28). Finally, it is also possible that another Opa protein receptor is present in the genital tracts of both women and female mice, but hormones regulate expression of this receptor. Such a receptor would not have been identified in the traditional tissue culture assays used to identify the HSPG and CEACAM molecules as Opa protein receptors. Progesterone itself may directly select against Opa-positive gonococci, based on a report that opaque gonococci were more

susceptible to direct killing by the gonadal hormone progesterone than their transparent counterparts (170). However, we have not been able to reproduce this result using defined Opa variants of strain FA1090 and several different assays (Simms and Jerse, unpublished). A third possibility is that hormonally-driven host factors in estradiol-treated mice may have contributed to the decreased isolation of Opa-positive gonococci at the mid phase of infection through an increase in the phase variation rate of the *opa* genes, leading to recovery of primarily Opa-negative gonococci. The suggestion is unlikely, based on our results with strain ANS100. In these experiments where “on” or “off” variants of strain ANS100 were cultured in vitro in the presence of sub-inhibitory concentrations of progesterone, no change in the frequency or rate of *opaB* phase variation was observed, leading us to conclude that progesterone secretion does not directly act on phase variation of the *opa* genes. Experiments using ovariectomized mice would allow us to determine if progesterone, either by direct actions on the bacteria or through downstream effects of the hormone on cellular morphology and receptor expression, accounted for the decrease in Opa-positive gonococci recovered from mice in the mid-phase of infection.

Significance of this work

Neisseria gonorrhoeae is a human-specific pathogen that is well equipped to avoid and, or use host factors encountered during urogenital infection. Interestingly, the lack of animal or environmental reservoirs, as well as its existence in the human population since biblical times (189), indicates that this pathogen has evolved highly effective adaptation mechanisms that promote transmission and survival of the organism

on human mucosal surfaces. One such adaptation mechanism that allows the gonococcus to survive and proliferate in the presence of damaging host factors is phase and antigenic variation of surface molecules. Phase and antigenic variation allows the gonococcus to create subpopulations that can withstand challenges presented by the host environment, as well as to allow the gonococcus to capitalize on opportunities that are present.

The Opa proteins are a family of phase variable outer membrane proteins expressed by the gonococcus, which play a role in adherence to specific host receptors and in evasion of host defenses. Opa protein expression during infection is dependent upon the body site, gender, and the phase of the menstrual cycle (55, 99, 100). Expression of the Opa proteins in the female genital tract appears to follow a cyclical pattern, with fluctuations in expression occurring over time during natural infection of women (99, 100). Here we have extended knowledge of Opa protein expression *in vivo*, using mice as an experimental model of female genital tract infection. We have shown that selection of a pre-existing population present in the inoculum is responsible for the early recovery of predominantly Opa-positive gonococci in female mice (Chapter 2). Additionally, our data indicate that expression of the Opa proteins in experimental infection of mice follows a cyclical pattern (Chapter 2). We have also shown that induction of *opa* gene phase variation does not occur via exposure to various conditions relevant to the host *in vitro* (Chapter 3). In our mouse infection experiments, no change in the frequency of *opaB* phase variation from “off to on” was detected (Chapter 3). However, in experiments examining the frequency of phase variation from “on to off”,

the dramatic recovery of “off” variants was suggestive of increased SSM due to in vivo conditions that we have not yet been able to mimic in vitro.

Several important avenues of investigation remain. Among these are the specific host factors that drive selection of Opa-positive gonococci early in infection, as well as those that mediate the cyclical pattern of Opa protein expression in mice; a few possibilities may be resistance to complement-mediated bacteriolysis, attachment to specific receptors not yet identified, or invasion of Opa-positive gonococci into the host epithelium. It is also possible that Opa protein expression in vivo may be selected against by cervical proteases, specific antibody, or progesterone. Finally, further studies using transcriptional and translational *opa* gene fusions in *N. gonorrhoeae* strain FA1090 would strengthen our conclusions regarding the induction of *opa* gene phase variation in response to host environmental stimuli.

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