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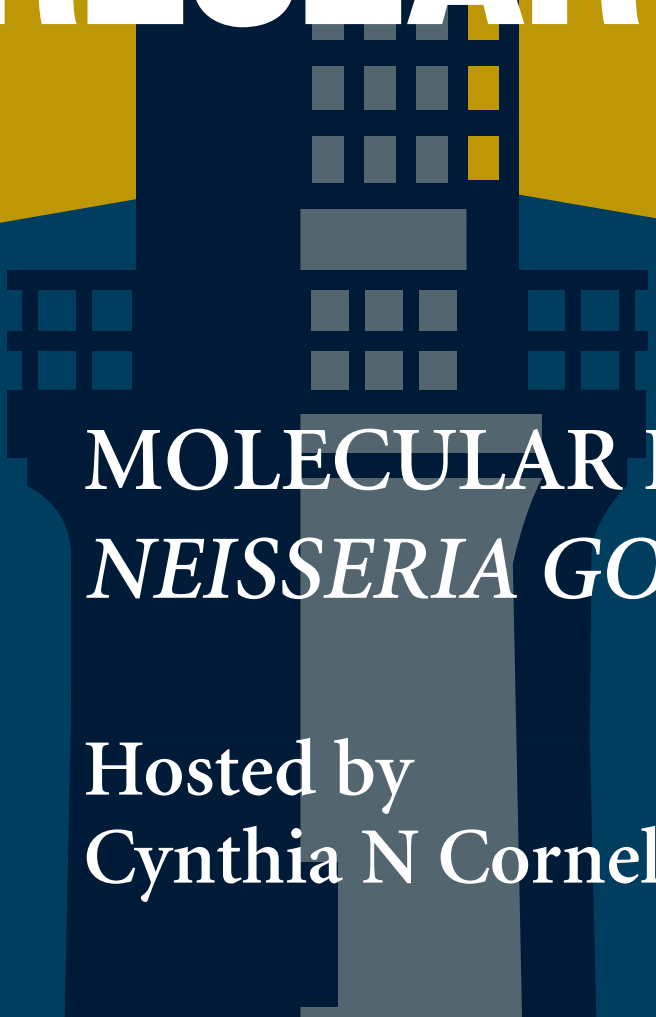

MOLECULAR PATHOGENESIS OF *NEISSERIA GONORRHOEAE*

Hosted by
Cynthia N Cornelissen

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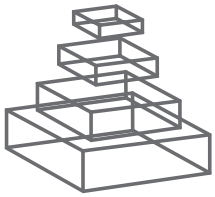

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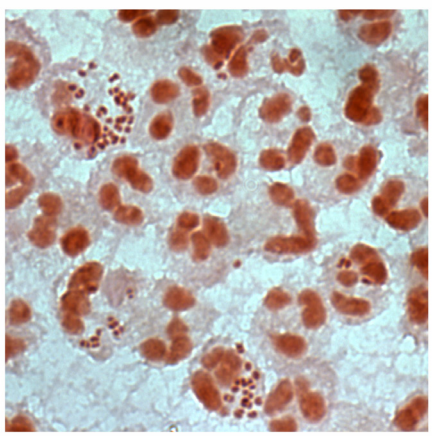
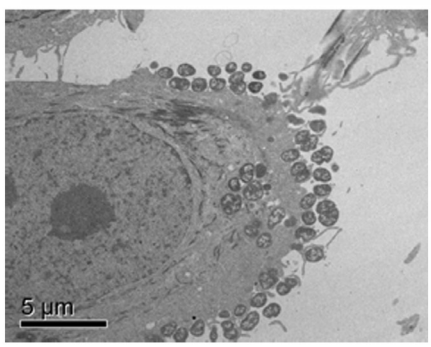
MOLECULAR PATHOGENESIS OF *NEISSERIA GONORRHOEAE*

Hosted By
Cynthia N Cornelissen, Virginia Commonwealth University School of Medicine, USA





This Research Topic is dedicated to our current understanding of the molecular pathogenesis of *Neisseria gonorrhoeae*. Topics in this issue include the following: human infection models for testing gonococcal virulence, animal models that mimic lower female genital tract infection, and an *ex vivo* system derived from the human female lower genital tract. Vaccine development efforts, and the impact of gonococcal infection on the host's immune response are also described. Also part of this issue are reviews of the molecular aspects of several important virulence factors, including: biofilm formation, neutrophil resistance, innate immune factor resistance, iron acquisition, and type 4 secretion systems.

Image:
Top: Gram stain of *Neisseria gonorrhoeae* within human polymorphonuclear leukocytes.
Bottom: Electron micrograph of *Neisseria gonorroheae* attached to and within a human cervical epithelial cell.

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Molecular pathogenesis of *Neisseria gonorrhoeae*

Cynthia N. Cornelissen*

Department of Microbiology and Immunology, Virginia Commonwealth University School of Medicine, Richmond, VA, USA
**Correspondence: cncornel@vcu.edu*

This Research Topic is focused on the molecular mechanisms of pathogenesis employed by the obligate human pathogen, *Neisseria gonorrhoeae*. The 10 articles cover a range of topics, including updates on important virulence factors, vaccine development efforts, immune evasion by the gonococcus, and current models for assessing virulence, treatments, and prophylactic measures. In the chapter by Balthazar et al. (2011) the authors present a research article that describes a random mutagenesis screen for gonococcal mutants with reduced sensitivity to the bactericidal action of normal human serum. This screen led to the identification of lipooligosaccharide biosynthesis and modification genes, which appear to play important roles in serum resistance as well as resistance to cationic antimicrobial peptides. The review by Ramsey et al. (2011) describes the characteristics and contributions of a type 4 secretion system that is expressed by 80% of gonococcal isolates. This system secretes single-stranded DNA into the external milieu, resulting in free DNA in the environment. Subsequent transformation of gonococci within the population with this DNA does not require cell to cell contact and results in spread of genetic information within the population, enhancing antigenic diversity, and potentially spread of antibiotic resistance. Cornelissen and Hollander (2011) review the current knowledge on gonococcal TonB-dependent transporters and their contribution to iron acquisition. The transferrin–iron acquisition system is one of several transport systems that is comprised of a TonB-dependent transporter and an associated lipoprotein. These surface-exposed transferrin binding proteins are ubiquitously expressed by all gonococci, not subject to antigenic variation, and important for growth *in vivo*; therefore, these proteins are being pursued as potential vaccine antigens. Other TonB-dependent transporters are of unknown function, but their characteristics and potential functions are summarized. In the review by Falsetta et al. (2011) the authors describe the gonococcal requirements for biofilm formation on glass and on epithelial cell surfaces. Formation of a biofilm on epithelial cell surfaces is a survival strategy employed under stressful environmental conditions like those encountered in the human host. The gonococcal biofilm matrix is composed largely of DNA, and formation of the biofilm requires the release of membrane blebs, which contain DNA. The genes that are differentially regulated upon biofilm development are summarized, along with their individual contributions to the formation of robust biofilms. Gonococcal infections are characterized by an infiltration of PMNs to the site of infection; however, gonococci survive this assault and, in fact, can thrive within PMNs. Johnson and Criss (2011) review the mechanisms that allow the gonococcus to resist clearance by polymorphonuclear leukocytes (PMNs) including resistance to both oxidative and non-oxidative killing mechanisms. Gonorrhea infections do not elicit protective immunity, leaving persons who suffer from this disease susceptible to subsequent infections. In this issue, Liu et al. (2011) summarize our current understanding of how the gonococcus subverts the immune system, and takes advantage of the immune privilege of the genital tract to foster an environment that is hospitable to this pathogen. Since infection does not elicit a protective response, a gonococcal vaccine has been sought for many years. Zhu et al. (2011) present a summary of gonococcal vaccine development efforts, including a previously unpublished study testing the efficacy of a viral replicon particle (VRP) expressing the PorB antigen. Several models have been developed with which to study gonococcal pathogenesis, and to examine the effectiveness of vaccination or treatment strategies. The review by Edwards and Butler (2011) describes an *ex vivo* model employing primary human cervical epithelial cells, and summarizes what is known about the pathobiology of gonococcal infection within the microenvironment of the lower human reproductive tract. The review focuses on adherence, invasion, host cell signaling events, and intracellular survival of the gonococcus within human epithelial cells of genital tract origin. Although the gonococcus is highly adapted to the human host, a mouse model of lower female genital tract colonization has been developed by Jerse and colleagues. In the review by Jerse et al. (2011) the characteristics of the estradiol-treated mouse model are summarized along with the various gonococcal mutants that are defective for colonization in this model. The utility of the mouse model for testing vaccines, antibiotics and microbicides, and the synergy between chlamydial and gonorrheal infections is also discussed. In the final chapter, Hobbs et al. (2011) summarize the results from human infection models, which have been used for decades to evaluate the molecular determinants to gonococcal virulence. This review also describes the strengths and weaknesses of the human model and its potential for testing vaccine candidates. This Frontiers Research Topic, dedicated to gonococcal pathogenesis, summarizes the progress that has been made over the past few years in this field, and highlights potential opportunities for future research. While much has been accomplished, much remains to be understood. With the incidence of gonococcal disease still unacceptably high, limited options for antimicrobial treatments, and no modes for immunoprotection, continued research in this field is critical. These reviews put into context and point the way toward key avenues of research, which could lead to decreases in incidence, novel treatment modalities, or long term protection against gonococcal disease.

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Lipooligosaccharide structure is an important determinant in the resistance of *Neisseria gonorrhoeae* to antimicrobial agents of innate host defense

Jacqueline T. Balthazar[1,2], Asiya Gusa[2], Larry E. Martin[1,3], Biswa Choudhury[4†], Russell Carlson[4] and William M. Shafer[1,2]*

[1] *Laboratories of Bacterial Pathogenesis, VA Medical Research Service, VA Medical Center, Decatur, GA, USA*
[2] *Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA*
[3] *Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA*
[4] *Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA*

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Sanjay Ram, University of Massachusetts Medical Center, USA
Christopher Davies, Medical University of South Carolina, USA

***Correspondence:**
William M. Shafer, Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322, USA. e-mail: wshafer@emory.edu

†Current address:
Biswa Choudhury, Glycotechnology Core Resource, University of San Diego, La Jolla, CA 92093-0687, USA.

The strict human pathogen *Neisseria gonorrhoeae* has caused the sexually transmitted infection termed gonorrhea for thousands of years. Over the millennia, the gonococcus has likely evolved mechanisms to evade host defense systems that operate on the genital mucosal surfaces in both males and females. Past research has shown that the presence or modification of certain cell envelope structures can significantly impact levels of gonococcal susceptibility to host-derived antimicrobial compounds that bathe genital mucosal surfaces and participate in innate host defense against invading pathogens. In order to facilitate the identification of gonococcal genes that are important in determining levels of bacterial susceptibility to mediators of innate host defense, we used the *Himar I mariner in vitro* mutagenesis system to construct a transposon insertion library in strain F62. As proof of principle that this strategy would be suitable for this purpose, we screened the library for mutants expressing decreased susceptibility to the bacteriolytic action of normal human serum (NHS). We found that a transposon insertion in the *lgtD* gene, which encodes an *N*-acetylgalactosamine transferase involved in the extension of the α-chain of lipooligosaccharide (LOS), could confer decreased susceptibility of strain F62 to complement-mediated killing by NHS. By complementation and chemical analyses, we demonstrated both linkage of the transposon insertion to the NHS-resistance phenotype and chemical changes in LOS structure that resulted from loss of LgtD production. Further truncation of the LOS α-chain or loss of phosphoethanolamine (PEA) from the lipid A region of LOS also impacted levels of NHS-resistance. PEA decoration of lipid A also increased gonococcal resistance to the model cationic antimicrobial polymyxin B. Taken together, we conclude that the *Himar I mariner in vitro* mutagenesis procedure can facilitate studies on structures involved in gonococcal pathogenesis.

Keywords: *Neisseria gonorrhoeae*, mutagenesis, complement, antimicrobial, peptide, resistance, lipooligosaccharide

INTRODUCTION

Neisseria gonorrhoeae is a strict human pathogen that has caused the sexually transmitted disease gonorrhea for thousands of years (Sparling et al., 1990). Over the millennia, gonococci have developed a number of mechanisms to escape both innate and adaptive immune responses of the human host, which likely explains why immunity to re-infection does not occur. The ability of gonococci to alter the structure of a number of surface antigens or to variably produce them or to express surface structures that are similar to host antigens ("molecular mimicry") have been invoked (Sparling et al., 1990) as mechanisms by which this pathogen evades both innate and adaptive host defense systems.

We are interested in the genetic basis for how gonococci can evade antimicrobial agents that it encounters during infection of mucosal surfaces or when growing in the bloodstream. In this respect, our previous work showed that the MtrC-MtrD-MtrE efflux pump can export host-derived antimicrobial agents such as cationic antimicrobial peptides (CAPs; Shafer et al., 1998), as well as certain classical antibiotics (Veal et al., 2002). In addition to energy-dependent efflux, gonococcal resistance to CAPs has been linked to the decoration of lipid A by phosphoethanolamine (PEA; Lewis et al., 2009), which likely interferes with the ability of CAPs to bind to negatively charged groups on the bacterial surface.

In order to identify additional gonococcal determinants important for bacterial resistance to host-derived antimicrobials, we constructed a *Himar I mariner* transposon (Pelicic et al., 2000) mutant library of strain F62. We screened this library for mutants expressing decreased susceptibility to normal human serum (NHS) because the ability of gonococci to escape such killing is of likely importance in its capacity to proliferate if it enters the blood stream or if it encounters lethal levels of complement components and natural antibody at mucosal surfaces (Schoolnik et al., 1976; Rice et al., 1980; Rice, 1989; Shafer et al., 1982, 1984) Since NHS killing of strain F62 (and other NHS-sensitive gonococci) can be

potent, we hypothesized that it could be used to directly select transposon-facilitated NHS-resistant mutants in a library of susceptible bacteria. NHS killing of gonococci is principally mediated by an antibody-dependent (IgM) mechanism involving the classical complement pathway (CCP; Shafer et al., 1982, 1984, 2002; Rice, 1989). While most strains of gonococci are NHS-sensitive, some strains can display a stable NHS-resistance phenotype and they are more frequently isolated from patients with disseminated gonococcal infection (DGI; Schoolnik et al., 1976, Rice et al., 1980). Stable NHS-resistance expressed by these gonococci has been associated with the production of a certain serotype of the major outer membrane porin (Por1A vs. Por1B; Cannon et al., 1983; Rice, 1989; Ram et al., 1998; Ram, 2001), production of a 3.6-kDa lipooligosaccharide (LOS; Schneider et al., 1985; Shafer et al., 2002), and decoration of lipid A by PEA (Lewis et al., 2009).

In the present study we obtained additional evidence that LOS structure is important in determining whether gonococci can resist killing by NHS. This was surmised because a stable *Himar I mariner* transposon insertion in a gene (*lgtD)* encoding a glycosyltransferase that adds *N*-acetyl-galactosamine (GalNAc) to the terminal galactose in the LOS α-chain rendered strain F62 less susceptible to such killing. *lgtD* is a phase variable gene within the *lgtABCDE* operon that switches from phase-on to phase-off (and vice versa) at high frequencies due to a homopolymeric repeat within the coding sequence (Gotschlich, 1994). This mutant encouraged us to more closely evaluate the role of LOS in determining the ability of strain F62 to resist host defensive agents. Importantly, this work shows that the *Himar I mariner* transposon mutagenesis procedure, previously employed with success in *Neisseria meningitidis* (Pelicic et al., 2000), can be used to study mechanisms of pathogenesis in gonococci.

MATERIALS AND METHODS

BACTERIAL STRAINS, CULTURE CONDITIONS, AND BACTERICIDAL ASSAYS

Neisseria gonorrhoeae strain F62, which was kindly provided by S. Morse (Centers for Disease Control and Prevention, Atlanta, GA, USA), was the primary strain employed in this study and was cultured from a lyophilized stock prepared by Kellogg in 1962 (Kellogg et al., 1963). It was routinely cultured on GCB agar plates containing defined supplements I and II (Shafer et al., 1982) in 3.8% (v/v) CO_2 at 37°C as a piliated (P+), opacity-negative (Opa−) variant for transformation experiments and as a P− Opa− variant for serum bactericidal assays (SBA). The SBA employed pooled NHS and heat-inactivated NHS (ΔNHS), as described previously (Shafer et al., 1982). Gonococci were incubated with serum samples for 45 min at 37°C prior to plating onto GCB agar. In this assay, NHS-resistance is defined as >30% survival calculated on the input (10^5 cfu) of gonococci in the reaction mixture. Naturally NHS-resistant strain FA19 was used as a control since it can survive in 50% (v/v) NHS concentrations (Shafer et al., 1982, 2002). All assays were repeated in triplicate and the data were calculated as average values ± SD. The significance of the results was determined by Student's *t*-test. Serum was obtained by venipuncture from healthy volunteers after written informed consent and the collection of such serum was approved by the Institutional Review Board of Emory University. The agar dilution method for determining the minimal inhibitory concentration (MIC) for polymyxin B (PMB) was as described previously (Lewis et al., 2009).

CONSTRUCTION AND ANALYSIS OF A *HIMAR I MARINER* MUTANT LIBRARY OF STRAIN F62 AND ISOLATION AND MOLECULAR CHARACTERIZATION OF AN NHS-RESISTANT MUTANT

Himar I transposase was purified (Pelicic et al., 2000) and used for *in vitro* transposition reactions with bulk DNA purified from strain F62, as described previously (Alexander et al., 2006). The transposition reaction mixture was used to transform piliated strain F62, as described previously (Gunn and Stein, 1996), and transformants were selected on GCB agar plates containing 100 μg of kanamycin (Kan)/mL. This procedure was repeated until a total of 10,000 transformants were obtained. All transformants were passed on GCB agar containing Kan and then resuspended in 100 μl of freezing media in 96 well microtiter plates and stored at −80°C. A pool of the library was also made and similarly stored. In order to determine whether random insertion of the *Himar I* transposon was achieved, DNA was prepared from 20 mutants. The DNA preparations were digested with *Cla*I and subjected to agarose gel electrophoresis followed by Southern hybridization analysis (Southern, 1975) using the *aphA-3* gene as a transposon-specific probe (Pelicic et al., 2000). The results showed that the DNA from these mutants had a single insertion and that the insertion was in distinct *Cla*I fragments (data not presented).

In order to isolate *Himar I* mutants of F62 expressing decreased susceptibility to NHS, the library was grown overnight on GCB agar plates containing Kan at 37°C under 3.8% (v/v) $C0_2$. The growth was removed and used to inoculate 50 mL. of GCB broth with supplements and grown as described above to late-logarithmic phase. The culture was diluted 10-fold in 0.2× GCB broth and exposed to NHS [final concentration of 12.5% (v/v)] for 30 min and survivors were recovered by dilution plating onto GCB agar. This concentration of NHS was used because in preliminary experiments we found that use of a lower concentration of NHS resulted in inefficient killing and gave a high background of surviving gonococci that did not differ from parental strain F62 with respect to level of susceptibility to NHS (data not presented). After overnight growth, the colonies that survived in the presence of 12.5% NHS were pooled and re-exposed to NHS as described above. This procedure was repeated for a third time and individual colonies were then screened along with parental strain F62 in the standard SBA. Ultimately, a mutant (WMS 100) that was less NHS-susceptible than parental strain F62 was identified. DNA was prepared from this mutant and used to transform F62 for Kan resistance and 10 individual transformants were screened in the NHS assay, all of which expressed the NHS-resistance profile of the original mutant.

Ligation-polymerase chain reaction (PCR) and DNA sequencing was used to map the site of the transposon insertion as described previously (Pelicic et al., 2000). The oligonucleotide primers used for the ligation-PCR were LMP1, LMP2, and IR1 (Pelicic et al., 2000). DNA sequencing was performed on a PCR product from a pBAD construct that contained the ligation-PCR product. This PCR product was sequenced using primer LGT19 (5′-GCGGTATTTGACCAAGGCTTC-3′) and IR1 (Pelicic et al., 2000).

CONSTRUCTION OF *lgtA* AND *lptA* INSERTIONAL MUTANTS

Transformants of strain F62 bearing inactivated *lgtA* and/or *lptA* genes were constructed by transformation using donor DNA from strains FA19 *lgtA::km* or FA19 *lptA::spc*, which have

been described (Shafer et al., 2002; Lewis et al., 2009). The *lgtA* mutation were confirmed by PCR using oligonucleotide primers LGT5 (5′-CCGGCCTGATTCCCTCTTTAA-3′) and LGT8 (5′-CCGGCAGTTCATACGCCGCT-3′). The *lptA* mutation was confirmed using LPTA4 (5′-GGCGGAAGGCTTTGGAAAAC-3′) and LPTA5 (5′-GGCGAGTGCCTCGACA ATAT-3′).

COMPLEMENTATION ANALYSIS

The pGCC4 complementation vector described previously (Skaar et al., 2002) was used for complementation of the *lgtD::km* mutation generated by *Himar I mariner* mutagenesis. *lgtD* was PCR-amplified from genomic DNA using oligonucletotide primers that contained a *Pac*I site (LGT15PAC; 5′-TTAATTAAGCCGTCTGAAGCCT-3′; restriction site is underlined) or *Pme*I site (LGT16Pme; 5′-GTTTAAACGCGGAACGGAAGC-3′) and cloned into *Pac*I–*Pme*I digested pGCC4. The plasmid construct, which contains an IPTG-inducible *lacZ* promoter (Skaar et al., 2002) was purified and used to transform strain WMS 100 (see **Table 1**) for resistance to chloramphenicol (1 μg/mL). The insert, positioned between the *lctP* and *aspC* chromosomal sites (Skaar et al., 2002; Folster et al., 2009), was verified by PCR using primers LGT23 (5′-CGGCTGGATTGAGAAAATCGT-3′) and LCTP (5′-GCGCGATCGGTG CGTTCT-3′). Expression of the insert was performed by growing the complemented strain (WMS 101) in GCB broth or on GCB agar containing 1 mM IPTG as described previously (Folster et al., 2009). RT-PCR (Folster et al., 2009) was performed to confirm expression of the ectopically expressed *lgtD* gene in WMS 101. The *lptA::spc* mutation was complemented using the previously described (Lewis et al., 2009) pGCC4 construct that contained the wild type gene.

LOS CHEMICAL ANALYSES

Lipooligosaccharide produced by gonococcal strains were visualized by subjecting proteinase K digests of whole cell lysates to Tricine SDS-PAGE (Lesse et al., 1990) followed by silver staining (Tsai and Frasch, 1982), or Western immunoblotting (Towbin et al., 1979) using anti-LOS monoclonal antibodies 4C4, 3F11, or 1-1-M as described (Gotschlich, 1994; Shafer et al., 2002), which recognize epitopes in the α-chain of the core oligosaccharide.

Lipooligosaccharide was purified from 12 L batch cultures of strains grown in GCB broth with defined supplements I and II under 3.8% CO_2 as described (Lewis et al., 2009). In order to remove contaminating phospholipids the dried bacterial pellets were extracted with a 9:1 ethanol–water mixture and then lyophilized. The oligosaccharides (OSs) were released from the LOS by mild acid hydrolysis [1% (v/v) acetic acid at 100°C for 2 h]. The lipid A portions were precipitated by low-speed centrifugation, and supernatants containing the OS fraction were lyophilized and used for chemical and structural analyses as described (Shafer et al., 2002; Lewis et al., 2009). The OSs were treated with 48% hydrogen fluoride (HF) at 4°C for 48 h to remove any phosphate groups; e.g., phosphate or PEA. The HF-treated material was neutralized by ice-cold ammonium hydroxide solution (approximately 30%) on an ice-water bath. The neutralized material was desalted by gel permeation chromatography using fine grade Bio-Gel P2 (Bio-Rad).

Glycosyl compositional analysis was performed by gas chromatography–mass spectrometry (GLC–MS) of trimethylsilyl (TMS) methyl glycosides with myoinositol used as an internal standard (York et al., 1985). The samples were hydrolyzed with methanolic 1 M HCl at 80°C for 18 h. The released monosaccharides were dried under a stream of dry air and *N*-acetylated with 3:1:1 methanol/pyridine/acetic anhydride (v/v/v) at 100°C for 1 h. After cooling, samples were dried-down and trimethylsilylated with Tri-sil reagent (Pierce) for 30 min at 80°C. The resulting TMS derivatives were analyzed by GLC–MS, on Hewlett–Packard HP5890/HP5970 MSD gas chromatograph/mass spectrometer equipped with Supelco DB-1 fused silica capillary column (30 m × 0.25 mm I.D.) with helium as the carrier gas.

Linkage analyses were carried out by the slurry NaOH method modified from that of Ciucanu and Kerek (1984). Samples were dissolved in 0.5 mL dimethyl sulfoxide (DMSO) by stirring overnight at room temperature under a N_2 atmosphere. After dissolution, a freshly prepared slurry of NaOH in DMSO was added (0.5 mL) and the reaction mixture was stirred for 2 h at room temperature. Methylation was performed by the sequential addition of iodomethane (250 μL followed by 100 μL) at 30 min intervals. The permethylated monosaccharide was extracted into the organic phase after partitioning the reaction mixture between water and chloroform. The organic phase was then removed by evaporation under a stream of N_2. The permethylated OS was hydrolyzed with 4 M TFA (100°C, 6 h), reduced with $NaBH_4$, acetylated and the resulting partially methylated alditol acetates (PMAAs) were dissolved in dichloromethane and analyzed by GLC/MS using an HP-1 (from Hewlett–Packard) capillary column (25 m × 0.25 mm).

Oligosaccharides were analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS) using a 4700 Proteomics Analyzer instrument (Applied Biosystems). The OS samples were dissolved in water (1 μg/μL), mixed in a 1:1 (v/v) ratio with 0.5 M 2,5-dihydroxybenzoic acid (DHB) in methanol matrix solution, and spotted on a stainless steel MALDI plate. Spectra were acquired in both the positive and negative acquisition modes. The acceleration voltage was set to 20 kV and data were acquired in the reflectron mode with a 200-ms delay.

Table 1 | Description and susceptibility of strains to NHS and PB.

Strain	Genotype	% Survival in 12.5% NHS	PB MIC (μg/mL)
F62	Wild type	2.15 (±1.24)	50
WMS 100	F62 *lgtD::km*	51.8 (±18.1)*	50
WMS 101	WMS 100 *lgtD*$^+$	1.65 (±1.45)**	50
WMS 102	F62 *lgtA::km*	87.2 (±7.05)*	50
WMS 103	WMS 100 *lptA::spc*	2.37 (±2.17)**	0.2
WMS 104	F62 *lptA::spc*	ND	0.2
WMS 105	WMS 104 *lptA*$^+$	ND	50

*$p < 0.001$ compared to F62; **$p < 0.001$ compared WMS 100.
ND, not determined.*

RESULTS

ISOLATION OF A *Himar I* INSERTION MUTANT OF STRAIN F62 WITH DECREASED NHS-SUSCEPTIBILITY

We employed strain F62 in this investigation for a number of important reasons. First, it was originally employed in the early 1960s for the development of a gonococcal typing scheme that

was based on the state of piliation (Kellogg et al., 1963; piliated T1 and T2 vs. non-piliated T3 and T4 colony types). Second, it was employed in human experimental infection studies that identified pili as being important for establishing a gonococcal infection in male volunteers (Kellogg et al., 1968). Third, the LOS species it naturally produces have been defined (Yamasaki et al., 1991). Finally, since it is highly sensitive to killing by NHS, which is mediated by an antibody (natural IgM)- and CCP-dependent mechanism (Shafer et al., 1982), we reasoned that the killing capacity of NHS would be sufficient to use it as a selective pressure when screening the transposon library.

In order to isolate stable transposon mutants of strain F62 in the *Himar I mariner* library bank that expressed decreased susceptibility to NHS, the entire bank was grown in batch culture and exposed to 12.5% (v/v) serum. This process was repeated twice and ultimately a transposon mutant (WMS 100) expressing decreased susceptibility to NHS (average of 51.8% survival), compared to parent strain F62 (average of 2.15 % survival), was identified (**Table 1**). A randomly picked *Himar I* mutant was used as a control in the NHS bactericidal assay and it was as NHS-susceptible as parent strain F62 (data not presented) indicating that the possession of the transposon *per se* was not responsible to the elevated NHS-resistance displayed by WMS 100.

In order to locate the site of the transposon insertion in WMS 100, we employed ligation-PCR and DNA sequencing (Pelicic et al., 2000). These procedures mapped the transposon insertion to the 5′-end of the *lgtD* coding sequence between nucleotide positions 61 and 62 (data not presented). The *lgtD* gene is part of a five gene operon (*lgtABCDE*) and it, as well as *lgtA* and *lgtC*, are phase variable due to polymeric repeat sequences (Gotschlich, 1994; Yang and Gotschlich, 1996; Shafer et al., 2002). Due to the phase variable property of *lgtD*, it was not clear if the observed NHS-resistance property of WMS 100 was due to the transposon insertion or spontaneous mutation in other genes. To determine if transposon insertion within *lgtD* was responsible for the increased NHS-resistance property of strain WMS 100, DNA from this mutant was used to transform parent strain F62 for Kan^R. Analysis of 10 individual transformants revealed that all expressed decreased susceptibility to NHS (data not presented) similar to WMS 100, indicating that the serum susceptibility phenotype was linked to the transposon insertion. Further verification that the transposon insertion in *lgtD* in WMS 100 was responsible for its decreased NHS-susceptibility phenotype was obtained by complementation analysis in which the wild type *lgtD* gene from parental strain F62 was expressed ectopically from the *lctP–aspC* region under the control of the *lac* promoter (Skaar et al., 2002). With complemented strain WMS 101, we found that expression of the wild type *lgtD* resulted in an NHS-susceptible phenotype (average of 1.65 % survival) similar to strain F62 (**Table 1**).

CHARACTERIZATION OF LOS DUE TO TRANSPOSON INSERTION IN *lgtD*

The major LOS species produced by strains F62 have been structurally characterized (Yamasaki et al., 1991). Herein, we determined whether the LOS species produced by strain F62 would differ from WMS 100 and if those produced by complemented strain WMS 101 would resemble that of F62. We first identified the major LOS species produced by these strains by subjecting proteinase K digests of their whole cell lysates to SDS-PAGE. Silver staining of the gels revealed that F62 and complemented strain WMS 101 produced two in predominant LOS species (data not presented but summarized in **Table 2**) that migrated at 4.5 and 4.8 kDa. In contrast, mutant strain WMS 100 produced a single LOS species that migrated at 4.5 kDa. The 4.5-kDa species was reactive with mAb 3F11 (which recognizes Gal-GlcNAc-Gal-Glc-HepI), while the 4.8-kDa species was reactive with mAb 1-1-M (which recognizes GalNAc-Gal-GlcNAc-Gal-Glc-HepI; **Table 2**); neither of these species bound mAb 4C4, which recognizes Gal-Glc-HepI in the 3.6-kDa species previously linked to stable NHS-resistance in strain FA19 (Shafer et al., 2002). Based on earlier LOS-epitope mapping experiments, the electrophoretic mobility of the LOS species, the reported structure of F62 LOS species (Yamasaki et al., 1991) and the deduced *N*-acetylgalactosamine transferase activity of LgtD (Gotschlich, 1994), we hypothesized that the LOS produced by F62 and WMS 100 would differ by the absence of a terminal *N*-acetylgalactosamine in the α-chain. Indeed, chemical and structural analyses (**Figure 1**; **Table 3**) of the LOS produced by F62 and WMS 100 confirmed this inference.

For structural analysis the OSs from F62 and WMS 100 LOSs were treated with aqueous HF to remove any phosphate groups, as described in the Methods, in order to facilitate glycosyl composition and linkage analyses as well as MS analysis of the OSs. Glycosyl composition analysis of the HF-treated OS fraction from wild type F62 showed the presence of glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), heptose (Hep), and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Composition analysis of the HF-treated OS from the *lgtD::km* mutant strain (WMS 100) showed the same glycosyl components with the exception that GalNAc was not detected. Glycosyl linkage analysis showed that the F62 OS fraction contained terminally linked GalNAc (t-GalNAc), t-Gal, t-GlcNAc, 4-linked GlcNAc (4-GlcNAc), 4-Glc, 3-Gal, 2-Hep, and 3,4-Hep. The OS from mutant strain WMS 100 contained all of the same residues except that it was completely devoid of t-GalNAc and the t-Gal: 3-Gal ratio was increased.

The HF-treated OS fraction from each strain was subjected to MALDI–TOF MS analysis and the results are shown in **Figure 1** and in **Table 3**. The wild type F62 OS fraction shows two ion clusters

Table 2 | Lipooligosaccharide species produced by gonococcal strains.

Strain	LOS species (kDa)	Reactivity with mAbs		
		3F11	1-1-M	4C4
F62	4.5	+	–	–
	4.8	–	+	–
WMS 100	4.5	+	–	–
WMS 101	4.5	+	–	–
	4.8	–	+	–
WMS 102	3.6	–	–	+

Lipooligosaccharide species are given in kilodaltons (kDa) as determined by Tricine SDS-PAGE analysis.

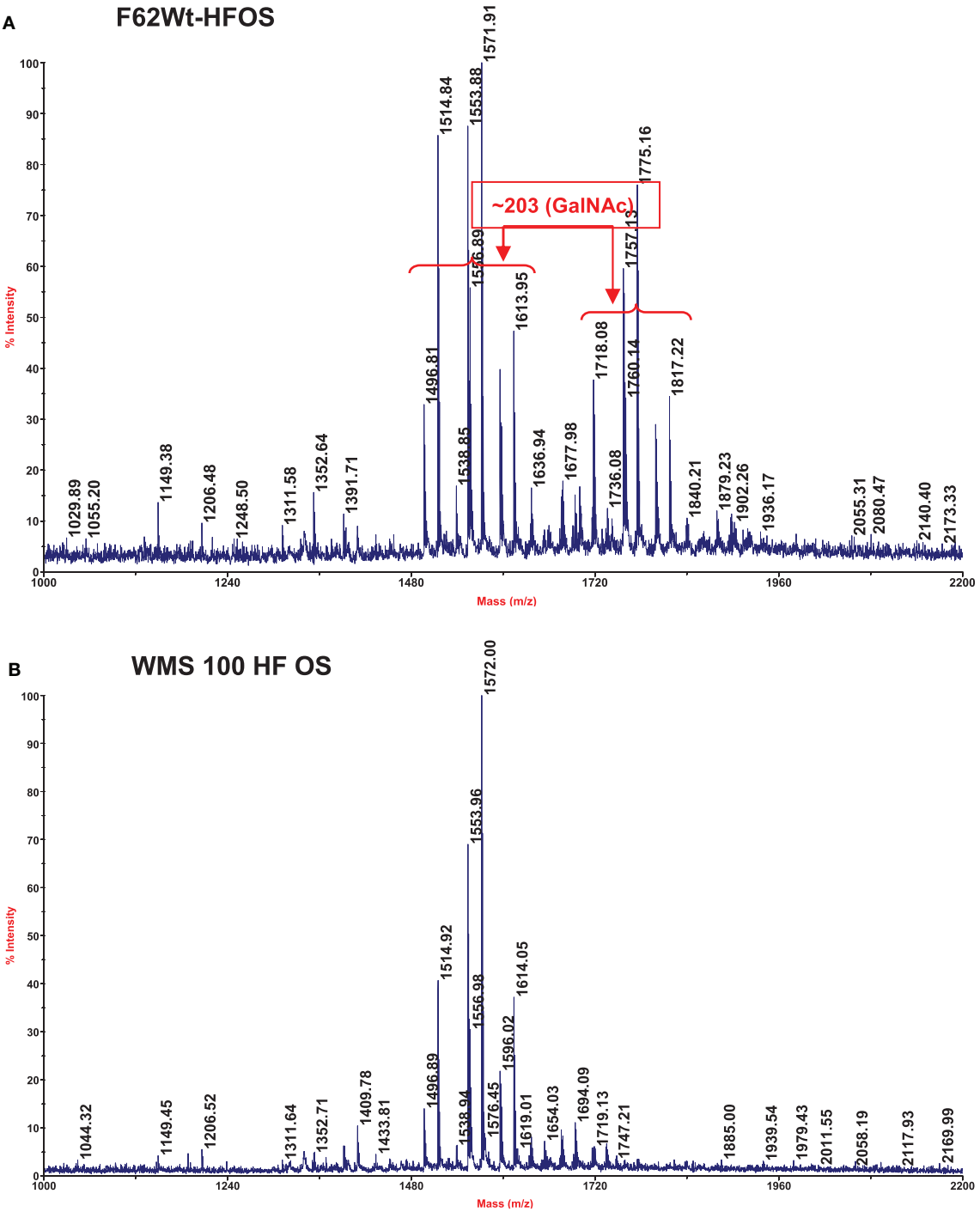

FIGURE 1 | Matrix-assisted laser desorption ionization–time of flight mass spectra, which was obtained in the negative mode, of the HF-treated OS from strains F62 (A) and WMS 100 (B) LOS.

with the higher molecular weight cluster differing from the lower molecular weight cluster by 203 mass units, the mass of a HexNAc residue (**Figure 1**). Both ion clusters also contained masses that differed by 57 mass units; the mass of glycine (Gly) indicated that Gly-containing structures with and without a HexNAc residue were present. The WMS 100 mutant OS fraction showed a single ion cluster that contains the same ions as observed for the lower molecular weight ions present in the wild type F62 OS fraction; i.e., only those ions that lack a HexNAc residue are present in the WMS 100 mutant OS. The proposed compositions, based on the glycosyl residue analysis described above, for the observed ions are shown in **Table 3** together with the observed and calculated *m/z* values.

Table 3 | Ions, $[M-H]^-$, observed on mass spectrometric analysis for the HF-treated oligosaccharides from wild type F62 and mutant WMS 100 lipooligosaccharides.

Obs. mass	Cal. mass	Proposed composition	F62	WMS 100
1556.9	1556.5	$Gal_2GlcGlcNAc_2Hep_2$.KdoOAc	+	+
1614.0	1613.6	$Gal_2GlcGlcNAc_2Hep_2$.KdoOAcGly	+	+
1514.8	1514.4	$Gal_2GlcGlcNAc_2Hep_2$.Kdo	+	+
1496.8	1496.4	$Gal_2GlcGlcNAc_2Hep_2$. Kdo (anhydro)	+	+
1571.9	1571.5	$Gal_2GlcGlcNAc_2Hep_2$.KdoGly	+	+
1553.9	1553.5	$Gal_2GlcGlcNAc_2Hep_2$. KdoGly (anhydro)	+	+
1760.1	1759.7	$GalNAcGal_2GlcGlcNAc_2Hep_2$KdoOAc	+	–
1817.2	1816.8	$GalNAcGal_2GlcGlcNAc_2Hep_2$KdoOAcGly	+	–
1718.1	1717.7	$GalNAcGal_2GlcGlcNAc_2Hep_2$Kdo	+	–
1775.2	1774.7	$GalNAcGal_2GlcGlcNAc_2Hep_2$KdoGly	+	–
1757.1	1756.7	$GalNAcGal_2GlcGlcNAc_2Hep_2$KdoGly (anhydro)	+	–

Shown are the observed (obs.) and calculated (cal.) masses for each OS species in strains.
F62 and WMS 100 with presence (+) or absence (–) of species designated.

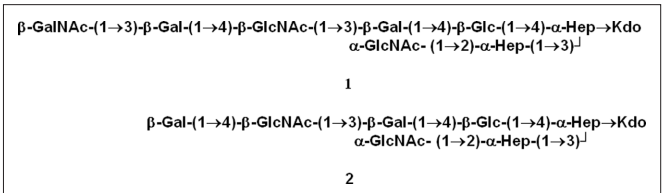

FIGURE 2 | The structures of the oligosaccharides reported by Yamasaki et al. (1991) for the LOS from strain F62. The composition, linkage, and MS results obtained in this report for F62 are consistent with these two structures and also with the conclusion that the *lgtD* mutant, WMS 100, contains only structure 2 which lacks the GalNAc residue.

The complete structures of the OSs for the F62 LOS have been reported (Yamasaki et al., 1991). These structures are shown in **Figure 2**. Our glycosyl composition, linkage, and MS results are consistent with this report by Yamasaki et al. (1991), which shows that the F62 LOS contains the two OSs shown in **Figure 2**, one with a t-GalNAc residue and one that lacks this residue. The lack of the t-GalNAc residue in the LOS from mutant WMS 100, as well as the MS, glycosyl composition, and linkage analyses is consistent with the conclusion that this LOS contains the OS structure shown in **Figure 2** that lacks the GalNAc residue and confirms that the *lgtD* gene encodes the GalNAc transferase.

PRODUCTION OF A 3.6-kDa LOS RESULTS IN INCREASED NHS-RESISTANCE IN STRAIN F62

Since our earlier work (Shafer et al., 2002) with the naturally NHS-resistant strain FA19 revealed that production of a 3.6-kDa LOS species with the LOS α-chain, which consists of Gal-Glc-HepI, was important in NHS-resistance, we next asked if production of a similarly truncated LOS by normally highly NHS-sensitive F62 would result in a level of NHS-resistance greater than that expressed by *lgtD* mutant strain WMS 100. We addressed this matter because our screening of the transposon library for NHS-resistant variants did not identify an insertion in *lgtA*, which based on our earlier work with strain FA19 (Shafer et al., 2002), is known to be important in NHS-resistance. In order to test the importance of *lgtA*, we introduced a non-polar *lgtA::km* mutation into strain F62 since LgtA is glycosyltransferase that adds GlcNAc to the terminal galactose in the α-chain of the 3.6-kDa LOS (Gotschlich, 1994; Shafer et al., 2002). A resulting *lgtA::km* transformant (WMS 102) was found to produce a single LOS species (3.6 kDa) that was reactive with mAb 4C4 (**Table 2**), but not 3F11 or 1-1-M. Importantly, WMS 102 expressed a level of NHS-resistance (average of 87.2% survival) that was substantially greater than that seen in the *lgtD* mutant WMS 100 (**Table 1**) or parental strain F62. However, incubation of WMS 102 (or WMS 100) in higher concentrations (>25%) of NHS resulted in less survival (<1%) of gonococci, while a naturally NHS-resistant strain (FA19) showed complete resistance (>100% survival). Thus, truncation of the LOS α-chain in F62 is not sufficient for complete NHS-resistance at higher serum levels presumably because it still possesses a Por1B protein as opposed to the Por1A produced by FA19 (Shafer et al., 2002). Nevertheless, since complement components can be at reduced levels at mucosal surfaces (Rice, 1989), phase-variants of naturally NHS-sensitive gonococci that produce truncated LOS species due to spontaneous mutations in *lgtA* or *lgtD* would have a survival advantage against this mechanism of innate host defense.

LOSS OF PEA MODIFICATION OF LIPID A INCREASES NHS-SUSCEPTIBILITY OF GONOCOCCI

Having determined that the length of the LOS α-chain is important in determining levels of NHS-susceptibility expressed by strain F62, we next examined whether the structure of its lipid A can also contribute to resistance. In this respect, we recently showed (Lewis et al., 2009) that loss of 4′ PEA from lipid A due to a null mutation in *lptA* could render normally NHS-resistant strain FA19 highly susceptible to NHS through a classical pathway-dependent mechanism. In order to test if such PEA modification of the lipid A was important in the ability of WMS 100 to express decreased susceptibility to NHS, we created an *lptA::spc* transformant (see strain WMS 103). The results (**Table 1**) showed that WMS 103 was substantially more NHS-susceptible (average of 1.3 % survival) than parent strain WMS 100. In fact, the *lptA* mutation negated the NHS-resistance profile afforded by the *lgtD* mutation. Thus, from our studies on the *Himar I* transposon mutant strain WMS 100, we conclude that both the length of the α-chain of the core oligosaccharide and 4′ lipid A PEA are important LOS structures that can determine the extent of gonococcal susceptibility to NHS.

LOS STRUCTURE AND CAP SUSCEPTIBILITY EXPRESSED BY GONOCOCCI

Cationic antimicrobial peptides are on the first line of innate host defense and can kill microbes directly by damaging their membranes or inhibiting intracellular processes (Brogden, 2005). They also indirectly kill microbes by virtue of their immunostimulatory, proinflammatory activities (Easton et al., 2009). Substantial evidence exists that alterations in lipid A structure can modulate levels of bacterial susceptibility to CAPs (Lee et al., 2004; Lewis et al., 2009). Accordingly, we tested if the LOS mutants described above would differ from parental strain F62 in their susceptibility to PMB, a model CAP. Although truncation of the LOS α-chain did not greatly influence the susceptibility of strain F62 to PMB, the presence of the *lptA::spc* mutation rendered strain F62 hypersusceptible to PMB (250-fold compared to parent strain F62; **Table 1**). Complementation of the mutant with the wild type *lptA* gene expressed from the *lacZ* promoter enhanced PMB-resistance by >60-fold (**Table 1**).

DISCUSSION

A long-standing interest in gonococcal research has been to identify and characterize the molecular mechanisms that explain how this strict human pathogen can resist innate and adaptive immunity systems. We have been interested in how the gonococcus can evade killing by the complement system and host defense peptides (e.g., CAPs). In previous studies, we and others identified genes of interest and constructed directed mutations for subsequent testing in antimicrobial assays. While this has proven to be a useful strategy, it is in fact biased for a select panel of genes. We have wanted an unbiased approach so that the killing power of host defense systems could be used to directly identify such mutants. To facilitate this effort, we employed the *Himar I mariner in vitro* transposon mutagenesis system for isolation of mutants of *N. gonorrhoeae* that manifest decreased susceptibility to complement-mediated killing by NHS. Through this strategy and with follow-up studies using gene-directed mutagenesis, we found that historically important strain F62 has LOS-dependent susceptibility to two components of innate host defense: the bactericidal action of NHS and CAPs. In this respect, the results presented herein and elsewhere (Shafer et al., 2002; Lewis et al., 2009) show that changes in the extension of the LOS α-chain, which can occur at high frequencies due to phase variable expression of genes in the *lgtABCDE* operon (Gotschlich, 1994; Yang and Gotschlich, 1996), can determine levels of gonococcal susceptibility to NHS (Shafer et al., 2002) while PEA decoration of lipid A is important in CAP-resistance (Lewis et al., 2009).

Normal human serum-resistance in gonococci has been linked to the serovar of the major outer membrane porin (Por1A vs. Por1B) produced. Por1B-producing gonococci are more frequently killed by NHS than Por1A strains. There is growing evidence, however, that the structure of LOS is important in NHS-resistance (Shafer et al., 2002; Ram et al., 2003) and other pathogenic steps used by gonococci during infection (van Putten, 1993), including bacterial attachment to host cells, stimulation of inflammatory responses and resistance to CAPs. Since an individual strain (and colony of that strain) can produce multiple LOS species due largely to the presence of phase variable expression of LOS biosynthesis genes (Apicella et al., 1987; Schneider et al., 1991; Preston et al., 1996; Burch et al., 1997; Tong et al., 2002), it has been difficult to ascribe with certainty an LOS chemotype with a particular pathogenic property. The results presented herein and that of earlier work on the role of the LOS α-chain and its extension by glycosyltransferases in determining whether gonococci can be killed by NHS (Shafer et al., 2002) demonstrate that extension of this chain past Gal-Glc-HepI-Kdo can result in NHS-susceptibility. The enhanced NHS-susceptibility observed in strains producing an extended LOS α-chain is likely due to natural IgM antibodies directed against epitopes present in such structures (Gotschlich, 1994). The NHS-resistance property displayed by transposon mutant WMS 100 may reflect a decrease in LOS affinity for such antibodies due to loss of the terminal *N*-acetyl galactosamine present in parental strain F62. Since *lgtD* is naturally phase variable, those gonococci having a phase-off gene might have a fitness advantage over those with a phase-on gene since they would have an enhanced ability to escape NHS-killing mediated by the CCP at sites where complement levels are limited. Under these circumstances, naturally occurring phase-off *lgtA* or *lgtD* mutants of Por1B-producing strains would have an advantage over parent PorIB strains with phase-on sequences of these genes. NHS-resistance expressed by gonococci also requires PEA modification of gonococcal lipid A and this decoration also enhances resistance to CAPs. We previously proposed (Lewis et al., 2009) that PEA decoration of lipid A influences the efficacy of complement regulatory protein C4BP binding to the surface of gonococci. C4BP binds to most gonococcal Por1A and select Por1B molecules. With respect to CAPs, loss of PEA from lipid A would enhance ionic interaction of positively charged groups in CAPs with phosphates at the 1 and 4′ positions of lipid A that become available, resulting in enhanced susceptibility of gonococci to CAPs. Using a murine model of vaginal infection, we are now testing whether mutations that impact LOS core oligosaccharide or lipid A structure and influence levels of resistance to mediators of innate host defense impact the *in vivo* fitness and survival of gonococci during infection.

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The gonococcal genetic island and type IV secretion in the pathogenic *Neisseria*

Meghan E. Ramsey, Katelynn L. Woodhams and Joseph P. Dillard*

Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI, USA

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Eric P. Skaar, Vanderbilt University, USA
Nathan John Weyand, BIO5 Institute, USA

****Correspondence:***
Joseph P. Dillard, Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, 6245 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706, USA.
e-mail: jpdillard@wisc.edu

Eighty percent of *Neisseria gonorrhoeae* strains and some *Neisseria meningitidis* strains encode a 57-kb gonococcal genetic island (GGI). The GGI was horizontally acquired and is inserted in the chromosome at the replication terminus. The GGI is flanked by direct repeats, and site-specific recombination at these sites results in excision of the GGI and may be responsible for its original acquisition. Although the role of the GGI in *N. meningitidis* is unclear, the GGI in *N. gonorrhoeae* encodes a type IV secretion system (T4SS). T4SS are versatile multi-protein complexes and include both conjugation systems as well as effector systems that translocate either proteins or DNA–protein complexes. In *N. gonorrhoeae*, the T4SS secretes single-stranded chromosomal DNA into the extracellular milieu in a contact-independent manner. Importantly, the DNA secreted through the T4SS is effective in natural transformation and therefore contributes to the spread of genetic information through *Neisseria* populations. Mutagenesis experiments have identified genes for DNA secretion including those encoding putative structural components of the apparatus, peptidoglycanases which may act in assembly, and relaxosome components for processing the DNA and delivering it to the apparatus. The T4SS may also play a role in infection by *N. gonorrhoeae*. During intracellular infection, *N. gonorrhoeae* requires the Ton complex for iron acquisition and survival. However, *N. gonorrhoeae* strains that do not express the Ton complex can survive intracellularly if they express structural components of the T4SS. These data provide evidence that the T4SS is expressed during intracellular infection and suggest that the T4SS may provide an advantage for intracellular survival. Here we review our current understanding of how the GGI and type IV secretion affect natural transformation and pathogenesis in *N. gonorrhoeae* and *N. meningitidis*.

Keywords: type IV secretion system, gonococcal genetic island, *Neisseria gonorrhoeae, Neisseria meningitidis*, natural transformation

INTRODUCTION

Neisseria gonorrhoeae (gonococcus, GC) and *Neisseria meningitidis* (meningococcus, MC) are both exclusively pathogenic to humans, causing the sexually transmitted disease gonorrhea and bacterial meningitis respectively. Approximately 80% of gonococcal strains carry a genomic island called the gonococcal genetic island (GGI; Dillard and Seifert, 2001). Recently, this island was also identified in some strains of *N. meningitidis*, although it has not been found in any of the commensal *Neisseria* species (Dillard and Seifert, 2001; Snyder et al., 2005). In *N. gonorrhoeae*, the GGI is 57 kb in size and is integrated into the gonococcal chromosome at the replication terminus (Dillard and Seifert, 2001; Hamilton et al., 2005).

Sequence characteristics of the GGI indicate that, like many other genomic islands in bacteria, the GGI was horizontally acquired (Hacker and Kaper, 2000; Hamilton et al., 2005). The G + C content of the GGI is 44%, significantly lower than the 51% average for the sequenced *N. gonorrhoeae* genomes. Dinucleotide frequencies also vary between the GGI and the core genome (Karlin, 1998; Hamilton et al., 2005). Additionally, horizontally acquired genetic islands are often flanked by short direct repeats (Hacker and Kaper, 2000). The GGI is flanked by copies of the *dif* site, and site-specific recombination at these sites may have resulted in the initial acquisition of the island (Hamilton et al., 2005; Domínguez et al., 2011). The GGI also differs from the core genome in the number of DNA uptake sequences (DUS) it contains. The DUS is required for DNA uptake during natural transformation and is present at an average frequency of one DUS per every 1.1 kb in neisserial genomes (Goodman and Scocca, 1988; Smith et al., 1999). In *N. gonorrhoeae* strain MS11, the GGI contains only six copies of the DUS, while the 57-kb region flanking the GGI contains 53 copies (Hamilton et al., 2005). Together, these observations suggest that the GGI was likely horizontally acquired and has stably integrated into the gonococcal chromosome.

Genetic islands often encode genes important for pathogenesis, metabolism, or ecological fitness and therefore may provide their host species with selective advantages (Hacker and Kaper, 2000; Gal-Mor and Finlay, 2006). The role of the GGI in meningococci remains unclear, but in gonococci, it has been shown that the GGI encodes a type IV secretion system (T4SS) that secretes DNA into the extracellular environment (Dillard and Seifert, 2001; Hamilton et al., 2005). Both *N. gonorrhoeae* and *N. meningitidis* are naturally competent, and the DNA secreted by the T4SS is effective for natural transformation in gonococci (Dillard and Seifert, 2001; Hamilton and Dillard, 2006). Therefore, the GGI-encoded T4SS contributes to horizontal gene transfer within *Neisseria* populations.

The role of the GGI in gonococcal pathogenesis is less clear, and several studies have investigated whether the presence of the GGI is correlated with different disease types. The mere presence of the GGI does not correlate with any particular disease presentation. The GGI was identified at a similar frequency in gonococcal strains isolated from women with either symptomatic or asymptomatic infection (Wu et al., 2011). The GGI is also present at similar frequencies in low-passage clinical isolates causing both pelvic inflammatory disease and local infection. However, the GGI is variable at the *traG/atlA* locus, and certain versions of the GGI have been correlated with disseminated gonococcal infections (DGI; Dillard and Seifert, 2001). TraG is a predicted T4SS protein, while AtlA is one of two peptidoglycanases encoded by the genetic island (Dillard and Seifert, 2001; Hamilton et al., 2001, 2005; Lawley et al., 2003; Kohler et al., 2007). Early work identified an allele of *traG* that included the *sac-4* locus, a locus that had previously been implicated in serum-resistance (McShan et al., 1987; Nowicki et al., 1997; Dillard and Seifert, 2001). The *sac-4* allele of *traG* is not present in all GGI variants, however, and the presence of the *atlA* gene is also variable (Dillard and Seifert, 2001). In a survey of low-passage clinical isolates, *atlA* and the *sac-4* allele of *traG* were each correlated with isolates causing DGI (Dillard and Seifert, 2001). These observations suggest that some versions of the GGI may be correlated with an increased ability of *N. gonorrhoeae* strains to cause systemic infection.

THE GGI ENCODES A TYPE IV SECRETION SYSTEM

The GGI contains 62 open reading frames, and many of these genes are homologous to T4SS genes (Hamilton et al., 2005; **Figure 1**). T4SSs have been identified in many Gram-negative and Gram-positive bacteria, and include both conjugation systems as well as effector systems that translocate proteins or DNA–protein complexes (Christie, 2001; Alvarez-Martinez and Christie, 2009). The F-plasmid conjugation system of *E. coli* and the VirB/D T4SS of *Agrobacterium tumefaciens* are among the best characterized. *A. tumefaciens* uses the VirB/D system to secrete both proteins and oncogenic T-DNA directly into a plant cell, leading to the formation of crown gall tumors (Zhu et al., 2000). Many human pathogens also encode T4SSs that are involved in pathogenesis including *Bordetella pertussis*, which secretes pertussis toxin using a T4SS, and *Legionella pneumophila*, which secretes numerous effectors important for intracellular survival in macrophages (Shrivastava and Miller, 2009; Hubber and Roy, 2010). Expression of the *cag* T4SS in *Helicobacter pylori* results in the secretion of the virulence factor CagA as well as increased NF-κB activation in host cells mediated by Nod1 detection of peptidoglycan fragments (Viala et al., 2004; Backert and Selbach, 2008).

The GGI encodes 23 proteins with significant similarity to T4SS proteins, most of them contained in the first 28 kb of the island (**Figure 1**). Many of these genes show similarity to genes from the *E. coli* F-plasmid conjugation system, and the order of the genes in the GGI is highly similar to the IncF family of conjugative plasmids (Hamilton et al., 2005). There are several notable differences in gene organization between the GGI and the F-plasmid conjugation system, however, including *ltgX*, which encodes a peptidoglycan transglycosylase similar to the *geneX* product (Orf169) from F-plasmid, as well as *traD* and *traI*, which encode the putative coupling protein and the relaxase, respectively (Frost et al., 1994; Hamilton et al., 2005). In the GGI, *ltgX* is the first gene in a predicted operon that contains the majority of the T4SS genes, while *traI* and *traD* are transcribed divergently (Hamilton et al., 2005; **Figure 1**). Interestingly, the relaxase encoded by the GGI is not phylogenetically related to the IncF family of relaxases but clusters instead with the IncH family of relaxases that includes relaxases from many integrative conjugative elements and genetic islands (Salgado-Pabón et al., 2007; Garcillan-Barcia et al., 2009).

In addition to the T4SS homologs, the GGI encodes 39 open reading frames, several of which are homologous to hypothetical genes (Hamilton et al., 2005). Some of these open reading frames do show sequence similarity to DNA-binding or processing proteins in sequence databases, including two DNA methylases (Ydg and YdhA), a helicase (Yea), a topoisomerase (TopB), and a single-stranded binding protein (Ssb; Hamilton et al., 2005). This region of the GGI has not been investigated as much as the region encoding the T4SS genes. However, this region might be a good place to look for as-yet unidentified protein substrates of the T4SS, additional unknown genes for T4S, or other factors that have caused the GGI to be maintained in 80% of gonococcal strains.

THE GONOCOCCAL T4SS SECRETES CHROMOSOMAL DNA

The gonococcal T4SS secretes chromosomal DNA into the extracellular environment in a contact-independent manner (Dillard and Seifert, 2001). Addition of DNaseI to the culture medium blocks the

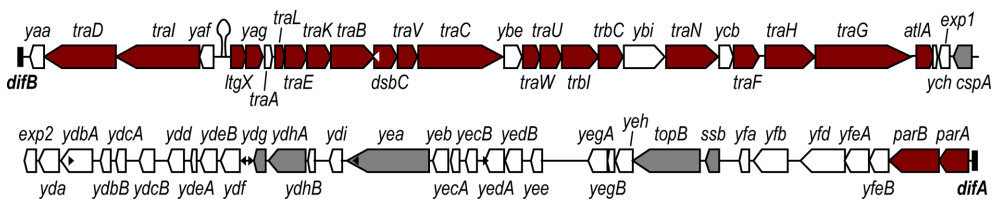

FIGURE 1 | Map of the gonococcal genetic island (GGI) from *N. gonorrhoeae* strain MS11. The GGI in *N. gonorrhoeae* strain MS11 is 57 kb and is inserted in the chromosome at the *dif* site. The GGI encodes many homologs of type IV secretion system genes (red), as well as 33 genes of unknown function (white). There are six genes encoding homologs for DNA-binding or processing proteins (gray). In this map, *traA* is colored white despite encoding a homolog for the conjugative pilin subunit because it is not required for DNA secretion. The genes for the lytic transglycosylase AtlA and the putative peptidoglycan binding protein Yag are colored red because they are required for DNA secretion in gonococci even though they are not similar to known T4SS genes in other systems. The sequences of *traA*, *traG*, and *atlA* are variable between strains. The GGI contains six copies of the DNA uptake sequence (DUS, white and black triangles), significantly fewer copies than the flanking 57 kb of chromosomal DNA. A putative origin of transfer (*oriT*) has also been identified in the intergenic region between the *yaf* and *ltgX* genes (stem-loop).

transfer of a genetic marker during co-culture, indicating that the secreted DNA is exposed to the extracellular environment and is not directly transferred between cells (Dillard and Seifert, 2001). Based on the susceptibility of the secreted DNA to different nucleases, it was determined that the DNA secreted by the gonococcal T4SS is single-stranded and blocked at the 5′ end (Salgado-Pabón et al., 2007). We hypothesize that the DNA remains bound to the relaxase at the 5′ end, as is the case for many other T4SSs (Young and Nester, 1988; Pansegrau et al., 1990). Although it is likely that the relaxase remains bound to the 5′ end of the secreted DNA, we currently have no direct evidence for protein secretion through the gonococcal T4SS. However, the secretion of proteins is highly probable. In fact, one model regarding DNA-secretory T4SSs is that they are actually protein secretion machines in which associated DNA comes along for the ride (Hazes and Frost, 2008). The contact-independent secretion of DNA by gonococci is unique among characterized T4SSs. Although *B. pertussis* secretes pertussis toxin directly into the extracellular environment, all other characterized T4SSs that transport DNA secrete it directly into a recipient or host cell (Cascales and Christie, 2003; Alvarez-Martinez and Christie, 2009).

Measurement of the transfer of a genetic marker from a donor to a recipient strain in co-culture has been used to identify some of the genes required for T4S in gonococci (Dillard and Seifert, 2001; Hamilton et al., 2001; **Figure 2A**). In this co-culture assay, wild-type donor strains are compared to donor strains with putative T4SS genes mutated. The donor strains also carry a chloramphenicol resistance (Cm^R) marker and a mutation in either *recA* or *pilT* to ensure that they cannot also act as recipients in transformation. The donors are then co-cultured with a recipient strain that carries a spectinomycin resistance (Sp^R) marker. After a period of 2–4 hours, bacteria from the co-culture are plated to enumerate Cm^R Sp^R colonies. Co-culture with a donor carrying a mutation in a T4SS gene such as *traG* results in between 50 and 500-fold fewer Cm^R Sp^R transformants than co-culture with a wild-type donor (**Figure 2B**; Dillard and Seifert, 2001; Hamilton et al., 2001).

Gonococci readily undergo autolysis, and it was previously assumed that DNA released by autolysis was the primary source of DNA for natural transformation (Hamilton and Dillard, 2006). However, in the co-culture assay described above, most of the transformation occurs during log-phase growth, suggesting that DNA donation by T4S is independent of autolysis (Dillard and Seifert, 2001). Indeed, various measures of autolysis indicate that T4SS mutants are not defective in autolysis. Cell viability is comparable between T4SS mutants and wild-type cells, and T4SS mutants release similar levels of both RNA and the cytoplasmic protein chloramphenicol acetyltransferase (CAT) compared to wild-type cells (Dillard and Seifert, 2001; Hamilton et al., 2005; Kohler et al., 2007). Furthermore, even if co-culture transformation is allowed to progress into stationary phase when autolysis occurs, DNA transfer occurs at a much higher frequency with a wild-type donor compared to a T4SS mutant donor (Dillard and Seifert, 2001). Thus, although DNA released by autolysis can contribute to transformation, DNA donation by the T4SS is independent of autolysis and appears to work better for transformation. It is unclear why this should be the case. Single-stranded DNA and double-stranded DNA transform gonococci with similar efficiency (Stein, 1991). Perhaps proteins bound to the secreted DNA encourage its uptake or its recombination during natural transformation.

Little is known about the regulation of DNA secretion in gonococci, and the GGI does not encode homologs of the regulatory factors that have been described for F-plasmid (Frost et al., 1994; Hamilton et al., 2005). Recently, however, it was shown that DNA secretion is increased in gonococci producing type IV pili, and that this increased secretion is due in part to increased expression levels of the relaxase TraI and the coupling protein TraD (Salgado-Pabón et al., 2010). Because *traI* and *traD* are divergently transcribed from the majority of the T4SS genes, differential regulation of these genes may allow regulation of DNA secretion (Salgado-Pabón et al., 2010). In gonococci, type IV pili are involved in DNA uptake as well as mediating host attachment and twitching motility (Swanson, 1973, 1978; Biswas et al., 1977). It is unclear why DNA secretion should be associated with piliation. One hypothesis is that DNA secretion may occur in response to signals released by piliated gonococci indicating the presence of competent members of the population (Salgado-Pabón et al., 2010).

THE PROTEINS OF THE GONOCOCCAL T4SS

We propose a model of T4S in gonococci based on the similarity of the proteins encoded by the GGI with homologs from other characterized T4SSs (**Figure 3**). The T4SS proteins contained in the GGI fulfill several functions for T4S, including DNA processing, recruitment to the secretion apparatus, structural components of the apparatus, and possible pilus assembly.

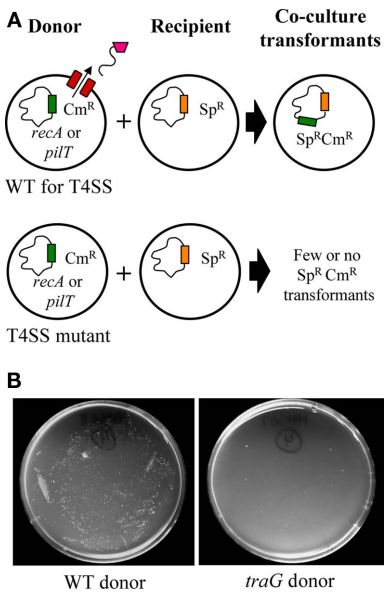

FIGURE 2 | The gonococcal T4SS secretes chromosomal DNA active in natural transformation. (A) To assay for DNA secretion, a donor strain that is either wild-type (WT) or mutant for various T4SS genes is grown in co-culture with a recipient strain. The donor strain carries a chloramphenicol resistance marker (Cm^R), while the recipient is marked with spectinomycin resistance (Sp^R). The donor strain also carries a mutation in either *pilT* or *recA* to render it transformation-deficient and to ensure that it cannot act as a recipient. Following co-culture, bacteria are plated to double-selective plates to enumerate the number of Cm^R Sp^R transformants. **(B)** Co-culture with a wild-type donor yields significantly more Cm^R Sp^R colonies (left) compared to co-culture using a *traG* mutant donor (right).

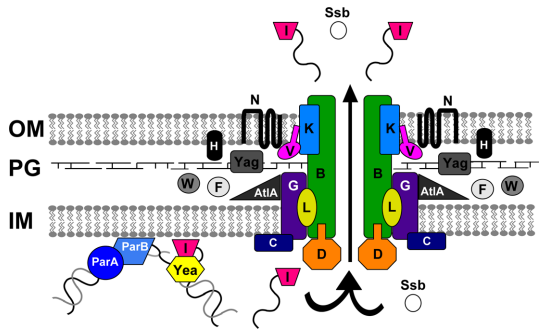

FIGURE 3 | Model of type IV secretion in gonococci. Predicted functions can be assigned to many of the proteins encoded by the GGI based on their similarity to other characterized type IV secretion system proteins. The putative partitioning proteins ParA and ParB may bring the DNA to the apparatus. TraI nicks the DNA at the origin of transfer (*oriT*), and a helicase, possibly Yea, unwinds it. The relaxase likely remains bound to the 5′ end of the DNA and pilots it to the secretion apparatus, where it may dock with the putative coupling protein, TraD. The putative ATPase TraC and the mating-pair stabilization homolog TraG likely contribute to the inner membrane pore. Periplasmic proteins such as Yag, TraW, TraF, TraH, and AtlA are likely involved in apparatus assembly or in making localized breaks in the peptidoglycan layer. The DNA is then secreted through the transmembrane apparatus, the core proteins of which are predicted to be TraB, TraK, and TraV. TraN may form part of the outer membrane pore. The DNA is secreted into the extracellular environment. Proteins such as single-stranded binding protein (Ssb) may also be secreted, but there is no evidence as-yet for protein secretion by the gonococcal T4SS. OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

DNA PROCESSING AND RECRUITMENT

The proteins responsible for DNA processing and recruiting to the secretion apparatus have been well-studied in other T4SSs. In gonococci, DNA secretion likely begins when the DNA is brought into proximity of the T4SS machinery for processing. The GGI encodes homologs for both ParA and ParB, the partitioning proteins required for proper segregation of chromosomal and plasmid DNA during cell division (Leonard et al., 2005). In *A. tumefaciens*, a ParA-like protein (VirC1) is involved in localizing the T-DNA to the T4S apparatus for secretion (Leonard et al., 2005; Atmakuri et al., 2007). In the GGI, ParA, and ParB are separated from the majority of the T4SS genes by approximately 30 kb (Hamilton et al., 2005; **Figure 1**). However, ParA is essential for DNA secretion in gonococci, and a point mutation in the putative Walker box abolished DNA secretion (Hamilton et al., 2005). We hypothesize that ParA and ParB are required for bringing chromosomal DNA to the secretion apparatus where it can be processed by the relaxase.

Relaxases bind to and nick double-stranded DNA at a specific sequence known as the origin of transfer (*oriT*; Grinter, 1981). The reaction is mediated by one or two catalytic tyrosines and can result in the covalent attachment of the relaxase to the 5′ end of the DNA (Pansegrau et al., 1990). Previously characterized relaxases contain three motifs: the catalytic tyrosines, a motif facilitating DNA–protein interactions, and a histidine-rich motif involved in metal coordination (Pansegrau et al., 1994). Like most characterized relaxases, two tyrosines have been identified in gonococcal TraI that are required for efficient DNA secretion (Y93 and Y201). Mutation of Y93 completely blocks DNA transfer, while mutation of Y201 results in an intermediate level of transfer (Salgado-Pabón et al., 2007).

However, the GGI-encoded TraI differs significantly from other previously characterized relaxases. Point mutations altering the histidine-rich motif of *N. gonorrhoeae* TraI do not affect DNA secretion, suggesting that this motif may not be required for T4S in gonococci (Salgado-Pabón et al., 2007). *N. gonorrhoeae* TraI also differs from other relaxases by the presence of an HD phosphohydrolase domain. HD phosphohydrolase domains can sometimes coordinate metals, and some mutations in this region of gonococcal TraI result in diminished DNA secretion, suggesting that this domain may be fulfilling the role of the histidine-rich motif (Salgado-Pabón et al., 2007). Finally, *N. gonorrhoeae* TraI is distinguished from other characterized relaxases by the presence of an N-terminal hydrophobic region. This hydrophobic region is predicted to form an amphipathic helix, and it has been shown that this region enables TraI to associate with the cell membrane (Salgado-Pabón et al., 2007).

DNA processing by the relaxase occurs at a specific sequence known as the *oriT*. A single putative *oriT* was identified within the GGI between the genes *yaf* and *ltgX* (**Figure 1**) that exhibits many of the characteristics of previously characterized *oriT* sequences. The sequence contains an inverted repeat and is located near the relaxase gene in an A-T rich region between divergent promoters (Lanka and Wilkins, 1995). An insertion disrupting this sequence reduced DNA secretion, while introduction of the inverted repeat region elsewhere on the chromosome restored DNA secretion. This result suggests that the *oriT* is located within the GGI between *yaf* and *ltgX* and that this region contains the only *oriT* on the gonococcal chromosome (Salgado-Pabón et al., 2007).

Once the DNA has been processed by the relaxase, the nucleoprotein complex must be targeted to the T4SS for secretion. This function is provided by coupling proteins – inner membrane ATPases that are widely conserved among T4SSs and couple the recognition of the substrate with the energy production necessary for translocation (de Paz et al., 2010). The GGI encodes the putative coupling protein TraD, and deletion of *traD* diminished DNA secretion (Salgado-Pabón et al., 2010). Thus, in a model of gonococcal T4S, it is likely that ParA and ParB bring the DNA to the secretion apparatus where it is processed by TraI at the *oriT* contained on the chromosome within the GGI. We hypothesize that, as is the case for Hfr transfer in *E. coli*, the DNA would then be unwound by a helicase, possibly Yea, prior to secretion, and strand-replacement synthesis would regenerate the chromosome in the donor cell (Willetts and Wilkins, 1984). Since only one *oriT* is present, it is predicted that this region would be secreted first followed by the rest of the chromosome, with secretion of the chromosome proceeding in a unidirectional manner until the *oriT* is reached again.

TRANSMEMBRANE APPARATUS

Following processing and recruitment, the DNA substrate must pass through the T4SS apparatus, which forms a continuous channel across the inner and outer membranes (Fronzes et al., 2009a). The inner membrane pore likely consists of the ATPase TraC, the coupling protein TraD, the mating-pair stabilization protein TraG, and the N-terminal domain of TraB. Mutations in both *traG* and *traD* result in reduced DNA secretion, supporting the idea that these proteins may contribute to the T4SS apparatus in gonococci

(Hamilton et al., 2001; Salgado-Pabón et al., 2010). The *traG* gene is highly variable between strains. In addition to the *sac-4* allele of *traG* that is correlated with isolates causing disseminated infection, an allele of *traG* followed by a putative endopeptidase-encoding gene *eppA* has also been identified (Dillard and Seifert, 2001; Kohler et al., 2007). The function of TraG in T4S in gonococci remains unclear.

Our understanding of the structural biology of the core T4SS apparatus has greatly increased with the first cryo-electron tomography and crystal structures of a core T4SS apparatus from the IncN conjugative plasmid pKM101 (Chandran et al., 2009; Fronzes et al., 2009b). In these structures, the core T4S complex was composed of 14 copies each of the pKM101 homologs of TraK, TraV, and TraB (Chandran et al., 2009; Fronzes et al., 2009b). TraK homologs belong to a family of secretin-like proteins, while TraV homologs are lipoproteins required to stabilize the secretin. TraK and TraV homologs have been localized to the outer membrane in many other T4SSs and form a heterodimer linked by disulfide bonds (Baron et al., 1997; Harris et al., 2001). TraK and TraV also interact in gonococci, and we predict that these proteins also contribute to the outer membrane pore of the gonococcal T4SS (Hackett et al., 2008). In the crystal structure from pKM101, a two-helix bundle in the C-terminal domain of the TraB homolog was found to cross the outer membrane and was surface-exposed, making it the first characterized protein to insert in both the inner and outer membranes (Chandran et al., 2009). The two-helix bundle is conserved in gonococcal TraB, and we predict that this portion of TraB may also be surface-exposed (our unpublished observation).

Because *N. gonorrhoeae* and *N. meningitidis* can only be found within human hosts, it is noteworthy that any surface-exposed proteins in the T4SS could be exposed to the immune system of the host. Predicted surface-exposed proteins include TraK and TraB, as well as the protein TraN, which plays a role in stabilizing interactions between mating-pairs in *E. coli* F-plasmid (Lawley et al., 2003). Since gonococci secrete DNA in a contact-independent manner, the formation of mating-pairs is unnecessary, and it is unclear what role TraN might be playing in gonococci. However, mutation of *traN* greatly reduces DNA secretion in gonococci, indicating that TraN is required for T4S (Hamilton et al., 2005). Many surface-exposed proteins in *Neisseria* undergo high-frequency antigenic or phase variation, including the opacity proteins, pilin, and porin (Stern et al., 1986; Gibbs et al., 1989; Fudyk et al., 1999; Hill and Davies, 2009). However, based on eight sequenced gonococcal genomes that contain the GGI as well as sequence data from two additional gonococcal strains, predicted surface-exposed T4SS proteins such as TraN, TraK, and TraB show only minor sequence differences between strains, suggesting that these proteins may be rare conserved surface proteins in *Neisseria* (GenBank accession no. CP001050 and http://www.broadinstitute.org/annotation/genome/neisseria_gonorrhoeae/GenomesIndex.html; Hackett et al., 2008).

PILUS ASSEMBLY

Type IV secretion systems such as *E. coli* F-plasmid or the *A. tumefaciens* VirB/D system construct conjugative pili (Kado, 2000; Lawley et al., 2003). The importance of the conjugative pilus in DNA transfer is unclear, although several models have been proposed. An early model proposed that the pilus could serve as a conduit for the DNA to travel between donor and recipient. Alternatively, the retraction of the pilus could facilitate close contact between the donor and recipient and allow the direct transfer of DNA (Kado, 2000). An interesting new hypothesis suggests that the pilus may help the donor cell to "sample" the surrounding environment for potential recipients (Silverman and Clarke, 2010). The GGI encodes a homolog for the pilin subunit TraA (**Figure 1**). The *traA* sequence is variable, with both a short allele and a long allele present in *Neisseria* strains, and the GGI in the gonococcal strain MS11 contains the short allele (Jain et al., 2008; Llosa et al., 2009). We predict that the longer allele encodes the full-length pilin protein, while the short allele may encode a pilin that cannot be assembled. However, TraA is not required for T4S in gonococci, since deletion of *traA* has no effect on DNA secretion (Immel et al., 2003). If the role of the conjugative pilus is to facilitate close contact between a donor and a recipient cell, then a pilus may not be necessary for gonococcal T4S since the DNA is secreted into the extracellular environment and the donor does not require contact with the recipient for natural transformation.

THE GGI IN *N. MENINGITIDIS*

When the GGI was first identified in *N. gonorrhoeae*, attempts were also made to detect the GGI in a panel of symbiotic *Neisseria* species and *N. meningitidis* using PCR or low-stringency Southern blotting for *traG* and *atlA*. However, *traG* and *atlA* are variable in the GGI, and it was not detected (Dillard and Seifert, 2001). The GGI was also absent in the genome sequences for meningococcal serotype A and B strains that had been published. Thus, it was unexpected when versions of the GGI were identified in meningococci in 2005 (Snyder et al., 2005). Snyder et al. (2005) used meningococcal chromosomal DNA to probe a microarray containing a single oligonucleotide for each gene in the GGI. Six meningococcal strains were found to have the GGI, and three island types were represented among the strains. No meningococcal strain had a GGI identical to that of gonococcal strain MS11, and three of the strains had large deletions of T4SS genes. The variant GGIs were found in serotype H, W-135, and Z strains. Although it appears the GGI is not present in most strains of *N. meningitidis*, it will be interesting to learn what portion of the meningococcal population carries a version of the GGI and whether it is present in strains from sequence types common in invasive disease or symbiotic colonization.

Recently, a draft genome from meningococcal strain α275, which contains the GGI, has become available (GenBank accession no. AM889138). Analysis of the sequence shows that the GGI from this strain is 64 kb and differs from the GGI in *N. gonorrhoeae* strain MS11 in several ways (**Figure 4**). The GGI in α275 contains the long allele of *traA*, a variant allele of *traG*, and the putative endopeptidase-encoding gene, *eppA*. Each of these variations is also present in some gonococcal strains. Additionally, a deletion from within *traD* results in the truncation of *traD*, and *difB* being contained within the *traD* coding region. Another significant difference is the presence of an insertion of IS*1655* just after the start codon of *traK*. Analysis of the inserted sequence did not reveal a possible ribosome binding site or in-frame start for the interrupted *traK* (our unpublished observation). As a result of the insertion in *traK* and deletion in *traD*, it is unlikely that α275 is capable of making a functional T4SS or secreting DNA.

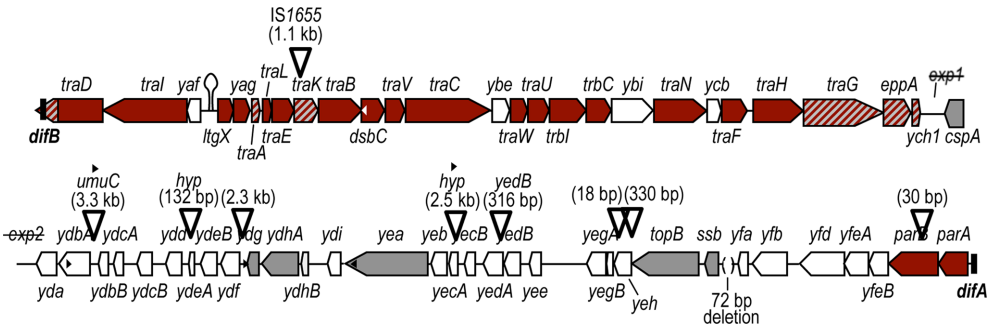

FIGURE 4 | Map of the GGI from *N. meningitidis* strain α275. The GGI in α275 is 64 kb and differs in several ways from the GGI in *N. gonorrhoeae* MS11. Insertions in the α275 GGI are indicated by inverted triangles, with the text above the triangle indicating both the size of the insertion as well as the identity of the sequence, if known. Two insertions showed similarity to hypothetical genes from *N. gonorrhoeae*, *N. meningitidis*, *N. mucosa*, and *N. flava* (*hyp*). If no sequence is indicated above a triangle, the insertion is not similar to any sequence in GenBank. Genes that are significantly different from homologs in the GGI of *N. gonorrhoeae* MS11 are indicated by red and gray diagonal lines. In α275, a deletion in *traD* results in the truncation of *traD*, and *difB* is contained within the *traD* coding region. This GGI contains the long allele of *traA*, and IS*1655* is inserted just after the start codon of *traK*. A truncated allele of *traG* is present followed by the hypothetical endopeptidase *eppA* (also found in some gonococci). There is a deletion of *exp1*. The α275 GGI also has an insertion of partial DNA polymerase V subunit *umuC* at *ydbB* and an insertion of partial *yedB* at *yedA*. A small deletion upstream of *ssb* is indicated by parentheses. DNA uptake sequences (DUS) are indicated by small black or white triangles. In addition to the four DUS indicated in the figure, two DUS are located in the inserted sequences (black triangles over the inserted sequence).

Based on the sequence of the GGI in α275 and the hybridization studies of Snyder et al. (2005), it appears that several of the meningococcal GGIs are degrading, acquiring deletions and insertions. It is unlikely that they encode functional secretion systems. However, three of the GGIs identified in meningococci do carry most of the T4SS genes and might yet produce a functional T4SS (Snyder et al., 2005). It is unclear whether these secretion systems would function for DNA secretion as the T4SS does in gonococci. None of the meningococcal GGIs carry *atlA*, which encodes a peptidoglycanase essential for DNA secretion in gonococci (Dillard and Seifert, 2001; Kohler et al., 2007). One of the three mostly intact GGI variants has *parB* deleted, a gene which would be expected to be required for DNA secretion, since a functional ParA is required (Hamilton et al., 2005). Contrary to what was reported by Snyder et al. (2005) in their hybridization studies, PCR analysis and sequencing data indicate that all of the meningococcal GGIs do encode ParA (our unpublished observation). It is possible that these T4SSs may have some function other than DNA secretion. They could secrete proteins, or the apparatus might serve an adhesive function, as was seen for a T4SS in *Bartonella* (Vayssier-Taussat et al., 2010).

THE T4SS ALLOWS TON-INDEPENDENT SURVIVAL OF GONOCOCCI DURING INTRACELLULAR INFECTION

The Ton complex, consisting of TonB, ExbB, and ExbD, is required for iron transport into both gonococci and meningococci. Previous work in gonococci and meningococci established that the Ton complex is required for intracellular survival during epithelial cell infection (Larson et al., 2002; Hagen and Cornelissen, 2006). However, Zola et al. (2010) found that gonococcal strain MS11 was able to survive intracellularly without a functional Ton complex. A strain of MS11 deleted for the GGI and without a functional Ton complex was not able to survive intracellular infection. Intracellular survival could be restored by addition of iron to the medium, confirming that it was the inability of the bacteria to acquire iron that resulted in the bacterial death. Experiments using strains with mutations in individual T4SS genes demonstrated that the T4SS apparatus is required for Ton-independent survival, but DNA secretion is not required (Zola et al., 2010). Although the mechanism of survival is unknown, several hypotheses could explain these results. First, it is possible that the T4SS secretes an unknown factor that is able to bind iron so that it can be taken up by gonococci or stimulate the release of iron sequestered by the host (**Figure 5A**). Second, iron could be entering the cell through the T4SS apparatus (**Figure 5B**). There are precedents for molecules sneaking in through such apparatuses. Antibiotics and heme can enter gonococci through the pilus pore in certain *pilQ* variants (Chen et al., 2004). Also, the T4SS of *L. pneumophila* makes the bacteria sensitive to salt in the medium (Sadosky et al., 1993). The third hypothesis is that the T4SS could affect gonococcal trafficking within epithelial cells so that cells expressing the T4SS traffic to an iron-rich niche (**Figure 5C**). Other bacteria use T4SSs to interact with and affect host cell trafficking, so it is possible that the gonococcal T4SS is serving a similar function (Isberg et al., 2009).

THE GONOCOCCAL GENETIC ISLAND AS A MOBILE ELEMENT

The GGI shows evidence of horizontal acquisition from another bacterial species, and it appears that the bacterium's own site-specific recombinase was responsible for incorporating this foreign DNA into the genome (Domínguez et al., 2011). The data from several experiments suggest that the site-specific recombinase XerCD integrated the GGI into the gonococcal chromosome. The direct repeats flanking the GGI, *difA* and *difB*, are both recognition sites for XerCD, with *difA* being a consensus sequence and *difB* having four mismatches to the consensus (Hamilton et al., 2005; Domínguez et al., 2011). The function of XerCD is usually to resolve chromosomal dimers that arise during replication. During chromosome replication homologous recombination can occur between the daughter chromosomes resulting in the two chromosomes being linked as a single molecule, a head-to-tail dimer. XerCD mediates a site-specific recombination reaction that separates the

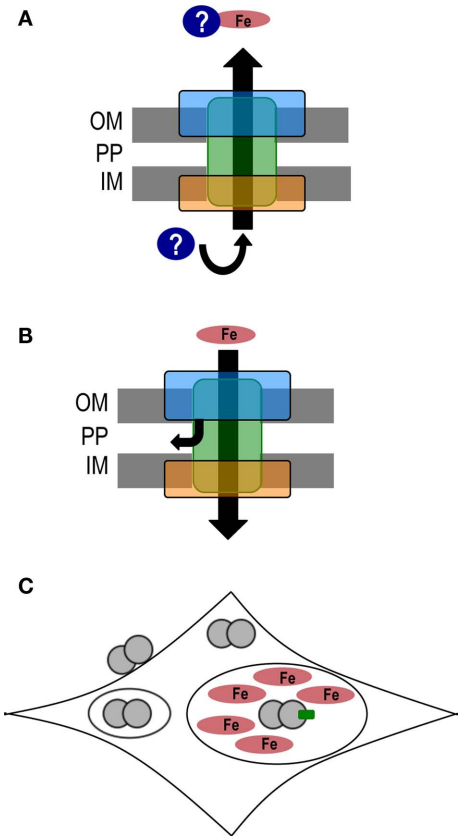

FIGURE 5 | Potential methods of Ton-independent iron acquisition. (A) The type IV secretion system could secrete a factor that either binds iron or stimulates iron release by the host, thereby facilitating iron uptake by gonococci. **(B)** The T4SS apparatus may act as a pore for iron to pass into either the periplasm or the cytosol. The T4SS pore is represented in a simplified fashion by the outer membrane protein complex (blue rectangle), the transmembrane proteins (green rectangle), and the inner membrane protein complex (orange rectangle). **(C)** The T4SS could affect gonococcal trafficking within host epithelial cells so that gonococci expressing a T4SS (diplococcus with green box) go to an iron-rich niche. OM, outer membrane; PP, periplasm; IM, inner membrane.

chromosomes (Carnoy and Roten, 2009). A similar type of reaction can occur in a chromosome that carries the GGI. XerCD can act at the two *dif* sites to excise the GGI from the chromosome, creating a circular form of the GGI. However, the reaction can also go in the opposite direction, and XerCD can integrate DNA into the chromosome if the DNA carries a *dif* site. This type of integration was demonstrated for gonococci by cloning the *difA* site plus 90 bp from the GGI and transforming the resulting plasmid into a gonococcal strain that did not carry the GGI. The plasmid integrated into the chromosome at the *dif* site (Hamilton et al., 2005). We hypothesize that the GGI was similarly acquired, that it was introduced into gonococci as a plasmid, and like some known plasmids (Blakely et al., 1991), it carried a *dif*-like site. XerCD could then have integrated it into the chromosome where it became mostly stabilized through acquisition of mutations at *difB*.

Mutations at *difB* and mutation of *xerD* demonstrated the involvement of the *dif*–XerCD system in GGI recombination. In strain MS11, the GGI was found to be lost from the cells at a frequency of 10^{-6} after 18 h of growth. However, if the *difB* sequence was mutated to a consensus *difA* sequence (resulting in the GGI being flanked by two consensus *difA* sequences), then the GGI was lost at a frequency of 10^{-3}. By contrast, a mutation in *xerD* prevented GGI loss even in the strain with two *difA* flanking sequences (Domínguez et al., 2011). These results suggest that XerD is necessary for GGI excision and loss and that increased similarity of the GGI-flanking sequences to the XerCD binding sequences facilitates higher frequency excision and loss of the GGI. The loss of the GGI from cells in the population indicates that the GGI cannot replicate on its own, or if it can, it does so poorly. PCR analyses to detect the GGI as a circle could easily detect the circle in the double *difA*$^+$ strain but could barely detect the circle in the wild-type strain, and then only after a high number of amplification cycles (Domínguez et al., 2011). However, the GGI was easily detected in the chromosome. Thus, the GGI is nearly always in the chromosome and only rarely excises to form an extrachromosomal circle.

It is possible that the ability of the GGI to circularize could allow it to be transferred to another bacterium. When the GGI is secreted by the T4SS as part of the chromosome, the *oriT* within the GGI is the first region to be secreted, and it is not until the entire chromosome has been secreted that the remainder of the GGI is exported. However, if the GGI excises from the chromosome, then the GGI could be secreted as a smaller DNA molecule. It would still be difficult for the GGI to be transferred as this smaller molecule, since it would need to be taken up in its entirety and re-circularized in the recipient. The recircularization might not be as unlikely as it would first seem, however, since relaxases of conjugative plasmids perform such a reaction in the recipient, and the gonococcal TraI might do so as well (Garcillan-Barcia et al., 2007).

It is curious that all gonococci examined have been found to have the same *difB* sequence with the same four mismatches compared to the consensus *dif* (Domínguez et al., 2011). If the utility of sequence changes in *dif* is to stabilize the GGI in the chromosome, why are the same four changes always present? Different mismatches are present in *N. meningitidis*, so it is unlikely that these mismatches were present in an ancestral *Neisseria* species that acquired the GGI. Perhaps excision of the GGI could sometimes be advantageous (at least for GGI propagation if not for *N. gonorrhoeae* itself), and the *difB* sequence is an escape hatch. The GGI would be maintained and replicated in the chromosome most of the time but occasionally could be excised and transferred.

Unlike the GGI in *N. gonorrhoeae*, some versions of the GGI appear to be stuck in *N. meningitidis* chromosomes and are unable to excise. The *difB2* allele is found in some *N. meningitidis* strains and has eight mismatches compared to the *dif* consensus (Domínguez et al., 2011). The *difB2* sequence appears to have arisen from a deletion occurring between *difB* and the *traD* coding sequence. It is unlikely that this *dif* sequence would function for XerCD-mediated recombination. The inability of the GGI to excise in these strains may explain why some *N. meningitidis* strains maintain a GGI that is unlikely to produce a functional secretion system.

CONCLUSION

The GGI is a large, horizontally acquired genomic island present in most strains of *N. gonorrhoeae* and in some strains of *N. meningitidis*. As is the case for many horizontally transferred genetic

islands in bacteria, the origin of the GGI is unknown. However, since *N. gonorrhoeae* and *N. meningitidis* are found exclusively inside a human host, we hypothesize that the GGI was acquired from another human colonizer, just as *Haemophilus* genes have been found to be acquired by *Neisseria* (Kroll et al., 1998). The GGI is not similar to conjugative plasmids found in some gonococcal strains (Pachulec and van der Does, 2010). In gonococci, the GGI-encoded T4SS acts to secrete chromosomal DNA. This DNA secretion likely increases DNA transfer in the gonococcal population, increasing genetic diversity, and creating new recombinants that may avoid the host immune response. DNA transfer through the T4SS may also facilitate the spread of antibiotic resistance genes, a continual problem for the treatment of gonorrhea. The mechanism of transfer appears similar to that of a conjugation system or Hfr transfer in *E. coli*. The chromosome is cut at a single site (*oriT*) by a relaxase, TraI, unwound, and transported as a single-strand through the T4SS, with DNA at the *oriT* transferred first, followed by the rest of the chromosome in an ordered fashion. An important difference between the gonococcal T4SS and conjugation systems is that in gonococci, DNA is secreted into the surrounding milieu and is not transferred directly from cell to cell. It is likely that the T4SS also transports other substrates in addition to DNA, such as proteins, but none have yet been identified.

There are many unanswered questions regarding the role of the gonococcal T4SS in host–pathogen interactions. It is clear from the TonB experiments that the T4SS is expressed when gonococci are intracellular. However, the T4SS may be expressed at other times during infection as well, and it is unclear what impact the T4SS might have on gonococcal pathogenesis or biology under these conditions. For example, the fact that certain forms of the GGI are correlated with disseminated infections may suggest that there is a role for T4S during systemic infection. Although secreted protein effectors have not yet been identified, it is easy to imagine that such effectors might be involved in host immune modulation, intracellular trafficking, nutrient acquisition, or other functions that enable gonococci to be so well-adapted for life in a human host. Regardless of whether the T4SS secretes proteins in addition to a DNA–protein complex, however, any transported substrates will necessarily come in contact with host cells and may affect gonococcal infection. The secreted DNA could possibly be recognized by pattern recognition receptors, thereby inducing an inflammatory response. Additionally, the secreted DNA may affect binding or other interactions of gonococci with each other or with host cells.

Dissecting the role of T4S in gonococcal pathogenesis is an important topic for ongoing research, and as we continue to develop tools for studying the T4SS in the pathogenic *Neisseria*, such as antibodies to T4SS proteins and strains with regulated expression of the T4SS genes, we will be better able to address this question. The role of the GGI in *N. meningitidis* also remains a mystery and an important topic of investigation. In some meningococcal strains, the GGI is a scrap-heap of mutated genes. In other strains, many of the T4SS genes are intact, but genes needed for DNA secretion are missing. In these cases, it is still possible that the T4SS in meningococci may secrete proteins or that the T4SS may have some other role in infection. Understanding how the GGI is affecting both *Neisseria* populations and the function of the individual cell will lead us to a better understanding of *N. gonorrhoeae* and *N. meningitidis* horizontal gene transfer and pathogenesis.

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TonB-dependent transporters expressed by *Neisseria gonorrhoeae*

Cynthia Nau Cornelissen* and Aimee Hollander

Department of Microbiology, Virginia Commonwealth University Medical Center, Richmond, VA, USA

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Susan K. Buchanan, National Institutes of Health, USA
Shelley M. Payne, University of Texas, USA
Jorge Crosa, Oregon Health and Science University, USA

****Correspondence:***
Cynthia Nau Cornelissen, Department of Microbiology, Virginia Commonwealth University Medical Center, Richmond, VA 23298-0678, USA.
e-mail: cncornel@vcu.edu

Neisseria gonorrhoeae causes the common sexually transmitted infection, gonorrhea. This microorganism is an obligate human pathogen, existing nowhere in nature except in association with humans. For growth and proliferation, *N. gonorrhoeae* requires iron and must acquire this nutrient from within its host. The gonococcus is well-adapted for growth in diverse niches within the human body because it expresses efficient transport systems enabling use of a diverse array of iron sources. Iron transport systems facilitating the use of transferrin, lactoferrin, and hemoglobin have two components: one TonB-dependent transporter and one lipoprotein. A single component TonB-dependent transporter also allows *N. gonorrhoeae* to avail itself of iron bound to heterologous siderophores produced by bacteria within the same ecological niche. Other TonB-dependent transporters are encoded by the gonococcus but have not been ascribed specific functions. The best characterized iron transport system expressed by *N. gonorrhoeae* enables the use of human transferrin as a sole iron source. This review summarizes the molecular mechanisms involved in gonococcal iron acquisition from human transferrin and also reviews what is currently known about the other TonB-dependent transport systems. No vaccine is available to prevent gonococcal infections and our options for treating this disease are compromised by the emergence of antibiotic resistance. Because iron transport systems are critical for the survival of the gonococcus *in vivo*, the surface-exposed components of these systems are attractive candidates for vaccine development or therapeutic intervention.

Keywords: iron, *Neisseria gonorrhoeae*, TonB, transferrin, xenosiderophores

INTRODUCTION

NEISSERIA GONORRHOEAE AND IRON "PIRACY"

Neisseria gonorrhoeae causes a very common, but often asymptomatic sexually transmitted infection (STI). The pathogen gains a foothold in the human host via the lower genital tract, but can ascend into the upper genital tract and beyond, disseminating through the bloodstream to the joints and skin. In rare cases, *N. gonorrhoeae* infections can even result in meningitis (Hook and Handsfield, 2008). Neonatal conjunctivitis, or ophthalmia neonatorum, results from transmission of the bacterium from an infected mother to her newborn during a vaginal delivery (Kohlhoff and Hammerschlag, 2008). These diverse manifestations require that the gonococcus thrive in a multitude of human environments, including in the blood, in semen, on mucosal surfaces, in joint fluid, and on the conjunctiva. In each niche, the pathogen must acquire all of the nutrients, including iron, that are necessary for multiplication.

Unlike most bacteria, the *Neisseria* species do not produce siderophores in an effort to acquire iron from the environment (West and Sparling, 1985). As well-adapted human pathogens, these bacteria instead rely entirely on iron sources obtained within the human host. Iron sources utilized by *N. gonorrhoeae* include transferrin (Mickelsen and Sparling, 1981) and lactoferrin (Mickelsen et al., 1982), both of which are present in micromolar concentrations in semen, the normal transmission fluid for an STI pathogen (Anderson et al., 2003). Hemoglobin (Dyer et al., 1987) and heme can also be employed by the gonococcus as sole iron sources, both of which are present periodically in the female genital tract during menses. Heterologous siderophores produced by other bacteria (xenosiderophores) are available to the gonococcus within the context of cervical or rectal infections. It is anticipated that bacteria co-inhabiting these niches produce siderophores under iron stressed conditions, and the gonococcus takes advantage of this iron-sequestering attempt by hijacking the ferric complexes en route to the producer. Aerobactin (West and Sparling, 1987) and enterobactin (Carson et al., 1999), both produced by enteric bacteria, have previously been demonstrated to support the growth of *N. gonorrhoeae*.

Human transferrin is a glycoprotein responsible for binding to and transporting ferric iron throughout the human body. The protein is found at highest concentrations in the serum, cerebral spinal fluid, and joint fluid, but can also be detected on mucosal membranes, particularly in inflamed tissue. Lactoferrin is found in milk, secretions, and polymorpholeukocytes. Lactoferrin is believed to be primarily responsible for iron scavenging, rather than transport, and therefore is very poorly saturated with iron *in vivo*. By contrast, transferrin is approximately 30% saturated with iron in the serum (for a recent review, see Wally and Buchanan, 2007). Early studies of iron use by *N. gonorrhoeae* demonstrated that both human glycoproteins can be efficiently employed by the gonococcus as a sole source of iron. Low saturation levels did not hinder access to the bound iron. Moreover, these seminal studies demonstrated that iron is internalized, but the iron-binding protein remains intact outside of the cell. The process of iron

removal was shown to require metabolic energy and direct contact between the glycoprotein and the bacterial cell surface (McKenna et al., 1988).

TWO-COMPONENT SYSTEMS FOR IRON TRANSPORT FROM HOST PROTEINS

TRANSFERRIN–IRON ACQUISITION

The mechanism of transferrin–iron acquisition by *N. gonorrhoeae* has been the subject of a great deal of study. The precise details of lactoferrin and hemoglobin utilization, while expected to be similar, are less well-developed. The initial steps in characterization of the gonococcal transferrin–iron acquisition system involved the identification of two membrane proteins that bound to human transferrin (Cornelissen et al., 1992). Expression of both proteins was decreased under iron-replete conditions, consistent with a role in iron internalization. The first transferrin binding protein (Tbp) to be identified and characterized in *N. gonorrhoeae* was TbpA (Cornelissen et al., 1992; **Figures 1 and 2**). The gene that encodes this protein was sequenced and found to be similar to those encoding a family of iron transport proteins in Gram-negative bacteria known as TonB-dependent transporters. These proteins share sequence similarity primarily at their N- and C-termini, consistent with their shared localization and function as large outer membrane transporters (Noinaj et al., 2011). Expression of gonococcal recombinant TbpA in *Escherichia coli* resulted in surface presentation of the transporter and rendered *E. coli* capable of binding transferrin in a species-specific manner; only human transferrin was recognized (Cornelissen et al., 1993). The second Tbp (**Figure 1**), is encoded by the gene directly upstream of *tbpA* (Anderson et al., 1994). TbpB contains a typical "lipobox," which is necessary for N-terminal lipid modification and outer membrane localization. The genes encoding the Tbps are preceded by a typical iron-regulatory sequence (see below) and are transcribed as an iron-repressed bicistronic operon (Ronpirin et al., 2001). However, an intergenic region between *tbpB* and *tbpA* is capable of encoding an mRNA species with strong secondary structure potential. The observation that *tbpB* transcripts outnumber *tbpA* transcripts by 2:1 at steady state, suggests that this intervening sequence is important for maintenance of the optimum stoichiometry of the system (Ronpirin et al., 2001).

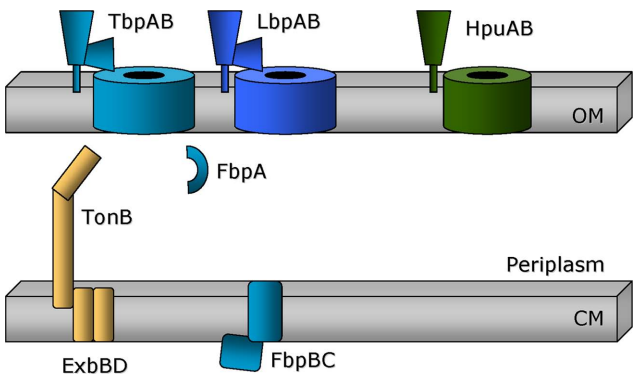

FIGURE 1 | Two component gonococcal systems for acquisition of iron from host proteins. The TonB dependent outer membrane transporters are shown as barrels traversing the outer membrane (OM). The lipid-modified companion proteins are shown tethered to the outer membrane surface. TonB, ExbB, and ExbD (gold) are depicted as attached to or imbedded within the cytoplasmic membrane (CM). The periplasmic binding protein, FbpA, is responsible for transporting iron from the outer membrane transporters, TbpA and LbpA, to the cytoplasmic membrane permease, comprised of FbpB. FbpC is the ATPase that energizes transport through FbpB.

All gonococcal isolates characterized and sequenced to date possess both *tbp* genes. However, transposon insertion mutants of *N. gonorrhoeae* were generated to test the function of these proteins in transferrin iron acquisition. Mutants lacking both proteins were incapable of growth on transferrin as a sole iron source, although growth on other iron sources was unimpaired (Anderson et al., 1994). Mutants lacking the ability to express TbpA were incapable of growth on transferrin and were unable to internalize any iron from transferrin (Cornelissen et al., 1992). By contrast, *tbpB* mutants were still capable of growth on transferrin, but were only able to internalize approximately 50% of wild-type levels of iron from transferrin (Anderson et al., 1994). These observations led us to suggest that TbpA, the TonB-dependent transporter, was the necessary portal for iron through the outer membrane whereas TbpB, the surface-tethered lipoprotein, was important for increased efficiency of the iron acquisition system (**Figure 1**).

TonB-dependent transporters share a common topology. Twelve different TonB-dependent transporters have been crystallized to date (Noinaj et al., 2011) and all share two characteristic domains: a beta-barrel comprised of 22 amphipathic beta-strands, and a globular plug domain that is folded up inside the barrel. After the first TonB-dependent transporter was crystallized (Buchanan et al., 1999), we developed a 2D model of TbpA, employing a combination of bioinformatics approaches and sequence diversity (**Figure 2**). Twenty-two putative amphipathic beta-strands were predicted by their similarity with the known transmembrane domains of FepA (Buchanan et al., 1999). The sequence diversity detected among five different gonococcal TbpA sequences (Cornelissen et al., 2000) was localized to regions within 11 putatively surface-exposed loops (L1–L11 in **Figure 2**). Likewise, three pairs of cysteine residues were localized to three extracellular loop regions (L2, L4, and L5 in **Figure 2**).

To begin testing the TbpA topology model, we deleted three predicted loop regions (L4, L5, and L8; Boulton et al., 2000). Mutants expressing TbpA deleted of predicted loops 4 or 5 were rendered unable to bind transferrin or use transferrin as a sole iron source. Mutants unable to express loop 8 of TbpA bound transferrin with a lower affinity but were nonetheless incapable of transferrin iron utilization. These results suggested that loops 4 and 5 were critically important ligand binding regions and were therefore crucial for transferrin–iron internalization. The phenotype of the loop 8 deletion mutant suggested that high affinity interactions between TbpA and transferrin were necessary for iron internalization and perhaps for iron removal from transferrin. We subsequently cloned regions encoding several predicted, surface-exposed loops of gonococcal TbpA (Masri and Cornelissen, 2002). Each predicted loop region was expressed as a fusion with a heterologous cellulose binding protein. The recombinant proteins were tested for their ability to bind human transferrin and amazingly two loop regions retained this ligand binding capability. Recombinant proteins comprised of predicted loop 5 or loops 4 and 5 together specifically bound

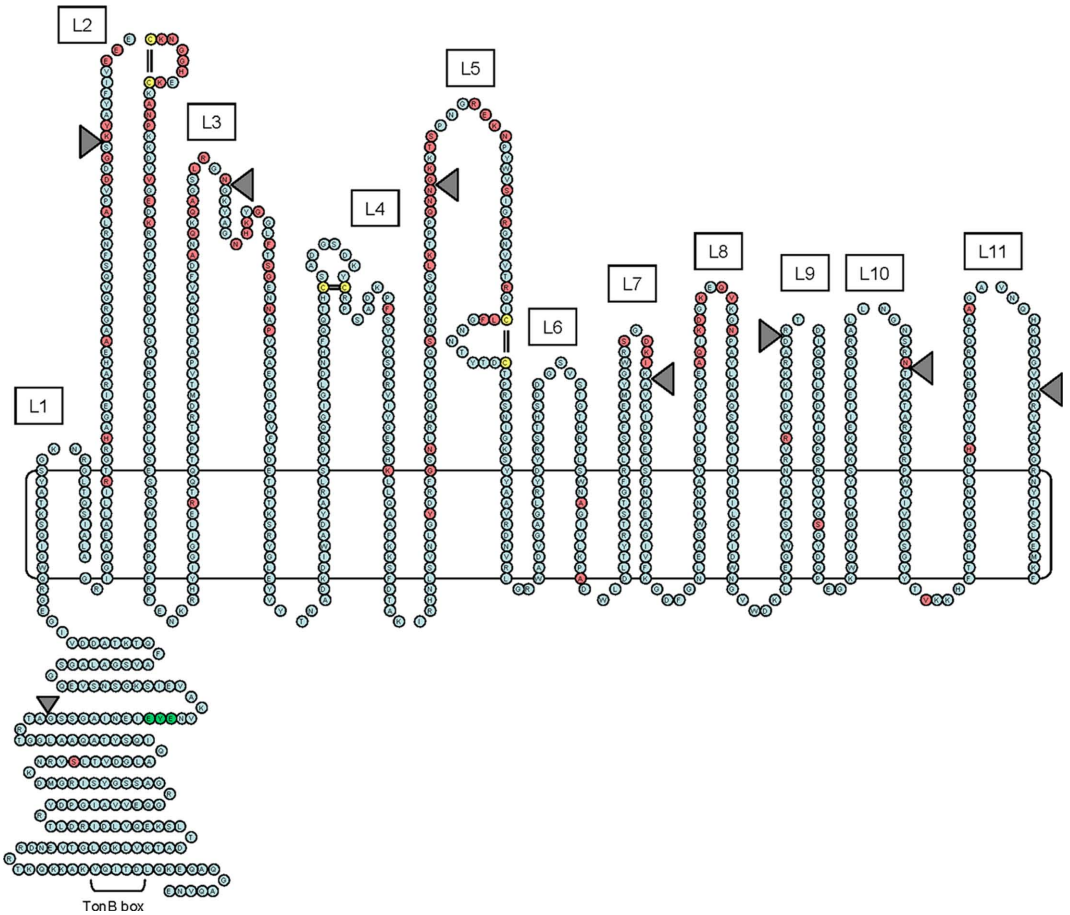

FIGURE 2 | Hypothetical two-dimensional topology model of gonococcal TbpA. The sequence shown is of the TbpA protein from gonococcal strain FA19. The model includes an amino-terminal plug domain of 161 residues, 11 surface-exposed loops (numbered L1–L11), and 22 transmembrane beta-strands. Residues highlighted in red are those that are divergent among a panel of five gonococcal TbpA sequences. Cysteine residues are highlighted in yellow. The EYE residues that were mutated within the plug domain are highlighted in green. The position of HA epitopes inserted into putative loops and the plug domain are shown as gray-shaded triangles. The TonB-box region within the plug domain is also indicated.

human transferrin, even out of context of the intact outer membrane protein. These data supported our earlier findings that loops 4 and 5 contained critical binding determinants.

We developed polyclonal antibodies against the over-expressed loop regions, and found that loops 2 and 5 (**Figure 2**) were indeed surface exposed on intact gonococci (Masri, 2003). We also determined that antibodies generated against the plug region of TbpA were also capable of specific binding to the cell surface of gonococci. However, none of these antibodies blocked transferrin binding in liquid phase binding assays. We propose that the binding affinity of human transferrin to TbpA (K_d ~10 nM) is greater than that of these epitope-specific antibodies and therefore we detected no inhibition. Competition between the natural ligand (human transferrin) and epitope-specific antibodies also may have led to our inability to detect bactericidal activity by these antibodies in the presence of human complement. Loop 5-specific antibodies were bactericidal when baby rabbit complement was employed, suggesting that the non-human species of transferrin present in the rabbit serum allowed for antibody binding and subsequent complement fixation and bactericidal activity (Masri, 2003).

We also employed an epitope insertion strategy to test the 2D model of TbpA topology (**Figure 2**). We generated fusion proteins, expressed in single copy by *N. gonorrhoeae*, in which the HA epitope was fused at various points within the TbpA protein (Yost-Daljev and Cornelissen, 2004). We inserted the HA epitope within seven putatively exposed surface loops, within two putative beta-strands, within two predicted periplasmic turns, and within the plug region. We determined that the HA epitopes localized in putative loops 2, 3, 5, 7, and 10 were surface exposed, confirming the extracellular localization of these regions (**Figure 2**). None of the HA epitopes in periplasmic turns or beta-strands were surface accessible, consistent with the model. The HA epitope tag was accessible to the surface when expressed in the plug domain, consistent with other data suggesting surface exposure of the plug domain. Contrary to the model predictions, HA epitopes in predicted loops 9 and 11 were not surface accessible by this analysis. Overall, these data largely supported the 2D model and confirmed the extracellular location of six of the predicted loop regions. Interestingly, only two epitope insertions (L3 and β9; **Figure 2** and not shown) resulted in a defective TbpA transporter; none of the other insertions resulted

in defects in transferrin binding or transferrin–iron utilization. The exception was the plug HA epitope insertion, which resulted in decreased binding affinity and abolished transferrin iron utilization. Another interesting class of mutants was exemplified by the L2, L9, and L11 HA insertion mutants. These mutants remained capable of transferrin binding and use of transferrin as a sole iron source, as long as TbpB was co-expressed. If *tbpB* was also mutated, the double mutants were rendered completely incapable of transferrin–iron internalization. The phenotypes of these mutants suggest that L2, L9, and L11 represent regions of TbpA that are important for iron removal from transferrin, without which TbpA is unable to function in the absence of TbpB.

TbpB shares similarity with other surface-exposed lipoproteins, but until recently none had been crystallized. From sequence analysis, it was clear that the gonococcal TbpB was comprised of two domains that shared limited sequence similarity, reminiscent of the ligand to which it binds, transferrin. We demonstrated that the amino-terminal half of gonococcal TbpB retained the ability to bind human transferrin following SDS–PAGE and transfer to solid support (Cornelissen et al., 1997a). Truncation of this binding domain from either end abolished binding. We further demonstrated that the amino-terminal half of TbpB contained most of the sequence diversity when five gonococcal sequences were compared. There were, however, six regions of sequence conservation that are probably important for export to the cell surface or for transferrin binding.

We employed the same approach to mapping gonococcal TbpB membrane topology as was described above for TbpA. We expressed TbpB-HA fusion proteins in the gonococcus, each of which contained an HA epitope at a distinct position (DeRocco and Cornelissen, 2007). All of the HA epitopes were accessible on the gonococcal cell surface, consistent with complete surface exposure of TbpB beyond the lipid modified, mature amino-terminal cysteine residue. There were only four epitope insertions that decreased transferrin binding by TbpB. Three insertion points were within the amino-terminal lobe that is responsible for efficient transferrin binding following SDS–PAGE and electroblotting to solid support. The fourth insertion point was within the C-terminal half of the protein, disruption of which resulted in decreased transferrin binding to the whole cell surface. When mutations in the amino- and carboxy-terminal halves were combined in the same strain, the resulting mutant TbpB was completely devoid of transferrin binding capabilities. These results suggest that the amino- and carboxy-terminal lobes of TbpB share both sequence and functional redundancy. Elimination of binding to one lobe only decreased transferrin interaction with the cell surface, while elimination of both domains abrogated all binding. Recently, the crystal structure for the smaller but related TbpB from *Actinobacillus pleuropneumoniae* was described (Moraes et al., 2009). The gonococcal protein is expected to assume a similar conformation, which is reminiscent of other lipid-modified neisserial proteins, including the factor H binding protein (Schneider et al., 2009). The *A. pleuropneumoniae* TbpB has two lobes, each of which has two distinct domains: a small beta-barrel and a curved, β-strand rich region denoted as a "handle" (Moraes et al., 2009). The amino- and carboxy-terminal domains of gonococcal TbpB are expected to resemble those of *A. pleuropneumoniae* TbpB and are depicted in **Figure 1** as trapezoid shapes. Both lobes of gonococcal TbpB retain transferrin binding domains (DeRocco and Cornelissen, 2007) and thus both are expected to participate in the process of iron acquisition from transferrin.

The gonococcal Tbp proteins are expected to form a complex, particularly in the presence of transferrin. We have detected TbpB in pull-down assays with TbpA and TbpA-specific antisera (Kenney, 2002), suggesting that even in the absence of transferrin the two proteins associate with each other. Surface exposure characteristics and binding kinetics differ depending upon whether the Tbp proteins are expressed independently or together, again suggesting complex formation at the cell surface (Cornelissen and Sparling, 1996; Cornelissen et al., 1997b). The transferrin binding affinities of both proteins for human transferrin are in the nanomolar range; however, TbpB specifically associates with the ferrated form of transferrin whereas TbpA interacts with both ferrated and apo forms of the glycoprotein (Cornelissen and Sparling, 1996). This observation led us to test the association and dissociation rates for the individual proteins. We found that both association and dissociation was facilitated by the presence of TbpB, suggesting that the ability of TbpB to discriminate between ferrated and apo-transferrin enhances both association and dissociation kinetics (DeRocco et al., 2008). Employing an *in vitro* technique and gonococcal membrane fragments, we determined that both TbpA and TbpB have the ability to remove iron from human transferrin (Siburt et al., 2009). In this *in vitro* system, the ferric binding protein, FbpA, was freely available in solution and served as the ferric iron acceptor molecule. Cumulatively, our studies suggest that while both TbpA and TbpB have the capacity to strip ferric iron from transferrin at the cell surface, in an intact cell, only TbpA has the capacity to transport iron across the outer membrane and into the periplasm.

While the precise mechanism of iron removal from transferrin and subsequent transport into the periplasm is not completely understood, our current model (**Figure 3**) involves both TbpA

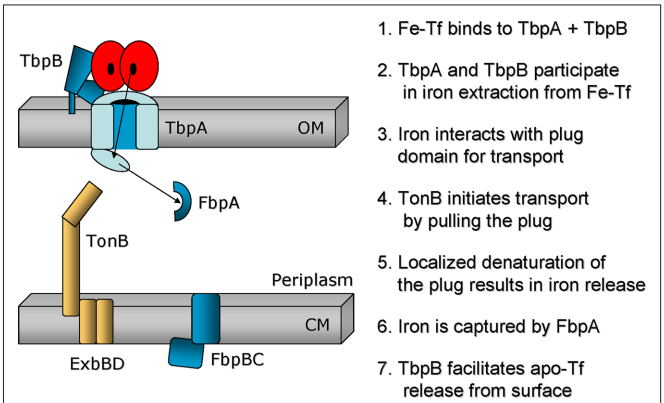

FIGURE 3 | Model of the mechanism of iron transport from transferrin through the gonococcal outer membrane. TbpA is shown as a bisected barrel with an amino-terminal plug domain exposed in the periplasm. TbpB is shown as a bi-lobed protein protruding from the gonococcal cell surface, tethered to the outer membrane by an amino-terminal lipid moiety. Human transferrin is shown as a bi-lobed structure, each lobe of which is capable of binding an atom of iron. Seven putative steps in iron acquisition from transferrin are listed on the right. The arrows depict the direction of iron import and the hypothetical, transient interaction between iron and the plug domain.

and TbpB playing roles in iron removal from transferrin at the cell surface (Siburt et al., 2009). After iron is extracted, we propose that the ferric ion interacts with the plug domain of TbpA. The HA epitope insertion in the plug domain resulted in a mutant that bound transferrin with lower affinity but was incapable of iron internalization (Yost-Daljev and Cornelissen, 2004). This observation prompted us to create a series of site-specific mutations in conserved, charged residues of the plug domain near the point of insertion of the HA epitope (Noto and Cornelissen, 2008). A triple mutant in which a conserved EYE motif was replaced by three alanine residues was generated. This mutant strain was incapable of transferrin–iron utilization unless TbpB was co-expressed. This phenotype is consistent with a role for this region in iron extraction and/or iron binding by the plug domain. The model of transport of iron through TonB-dependent transporters, developed by study of the vitamin B_{12} transporter of *E. coli* (Gumbart et al., 2007) suggests that plug unfolding upon interaction with TonB (see below) results in presentation of substrate at the periplasmic face of the beta-barrel. We hypothesize (**Figure 3**) that a similar mechanism would result in transport of iron, bound by the plug, through the barrel and allow the presentation of iron at the periplasmic face of TbpA.

PERIPLASMIC BINDING PROTEIN-DEPENDENT, ABC TRANSPORT OF FERRIC IRON FROM TRANSFERRIN

After ferric iron has been transported through the TbpA beta-barrel, the ferric binding protein, FbpA, sequesters the ion in the periplasm (Chen et al., 1993). FbpA has been called a "bacterial transferrin" because binding by this 37 kDa protein to iron resembles that of a single lobe of transferrin in terms of coordinating residues and the need for a synergistic anion (Nowalk et al., 1994; Taboy et al., 2001; Dhungana et al., 2003). Using a modified H/D exchange approach, we demonstrated that apo-FbpA binds directly and specifically to TbpA (Siburt et al., 2009). This interaction would allow for an efficient hand-off from the TbpA plug domain to the associated apo-FbpA. After FbpA becomes ferrated, its affinity for TbpA is decreased, facilitating liberation of the holo-FbpA and passage of the sequestered iron through the periplasmic space. Holo-FbpA then associates with the cytoplasmic permease protein, FbpB, which accomplishes transport from the periplasm into the cytoplasm employing the FbpC protein and ATP hydrolysis for energization. One publication reported that iron transport into the gonococcus was accomplished in the absence of a functional FbpC protein (Sebastian and Genco, 1999). This observation suggests that other ATP binding proteins, of which there are many predicted in the gonococcal genomes, might be able to replace FbpC in this energization step.

LACTOFERRIN–IRON ACQUISITION

As shown in **Figure 1**, the lactoferrin–iron acquisition system, comprised of LbpA and LbpB, resembles the transferrin iron acquisition system. LbpA is a TonB-dependent transporter (Biswas and Sparling, 1995); LbpB is a lipoprotein (Biswas et al., 1999). Gonococcal LbpA from strain FA19 is 46% identical and 65% similar to gonococcal TbpA from the same strain (Biswas and Sparling, 1995). LbpB is 31% identical to TbpB; however LbpB contains two unique stretches of anionic residues, which are absent from TbpB (Biswas et al., 1999). As is the case for the Tbps, the Lbps are encoded by contiguous genes with the *lbpB* gene located upstream of the *lbpA* gene. In contrast to the *tbp* operon, the *lbpB* and *lbpA* genes actually overlap slightly and are not separated by a region of potential secondary structure. About half of gonococcal isolates have lost the capacity to express the Lbps due to the presence of a large deletion that removes the entire *lbpB* gene and the 5′ terminus of the *lbpA* gene (Anderson et al., 2003). In addition, the *lbpB* gene, if present, is subject to phase variation due to a poly C-tract within the coding region. While LbpA is required for utilization of human lactoferrin as a sole iron source, LbpB is not (Biswas et al., 1999). Variants in which LbpB is not expressed due to phase variation, retain the ability to express LbpA, and the ability to grow on lactoferrin. LbpB does not apparently bind lactoferrin independent of LbpA (Biswas et al., 1999), in contrast to the situation with the Tbps, which both retain the ability to bind specifically to human transferrin. Detailed structure–function analyses have not been conducted to date with the gonococcal Lbps. Thus the mechanism of lactoferrin binding, iron extraction, TonB energization and iron transport into the periplasm is unclear. The FbpABC system is known to receive and transport lactoferrin-bound iron (Chen et al., 1993) through the periplasm and across the cytoplasmic membrane as shown in **Figure 1**.

HEMOGLOBIN–IRON ACQUISITION

Neisseria gonorrhoeae is capable of utilizing both free heme and heme bound to hemoglobin to fulfill its iron requirement. No specific receptor has been defined for free heme; however, hemoglobin is employed by virtue of expression of a third two-component iron acquisition system (Chen et al., 1996, 1998). As shown in **Figure 1**, the topology of the hemoglobin–iron acquisition system, comprised of HpuA and HpuB, is expected to resemble those employed for transferrin– and lactoferrin–iron utilization. HpuB is encoded downstream of HpuA; the *hpuB* gene encodes the TonB-dependent transporter whereas the *hpuA* gene encodes the lipidated component of the system. The HpuB protein at ~90 kDa (Chen et al., 1996) is somewhat smaller than either TbpA or LbpA (~100 kDa), but is similar in size to most other TonB-dependent transporters (**Table 1**). Similarly, the lipoprotein component, HpuA, is approximately half the size of either TbpB or LbpB (Chen et al., 1998). Given the recent insights into the structure of TbpB (Moraes et al., 2009), one could speculate that HpuA is composed of a single lobe with only one beta-barrel and a single "handle" domain (as shown in **Figure 1**). Interestingly, and in contrast to the transferrin– and lactoferrin–iron acquisition systems, hemoglobin binding requires the expression of both HpuA and HpuB, consistent with the hypothesis that the two proteins form a heteromultimer in order to constitute the obligate hemoglobin binding pocket (Chen et al., 1998). Also consistent with the single binding pocket hypothesis is the observation that hemoglobin–iron utilization is dependent upon expression of both HpuA and HpuB, unlike the TbpAB system which only requires expression of TbpA. Also in stark contrast to the transferrin and lactoferrin binding proteins, HpuA and B do not exclusively interact with human hemoglobin as these gonococcal proteins recognize the hemoglobins from non-human sources as well. Point mutations in HpuB allow

Table 1 | TonB-dependent transporters encoded in the gonococcal genome.

Name	Locus in FA1090[1]	Predicted size[2]	Closest homologs	Linked genes[3]	Distribution[4]	Function or comments
TbpA	NG1495	912 (102 kDa)	N/A[5]	TbpB	Most *Neisseriae*	Transferrin–iron utilization; iron repressed
LbpA	NG0260	N/A	N/A	LbpB	Most *Neisseriae*; deleted genes common among *N. gonorrhoeae*	Lactoferrin–iron utilization; genes deleted in FA1090; iron repressed
HpuB	NG2109	809 (90 kDa)	N/A	HpuA	Most *Neisseriae*; subject to phase variation	Hemoglobin–iron utilization; iron repressed
FetA	NG2093	713 (79 kDa)	Siderophore transporters	Periplasmic binding protein and ABC transport system	All *Neisseriae*; subject to phase variation	Enterobactin, multimers of DHBS[6] and salmochelin S2 utilization; iron repressed
TdfF	NG0021	725 (80 kDa)	Siderophore transporters	Periplasmic binding protein encoded upstream	*N. meningitidis* and *N. gonorrhoeae* (pathogens only)	Important for intracellular survival of FA1090; iron repressed
TdfG	NG0553	1206 (136 kDa)	Heme transporters	None	*N. gonorrhoeae* only	Iron repressed
TdfH	NG0952	922 (104 kDa)	HasR and heme transporters	None	Most *Neisseriae*	Not iron regulated
TdfJ	NG1205	764 (86 kDa)	Siderophore and heme transporters	None	All *Neisseriae*	Meningococcal homolog suggested to be zinc transporter; Iron induced

[1]Locus corresponds to the gene ID in the following database: stdgen.northwestern.edu. Genome sequence of FA1090 was determined at the University of Oklahoma. [2]Predicted size in number of amino acids followed by predicted molecular weight in parentheses. [3]Linked genes that have homologs known to be involved in iron transport. [4]Distribution indicates whether the gene is possessed by N. gonorrhoeae, both pathogenic Neisseria species or all Neisseriae, including the commensal species. [5]N/A, not applicable. [6]Dihydroxybenzoylserine.

hemoglobin utilization in the absence of the lipoprotein partner protein, HpuA (Chen et al., 2002). The Δ*hpuA*, *hpuB* point mutants grew slightly better than the Δ*hpuA*, *hpuB*+ parent in the presence of limiting amounts of heme. The point mutations mapped to the beta-barrel portion of the TonB-dependent transporter, suggesting that transmembrane passage of the heme was enhanced in the mutants. Finally, in the absence of HpuA, growth by the hemoglobin+ point mutants was abrogated in the presence of albumin, which binds and makes inaccessible heme outside the cell. The authors therefore concluded that an important function for HpuA is to retain heme in close proximity to the cell to make transport through the TonB-dependent transporter more efficient (Chen et al., 2002). Beyond these studies, detailed structure–function analyses have not been conducted on the gonococcal HpuAB proteins. Subsequent to transport through HpuB, heme is presumably bound by a periplasmic binding protein (PBP) for transit to the cytoplasmic membrane. Likewise, a heme-specific cytoplasmic membrane permease is expected to be necessary for entry into the cytoplasm. However, no such PBP-dependent, ABC transport system has yet been described.

A second TonB-dependent hemoglobin–iron transporter is expressed by the closely related *N. meningitidis* (Stojiljkovic et al., 1996). HmbR is similar to HpuB, but in contrast to the systems described above, meningococcal HmbR is not encoded proximate to a companion lipoprotein. Moreover, none of the gonococcal strains sequenced to date have the capacity to express HmbR as the loci that would encode this protein are pseudogenes.

SINGLE COMPONENT SYSTEMS FOR IRON TRANSPORT

FetA

The *fetA* gene was formerly called *frpB* (Beucher and Sparling, 1995) and encodes a TonB-dependent transporter of approximately 80 kDa. The *f*erric *e*nterobactin *t*ransporter (FetA) protein was renamed when Carson et al. (1999) demonstrated that binding and acquisition of ferric-enterobactin was enhanced when FetA was expressed. Enterobactin is a catecholate-type siderophore that is produced by a variety of bacteria, including *E. coli* (O'Brien and Gibson, 1970), *Salmonella* species (Pollack and Neilands, 1970), and *Klebsiella* species (Perry et al., 1979). The gonococcal *fetA* gene lies immediately upstream of a cluster of genes that putatively encode components of a PBP-dependent, ABC transport system. Carson et al. (1999) demonstrated that *fetB* mutants were defective in enterobactin utilization. The gene products encoded downstream of FetA are shown in **Figure 4** and are labeled as FetB, the PBP, and FetCDEF, putative members of the ABC transport system. There is one ORF (NG2089) within the cluster that has no homologs but is conserved among the *Neisseria* species; therefore, this protein's role in iron uptake is not obvious. This ORF corresponds to the FetE protein shown in **Figure 4**. While FetA was known to transport ferric-enterobactin (Carson et al., 1999), we recently demonstrated (Hollander et al., in revision) that this transporter is also critical for import of other catecholate siderophores including dimers and trimers of dihydroxybenzoylserine (DHBS) and salmochelin S2 (the linearized form of salmochelin). Salmochelin is a diglucosylated form of enterobactin that is made by some pathogens,

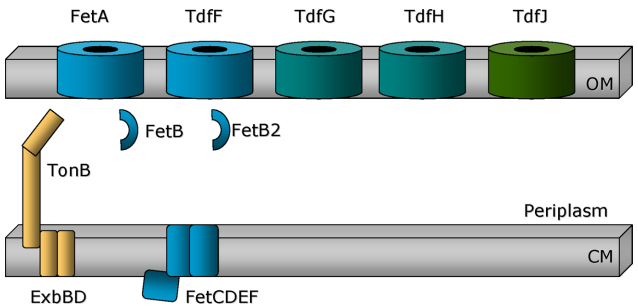

FIGURE 4 | Single component iron transport systems. FetA and the uncharacterized TonB-dependent transporters (TdfF, TdfG, TdfH, and TdfJ) are shown as barrels traversing the outer membrane. TonB, ExbB, and ExbD (gold) are depicted as attached to or imbedded within the cytoplasmic membrane (CM). The periplasmic binding protein FetB is encoded downstream of FetA and is expected to transport ferric-siderophores across the periplasm to a cytoplasmic membrane permease. Genes located immediately downstream of that encoding FetB are hypothesized to fulfill this function. Encoded near TdfF is a putative periplasmic binding protein annotated as FetB2, reflecting its sequence similarity with FetB.

including uropathogenic *E. coli* (Hantke et al., 2003). Thus, the FetA transporter appears to have broad specificity for catecholate-type xenosiderophores. FetA expression is iron repressed and also subject to phase variation. A poly C-tract within the promoter region preceding the *fetA* gene results in modulated expression levels from very high to very low (Carson et al., 2000).

TdfF

When the first gonococcal genome sequence was being completed, Turner et al. (2001) identified three uncharacterized TonB-dependent transporters by their similarity to other proteins in this family. These putative transporters were named *T*onB-*d*ependent *f*unction (Tdf) F, G, and H (**Figure 4**). As shown in **Table 1**, these transport proteins were similar to siderophore and heme transporters from other Gram-negative bacteria. With subsequent sequence analysis, we identified a fourth putative transporter, which we named TdfJ (**Table 1**; Hagen and Cornelissen, 2006).

To discern whether any of the TonB-dependent transporters were important for gonococcal survival within human epithelial cells, we modified an assay developed by Larson et al. (2002) to evaluate intracellular survival of *N. meningitidis*. We first tested whether a *tonB* mutant of gonococcal strain FA1090 was inhibited for growth within human cervical epithelial cells. We found that the *tonB* mutant was indeed impaired for survival and that the addition of iron rescued the mutant (Hagen and Cornelissen, 2006), suggesting that the defect was related to an iron acquisition defect in the *tonB* mutant. We then tested both characterized TonB-dependent transporters (TbpA and FetA) and the uncharacterized TonB-dependent transporters (TdfF, G, H, and J) for their participation in this survival phenotype. The only mutant that was defective for intracellular survival in these assays was the *tdfF* mutant (Hagen and Cornelissen, 2006). Like the *tonB* mutant, addition of excess iron overcame the survival defect, suggesting that the TdfF transporter participated in intracellular iron acquisition and that excess iron could bypass the need for high affinity transport through TdfF.

Upstream of the *tdfF* gene is a gene that putatively encodes a PBP related to iron transport. This gene is annotated in the FA1090 genome database as *fetB2* due to its similarity to the protein encoded within the *fet* gene cluster. TdfF expression is iron regulated, but is only detected when gonococci are grown in cell culture medium plus serum (Hagen and Cornelissen, 2006). This is consistent with the inability of Turner et al. (2001) to detect TdfF expression in gonococcal growth medium, even under iron stressed conditions. Likewise, in microarray analyses of the iron-dependent gonococcal transcriptome, *tdfF* expression has never been reported (Ducey et al., 2005; Jackson et al., 2010). We hypothesize that the putative AraC-like transcriptional regulator, MpeR, encoded in close proximity to *tdfF* may activate expression of TdfF in the presence of the appropriate inducing stimulus. The molecular identity of this stimulating molecule(s) is currently under investigation. The entire locus between *tdfF* and *mpeR* is unique to the pathogenic *Neisseria* species as it is completely absent from commensal *Neisseriae* (Snyder and Saunders, 2006; Marri et al., 2010). This observation suggests that iron acquisition via the TdfF-dependent pathway and optimized MpeR-dependent gene regulation are important virulence factors, distinguishing the pathogens from the commensals.

OTHER UNCHARACTERIZED TonB-DEPENDENT TRANSPORTERS

TdfG is an extraordinarily large TonB-dependent transporter at ~136 kDa and is encoded by all gonococcal genomes; however, protein expression was only detected by Turner et al. in 17% of gonococcal strains (Turner et al., 2001). TdfG is most similar to heme transporters and was shown by Turner et al. (2001) to be iron repressed. A knock-out mutant of strain FA1090 that was unable to express TdfG retained the ability to utilize heme as a sole iron source. All gonococcal genomes sequenced to date have an intact *tdfG* gene; however the sequences fall into two distinct clades, showing only 48–49% sequence similarity to each other (data not shown). The biological significance of these different *tdfG* gene families among different gonococcal strains is unclear but may have contributed to the inability to detect protein expression in the majority of gonococci using an antibody probe despite the presence of full-length genes. TdfH is also a large TonB-dependent transporter (~104 kDa) with similarity to heme transport systems. TdfH mutants similarly retained the ability to grow on free heme as a sole iron source (Turner et al., 2001). Moreover, expression of recombinant TdfH in a *hemA* mutant of *E. coli* was not sufficient to allow heme utilization, even when the neisserial TonB, ExbB and ExbD proteins were co-expressed. Cumulatively, these data suggest that neither TdfG nor TdfH is a TonB-dependent transporter for free heme (Turner et al., 2001); however, the genes were not simultaneously inactivated in the gonococcus. If the functions of TdfG and TdfH are redundant with respect to iron acquisition, their phenotypes may only be obvious in a mutant lacking both transporters.

TdfJ is an 86 kDa TonB-dependent transporter that shares similarity with siderophore and heme transporters. The *tdfJ* gene is present in all *Neisseriae*, including pathogens and commensals. Microarray studies (Ducey et al., 2005; Jackson et al., 2010) and our unpublished, confirmatory RT-PCR experiments demonstrate that *tdfJ* expression is iron induced. A recent publication indicates that the meningococcal TdfJ homolog (NMB0964) facilitates zinc

acquisition and thus the authors renamed the meningococcal protein ZnuD (Stork et al., 2011). ZnuD is regulated by the putative zinc-dependent regulator, Zur, but expression of ZnuD in the meningococcus was not subject to iron regulation. Similar studies have not been reported for gonococcal TdfJ, so the function of this protein and its contribution to growth of the gonococcus is not currently known. However, the mechanisms that regulate expression of these TonB-dependent transporters in the two pathogenic *Neisseriae* appear to be distinct.

TonB and iron transport across the gonococcal outer membrane

While the mechanism by which TonB harnesses energy and transduces it to energize the outer membrane iron transporters has not been completely defined in any system, it is well established that TonB, in complex with ExbB and ExbD is charged by the proton motive force generated at the cytoplasmic membrane (Postle and Larsen, 2007). TonB, in its energized state, physically interacts with TonB-dependent transporters. One conserved domain within the plug domain of the transporters has been called the "TonB-box" (Cadieux et al., 2000). Defined domains of TonB have been demonstrated to interact directly with the TonB-box (Pawelek et al., 2006) and in so doing, facilitate substrate transport across the outer membrane. We generated a TonB-box (**Figure 2**) mutant of gonococcal TbpA and showed that while transferrin binding was unaffected, transferrin–iron acquisition was completely prevented (Cornelissen et al., 1997b). The TonB-box mutant presented TbpB at the cell surface in a distinct conformation, resulting in a new protease sensitivity pattern. This observation allowed us to conclude that the energization state of TbpA resulted in a conformational change in TbpB, consistent with a physical interaction between the two outer membrane proteins. Additionally, the TonB-box mutant was incapable of transferrin release from TbpA, suggesting that the energy-dependent step in transferrin–iron transport is necessary for ligand release to occur. TonB directly interacted with TbpA, but the association was inhibited when the TonB box of TbpA was mutated (Kenney and Cornelissen, 2002). The ability of TonB to co-purify with TbpA did not depend upon the presence of the natural ligand, transferrin (Kenney and Cornelissen, 2002), prompting us to conclude that the natural signal triggering TonB interaction with TbpA was in fact iron binding by the plug domain.

Most current models of TonB function are based upon the known crystal structures of TonB-dependent transporters and TonB. These models suggest that the most efficient means to accomplish vectorial ligand transport through the beta-barrel is by TonB first binding to the TonB-box and then initiating a pulling force on the plug domain (Shultis et al., 2006). This would result in a localized denaturation or unfolding of the plug, allowing movement of substrate through the barrel and presentation at the periplasmic face of the transporter. With regard to gonococcal transferrin–iron acquisition, we propose (**Figure 3**) that both TbpA and TbpB participate in iron removal at the cell surface. The plug domain of TbpA may be at least partially surface accessible and therefore may also participate in iron removal from transferrin. We postulate that the plug domain interacts with the extracted ferric iron and that the EYE motif in some way participates in this process. We further propose that TonB then interacts with the TonB-box of TbpA, a pulling force is exerted resulting in plug unraveling, at which point the ferric ion is presented to apo-FbpA, which we demonstrated interacts with TbpA. Apo-transferrin release would re-set the system to baseline but would not occur if all of the previous steps had not been accomplished, as in the TonB-box mutant.

TonB-INDEPENDENT USE OF IRON AND XENOSIDEROPHORES

While use of ferric siderophores in other Gram-negative bacteria is accomplished via TonB and TonB-dependent transporters, several iron sources are employed by *N. gonorrhoeae* in a Ton-independent manner. Ferric citrate, free heme (Biswas et al., 1997), and some xenosiderophores (Strange et al., 2011) are acquired by the gonococcus in pathways that do not depend upon expression of TonB or any of the individual TonB-dependent transporters. The mechanisms by which ferric citrate and heme are internalized have not been explored, but we recently demonstrated that gonococcal strain FA19 utilizes the xenosiderophores ferric enterobactin, DHBS, and salmochelin S2 in a pathway that is TonB-independent (Strange et al., 2011). Use of the xenosiderophores by strain FA19 is limited to an inefficient, Ton-independent system, which requires expression of the FbpABC proteins (**Figure 5**). When the Fbp proteins were not expressed, xenosiderophore utilization was completely prevented. Since mutants lacking the outer membrane protein complexes Mtr or PilQ remained competent for Ton-independent iron acquisition (Strange et al., 2011), we suggest that the xenosiderophores permeate the outer membrane passively, perhaps through porins (**Figure 5**). This is in contrast to utilization of enterobactin, DHBS, and S2 by gonococcal strain FA1090, which occurs in a TonB- and FetA-dependent pathway (**Figures 4 and 5**; Carson et al., 1999; Hollander

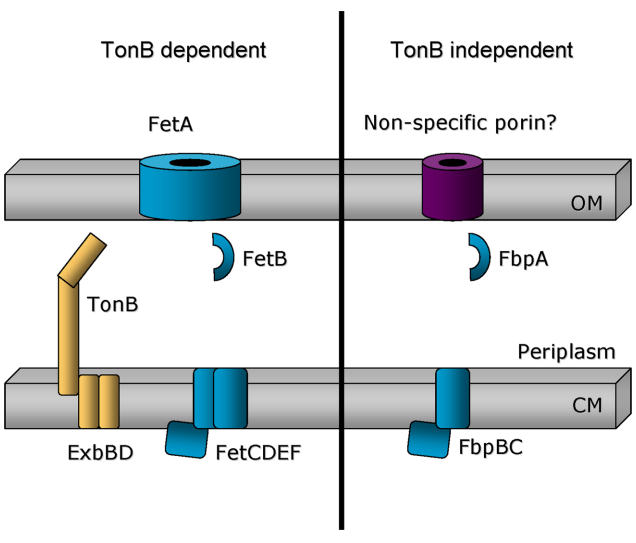

FIGURE 5 | Model comparing TonB-dependent and TonB-independent iron acquisition systems. Left: Ferric-catecholate siderophores are imported through the outer membrane in a TonB- and FetA-dependent mechanism. Use of these ferric-siderophores is expected to require proteins encoded by genes located downstream of *fetA*. Right: In gonococcal strain FA19, use of ferric-catecholate siderophores is limited to a TonB-independent mechanism that involves the participation of the FbpABC system. Gonococcal strain FA1090 appears to be able to employ both TonB-dependent and TonB-independent mechanisms for the use of ferric siderophores, including enterobactin, DHBS, and salmochelin.

et al., in revision). We propose that all gonococcal strains have the capacity to internalize iron from xenosiderophores by both FetA-dependent and FetA-independent pathways. In FA1090, xenosiderophore-dependent growth by the *tonB* and *fetA* mutants was diminished relative to wild-type, but not eliminated, suggesting that both TonB-dependent and TonB-independent pathways are functional. FA19 however is limited to the TonB-independent pathway. Why might FA19 be incapable of Fet-mediated internalization of enterobactin and its derivatives? One reason could be because FetA expression varies between strains and isolates due to the poly C-tract in the promoter region (Carson et al., 2000). Another possible explanation could be related to the observation that the genome sequence of gonococcal strain FA19 contains a frame-shift mutation within *ng2090* (encoding FetD in **Figure 5**). We have confirmed by direct sequencing of a PCR product amplified from FA19 chromosomal DNA that this lesion represents a genuine mutation and is not a sequencing error (data not shown). This mutation would result in a truncated FetD protein and may prevent expression of downstream genes as well. We hypothesize that one or both of these genetic differences between strains FA1090 and FA19 result in a defective Fet system and a reliance on the TonB-independent uptake pathway for uptake of iron from xenosiderophores in strain FA19.

We have also detected strain-specific, Ton-independent growth phenotypes with respect to intracellular survival. While we demonstrated that intracellular survival of strain FA1090 was TonB- and TdfF-dependent (Hagen and Cornelissen, 2006), we found that this was not the case in two other gonococcal strains. Intracellular survival of strains FA19 and MS11 did not depend upon expression of either TonB or TdfF (Zola et al., 2010). This suggested that something unique to these strains allowed a bypass of the need for high affinity iron acquisition through the TonB system and TonB-dependent transporters. Both FA19 and MS11 differ from FA1090 by possessing the gonococcal genetic island (GGI), which encodes a type IV secretion system (Hamilton et al., 2005). In collaboration with Joe Dillard's group, we created double mutants lacking both the Ton system (a polar mutation in *tonB*) and the GGI. This double mutant was incapable of intracellular survival whereas the single mutants lacking either the Ton system or the GGI remained competent to survive within cervical epithelial cells (Zola et al., 2010). We mapped the key region of the GGI to the structural genes that encode critical components of the type IV secretion apparatus. None of the accessory proteins or known secreted proteins were involved in the TonB bypass mechanism. We proposed three possible explanations for these observations. First, the type IV secretion apparatus may secrete a reductase allowing the gonococcus to acquire ferrous iron in a TonB-independent pathway. Second, the type IV secretion apparatus may simply form an indiscriminate pore through the cell envelope, allowing ferric iron entry. And finally, the type IV secretion system may facilitate the entry of the gonococcus into an intracellular niche that does not require high affinity iron acquisition via the Ton system.

CONTROLLED EXPRESSION OF IRON ACQUISITION SYSTEMS

REPRESSION

All of the characterized iron acquisition systems are subject to iron-dependent repression. The most common mechanism employed for iron-dependent repression is mediated through the function of the *f*erric *u*ptake *r*egulator (Fur) protein. Iron-regulated expression of the transferrin and lactoferrin binding proteins is known to be Fur dependent (Thomas and Sparling, 1996). As is the case in other Gram-negative bacteria (Escolar et al., 1999) when Fur is complexed with ferrous iron, the protein dimerizes and binds to a DNA sequence called a "Fur box," which generally overlaps with the promoter region preceding iron-repressed genes (Sebastian et al., 2002; Jackson et al., 2010). However, there are examples of Fur binding sites within structural genes or overlapping transcriptional start sites. In any event, binding of Fur results in the inaccessibility of the promoter to the RNA polymerase or the inability of the RNA polymerase to move processively down the template strand to transcribe the entire gene. When iron is limiting, the apo-Fur protein no longer functions as a repressor, thus gene expression is enabled. This simple mechanism results in expression of iron acquisition systems only under conditions of iron depletion.

ACTIVATION

There are no characterized activators of gonococcal iron transport systems. However, we recently discovered that an iron-repressed, AraC-like regulator activates expression of *fetA* (Hollander et al., in revision). MpeR was previously shown to regulate the expression of *mtrF*, which in turn controls the activity of the MtrCDE efflux pump (Folster and Shafer, 2005). We subsequently observed that MpeR is encoded upstream of TdfF and an iron-related PBP annotated as FetB2 (see above). When *mpeR* was inactivated, we were surprised to discover that another TonB-dependent transporter, FetA, was no longer expressed. We determined that MpeR binds to the region upstream of the *fetA* gene, consistent with a direct mechanism of activation. These observations cumulatively link expression of antimicrobial efflux systems with iron transport systems through the iron-regulated expression of a key regulator, MpeR. As noted above, the *mpeR – tdfF* locus is unique to the pathogenic *Neisseria* species, which suggests that optimum expression of efflux systems and iron transport systems represents an important virulence mechanism.

TRANSLATIONAL IMPLICATIONS

HUMAN AND ANIMAL INFECTION EXPERIMENTS

The transferrin–iron acquisition system was the first to be tested for its influence on the outcome of experimental human infections. Gonococcal strain FA1090 was chosen for human infection experiments because the Opa protein profile has been well characterized in this background (Connell et al., 1990; Jerse et al., 1994). FA1090 is also naturally unable to utilize lactoferrin as a sole iron source due to the inability to express either of the lactoferrin binding proteins. We created a mutant derivative of gonococcal strain FA1090 that no longer expressed either of the Tbps, effectively generating a double mutant lacking the ability to employ either transferrin or lactoferrin as sole iron sources. This mutant was completely unable to initiate urethritis in a human male infection model, whereas infection with the wild-type FA1090 resulted in urethritis within 3–4 days of inoculation (Cornelissen et al., 1998). These results indicated that expression of the Tbps was critical for the first steps in initiating infection on a human male urethra. While suggestive, these findings do not definitively implicate the use of transferrin as a key for initiating infection since the Tbps could conceivably serve multiple functions *in vivo*, as has been documented with other gonococcal

proteins. Subsequently, Anderson et al. (2003) generated a derivative of FA1090 in which the lactoferrin binding proteins were expressed, but the genes encoding the Tbps were deleted. Unlike the *tbp* mutant parent, the Lbp$^+$ variant was capable of causing urethritis in human male volunteers. These results suggested that expression of the Lbp proteins, while not universal among gonococci, was sufficient to restore infectivity. The reason that so many gonococci have lost the capacity to express the lactoferrin–iron acquisition system, in spite of its obvious *in vivo* utility, remains unclear.

The hemoglobin–iron acquisition system, comprised of HpuA and B described above, is subject to phase variation due to a poly G tract within the HpuA coding region. Depending upon the number of G residues, the HpuA gene is either in- or out-of-frame. Anderson et al. (2001) demonstrated that variants capable of expressing HpuA were selected for *in vivo*, but only under specific conditions. By screening gonococcal strains isolated directly from infected men and women, these investigators determined that women in the early phase of their menstrual cycle disproportionately harbored gonococcal isolates that expressed HpuA and grew on hemoglobin. These results suggest that the ability to grow on hemoglobin is selected for by the intermittent presence of this protein in the human genital tract.

Jerse and colleagues (Jerse, 1999) have developed a mouse model of lower female genital tract colonization to facilitate studies of gonococcal pathogenesis and vaccine development. While the gonococcus is clearly species restricted to humans for normal genital tract infection, the mouse model mimics lower genital tract infection in humans in many ways. We tested gonococcal mutants of strain FA1090 individually lacking the ability to employ transferrin, lactoferrin, hemoglobin or ferric enterobactin for their ability to colonize the genital tract of female mice (Jerse et al., 2002). All mutants were competent for colonization, including a gonococcal *tonB* mutant (data not shown). These results imply that other sources of iron are available in the female genital tract, and that gonococcal access to this nutrient in the mouse does not depend upon expression of TonB-dependent systems or transporters. Perhaps due to the lower pH of the female genital tract and the co-localization of lactobacilli, iron may be more accessible, and thereby may not require high affinity acquisition systems for entry.

VACCINE DEVELOPMENT

As described above, many of the characterized TonB-dependent transport systems are subject to high frequency phase variation or are not universally expressed by gonococci. The exception to this observation is the transferrin–iron acquisition system, which is ubiquitously expressed by gonococci. The Tbps are obviously expressed in humans during infection; if this were not the case, the *tbp* mutant would have been without a phenotype in the human infection model. We detected low but measurable antibody titers in the serum and vaginal wash specimens from naturally infected women and men (Price et al., 2004). These antibody levels were well below those associated with a protective response, as was detected against tetanus toxoid. While the Tbps are clearly expressed *in vivo* (Agarwal et al., 2005), the mere presence of these proteins within the context of a natural infection is insufficient to initiate a high-titer, protective immune response (Price et al., 2004), consistent with the lack of immunity following natural infections.

A gonococcal vaccine has been sought for many years, but due to high frequency phase and antigenic variation and immune evasion and subversion strategies, no vaccine formulation of protein antigens or surface structures has met with any success. Because the gonococcal Tbps are ubiquitously expressed, not subject to high frequency variation and necessary to initiate infection in human males, we have focused vaccine development efforts on these proteins. When conjugated to the B subunit of cholera toxin, both TbpA and TbpB generated IgG in the serum of intranasally vaccinated mice (Price et al., 2005). TbpB-specific antibody levels were significantly higher than those generated against TbpA. Antibodies elicited against the Tbp proteins were bactericidal in the presence of human complement. Mucosal antibodies, detected in the genital tract, were also generated against the Tbp proteins. We have also immunized mice with portions of the Tbp proteins, genetically fused to the A2 domain of cholera toxin (Price et al., 2007). Co-expression of the fusion proteins with the B subunit of cholera toxin resulted in a complex comprised of the B pentamer and immunogenic regions of the Tbps. We chose the amino-terminal lobe of TbpB and surface-exposed loop 2 of TbpA for this analysis. Antibodies generated following intranasal vaccination with these epitope-specific antigens were detected in the serum and mucosal secretions. Serum antibodies in the presence of human complement were bactericidal against three different gonococcal strains and interestingly, vaginal secretions were growth inhibitory when human transferrin was provided as a sole iron source *in vitro*. These studies suggest that the Tbps can elicit antibodies with important and potentially protective biological properties. We are currently testing whether Tbp-specific antibodies are protective in a mouse model of lower female genital tract colonization.

CONCLUSIONS

TonB dependent transporters enable the efficient use of iron sources that are otherwise unavailable due to insolubility, low concentration, or chelation by iron-binding proteins. *N. gonorrhoeae* has the capacity to encode up to eight transporters, facilitating the use of a variety of iron compounds derived from or produced in the human host. The genes encoding the lactoferrin–iron acquisition system are intact only in a minority of gonococcal strains, suggesting that selective pressures *in vivo* have resulted in the loss of lactoferrin binding capability over time. The hemoglobin–iron acquisition system is off, due to phase variation, in most gonococcal strains. However, use of hemoglobin-derived iron through expression of this system seems to be selected for *in vivo* in women during the early phase of the menstrual cycle. The enterobactin– and salmochelin–iron acquisition system is also subject to phase variation, but expression is tuned up or down by alteration of promoter strength, suggesting that the presence of xenosiderophores in the human genital tract could select for variants with enhanced capability to internalize these iron chelates. Substrates and gene expression characteristics have not been defined for the four recently recognized TonB-dependent transporters (TdfF, G, H, and J), the genes for which are found in all gonococcal strains sequenced to date. While the substrate has not yet been defined, TdfF appears to play an important role in gonococcal survival inside of human cervical epithelial cells. The transferrin–iron acquisition system is ubiquitously expressed by all gonococcal isolates and is

the best characterized of all the gonococcal TonB-dependent transport systems. Cumulatively, studies of this iron transport system lead to the following model of vectorial transport of iron from human transferrin to the periplasm (**Figure 3**). The gonococcal TonB-dependent transporter, TbpA, is sufficient for iron acquisition from transferrin but is aided in this process by the lipoprotein, TbpB. Both proteins bind human transferrin specifically but TbpB further differentiates between the ferrated and apo forms of the protein, allowing for selection of the proper iron-loaded ligand and efficient release of the used transferrin subsequent to iron extraction. Both TbpA and TbpB participate in iron extraction at the cell surface, which results in free iron being captured by the TbpA plug domain. This interaction presumably effects the exposure of the TonB-box domain of TbpA, signaling to TonB that ligand, in this case free iron, is bound and ready for transport. TonB, in its energized form, tugs on the plug domain, causing a localized rearrangement and presentation of the iron atom at the inner orifice of the barrel, whereupon FbpA sequesters the iron for transport across the periplasm. Because expression of the transferrin–iron acquisition system is conserved among strains and necessary to initiate human male infection, the proteins that comprise this system are considered viable vaccine candidates. Initial studies suggest that vaccination with these proteins elicits biologically functional antibodies engendering hope that a gonococcal vaccine containing these proteins (and perhaps others too) could be efficacious and protective against the common STI caused by *N. gonorrhoeae*.

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The composition and metabolic phenotype of *Neisseria gonorrhoeae* biofilms

Megan L. Falsetta[1†], Christopher T. Steichen[1†], Alastair G. McEwan[2], Christine Cho[1], Margaret Ketterer[1], Jianqiang Shao[1], Jason Hunt[1], Michael P. Jennings[3] and Michael A. Apicella[1]*

[1] *Department of Microbiology, The University of Iowa, Iowa City, IA, USA*
[2] *School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia*
[3] *Institute for Glycomics, Griffith University, Gold Coast, QLD, Australia*

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Robert A. Nicholas, University of North Carolina at Chapel Hill, USA
Virginia Clark, University of Rochester, USA

***Correspondence:**
Michael A. Apicella, Department of Microbiology, The University of Iowa, 51 Newton Road, BSB 3-403, Iowa City, IA 52242, USA.
e-mail: michael-apicella@uiowa.edu

†Megan L. Falsetta and Christopher T. Steichen have contributed equally to this work.

Neisseria gonorrhoeae has been shown to form biofilms during cervical infection. Thus, biofilm formation may play an important role in the infection of women. The ability of *N. gonorrhoeae* to form membrane blebs is crucial to biofilm formation. Blebs contain DNA and outer membrane structures, which have been shown to be major constituents of the biofilm matrix. The organism expresses a DNA thermonuclease that is involved in remodeling of the biofilm matrix. Comparison of the transcriptional profiles of gonococcal biofilms and planktonic runoff indicate that genes involved in anaerobic metabolism and oxidative stress tolerance are more highly expressed in biofilm. The expression of *aniA*, *ccp*, and *norB*, which encode nitrite reductase, cytochrome *c* peroxidase, and nitric oxide reductase respectively, is required for mature biofilm formation over glass and human cervical cells. In addition, anaerobic respiration occurs in the substratum of gonococcal biofilms and disruption of the *norB* gene required for anaerobic respiration, results in a severe biofilm attenuation phenotype. It has been demonstrated that accumulation of nitric oxide (NO) contributes to the phenotype of a *norB* mutant and can retard biofilm formation. However, NO can also enhance biofilm formation, and this is largely dependent on the concentration and donation rate or steady-state kinetics of NO. The majority of the genes involved in gonococcal oxidative stress tolerance are also required for normal biofilm formation, as mutations in the following genes result in attenuated biofilm formation over cervical cells and/or glass: *oxyR*, *gor*, *prx*, *mntABC*, *trxB*, and *estD*. Overall, biofilm formation appears to be an adaptation for coping with the environmental stresses present in the female genitourinary tract. Therefore, this review will discuss the studies, which describe the composition and metabolic phenotype of gonococcal biofilms.

Keywords: *Neisseria gonorrhoeae*, biofilm, DNA, thermonuclease, matrix, anaerobic, enzymes

INTRODUCTION

Gonococcal infection has been recognized by different human societies as a distinct disease for over 4,000 years (Handsfield and Sparling, 2005). Gonorrhea, the infection caused by the *Neisseria gonorrhoeae*, is the second most commonly reported notifiable disease in the United States today with 355,991 cases of gonorrhea reported in 2007 (Anonymous, 2008). The infection rate in the United States has not changed since 1994 (Anonymous, 2008). Gonococcal disease in young women (age 15–25) has many consequences including increasing the risk for infertility, ectopic pregnancy, tubo-ovarian abscesses, and HIV infection (Holmes et al., 1999). In some cities in the US, infection rates in this age group approaches and exceeds 1.5% of the population (Jennings et al., 2010). Annually worldwide, approximately 69 million new cases of gonorrhea occur with the greatest number in Southeast Asia and Sub-Saharan Africa (Gerbase et al., 1998). Infection in 99% of men is symptomatic and treatment is sought quickly (Holmes et al., 1999). In contrast, up to 40% of infected women frequently exhibit no noticeable symptoms and are susceptible to chronic complications from undiagnosed gonorrhea (Holmes et al., 1999; Anonymous, 2008). The reservoir of asymptomatic infected individuals and antimicrobial resistance are two of the major contributors to the spread of gonorrhea (Holmes et al., 1999).

Our laboratory has shown that cervical gonorrhea involves a biofilm component (Greiner et al., 2005; Steichen et al., 2008). This contributes to persistence, and it has been established in the literature that biofilms are inherently resistant to antimicrobials (Ceri et al., 1999; Schierholz et al., 1999; Dunne, 2002), although this has not been directly tested for *Neisseria*. We now have evidence that the gonococcal biofilm matrix is composed of shed *N. gonorrhoeae* outer membrane and DNA, which can be remodeled by a chromosomally encoded nuclease. In addition, nitric oxide (NO) appears to be a factor that stimulates biofilm dispersal (Falsetta et al., 2010). The consequences of gonorrhea are significant, negatively affecting the reproductive health of infected individuals and helping to increase the spread of other sexually transmitted diseases, such as HIV1 (Holmes et al., 1999). Since this organism is an obligate human pathogen, the potential to eliminate gonorrhea with an effective vaccine is theoretically possible. However, through its close association with the human, the organism has evolved a repertoire of mechanisms to evade the human immune system including

antigenic and phase variation, molecular mimicry, resistance to host oxidative processes and the ability to incorporate DNA from its environment all of which make effective vaccine development problematic and unlikely to occur in the near future (Handsfield and Sparling, 2005). Two features of this infection make eradication very difficult: asymptomatic carriage and an increasing number of antimicrobial resistance strains (Holmes et al., 1999). Every decade since the 1950s, the CDC recommendations for the treatment of gonorrhea have become more aggressive and more expensive, from low dose penicillin G in the 1950s to comparatively expensive, injectable, long acting cephalosporins today (Anonymous, 2008). Resistance to these cephalosporins is now being reported (Bala and Sood, 2010; Golparian et al., 2010). Novel approaches to therapy are needed, and it is possible that greater understanding of the critical points in the pathogenesis of human infection, as well as improving the methods of diagnosis in asymptomatic women, may enable better multi-drug therapies to be applied. New multi-drug therapies would ideally reduce the emergence of resistance and eliminate the asymptomatic (female) carrier.

Since its discovery in 1879 until the 1990s, *N. gonorrhoeae* was considered an extracellular pathogen. Our work, and that of others, has shown that this organism can infect human genital cells in men and women and that these processes are crucial in pathogenesis (Edwards and Apicella, 2004). We have recently shown that the organism has the ability to form biofilms on the cervical epithelial cell surface in women (Steichen et al., 2008). It is well known that organisms within biofilms can be significantly more resistant to antimicrobials than planktonic organisms (Tart and Wozniak, 2008). As we will discuss in this review, the gonococcal biofilm matrix is composed of shed outer membranes and a matrix composed of DNA released from these blebs (Steichen et al., 2008). During the course of our work, we have identified a nuclease encoded in the gonococcal chromosome, which appears to be involved in remodeling of the biofilm matrix. We believe, but have not proven, that the activity of this nuclease is regulated. Complementary studies performed in our laboratories have examined the metabolic phenotypes of gonococcal biofilms, subsequently identifying a number of enzymes crucial for biofilm development (Seib et al., 2004, 2005, 2006, 2007; Wu et al., 2005, 2006, 2010; Lim et al., 2008; Potter et al., 2009a,b; Falsetta et al., 2010). In addition, our laboratories have shown that nitric oxide plays a dual role in biofilm formation. In the predominately anaerobic environment of the human cervix, nitric oxide (NO) plays a role as an electron acceptor in a respiratory pathway necessary for the survival of the gonococcus. Our studies have shown that nitric oxide reductase (NorB) is critical for production of a biofilm (Falsetta et al., 2009, 2010). NorB is involved in the reduction of nitric oxide (NO) to nitrous oxide (N_2O) and mutants in *norB* accumulate NO resulting in biofilm dispersal (Householder et al., 2000; Falsetta et al., 2009, 2010), which can be reversed by an NO scavenger. Our studies have also shown that NO is involved in biofilm dispersal (Falsetta et al., 2009, 2010). Moreover, transcriptomic and proteomic analyses of *N. gonorrhoeae* biofilm formation using *in vitro* models have shown a dynamic reorganization of gene and protein expression profiles between planktonic and biofilm populations. These data have provided us with new insights into the underlying biology of biofilm formation and have identified sets of proteins that are up- and/or down-regulated in these two states. For example, proteins such as nitrite reductase (AniA) and the RpiR transcriptional regulator are found to significantly increase in biofilms, while ferric enterobactin receptor (FetA) and transferrin-binding protein A (TbpA) appear more highly expressed in planktonic organisms. This review will focus on the available studies of *N. gonorrhoeae* biofilm, which have been conducted almost exclusively by our laboratories. Here we describe the unique structure, composition, and metabolic phenotype of gonococcal biofilms, which contributes to colonization and persistence in women, and may be paramount to its success as a pathogen in the cervical environment.

THE STRUCTURE AND COMPOSITION OF GONOCOCCAL BIOFILMS

We have investigated the matrix components of the *N. gonorrhoeae* biofilm. The organism lacks genes capable of producing exopolysaccharides. Instead, our studies have shown that membrane blebbing is necessary for mature biofilm formation (Steichen et al., 2008). Previously, Dorward et al. (1989) showed that *N. gonorrhoeae* membrane blebs were associated with large amounts of DNA. This prompted us to examine the gonococcal biofilm for the presence of DNA. DNA has been shown to be a major constituent of *Pseudomonas aeruginosa* and non-typeable *Haemophilus influenzae* biofilms. Our experiments showed bovine pancreatic DNaseI can rapidly disrupt established *N. gonorrhoeae* biofilms under continuous-flow conditions (**Figure 1**). In addition, confocal microscopy and electron microscopic studies demonstrated that DNA is a major component of the *N. gonorrhoeae* biofilm (**Figure 2**). Concomitant with this, we discovered that a hypothetical protein (NGO0969) encoded in *N. gonorrhoeae* 1291 genome had 25% identity and 40% similarity to the *Staphylococcus aureus* secreted thermonuclease that we have denoted Nuc (Steichen et al., 2011). The *nuc* gene appears to be co-transcribed in an operon containing at least six other genes, NGO0968–NGO0974. In order to determine if the protein encoded by the NGO0969 open reading frame (ORF) is in fact able to degrade DNA, we expressed this protein, designated Nuc, in *Escherichia coli.* The ORF was amplified starting from codon #36 as the online signal sequence predictor, SignalP 3.0, predicts the first 34 amino acids to be a TAT dependent signal peptide. Our results using the purified Nuc demonstrated it is ability to digest a single stranded DNA oligonucleotide as well as chromosomal DNA from a variety of prokaryotic and eukaryotic sources. To determine if activity of the Nuc protein requires the presence of divalent cations, identical experiments were run with EDTA (added as a chelating agent) to a final concentration of 4 mM. The addition of EDTA completely inhibited the activity of Nuc and no increase in fluorescence was observed. This indicates that the divalent cations (Ca^{2+} and/or Mg^{2+}) supplied in the buffer are necessary for Nuc DNA digestion activity. No nuclease activity was detected enzymatically in planktonic runoff or broth supernates, yet activity was present in *N. gonorrhoeae* lysates. Our biochemical studies also indicate that the methylation state of the DNA in the gonococcal biofilm is a factor in determining the susceptibility of gonococcal DNA to digestion (Steichen et al., 2011). The gonococcus is known to actively secrete DNA (Hamilton et al., 2005). Given that Nuc has a N-terminal *tat* dependent signal peptide, we postulate that as

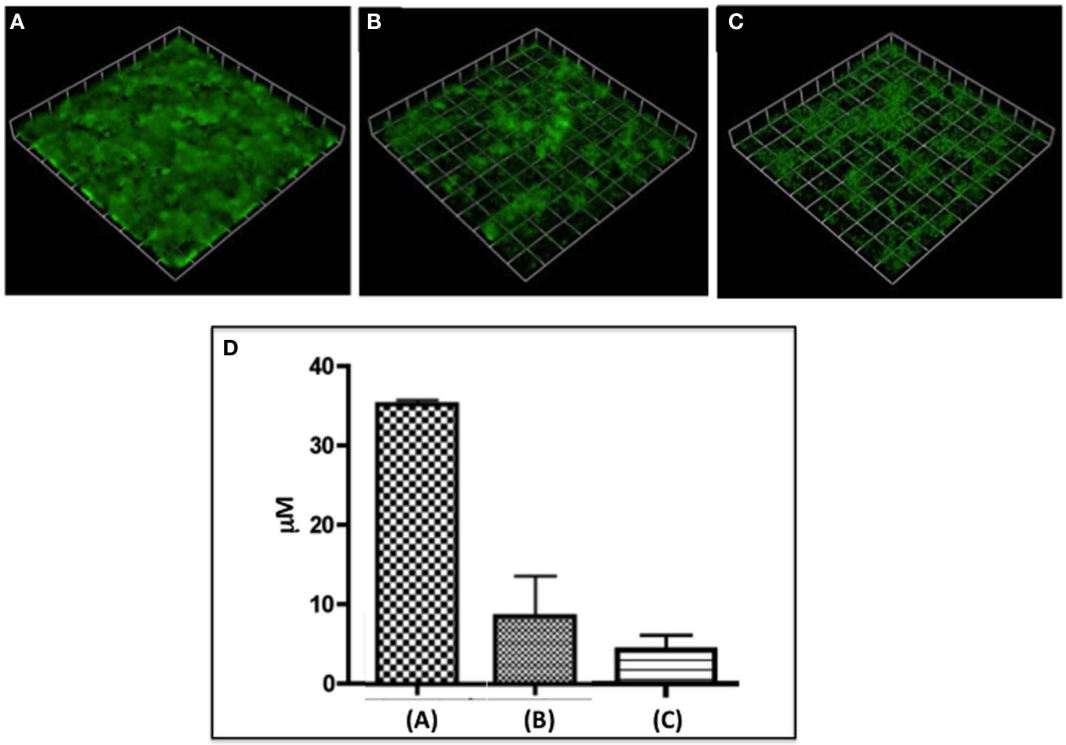

FIGURE 1 | It shows *N. gonorrhoeae* 1291 biofilms either untreated (A) or treated for 5 h with 2 U/ml DNaseI (B) or 5 μgm/ml *Neisseria* nuclease (C). **(D)** Shows a Comstat analysis of the average height of the respective biofilms. This figure demonstrates significant loss of biofilm after treatment with either nuclease.

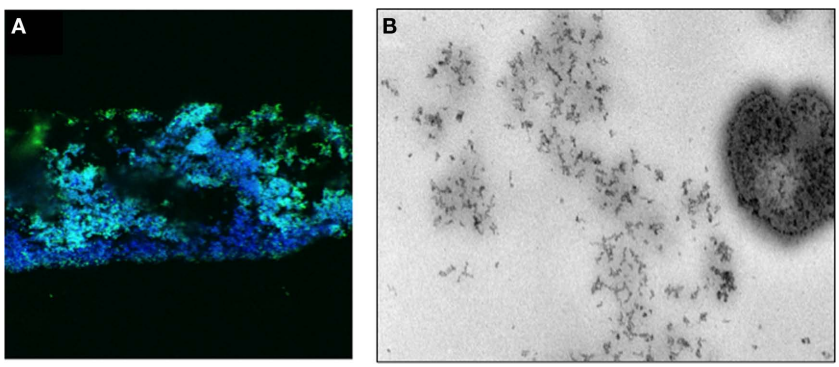

FIGURE 2 | (A) shows a confocal micrograph of a frozen section of a biofilm of *N. gonorrhoeae* 1291 expressing pGFP (green) and stained with DAPI (blue). **(B)** Shows an electron micrograph of a cryosection of a 48-h *N. gonorrhoeae* 1291 biofilm stained propidium iodide. The section was treated with RNase and proteinase K sequentially prior to staining with propidium iodide. The speckled DNA staining can be seen in the matrix substance in the biofilm and in the bacteria seen in the image.

DNA is secreted by the gonococcus, it is modified by the nuclease in the periplasm prior to secretion. The degree of remodeling determines the amount of DNA incorporated into the biofilm. We have evidence that this is a regulated process, as we have found that the abundance of DNA varies depending on the age and developmental state of the biofilm. Using DAPI staining, we demonstrated that 48 h *N. gonorrhoeae* 1291 biofilms contain DNA in the matrix, and that the amount of DNA is elevated in the 1291 *nuc* mutant (**Figure 2**). In separate experiments, quantitative analysis of a collected *z*-series was performed using COMSTAT software, to obtain numeric values for the total biomass and average thickness of these biofilms. Statistical analysis of these *z*-series using Student's *t*-test indicated that the *nuc* mutant produces a biofilm with greater biomass and average thickness than that of the parent strain $p = 0.01$. Chromosomal complementation of the *nuc* mutant with *nuc* results in biofilms with height and mass similar to the wild type strain and significantly different than the *nuc* mutant ($p < 0.001$).

THE METABOLIC PHENOTYPE OF GONOCOCCAL BIOFILMS

Prior to our work, little was understood about the mechanisms that contribute to or govern biofilm formation in *N. gonorrhoeae*. Much of the current biofilm literature suggests that biofilms exhibit unique patterns of gene expression that dictate community behaviors, including resistance to antimicrobials and host immune defenses (Whiteley et al., 1999; Beloin and Ghigo, 2005; An and Parsek, 2007). Using microarray analysis, 83 genes were identified that met our criteria for differential expression (fold-change ≥ 2.0 and $p \leq 0.05$) when biofilms grown over glass were compared to planktonic cells filtered from the biofilm effluent, the vast majority of these genes were hypothetical (57.8%), although many of the proteins encoded by these genes could play important roles in biofilm formation (Falsetta et al., 2009). We found that genes involved in anaerobic respiration (*aniA*, *ccp*, and *norB*) were highly up-regulated during biofilm formation, while genes involved in aerobic respiration were down-regulated (*nuo* operon; Falsetta et al., 2009). qRT-PCR was used to confirm expression of these genes over glass and to examine expression in a more relevant model system (growth over transformed human cervical epithelial cell, THCEC). We found that the pattern of regulation was similar over glass and over THCEC (Falsetta et al., 2009). This finding would suggest that expression of these genes is relevant to infection. These data also indicate that our continuous-flow system for biofilm growth over glass may be a suitable model for biofilm formation when the experimental design does not permit the use of cervical cells. For example, this system may be a useful tool for modeling biofilm behaviors during treatment with compounds that are toxic to or impair the growth of primary cervical cells.

The pathogenic *Neisseria* have evolved a number of mechanisms to deal with oxidative stress (**Figure 3**). High levels of expression of *aniA* and *norB* during growth as a biofilm indicates that anaerobic respiration occurs in gonococcal biofilms (Householder et al., 1999, 2000). *N. gonorrhoeae* is often isolated in the presence of obligate anaerobes and the cervical environment is presumed to be oxygen limited (Smith, 1975). Thus, anaerobic growth is important for gonococcal survival *in vivo*. Anaerobic respiration also contributes to the ability of the gonococcus to tolerate oxidative stress, as NorB can rapidly achieve a NO steady-state that reduces proinflammatory concentrations of NO to concentrations that are not inflammatory (Cardinale and Clark, 2005). Like NorB, Ccp also reduces an oxidant present in the cervical environment. Ccp reduces H_2O_2, which is produced by host PMNs and the *Lactobacillus* species that also inhabit the female genitourinary tract (Carreras et al., 1994b). Ccp expression only occurs under anaerobic conditions (Turner et al., 2003), and this would be consistent with the use of hydrogen peroxide as an electron acceptor when oxygen is limited.

The role of Ccp in the defense against hydrogen peroxide is redundant, as gonococcal catalase can also reduce H_2O_2 (Seib et al., 2006). If mutations are made in either the *ccp* or the *katA* (catalase) gene, the resulting strains become sensitive to H_2O_2-mediated killing (Seib et al., 2006). Moreover, a *ccp*/*katA* double mutant is more sensitive to H_2O_2 than either single mutant (Seib et al., 2006). This may help to account for the observation that the *ccp*::kan mutant is only partially impaired in its ability to form biofilms over glass. Thus, we would expect that a *ccp*/*katA* mutant may be further impaired in its ability to form biofilm, although this has not been directly tested. The role of AniA is not functionally redundant and yet the biofilm phenotype of the *aniA*::kan mutant is indistinguishable from the phenotype of the *ccp*::kan mutant. Although a partially attenuated phenotype in the *ccp*::kan mutant may be simply explained by functional redundancy, this does not account for a similar phenotype in the *aniA*::kan mutant. Other organisms also catalyze anaerobic respiration during growth as a biofilm (Hassett et al., 2002; Filiatrault et al., 2006) and our observations would be consistent with a significant role for energy generation via nitrite in the gonococcal biofilm. It has become increasingly evident that anaerobic respiration is critical for biofilm formation in a number of organisms, including the biofilm paradigm organism, *P. aeruginosa* (Hassett et al., 2002; Yoon et al., 2002; Filiatrault et al., 2006; Van Alst et al., 2007). Evidence suggests that anaerobic respiration is the primary mode of growth for *P. aeruginosa* during cystic fibrosis infection. This mode of growth may contribute to the antimicrobial resistance of *P. aeruginosa* biofilms, as cells that undergo anaerobic respiration are concentrated near the biofilm substratum, are less metabolically active, and are likely less susceptible to antimicrobials that target active cellular processes (Werner et al., 2004; Rani et al., 2007). Oxygen only penetrates approximately the first 50 μM of *P. aeruginosa* biofilms (Werner et al., 2004), although these biofilms typically achieve thicknesses of approximately 100 μM or more (Davies et al., 1998; Sauer et al., 2002). This observation may help to explain why the *aniA*::kan

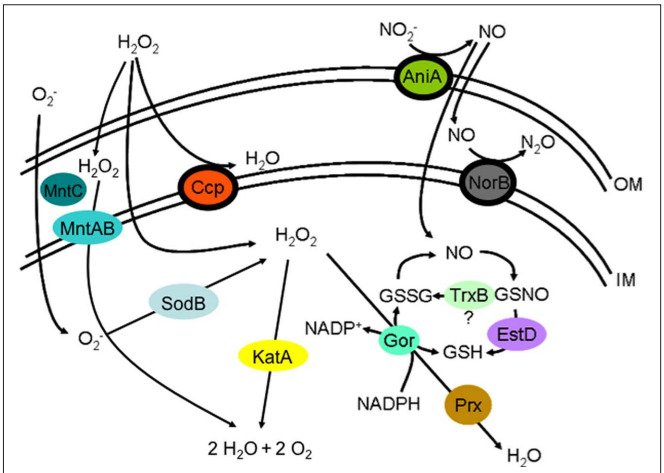

FIGURE 3 | The following is an illustration of the oxidative stress defenses present in *N. gonorrhoeae*. The outer membrane is designated OM, while the inner membrane is designated IM. The space between the two membranes is the periplasm, and the space below the IM is the cytoplasm. Reactive oxygen and nitrogen species and their intermediates are designated as follows: O_2^-, superoxide; H_2O_2, hydrogen peroxide; NO_2^-, nitrite; NO, nitric oxide; N_2O, nitrous oxide; H_2O, water; O_2, oxygen; GSSG, reduced glutathione; GSH, glutathione; GSNO, *S*-nitrosoglutathione; NAPD+, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate. Proteins that catalyze reactions in the oxidative stress pathways are represented as colored ovals and are placed in the positions where they are located in the cell. The arrows denote reactions, and the proteins that catalyze these reactions are placed near or on the appropriate arrows. These proteins are designated as follows: MntABC, manganese ABC transporter; KatA, catalase; SodB, superoxide dismutase; Ccp, cytochrome *c* peroxidase; NorB, nitric oxide reductase; AniA, nitrite reductase; EstD, esterase D; TrxB, thioredoxin oxidase; Gor, glutathione reductase; Prx, peroxiredoxin.

mutant forms biofilms with biomasses that are similar to wild type, yet these biofilms are significantly thinner than the wild type. If oxygen cannot penetrate the entire depth of the biofilm, the thicknesses of the biofilm may be limited by the availability of oxygen in mutants that cannot respire anaerobically. The thickness of an *aniA*::kan mutant biofilm does exceed 60 μM, which appears to correlate with the findings in *P. aeruginosa* (Werner et al., 2004).

Biofilm formation was most severely attenuated in the *norB*::kan mutant, as this mutant had significantly less biomass and lower average thicknesses than the wild type (Falsetta et al., 2009). This was initially puzzling, as *norB* and *aniA* encode components of the partial denitrification pathway found in pathogenic *Neisseria* and previous studies demonstrated that mutations in either *norB* or *aniA* resulted in strains that were unable to respire anaerobically (Householder et al., 1999, 2000). However, there was one obvious difference in these two mutant strains. AniA reduces nitrite to NO (Mellies et al., 1997), which is often toxic to many bacterial species (Fang, 1997; MacMicking et al., 1997; Zumft, 1997; Davies et al., 1998). NorB then reduces NO to nitrous oxide (Householder et al., 2000), which is not generally considered to be toxic (Seib et al., 2006). A mutation in *norB* would render the gonococcus unable to reduce AniA-generated NO. Thus, we considered the possibility that the accumulation of NO may be toxic or could impair biofilm formation in the *norB*::kan mutant through another mechanism, especially considering that NO is an important signaling molecule in eukaryotes (MacMicking et al., 1997; Davis et al., 2001). *N. gonorrhoeae* is presumed to be inherently resistant to NO, and a *norB*::kan mutant survives incubation under anaerobic conditions, which should result in the accumulation of AniA-generated NO (Householder et al., 2000). Thus, it would be unlikely that the accumulation of NO simply impairs biofilm formation through cytotoxicity. Furthermore, the gonococcus produces a variety of proteins that are involved in resistance to nitrosative stress, including esterase D, which is regulated by NmlR and is involved in the tolerance of nitrite and *S*-nitrosogluthathione, as *estD*::kan mutant is sensitive to both (Potter et al., 2009a).

We also used a scanning electron microscopy (SEM, **Figure 4**) technique developed by our laboratory to evaluate the structure of the mutant biofilms (Srikhanta et al., 2009). We found that the architecture of the mutant biofilms was distinctly different from that of wild type biofilms (**Figure 4**). Specifically, wild type biofilms were confluent over the glass surface of attachment and the ability to visualize individual biofilm cells was obscured by copious amounts membrane blebs, which are a large constituent of the biofilm matrix. The *ccp*::kan and *aniA*::kan mutants were both less confluent and contained fewer membrane blebs than the wild type, and the majority of the biofilm cells were exposed and not enmeshed within the biofilm matrix. The *norB*::kan mutant was much more severely attenuated than the *aniA*::kan or *ccp*::kan mutant. Few *norB*::kan cells were adherent to the surface of attachment and almost no matrix material was associated with the cells, although there did appear to be blebs that were associated with the glass surface. Cells in the *norB*::kan mutant biofilms also exhibited an unusual morphology, where these cells appeared to be slightly elongated and smaller in size than the cells in the *aniA*::kan and *ccp*::kan biofilms. The accumulation of NO may also explain the striking morphology of the *norB*::kan mutant biofilms. These findings again confirm that the *norB*::kan mutant is more severely attenuated than either the

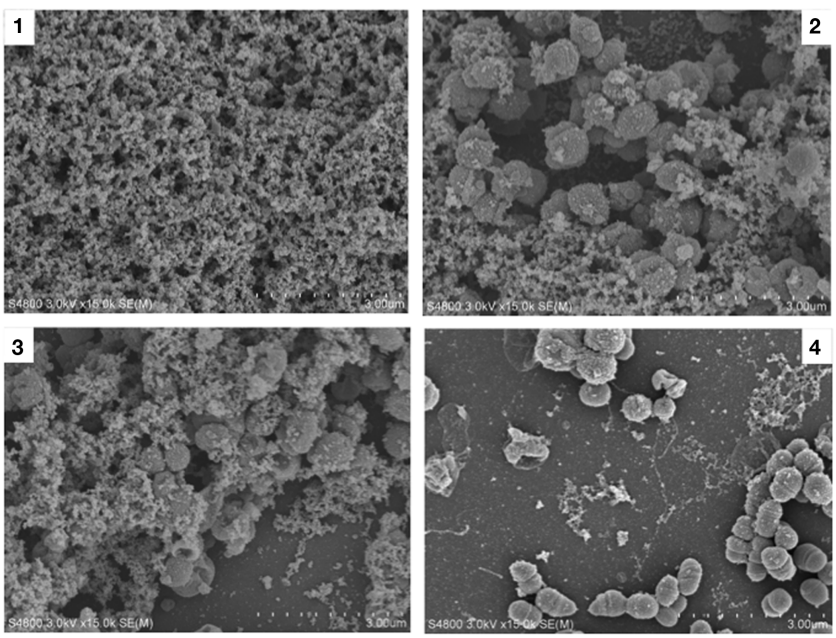

FIGURE 4 | Scanning electron microscopic examinations of biofilms produced by the wild type, *aniA, ccp,* and *norB* mutant. Panel 1 is a scanning electron micrograph of a *N. gonorrhoeae* 1291 48-h wild type biofilm taken at 15,000× magnification. Panel 2 depicts the *aniA*::kan mutant, while panel 3 depicts the *ccp*::kan mutant, and panel 4 depicts the *norB*::kan mutant. The morphology of all three mutants is distinctly different that the wild type, however it is apparent that *norB*::kan mutant is more severely attenuated than the *aniA*::kan and *ccp*::kan mutant.

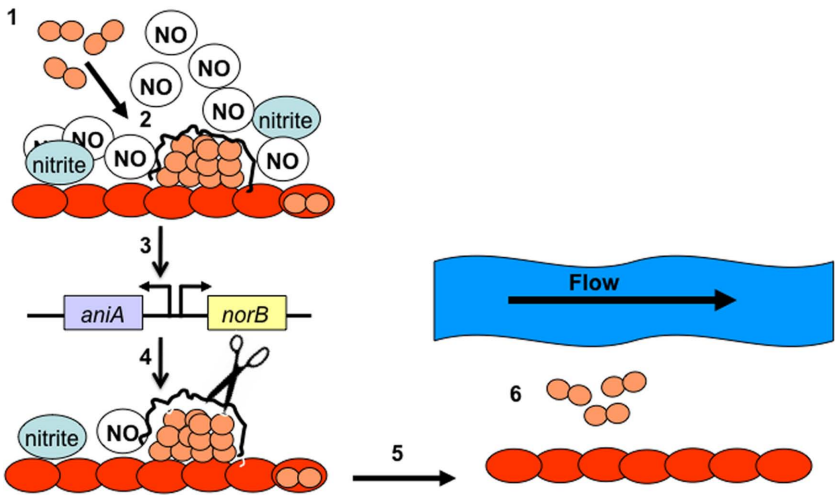

FIGURE 5 | It shows our theoretical model of the roles of AniA, NorB, and NO in remodeling of biofilm structure. During step 1, *N. gonorrhoeae* (depicted in pink) binds to the surface of the cervical cells (depicted in red). During step 2, these cells differentiate into a biofilm, producing a biofilm matrix. In step 3 biofilm formation turns on transcription of *aniA* and *norB*, which produces and modulates the concentration of NO in the surrounding media. In step 4, low levels of NO signal dispersal of the biofilm, which likely occurs through activation of the gonococcal nuclease and degradation of the biofilm DNA matrix (step 5). In step 6, cells that are released from the biofilm may be swept away in bodily secretions, allowing these cells to potentially colonize new areas of cervical tissue.

aniA::kan or *ccp*::kan mutant when grown under continuous-flow conditions in a biofilm. To also test biofilm formation in a more relevant system, we infected THCEC with our mutant and wild type strains. We found that all three mutants had significantly reduced biomasses and average thicknesses compared to the wild type when grown over THCEC for 48 h (Falsetta et al., 2009). This suggests that the role of these genes is more critical in an infection model, which better resembles the conditions present in the host environment.

The striking phenotype of the *norB*::kan mutant led us to investigate the role of NO in *N. gonorrhoeae* biofilms. We hypothesized that NO affected biofilm formation through another mechanism. The *P. aeruginosa* biofilm literature suggested that sublethal concentrations of NO may prevent biofilm formation or facilitate biofilm dispersal (Barraud et al., 2006). Therefore, we attempted to rescue biofilm formation in the *norB*::kan mutant by treating these biofilms with an NO quencher. We found that treatment with PTIO, an NO scavenger, improved biofilm formation in this mutant (Falsetta et al., 2009). Although PTIO improved biofilm formation, biofilms were not completely restored to wild type levels in the presence of PTIO (Falsetta et al., 2009). Wild type biofilms were also treated with the NO donor, sodium nitroprusside (SNP), at the beginning of biofilm formation and after 24 h of biofilm formation to determine if NO could also impair biofilm formation in the wild type. SNP was initially selected, because analogous biofilm studies performed with *P. aeruginosa* demonstrated that SNP was an effective NO donor that elicited biofilm dispersal. We found that treatment at the start of biofilm impaired biofilm formation, but did not completely inhibit biofilm formation. Treatment with SNP after 24 h of biofilm formation resulted in biofilms with less biomass and lower average thicknesses than untreated biofilms (Falsetta et al., 2009). This indicates that the introduction of NO after 24 h of biofilm either halts biofilm development or causes the biofilm to disperse. We also considered the possibility that NO could inhibit cytochrome oxidase, thus impairing aerobic respiration in the *norB*::kan mutant. However, we found that there was no defect in the growth of the *norB*::kan mutant when it was cultured under oxygen tension conditions that were similar to those present in our biofilm system (Falsetta et al., 2009). Treatment with 20 μM concentrations and higher (up to 1 mM) of SNP completely prevented biofilm formation, if it was administered at the start of the biofilm (Falsetta et al., 2009). These findings indicate that higher concentrations of NO could completely block biofilm formation in *N. gonorrhoeae*. *P. aeruginosa*, which is inherently more sensitive to NO, can form better biofilms in the presence of high concentrations of NO (Barraud et al., 2006).When we considered this, it occurred to us that anaerobic respiration would not be immediately induced in gonococcal biofilms, as *aniA* and *norB* are repressed under aerobic growth conditions (Householder et al., 2000; Barraud et al., 2006). Thus, we constructed a fluorescent transcriptional fusion to the *aniA* gene (*aniA′-′gfp*) to monitor the induction of anaerobic respiration in biofilms (Falsetta et al., 2010). We used light microscopy to monitor induction in overnight plate cultures, the biofilm inoculum, and biofilms grown for 24 and 48 h. We were unable to detect fluorescence in plate cultures or the biofilm inoculum. However, we could readily detect GFP in biofilms grown for 24 and 48 h. These data indicate that *N. gonorrhoeae* biofilms become anaerobic over time, as *aniA* expression was induced between 0 and 24 h of biofilm formation (Falsetta et al., 2010). Gradual induction of anaerobic respiration in biofilms could explain why immediate treatment with low concentrations of NO was partially inhibitory, while high concentrations completely inhibited biofilm formation. However, we observed that low doses of SNP halted biofilm formation in 24-h biofilms that were likely expressing *aniA* and undergoing anaerobic respiration (Falsetta et al., 2010). This suggests that the effect of NO on

N. `gonorrhoeae biofilms is not dictated solely by NO toxicity. This agrees with our data that indicate that the *norB*::kan mutant is not impaired in its ability to grow under microaerobic conditions. Microaerobic conditions classically been defined as those of low oxygen tension (e.g., between 5 and 27 mm Hg).

We also used the *aniA′-′gfp* fusion strain to evaluate the profile of anaerobic metabolism in biofilm. *P. aeruginosa* biofilms are stratified and use a combination of anaerobic and aerobic metabolism (Rani et al., 2007). Protein synthesis occurs in the uppermost 30–60 μm of biofilm, while anaerobic/metabolically inactive cells comprise the majority of the biofilm and are localized near the substratum (Werner et al., 2004). To determine if this was similar for *N. gonorrhoeae*, we grew the *aniA′-′gfp* fusion strain for 48 h and then stained the biofilm with 2C3 (H.8 antibody) to visualize all cells in the biofilm. We found that the majority of the biofilm cells expressed *aniA*, and that cells expressing *aniA* were located near the surface of attachment in the substratum of biofilm (Falsetta et al., 2010). The uppermost region of the biofilm was almost entirely comprised of cells that did not express *aniA* (Falsetta et al., 2010). This indicates that cells near the fluid-flow interface primarily use aerobic metabolism. This pattern is similar to the patterns observed in *P. aeruginosa* biofilms (Werner et al., 2004; Rani et al., 2007) *aniA* expression also did not localize to areas where there were gaps in the biofilm, indicating the presence of water channels. Transcripts of *aniA* and *norB* are virtually undetectable under aerobic growth conditions (Householder et al., 1999, 2000; Whitehead et al., 2007). Thus, it is no surprise that *aniA* is not expressed at the biofilm-fluid-flow interface where oxygen would be abundant. The dissolved oxygen concentrations of media entering and exiting the biofilm chamber were calculated and it was found that the concentration of oxygen was well above anaerobic or microaerobic concentrations (Falsetta et al., 2009). Although mutations in anaerobic respiratory genes can cause severe impairment of biofilm formation, especially when cultured in the presence of host cells, these mutations do not completely prevent biofilm formation (Falsetta et al., 2009, 2010). This finding reflects the metabolic profile of *N. gonorrhoeae* biofilms, which catalyze both anaerobic and aerobic respiration. Expression of *aniA* is not induced in the initial biofilm inoculum, which suggests that biofilms may rely on aerobic respiration early during an infection, prior to the establishment of a biofilm and transcription of the anaerobic respiratory genes.

To further examine the effect of NO on biofilm formation, a method was devised to induce anaerobic respiration in biofilms before evaluating the effect of NO. We grew biofilms for 24 h in the presence of nitrite, then transitioned these biofilms to media without nitrite, which either was or was not supplemented with 20 μM SNP. We previously determined that growing biofilms in the presence of nitrite for 24 h induces expression of *aniA* (Falsetta et al., 2009). Biofilm formation was enhanced by the addition of SNP under these conditions (Falsetta et al., 2010). It appears that treatment with high doses of SNP can enhance biofilm formation, if anaerobic respiration has been initiated. Anaerobic respiration likely allows *norB* to be transcribed at the levels necessary to effectively reduce NO concentrations. This observation further supports the hypothesis that *N. gonorrhoeae* biofilms are important for and contribute to oxidative stress tolerance during infection.

In our aim to develop a better model for studying the impact of NO on biofilm formation, another NO donor (DETA/NO) was selected for the treatment of biofilms. SNP has a relatively short half-life (on the order of minutes) and generates both NO and cyanide, the later of which could be toxic to biofilm cells. However, we did measure the concentrations of NO donated from our SNP stocks and determined that authentic NO was generated and that the concentration is not likely to exceed to 20 nM in the biofilm media (Falsetta et al., 2010). SNP was initially selected, so we could perform experiments that would parallel those published for *P. aeruginosa* biofilms, and it can easily be obtained in quantities sufficient to treat biofilm. However, DETA/NO is a more ideal NO donor, as it has a half-life of 20 h (Cardinale and Clark, 2005) and should donate NO gradually during the initial stages of biofilm development. This would presumably better reflect the conditions present *in vivo* where consistent (low) concentrations of NO would be supplied by cervical cells and PMNs (Carreras et al., 1994b; Fang, 1997; Seib et al., 2006). Cardinale and Clark (2005) have shown that the gonococcus can achieve and maintain a NO steady-state of approximately 85 nM after addition of 0.3 mM DETA/NO, when the cells are grown on plates. They also demonstrated that the steady-state achieved is independent of pH, but is dependent on the concentration of nitrite, if the initial concentration of nitrite supplied is below 1 mM. Due to the substantial differences in experimental design between our study and that of Cardinale and Clark (2005, i.e., growth as biofilm versus growth on plates), it is difficult to draw specific conclusions about the NO steady-state present in our biofilm system. However, we did measure the concentration of NO as it was donated from our DETA/NO stocks, and we determined that the average concentration supplied to the biofilm is between 15 and 22 nM. We are not aware of any studies that have directly measured the NO concentrations present in the cervical environment. However, the concentrations of NO supplied by DETA/NO are unlikely to be greater than those present *in vivo*, and based on the work of Cardinale and Clark (2005) these concentrations would not likely be detrimental to the growth *N. gonorrhoeae.*

We found that 20 μM DETA/NO did not prevent biofilm formation, but rather enhanced biofilm formation in the absence of nitrite (Falsetta et al., 2010). This finding suggests that DETA/NO donates NO at a rate that is easily tolerated during the early stages of biofilm development, before the induction of anaerobic respiration. It is obvious that the concentration of NO, the rate at which it is donated, and the time at which it is administered determines the effect on biofilm formation. Low concentrations of NO prevent biofilm formation, even in the presence of nitrite, while high concentrations enhance biofilm formation if a slow-release NO donor is used or anaerobic respiration is occurring in biofilm (Falsetta et al., 2009, 2010). A model has been proposed that illustrates the potential effects of NO on biofilm formation (**Figure 5**). This model proposes that NO is abundant when the gonococcus encounters the cervical environment, which elicits biofilm formation, leading to the expression of anaerobic respiratory genes (e.g., *aniA* and *norB*). Expression of *norB* would result in a reduction of the local NO concentration, signaling dispersal of the biofilm, which would likely be mediated by DNA nuclease (Falsetta et al., 2010). However, additional evidence is needed to either support or refute this model, and it is not clear what protein(s) may be sensing the concentration

of NO in biofilm. We would propose that the NsrR regulator may play a role in sensing the concentration of NO in biofilm, as NsrR is sensitive to NO (NO-sensitive repressor; Whitehead et al., 2007). When NO is present, NsrR is unable to function as a negative regulator of *aniA* and *norB*, which results in the de-repression of *aniA* and *norB* expression (Whitehead et al., 2007). It may be possible that NsrR regulates other previously unidentified targets that could play a role in the NO response. Further study is warranted to investigate this hypothesis.

Biofilms treated with DETA/NO from the start of biofilm formation or treated with SNP after 24 h of growth resembled those grown in the presence of nitrite for 48 h (Falsetta et al., 2009, 2010). Prior to determining that *N. gonorrhoeae* biofilms catalyze anaerobic respiration, it was observed that nitrite enhanced biofilm formation, although biofilms could form in the absence of nitrite (Greiner et al., 2005). Biofilms grown in the absence of nitrite develop at slower rate than those grown in the presence of nitrite (Greiner et al., 2005). Thus, supplementation of biofilms with nitrite allowed mature biofilms to form by 48 h post-inoculation. *N. gonorrhoeae* was initially considered to be incapable of anaerobic growth, due to the inability to culture cells under anaerobic conditions (James-Holmquest et al., 1973). This led to the finding that *N. gonorrhoeae* is unique in that it uses nitrite as a terminal electron acceptor for anaerobic growth, and it is incapable of using nitrate (Knapp and Clark, 1984). However, we found that biofilm growth can be partially restored in an *aniA*::kan mutant by adding NO to the biofilm media (Falsetta et al., 2009, 2010). This mutant cannot reduce nitrite and similar mutants were considered incapable of anaerobic growth. Previous mutants survived, but did not grow under anaerobic conditions (Householder et al., 1999). Our results suggest that this mutant may be able to grow under anaerobic conditions using NO. NO was not used to supplement the culture media in earlier studies that concluded that an *aniA* insertion mutant is unable to respire anaerobically (Householder et al., 1999). Although oxygen is abundant in the media of our biofilm system, the majority of the cells in these biofilms use anaerobic respiration (Falsetta et al., 2009, 2010). Anaerobic respiration occurs in more than two-thirds of the total thickness of *N. gonorrhoeae* biofilms, as visualized by *aniA* expression (Falsetta et al., 2010). Thus, the partial restoration of biofilm formation in the *aniA*::kan mutant strongly suggests that these biofilms are able to undergo anaerobic respiration, as the thickness of these biofilm exceeds the thickness of the aerobic portion of gonococcal biofilms. However, supplementation with NO does not fully restore biofilm formation in the *aniA*::kan mutant (Falsetta et al., 2010). This indicates that we may not have supplemented our media with the optimal concentration of NO, or that nitrite may be the preferred substrate for anaerobic metabolism. The ability to use both nitrite and NO to support anaerobic growth would be of advantage to gonococcal biofilms. If this were the case, NO could be used to support anaerobic growth if the function of AniA was impaired. In support of this hypothesis, some *N. meningitidis* strains possess a frameshift mutation in *aniA* (Pitcher and Watmough, 2004; Potter et al., 2009a), but are still able to respire anaerobically (Rock et al., 2005, 2007; Deeudom et al., 2006). *N. gonorrhoeae* biofilms are metabolically heterogeneous and may be able to use a variety of substrates to catalyze anaerobic and aerobic respiration. Biofilm heterogeneity confers advantages for biofilm survival, including the ability of metabolically inactive cells to resist antimicrobial treatment and the host immune response (Werner et al., 2004; Barraud et al., 2006).

If oxygen is abundant in our biofilm system, why do *N. gonorrhoeae* biofilms predominantly use anaerobic metabolism? The cbb_3 type family of cytochrome oxidases has a high affinity for oxygen (Pitcher and Watmough, 2004) and it has been speculated that the oxygen concentration *in vivo* would have to be considerably lower than the predicted concentration in order to hinder aerobic growth of the gonococcus. Studies that examined the oxygen profiles of *P. aeruginosa* biofilms determined that oxygen is limited in its ability to diffuse into the biofilm (Werner et al., 2004; Barraud et al., 2006). This may be the simplest explanation for the metabolic profile of *N. gonorrhoeae* biofilms, which catalyze aerobic respiration at the fluid-flow interface and anaerobic respiration in the depths of the biofilm. However, the matrix of *N. gonorrhoeae* biofilm is dramatically different than *P. aeruginosa* biofilm, and it is not clear as to whether the diffusion of oxygen is limited in gonococcal biofilms (Costerton et al., 1999; Steichen et al., 2008). Our results suggest that diffusion of oxygen into *N. gonorrhoeae* biofilms is limited. This may be a product of slowed diffusion due to the presence of the biofilm matrix and/or aerobic respiration at the biofilm-fluid-flow interface, which may consume the oxygen available in the bulk fluid, subsequently impeding diffusion into the biofilm.

Another possibility is that there is a complex interaction between the partial denitrification pathway and aerobic respiration. Moir and co-workers (Rock et al., 2005) have shown that in *N. meningitidis* that accumulation of NO above 100–200 nM results in inhibition of oxygen respiration. Thus, it is possible that respiration with nitrite and NO is of central importance within the biofilm even when oxygen is available and the balance between partial denitrification and aerobic respiration may depend upon the local concentrations of NO in the biofilm. It is interesting to note that the CcoP subunit of the *N. gonorrhoeae* cytochrome cbb_3 is a triheme that contains an additional *c*-type cytochrome compared to homologs in other bacteria. The additional heme center seems to be important for electron transfer to the nitrite reductase but not the cytochrome cbb_3 (Hopper et al., 2009). The respiratory chain may be organized in this way to allow electron transfer to oxygen or nitrite down a common respiratory pathway that bifurcates just before the terminal reductases.

The induction of anaerobic genes in biofilm may play an important role in oxidative stress tolerance. NorB functions dually in anaerobic respiration and oxidative stress tolerance by reducing NO (Mellies et al., 1997), while Ccp contributes to oxidative stress tolerance by reducing H_2O_2 (Turner et al., 2003). Induction of these genes in biofilm suggests that biofilm formation may enhance oxidative stress tolerance in *N. gonorrhoeae*. Although we did not directly challenge gonococcal biofilms with oxidative stressors (other than NO), we determined that a variety of oxidative stress tolerance genes are required for biofilm formation including *trxB*, *estD*, *mntABC*, *oxyR*, *prx*, and *gor* (Seib et al., 2004, 2005, 2006, 2007; Wu et al., 2005, 2006, 2010; Lim et al., 2008; Potter et al., 2009a,b; Srikhanta et al., 2009). A reasonable future objective would be to assess the ability of biofilm to withstand oxidative stress as compared to planktonic cells. Ideal oxidants to test would be NO and H_2O_2. Despite the varied function of the proteins encoded by these

genes, all are required for normal biofilm formation. Inhibiting the function of the periplasmic binding protein (MntC) or intermembrane domain (MntAB) of the MntABC transporter severely attenuates biofilm formation over glass. Mutations in either portion of the transporter should impair the ability of the gonococcus to take up Mn. These findings indicate that the antioxidant properties of Mn help to protect gonococcal biofilms from oxidative stress. However, the *mntABC* mutants are not as severely impaired as other oxidative stress tolerance mutants, which includes members of the OxyR regulon. This may reflect the relative importance of different oxidative stress tolerance mechanisms in *N. gonorrhoeae*. The OxyR regulator and members of its operon (*prx* and *gor*) are also required for robust biofilm formation. *oxyR*::kan mutant biofilms are indistinguishable from *prx*::kan or *gor*::kan mutant biofilms, which suggests that disruption of any member of this operon is sufficient to hinder biofilm formation. The effects of the *gor* and *prx* mutations do not appear to be cumulative, although they are not functionally redundant (Seib et al., 2006). However, they do play complementary roles in the reduction of H_2O_2 (Seib et al., 2006). Although the gonococcus has several mechanisms for coping with H_2O_2 stress, disrupting a single gene involved in H_2O_2 tolerance (*gor*, *prx*, or *ccp*) can impair biofilm formation (Seib et al., 2005). This suggests that protection against H_2O_2 is paramount in biofilms, which may correspond to the prevalence of H_2O_2-producing *Lactobacillus* species in the female genitourinary tract and the use of H_2O_2 by the host immune system (Carreras et al., 1994a). Of all the mutants tested, the *estD*::kan mutant displayed the most unique phenotype. This mutant is not defective in its ability to form biofilm over glass, yet it is severely attenuated in its ability to form biofilm over THCEC. Although *aniA*::kan and *ccp*::kan are more severely attenuated over THCEC, both mutants have reduced average thicknesses compared to the wild type when cultured over glass. Cervical epithelial and endothelial cells produce NO (Carreras et al., 1994a; Tschugguel et al., 1999; Ledingham et al., 2000), which is reduced to *s*-nitrosoglutathione (GSNO) in the gonococcus (Seib et al., 2006). NO and subsequently GSNO would likely be more abundant in the THCEC culture system. GSNO is toxic and EstD may have a role in its metabolism in the cell (Seib et al., 2006). This may explain why an *estD*::kan mutant is severely attenuated over cells, but is not attenuated over glass. In our glass flow cell system, the most abundant source of NO would be anaerobic respiration in the gonococcus. However, NO is rapidly reduced by NorB *in vitro* (Cardinale and Clark, 2005). In contrast to the *estD*::kan mutant, a *trxB*::kan mutant is attenuated for biofilm formation over glass and THCEC. TrxB is also involved in NO tolerance, but NO is likely not abundant in our continuous-flow system over glass, as previously discussed. However, the *trxB*::kan mutant is likely attenuated in both systems, because it is impaired in its ability to undergo anaerobic respiration. We have determined that transcription of both *aniA* and *norB* is reduced in this mutant compared to the wild type. Thus, deficient biofilm formation is likely attributable to the reduced expression of *aniA* and *norB*. The phenotype may be more severe over cells where the NO concentration is higher, as the *trxB*::kan mutant would also lack the ability to efficiently reduce NO. Overall, our findings clearly demonstrate that the ability to tolerate oxidative stress is necessary for robust levels of biofilm formation.

Biofilm formation by *N. gonorrhoeae* may aid in oxidative tolerance during the cervical infection of women by positively regulating factors, such as *ccp* and *norB*, which reduce reactive oxygen and nitrogen species, respectively. Anaerobic respiration in biofilm may also represent an adaptation to oxygen limitation within the biofilm or the host environment. However, biofilm formation may play a more prominent role in oxidative stress defense, as we determined that the majority of the genes involved in these pathways are required for robust biofilm formation. The propensity for biofilms to form during natural cervical infection, the conditions present during male infection that are likely not conducive to biofilm formation, and the lack of evidence of biofilm formation in men, suggests that biofilm formation may be specific to the infection of women. Biofilm formation may confer properties to the gonococcus through the induction of the anaerobic metabolism and oxidative stresses defense pathways, enhancing the ability to cope with or evade the host immune response. The ability to do so likely contributes to the occurrence of persistent infection in women and may help to account for the greater likelihood of asymptomatic infection in women. Mechanisms that govern biofilm formation might be manipulated to improve treatment or diagnosis of *N. gonorrhoeae* infection in women.

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Resistance of *Neisseria gonorrhoeae* to neutrophils

M. Brittany Johnson and Alison K. Criss*

Department of Microbiology, University of Virginia, Charlottesville, VA, USA

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
William Shafer, Emory University School of Medicine, USA
Rick Rest, Drexel University College of Medicine, USA

****Correspondence:***
Alison K. Criss, Department of Microbiology, University of Virginia Health Sciences Center, Box 800734, Charlottesville, VA 22908-0734, USA.
e-mail: akc2r@virginia.edu

Infection with the human-specific bacterial pathogen *Neisseria gonorrhoeae* triggers a potent, local inflammatory response driven by polymorphonuclear leukocytes (neutrophils or PMNs). PMNs are terminally differentiated phagocytic cells that are a vital component of the host innate immune response and are the first responders to bacterial and fungal infections. PMNs possess a diverse arsenal of components to combat microorganisms, including the production of reactive oxygen species and release of degradative enzymes and antimicrobial peptides. Despite numerous PMNs at the site of gonococcal infection, *N. gonorrhoeae* can be cultured from the PMN-rich exudates of individuals with acute gonorrhea, indicating that some bacteria resist killing by neutrophils. The contribution of PMNs to gonorrheal pathogenesis has been modeled *in vivo* by human male urethral challenge and murine female genital inoculation and *in vitro* using isolated primary PMNs or PMN-derived cell lines. These systems reveal that some gonococci survive and replicate within PMNs and suggest that gonococci defend themselves against PMNs in two ways: they express virulence factors that defend against PMNs' oxidative and non-oxidative antimicrobial components, and they modulate the ability of PMNs to phagocytose gonococci and to release antimicrobial components. In this review, we will highlight the varied and complementary approaches used by *N. gonorrhoeae* to resist clearance by human PMNs, with an emphasis on gonococcal gene products that modulate bacterial-PMN interactions. Understanding how some gonococci survive exposure to PMNs will help guide future initiatives for combating gonorrheal disease.

Keywords: *Neisseria gonorrhoeae,* virulence factors, neutrophils, polymorphonuclear leukocytes, phagocytosis, reactive oxygen species, antimicrobial peptides, neutrophil proteases

GONORRHEAL DISEASE

Gonorrhea is a major global health problem, with greater than 62 million cases estimated to occur worldwide per year (Anonymous, 2001). Numbers of reported cases in the United States have remained at approximately 330,000 annually, but it is estimated that the actual number is at least twice as high, and rates of gonorrhea are rapidly increasing among men who have sex with men and young adults (Workowski and Berman, 2010). The cause of gonorrhea is the Gram-negative diplococcus *Neisseria gonorrhoeae* (the gonococcus or Gc). Gc is a human-specific pathogen that is transmitted via close sexual contact with an infected individual. Gonorrhea presents as an acute urethritis in men and cervicitis in women, but the pharynx and rectum can also be infected (Wiesner and Thompson, 1980). Because of the frequently asymptomatic nature of female infection, gonorrhea is a major cause of pelvic inflammatory disease, characterized by abdominal pain and tubal scarring that results in ectopic pregnancy and infertility; untreated infections in men also lead to sterility. Disseminated Gc infections can cause arthritis–dermatitis syndrome, endocarditis, and meningitis. Gc can also be vertically transmitted during childbirth and is still a leading cause of infectious neonatal blindness in the developing world (Wiesner and Thompson, 1980). Gc remains a major public health problem due to rapid acquisition of resistance to multiple antibiotics (Tapsall, 2009) and its ability to phase and antigenically vary its surface structures, preventing infected individuals from developing a protective immune response and hindering development of a protective vaccine (Virji, 2009).

Regardless of the anatomic site that is infected, Gc promotes an inflammatory response that is characterized by the recruitment of PMNs (**Figure 1**). In men, PMNs appear in urethral swabs and urine several days after infection and immediately prior to the onset of symptoms (Cohen and Cannon, 1999). The purulent exudate produced by infected men, described in the Bible and by Galen, is the best-known aspect of gonorrheal disease and is reflected in the translation of "gonorrhea" from Greek as "flow of seed" (Edwards and Apicella, 2004). The cervical secretions of women with gonorrhea also contain PMNs (Evans, 1977). Bacteria in gonorrheal secretions are attached to and within PMNs (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; Evans, 1977; King et al., 1978; Apicella et al., 1996). PMNs are the primary innate immune responders to bacterial and fungal infection and are capable of phagocytosing and killing a variety of microorganisms (Borregaard, 2010). Yet in spite of the numerous PMNs at the site of gonorrheal infection, viable Gc can be cultured from the exudates of infected individuals (Wiesner and Thompson, 1980), and a subset of Gc remain viable when Gc are exposed to PMNs *in vitro* (see below). We interpret these results to show that the PMN-driven innate immune response to Gc is ineffective at clearing a gonorrheal infection. The persistence of Gc in the presence of PMNs facilitates Gc's long-term colonization of its obligate human hosts, creating enhanced opportunity for dissemination and transmission of gonorrhea. In this review we will highlight our current knowledge about Gc resistance to PMN clearance, a critical aspect of the virulence of Gc.

PMN ANTIMICROBIAL ACTIVITIES

PMNs are the most abundant white cells in the peripheral blood of humans. They are professional phagocytes and the first line of defense of the innate immune system (Borregaard, 2010). In response to peripheral infection or damage, PMNs follow chemotactic cues to extravasate from the bloodstream and migrate through tissues to reach the target site. Mucosal epithelial cells and resident immune release chemokines for PMNs, including interleukin-8, interleukin-6, tumor necrosis factor-α, and interleukin-1 (Borregaard, 2010). These chemokines are released during human Gc infection (Ramsey et al., 1995; Hedges et al., 1998).

PMNs possess receptors to bind and phagocytose complement- and antibody-opsonized particles [e.g., complement receptor 3 (CR3), FcRs]. They can also engulf unopsonized particles through lectin-like interactions or using receptors that are specific for ligands on the particle surface (Groves et al., 2008). Interaction between PMNs and a target particle results in the mobilization of different subsets of cytoplasmic granules to the plasma or phagosomal membrane (**Figure 2**). Granule fusion enables the degradation and killing of microorganisms both intracellularly and extracellularly (Borregaard et al., 2007). PMN mechanisms of microbial killing include production of reactive oxygen species (ROS) via the NADPH oxidase enzyme (the "oxidative burst") as well as the oxygen-independent activities of degradative enzymes and antimicrobial peptides (**Table 1**). Human PMN granules are classified as azurophilic or primary granules, which contain myeloperoxidase, α-defensin peptides, and cathepsin G, among other antimicrobial components; specific or secondary granules containing the flavocytochrome b_{558} subunit of NADPH oxidase, LL-37 cathelicidin, lactoferrin, and CR3; and gelatinase or tertiary granules containing gelatinase (Borregaard et al., 2007). PMN granules release their contents in a set order. Initially, gelatinase granule contents degrade extracellular matrix, allowing PMNs to migrate across the tissues underlying the site of infection. Next, the release of specific granules at the target destination increases phagocytic potential due to presentation of CR3 on the PMN surface.

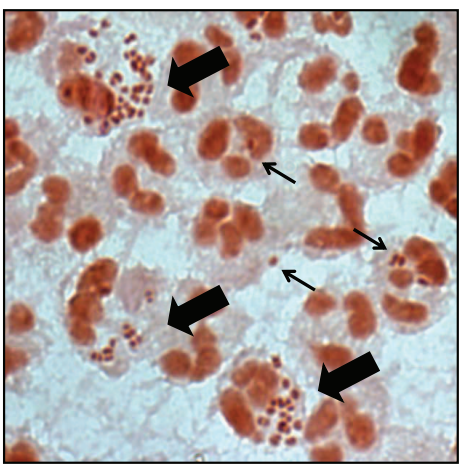

FIGURE 1 | Gonorrhoeal exudates contain numerous PMNs with associated Gc. Gram stain of the urethral exudate from a male with uncomplicated gonorrhea. Some PMNs associate with single diplococci (thin arrow), while others have multiple adherent and internalized Gc (thick arrow). Note that the majority of PMNs in the exudate are uninfected.

Finally, the release of both specific and azurophilic granules creates an environment that is generally hostile to microbial survival (Lacy and Eitzen, 2008). PMNs also release neutrophil extracellular traps (NETs) composed of DNA, histones, and selected granule components, which trap and kill microbes without requiring phagocytosis (Papayannopoulos and Zychlinsky, 2009). Thus PMNs combine oxygen-dependent and -independent mechanisms to combat intracellular and extracellular microorganisms.

The fact that gonorrheal exudates contain viable Gc indicates that PMNs are ineffective at completely clearing Gc infection. There are two mechanisms that could explain how Gc survives PMN challenge: Gc prevents PMNs from performing their normal antimicrobial functions (phagocytosis, granule content release), or Gc expresses defenses against oxidative and non-oxidative components produced by PMNs (**Figure 2**). As we will discuss, there is now substantial evidence for both mechanisms, which ultimately enable Gc to survive within a host and be transmitted to new individuals.

MODEL SYSTEMS FOR EXAMINING PMNS DURING Gc PATHOGENESIS

Four experimental approaches have been used to investigate the involvement of PMNs in gonorrheal disease. Each has contributed to our understanding of how PMNs are recruited during acute gonorrhea and how Gc withstands this onslaught.

THE MALE URETHRAL CHALLENGE MODEL

Experimental human infection is limited to male urethral inoculation, due to the potential for severe complications such as pelvic inflammatory disease in women with gonorrhea (Cohen and

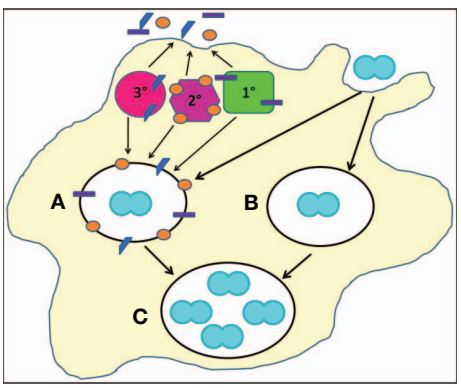

FIGURE 2 | Cellular mechanisms of Gc survival after exposure to PMNs. Gc (blue diplococcus) attaches to the surface of PMNs and is engulfed into a phagosome (white oval). PMNs possess three classes of granules (1°, 2°, and 3°), each of which contains a unique subset of antimicrobial compounds. Granules fuse with the nascent phagosome or plasma membrane to deliver their contents to invading microorganisms. We propose two mechanisms that allow Gc to survive after exposure to PMNs. (For illustrative purposes, only intracellular Gc survival is depicted.) First, PMN granules release their contents at the plasma membrane or into phagosomes containing Gc **(A)**. However, Gc virulence factors confer resistance to granules' antimicrobial compounds. Second, Gc prevents PMN granules from releasing their contents at the plasma membrane or into phagosomes, allowing the bacteria to avoid encountering PMN antimicrobial compounds **(B)**. Either mechanism would enable a fraction of Gc to survive and replicate in the presence of PMNs **(C)**.

Table 1 | Antimicrobial components housed in PMN granules.

Granule class	Granule components
Primary/azurophilic	***Cathepsin G, BPI, lysozyme,*** elastase, myeloperoxidase (MPO), α-defensins
Secondary/specific	***Flavocytochrome b_{558}, LL-37 (hCAP18), lysozyme,*** gelatinase, lactoferrin, CD11b/CD18 (CR3)
Tertiary/gelatinase	***Flavocytochrome b_{558}, lysozyme,*** gelatinase, CD11b/CD18 (CR3)

Proteins that have been shown to have or produce antimicrobial activity against Gc in vitro are bolded and italicized. Proteins to which Gc is resistant are indicated in red type.

Cannon, 1999). Urethral infection of male volunteers results in the release of proinflammatory cytokines and appearance of PMNs in the urogenital tract 2–3 days after infection, similar to what is seen in natural cases of gonococcal urethritis (Cohen and Cannon, 1999). As in natural infections, exudates from males with experimental Gc infection contain PMNs with associated Gc and occasional exfoliated epithelial cells. Electron microscopic analysis of these exudates revealed that a subset of Gc inside PMNs appear intact, providing the initial evidence that Gc may survive within PMN phagosomes (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; Apicella et al., 1996).

THE FEMALE MURINE GENITAL TRACT MODEL

Dr. Ann Jerse (Uniformed Services University of the Health Sciences) has developed a female mouse model of Gc genital tract infection, which allows gonorrheal infection to be examined in a genetically tractable host. In this model, estradiol-treated mice are inoculated vaginally with Gc, which allows over 80% of mice to be colonized with bacteria for over 1 week. Infected mice produce inflammatory cytokines, leading to rapid appearance of PMNs in the genital tract (Jerse, 1999). Experimental infection of female mice has provided insight into the selective advantage of opacity-associated (Opa) protein expression on Gc survival and the roles of Gc virulence factors conferring *in vitro* resistance to ROS and antimicrobial peptides in *in vivo* infection (Jerse, 1999; Jerse et al., 2003; Wu and Jerse, 2006; Wu et al., 2009; Cole et al., 2010). Because mice lack the human-specific receptors and other components that are likely to be important for gonorrheal disease, future studies could employ mice transgenic for human proteins of interest. Inbred mice that are transgenic for human carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) and CD46, receptors that are implicated in gonorrheal pathogenesis (Merz and So, 2000), have already been developed (Johansson et al., 2003; Gu et al., 2010), with additional mouse strains likely to be produced in the coming years.

IMMORTALIZED PMN-LIKE CELL LINES

The use of immortalized promyelocytic human cell lines to study the molecular mechanisms of Gc pathogenesis provides a system which is clonal, easy to maintain, and amenable to expression of transgenes. As one example, the leukemic HL-60 cell line can be differentiated into a PMN-like phenotype with retinoic acid (Collins et al., 1977; Newburger et al., 1979). Differentiated HL-60 cells can phagocytose and generate ROS in response to Opa-expressing Gc akin to primary human PMNs (Bauer et al., 1999; Pantelic et al., 2004). However, HL-60 cells do not possess the robust antimicrobial activity associated with primary cells, due in part to the absence of specific granules and other intracellular compartments (Le Cabec et al., 1997).

PRIMARY PMNs

Research on the molecular mechanisms underlying Gc infection of PMNs has mostly relied upon primary human cells, purified from freshly isolated human blood. The abundance of PMNs in human blood and the ease of purification make PMNs amenable to infection with Gc *in vitro*. The limitations of working with primary PMNs include their short half-life, their limited capacity for genetic manipulation, and the person-to-person variability intrinsic to primary human cells. However, primary human PMNs have been used to measure binding and phagocytosis of Gc, quantify Gc survival after PMN exposure, and assess the roles of Gc virulence factors in bacterial defense against PMNs (see below). Gc infection of murine PMNs has also been conducted (Wu and Jerse, 2006; Soler-Garcia and Jerse, 2007). Future studies using primary PMNs along with cultured epithelial cells from relevant anatomic sites may provide a means to examine the complex interactions between host cells that occur during gonorrheal infection.

Gc interaction with PMNs is influenced by the physiological state of the PMNs being used. Initial experimentation with primary human PMNs utilized cells and Gc suspended in buffered saline solutions (Densen and Mandell, 1978; Rest et al., 1982), but this is unlikely to reflect the transmigrated, primed state of PMNs in the genitourinary tract during acute infection. Research from the laboratory of Dr. Richard Rest (Drexel University) demonstrated that when PMNs were allowed to adhere to tissue culture-treated dishes, they released granule components and bound significantly more Gc than PMNs in suspension (Farrell and Rest, 1990). Dr. Michael Apicella's laboratory (University of Iowa) subsequently developed an assay using collagen-adherent PMNs, which generated a system for studying the role of selected Gc virulence factors in bacterial survival after PMN challenge (Seib et al., 2005; Simons et al., 2005). We adapted the Apicella protocol to include PMN treatment with the chemokine interleukin-8, which facilitates PMN activation (Borregaard, 2010). We used this system to demonstrate Gc survival inside PMNs and to identify Gc proteins that defend the bacteria from killing by PMNs (Stohl et al., 2005; Criss et al., 2009).

Gc SURVIVAL AND REPLICATION IN THE PRESENCE OF PMNs

Although the survival of Gc in association with PMNs was once hotly debated, there is now substantial evidence that gonococci survive and multiply within human phagocytes. Examination of urethral exudates by light and electron microscopy has repeatedly shown the presence of abundant PMNs with associated and internalized Gc (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; King et al., 1978; Apicella et al., 1996). The fact that viable gonococci can be cultured from urethral exudates or cervical swabs is strongly suggestive of Gc survival in the presence of PMNs (Wiesner and Thompson, 1980). *In vitro* studies from the Apicella laboratory using adherent human PMNs demonstrated that over 50% of Gc internalized by PMNs remained viable for

up to 6 h, as determined by viable bacterial counts and electron microscopy (Simons et al., 2005). Our group corroborated these findings and directly detected viable extracellular and intracellular Gc after PMN infection, using dyes that reveal the integrity of bacterial membranes (Criss et al., 2009). We conclude from these studies that a fraction of Gc can survive both extracellularly and intracellularly in the presence of PMNs.

There is evidence that Gc does not only persist within PMNs, but also uses the PMNs as a site for replication. Pioneering studies in the 1970s showed that Gc inside exudate-derived PMNs were sensitive to penicillin, which only kills replicating bacteria. In the presence of antimicrobial agents such as spectinomycin or pyocin that cannot permeate eukaryotic membranes, numbers of PMN-associated Gc increased over time, indicative of bacterial replication inside exudatous and *in vitro*-infected PMNs (Veale et al., 1976, 1979; Casey et al., 1979, 1980, 1986). Using electron microscopy and colony counts, the Apicella laboratory observed an increase in Gc within collagen-adherent human PMNs over a 6-h infection, results also suggestive of intracellular replication (Simons et al., 2005). Similarly, we used bacterial viability dyes to observe an increase in the number of viable Gc inside PMNs over time (Criss et al., 2009). While the advantage of Gc replicating inside terminally differentiated cells of a limited life span is questionable, the Apicella group showed that PMNs infected with Gc delay their spontaneous apoptosis (Simons et al., 2006). We anticipate that advances in cellular imaging will provide additional support for Gc replication inside PMNs and will give insight into the timing and extent of this event.

BINDING AND PHAGOCYTOSIS OF Gc BY PMNs

Since gonorrheal secretions contain PMNs associated with viable intracellular and extracellular bacteria, Gc must possess factors that promote attachment and phagocytosis by PMNs. Opsonic and non-opsonic interactions are the two basic means of phagocytosis, both of which may be utilized by Gc (Groves et al., 2008; **Figure 3**).

OPSONIC UPTAKE

The two major opsonins for PMN phagocytosis are immunoglobulins and complement, which bind to Fc receptors and complement receptors such as CR3, respectively (Groves et al., 2008). Patients with gonorrhea produce opsonic IgG and IgA directed against Gc surface-exposed components including porin, Opa proteins, pilin, iron-regulated outer membrane proteins, and lipooligosaccharide (LOS) (Brooks et al., 1976; McMillan et al., 1979; Tramont et al., 1980; Rice and Kasper, 1982; Siegel et al., 1982; Lammel et al., 1985; Schwalbe et al., 1985). Intriguingly, serum from individuals with no prior history of gonorrhea contains opsonic IgG against Gc porin and IgM against Gc LOS isotypes containing hexosamine; the non-Gc antigens recognized by these antibodies are not known (Sarafian et al., 1983; Griffiss et al., 1991). Many of the Gc surface structures that promote humoral immune responses are phase and antigenically variable and thus evade antibody-mediated immune surveillance (Virji, 2009). Also, Gc secretes an IgA protease that cleaves the polymeric IgA in mucosal secretions (Blake and Swanson, 1978). Thus complement rather than antibodies is likely to drive the opsonic phagocytosis of Gc by PMNs.

The complement system is a key component of the innate immune system comprised of more than 30 proteins. The complement system can be activated by three routes: the classical, the alternative, and the lectin pathway, but all three routes normally proceed to proteolytic activation of the major complement protein C3 and assembly of the membrane attack complex (Ram et al., 2010). Gc has multiple ways of resisting the bactericidal activities of complement in normal human serum. Gc binds the complement regulatory proteins C4b-binding protein (C4BP) and factor H (fH) on its surface via porins and sialylated LOS (Ram et al., 1998a,b, 2001; Gulati et al., 2005). C4BP restricts the amount of C3 which can be deposited by the classical complement pathway. fH is a cofactor for factor I-mediated cleavage of C3b to the hemolytically inactive iC3b. In the alternative pathway fH irreversibly dissociates factor Bb to limit C3 deposition and subsequent C5 cleavage (Ram et al., 2010). C4BP and fH provide defense against direct complement-mediated killing but concomitantly increase iC3b deposition on the Gc surface. iC3b is a ligand for CR3 (CD11b/CD18), which in PMNs drives actin-dependent particle engulfment into degradative phagolysosomes and production of ROS (Groves et al., 2008). Although it is assumed that Gc is complement-opsonized at mucosal surfaces, how opsonization impacts Gc survival after PMN exposure remains to be explored.

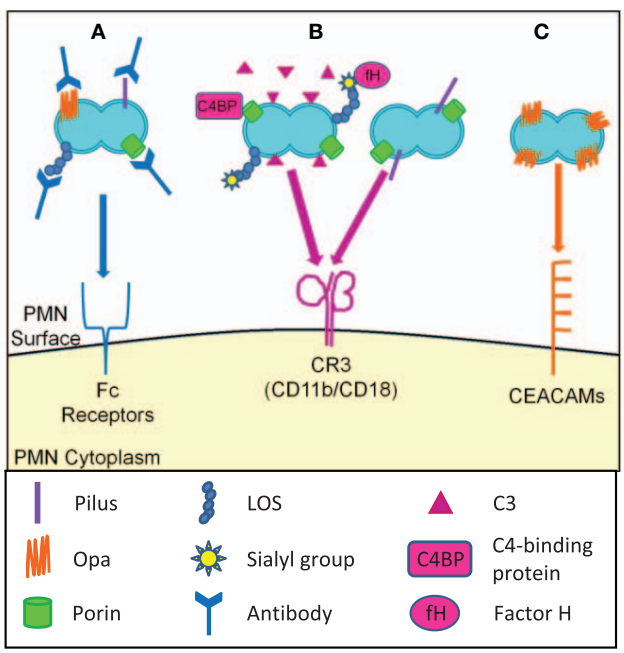

FIGURE 3 | Opsonic and non-opsonic phagocytosis of Gc by PMNs. (A) Antibodies that recognize Gc surface structures opsonize the bacteria and allow for phagocytosis via Fc receptors. The efficacy of immunoglobulin-mediated phagocytosis is questionable given the extensive phase and antigenic variation of Gc surface structures. **(B)** Gc binds factor H and C4 binding protein, resulting in opsonization of Gc with C3 and other complement components. Gc is then phagocytosed via the CR3 receptor. Gc pili and porin can cooperatively interact with CR3, which may mediate the non-opsonic phagocytosis of Gc by PMNs. **(C)** Selected Opa proteins bind to CEACAM family receptors expressed on PMNs, leading to non-opsonic phagocytosis of Opa$^+$ Gc.

NON-OPSONIC UPTAKE

In the absence of antibodies or complement, efficient binding and engulfment of Gc by PMNs is achieved via expression of colony Opa proteins (King and Swanson, 1978; Virji and Heckels, 1986; Fischer and Rest, 1988). Opa proteins, formerly known as "protein II," are a family of closely related, 20–30 kD outer membrane proteins that facilitate Gc binding and internalization by human cells, including PMNs (Sadarangani et al., 2011). Gc strains possess approximately 11 *opa* genes encoding 7–8 antigenically distinct Opa proteins (Connell et al., 1990; Dempsey et al., 1991). Each *opa* gene is phase-variable due to slipped-strand mispairing in a pentameric nucleotide repeat that places the gene in our out of frame (Murphy et al., 1989), such that individual Gc can express zero, one, or any possible combination of Opa proteins. Differential expression of Opa proteins can influence bacterial tropism for host cell types and provides a mechanism of immune evasion (Sadarangani et al., 2011).

Opacity-associated proteins bind heparan sulfate proteoglycans (HSPGs) and/or CEACAMs. Only those Opa proteins that bind CEACAMs are reported to influence Gc interactions with PMNs (Sadarangani et al., 2011). The Opa-binding CEACAMs on PMNs are CEACAM1, CEACAM3, and CEACAM6, with CEACAM3 expression exclusively restricted to PMNs. CEACAM1 and CEACAM3 are transmembrane proteins, while CEACAM6 possesses a glycosylphosphatidylinositol anchor (Gray-Owen and Blumberg, 2006). Binding of Opa proteins to any of the three CEACAMs results in Gc internalization, but via different signaling events (McCaw et al., 2004).

Opacity-associated protein expression is selected for in the male urethra, the female cervix during the follicular phase of the menstrual cycle, and in the murine cervix (James and Swanson, 1978; Swanson et al., 1988; Jerse et al., 1994; Jerse, 1999). However, Opa$^-$ Gc survives better after exposure to PMNs *in vitro* than isogenic Opa$^+$ Gc (Rest et al., 1982; Virji and Heckels, 1986; Criss et al., 2009). Opa protein expression increases Gc phagocytosis by PMNs and stimulates PMN ROS production, and both factors may influence bacterial survival after exposure to PMNs (Rest et al., 1982; Fischer and Rest, 1988).

Gc surface structures other than Opa proteins may contribute to adherence and phagocytosis by PMNs. Pili and porin cooperatively interact with CR3 on cervical epithelial cells (Edwards et al., 2002). It is not known if this interaction occurs on PMNs, but if it were to occur, it would drive non-opsonic uptake of Gc by PMNs. *In vitro* studies suggested that "type 1," virulent, piliated Gc were resistant to phagocytosis and killing by PMNs compared to "type 4," avirulent, non-piliated bacteria that arise after extensive laboratory passage (Ofek et al., 1974; Dilworth et al., 1975). We now know that type 1 and type 4 Gc vary in Opa expression as well as piliation, both of which could have contributed to these observations. Purified porins also decrease PMN actin polymerization, which may reduce the phagocytosis of Gc by PMNs (Bjerknes et al., 1995). Serogroup C strains of *N. meningitidis* with lacto-N-neotetraose (LNnT) on LOS are phagocytosed by neutrophils in an opsonin-independent manner (Estabrook et al., 1998); it has not been examined whether this LOS epitope on Gc affects phagocytosis by PMNs. Together, the combinatorial expression of Opa proteins, pili, porin, and LOS modulate Gc binding and internalization by PMNs.

Gc DEFENSES AGAINST PMN ANTIMICROBIAL ACTIVITIES

Whether they remain extracellular or are phagocytosed by PMNs, Gc must contend with the variety of oxidative and non-oxidative antimicrobial components produced by PMNs (**Figure 2**). Gc isolated directly from human material or guinea pig subcutaneous chamber fluid display increased survival in the presence of phagocytes compared to Gc grown *in vitro* (Witt et al., 1976; Veale et al., 1977), suggesting that Gc possesses factors necessary for defending against phagocyte killing that are lost or altered with extended *in vitro* culture. These Gc factors aid Gc in resisting the toxic activities of PMNs in two ways. First, Gc prevents PMNs from producing or releasing antimicrobial components. Second, Gc expresses virulence factors that defend against these components. As we will describe, many Gc gene products have been identified that protect Gc from purified ROS, proteases, or antimicrobial peptides, but in most cases their roles in defense against PMNs have not yet been investigated.

DEFENSES AGAINST OXIDATIVE DAMAGE

The major species of ROS include superoxide anion, hydrogen peroxide, and hydroxyl radical. These ROS have different reactivities and half-lives, but together they induce DNA, protein, and cell membrane damage that can lead to cell death (Fang, 2004). There are at least four potential sources of oxidative stress for Gc *in vivo*. (1) PMN NADPH oxidase transports electrons across the phagosomal or plasma membrane to generate superoxide, which spontaneously dismutates to hydrogen peroxide. In PMNs, the azurophilic enzyme myeloperoxidase uses hydrogen peroxide as a substrate to generate hypochlorous acid (bleach; Roos et al., 2003). Phagocytes can also produce reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite, but RNS appear to be of limited importance in human PMN antimicrobial activity (Fang, 2004). (2) Enzymes related to phagocyte NADPH oxidase are expressed in epithelial cells, and the survival defect of Gc antioxidant mutants inside primary cervical cells implies that epithelial cells may also be an important source of oxidative stress for Gc (Wu et al., 2005, 2006; Achard et al., 2009; Potter et al., 2009). (3) *Lactobacillus* species that generate hydrogen peroxide are normally found in the vaginal flora of women (Eschenbach et al., 1989). Women with inhibitory lactobacilli are less likely to be infected with Gc (Saigh et al., 1978), and lactobacilli inhibit Gc growth *in vitro* (Saigh et al., 1978; Zheng et al., 1994; St Amant et al., 2002). However, it appears that effects of lactobacilli on Gc may be independent of hydrogen peroxide production, since mucosal secretions can effectively quench lactobacilli-derived ROS (O'Hanlon et al., 2010). (4) Gc generate ROS during aerobic respiration, although this may be less of an issue *in vivo*, where the oxygen tension in the genitourinary tract is low (Archibald and Duong, 1986). Gc defenses against oxidative stress involve manipulation of the PMN oxidative burst, detoxifying or repair of oxidative damage, and transcriptional upregulation of antioxidant gene products (**Figure 4A**).

Gc manipulation of the PMN oxidative burst

In the absence of Opa protein expression, Gc fails to induce the PMN oxidative burst (Rest et al., 1982; Virji and Heckels, 1986; Fischer and Rest, 1988; Criss and Seifert, 2008). Even in the presence of Opa$^+$ Gc that induce ROS production in PMNs, the magnitude of ROS production is small relative to stimuli such as phorbol esters or other

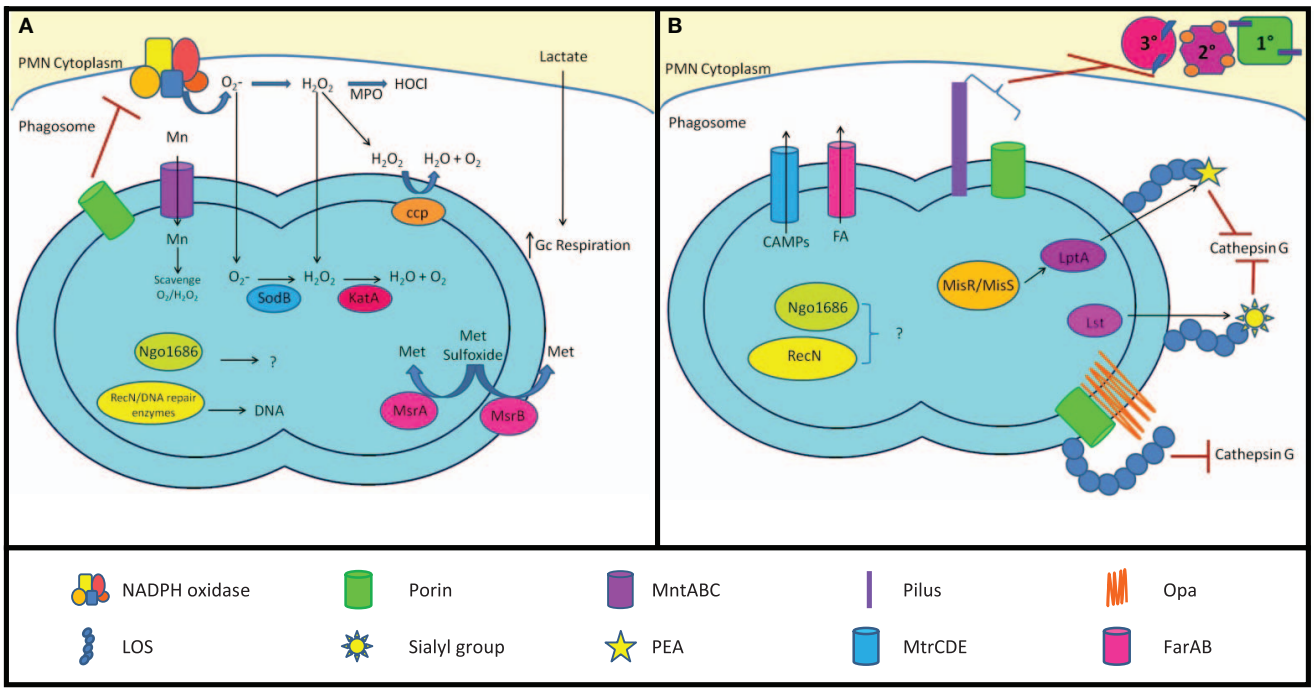

FIGURE 4 | Mechanisms of Gc survival after exposure to antimicrobial compounds produced by PMNs. (A) Resistance to oxidative damage. PMN NADPH oxidase generates superoxide (O_2^-) and hydrogen peroxide (H_2O_2) from O_2, which are converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). Gc prevents PMNs from generating ROS by lactate-mediated increase in Gc O_2 consumption and suppression of NADPH oxidase activity by porin or as-yet unidentified factors. Gc scavenges ROS through the activities of MntABC, superoxide dismutase (SodB), catalase (KatA), and cytochrome *c* peroxidase (Ccp). Gc can also repair damage due to ROS through DNA repair enzymes (RecN), protein reductases (MsrA/B), and other proteins (Ngo1686). **(B)** Resistance to PMN non-oxidative damage. Gc pili and/or porins prevent PMN granules from releasing non-oxidative antimicrobial components. LOS protects Gc outer membrane proteins such as Opa and porin from proteolysis by cathepsin G. Sialylation of LOS by Lst and PEA modification of LOS by LptA increases bacterial resistance to cathepsin G and other antimicrobials. The MisR/MisS two-component regulator increases expression of LptA and other gene products that confer resistance to PMN non-oxidative damage. Ngo1686 and RecN also protect Gc from PMN non-oxidative damage. The MtrCDE and FarAB efflux pumps export cationic antimicrobial peptides (CAMPs) and long-chain fatty acids (FA) from the Gc cytoplasm, respectively. In most cases, the contribution of these virulence factors to Gc survival after exposure to PMNs remains to be determined.

bacteria (Simons et al., 2005). Gc utilizes three mechanisms to reduce the amount of ROS produced by PMNs. First, exposure to lactate that is released from PMNs undergoing glycolysis stimulates the rate of Gc oxygen consumption, reducing the amount available to PMNs as a substrate for NADPH oxidase (Britigan et al., 1988). Second, purified Gc porin inhibits PMN ROS production in response to Gc, yeast particles, and latex beads (Lorenzen et al., 2000), but not formylated peptides (Haines et al., 1988; Bjerknes et al., 1995). Whether porin has this effect in the context of whole Gc bacteria remains to be examined. Third, we reported that Opa$^-$ Gc suppresses the PMN oxidative burst induced by serum opsonized staphylococci and formylated peptides by a process requiring bacterial protein synthesis and bacteria-PMN contact; the bacterial products mediating this effect are not known at this time (Criss and Seifert, 2008).

Detoxification and repair of oxidative damage

Bacteria respond to oxidative stress by catalysis of superoxide to hydrogen peroxide by superoxide dismutase (SOD), which is then converted to water and molecular oxygen by catalases and peroxidases (Seib et al., 2006). Gc possesses a single cytoplasmic superoxide dismutase (SodB), one cytoplasmic catalase (KatA), and several genes annotated as peroxidases. SodB activity is low in Gc and does not play a significant role in protection against oxidative stress (Tseng et al., 2001). In comparison, KatA is crucial to Gc defense against ROS. Gc has approximately 100-fold higher levels of catalase than *E. coli* (Hassett et al., 1990). Disruption of *katA* significantly reduces Gc survival to hydrogen peroxide and superoxide *in vitro* (Johnson et al., 1993; Soler-Garcia and Jerse, 2004; Stohl et al., 2005) and reduces the survival of some strains of Gc in the female murine genital tract (Wu et al., 2009). Gc also has high peroxidase Gc activity due to the periplasmic cytochrome c peroxidase encoded by *ccp* (Archibald and Duong, 1986). *ccp* mutant Gc show slight sensitivity to hydrogen peroxide, which is markedly enhanced when *katA* is also inactivated (Turner et al., 2003). Gc also imports Mn(II) into its cytoplasm via the MntABC transporter, where it scavenges superoxide and hydrogen peroxide by a mechanism independent of SodB and catalase (Tseng et al., 2001). This system is similar to the manganese transport system in *Lactobacillus plantarum* (Archibald and Duong, 1984).

Gc can also repair oxidative damage to proteins and DNA. Gc expresses two forms of methionine sulfoxide reductase, which reverses the oxidation of methionine residues in proteins. The MsrA protein is localized to the cytoplasm, while MsrB is secreted to the outer membrane. A *msrAB* mutant is more sensitive to hydrogen peroxide and superoxide *in vitro* than its wild-type parent (Skaar et al., 2002).

Many Gc gene products involved in recombinational DNA repair, base excision repair, and nucleotide excision repair participate in Gc defense against ROS, such as the recombinase RecA and the DNA-binding protein RecN (Davidsen et al., 2005; Stohl et al., 2005; Stohl and Seifert, 2006; LeCuyer et al., 2010). The putative metalloprotease Ngo1686 helps protect Gc from hydrogen peroxide and the lipid oxidant cumene hydroperoxide, but the cellular targets with which Ngo1686 interacts are currently unknown (Stohl et al., 2005). Both *ngo1686* and *recN* mutants have significant survival defects after exposure to primary human PMNs, but a *recA* mutant does not (Stohl et al., 2005; Criss et al., 2009).

Transcriptional induction of antioxidant gene products

Gc pre-exposed to hydrogen peroxide survives PMN challenge significantly better than unexposed Gc (Criss et al., 2009). This finding implies that Gc possesses complex transcriptional circuitry that is important for defenses against ROS and/or PMNs. The transcriptome of Gc exposed to sublethal concentrations of hydrogen peroxide was defined and revealed the upregulation of transcripts encoding RecN, Ngo1686, and other antioxidants after oxidative challenge (Stohl et al., 2005). Antioxidant gene expression is regulated by selected transcriptional repressors. The OxyR protein represses KatA expression, which is relieved following oxidative stress in order to increase catalase production (Tseng et al., 2003). PerR is responsive to Mn(II) levels and represses expression of MntC, part of the Mn(II) transporter (Wu et al., 2006). Finally, Ngo1427, a LexA homolog, represses expression of RecN, which is relieved when a cysteine residue is oxidized (Schook et al., 2011).

PMNs PRIMARILY DIRECT NON-OXIDATIVE ANTIMICROBIAL COMPONENTS AGAINST Gc

Although Gc has complex mechanisms for detecting oxidative damage and responding to it, the importance of these processes in Gc survival to PMNs appears to be limited. Mutants in *katA*, *sodB*, *ccp*, or *mntABC*, alone or in combination, do not affect the percentage of Gc that can survive PMN challenge (Seib et al., 2005; Criss et al., 2009). Moreover, Gc survival is similar between normal PMNs and ROS-deficient PMNs obtained from patients with chronic granulomatous disease (CGD; Rest et al., 1982; Criss and Seifert, 2008), and PMNs maintained in anoxic conditions, as are likely to be found in the upper reproductive tract of females, are not impaired for antigonococcal activity (Casey et al., 1986; Frangipane and Rest, 1992). Our group showed that Gc survival was unaffected after exposure to PMNs treated with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase. DPI treatment or CGD PMNs did not increase the percent survival of *ngo1686* or *recN* Gc, nor did it enhance survival of Opa$^+$ Gc that induce ROS from PMNs (Criss et al., 2009). From these results, we conclude that PMNs primarily direct non-oxidative antimicrobial activities against Gc. The functional redundancy in Gc antioxidant defenses may be sufficient to counter PMN-derived ROS; alternatively, PMNs may not generate enough ROS during infection to affect Gc survival.

DEFENSES AGAINST NON-OXIDATIVE DAMAGE

Seminal research from the Rest and Shafer laboratories indicated that components found inside PMN granules display oxygen-independent antigonococcal activity (Rest, 1979; Casey et al., 1985; Rock and Rest, 1988). These components include the bactericidal/permeability-increasing protein ("hCAP57"), cathepsin G protease, and LL-37 antimicrobial peptide (Casey et al., 1985; Shafer et al., 1986, 1998). Unlike many Gram-negative bacteria, Gc are highly resistant (>0.2 mg/ml) to another class of antimicrobial peptides, the defensins (Qu et al., 1996), although the observed resistance varies depending on experimental conditions used (Porter et al., 2005). Of PMN non-oxidative granule components, cathepsin G and LL-37 have been the most actively studied for their effects on Gc.

Cathepsin G is a highly cationic serine protease which resides in PMN azurophilic granules. It enzymatically cleaves Gc outer membrane proteins including porin and Opa proteins (Rest and Pretzer, 1981; Shafer and Morse, 1987). However, heat and protease inhibitors do not impede cathepsin G's ability to kill Gc *in vitro*, indicating its antigonococcal activity is independent of its proteolytic activity (Shafer et al., 1986). Cathepsin G can insert into Gc membranes, but killing does not appear to be due to changes in membrane permeability; instead, cathepsin G may impede peptidoglycan biosynthesis (Shafer et al., 1990).

LL-37 is the active form of an 18 kD protein precursor ("hCAP18") which resides in specific granules. hCAP-18 is proteolytically processed to LL-37 by the azurophilic granule protein proteinase-3 (Sorensen et al., 2001). hCAP-18/LL-37 is also synthesized by mucosal epithelial cells, and is readily detected in cervicovaginal secretions (mean LL-37 concentration of 10 μg/ml) and seminal plasma (mean hCAP-18 concentration of 86 μg/ml; Malm et al., 2000; Tjabringa et al., 2005). Gc infection increases the levels of hCAP18/LL-37 by two- to four-fold in cervical and urethral washes (Porter et al., 2005; Tjabringa et al., 2005). These concentrations of LL-37 would be sufficient to exert antibacterial activity on Gc, since the mean inhibitory concentration of LL-37 for Gc is 6 μg/ml (Shafer et al., 1998). The antigonococcal mechanism of action of LL-37 remains enigmatic and may be related to its ability to form pores that disrupt the integrity of bacterial membranes (Brogden, 2005).

Although Gc are susceptible to cathepsin G and LL-37 *in vitro*, the ability of some percentage of Gc to survive PMN exposure suggests that the bacteria have evolved mechanisms to counter these antimicrobial components. These mechanisms involve direct modulation of PMN granule release, changes to the Gc surface to resist non-oxidative antimicrobial components, and active export of these components (**Figure 4B**).

Modulation of PMN granule release

Both pili and porin have been reported to reduce PMN granule fusion with the plasma membrane or phagosomes. When added to primary PMNs, purified porins inhibit azurophilic and specific granule exocytosis (Bjerknes et al., 1995; Lorenzen et al., 2000). "Type 1," piliated Gc was also reported to inhibit azurophilic granule exocytosis relative to "type 4," non-piliated Gc, but additional surface structures expressed on type 1 bacteria may have mediated this result (Densen and Mandell, 1978). More detailed studies with isogenic Gc strains are necessary to determine whether and how Gc surface structures influence granule mobilization.

Modifications to the Gc surface

Gc LOS is thought to mask proteins which are degraded by cathepsin G, since truncation or loss of LOS results in increased binding of cathepsin G and increased susceptibility to cathepsin G-mediated

killing (Shafer, 1988). Two modifications of LOS impact bacterial interactions with host cells and host defenses: phosphoethanolamine (PEA) substitution on lipid A or the oligosaccharide, and sialylation of the terminal Galβ1-4GlcNAc epitopes of the oligosaccharide (Mandrell et al., 1990; Plested et al., 1999). PEA addition to the heptose group on the beta chain of the core oligosaccharide enhances Gc serum resistance but does not affect susceptibility to antimicrobial peptides (Lewis et al., 2009). In contrast, PEA addition to lipid A by the LptA enzyme increases resistance to both normal human serum and cationic antimicrobial peptides, indicating that structural changes in LOS contribute to the ability of gonococci to resist the bactericidal action of these innate immune components (Lewis et al., 2009). In the related bacterium *N. meningitidis*, expression of *lptA* is positively regulated by the *misR/misS* two-component regulatory system (Newcombe et al., 2005; Tzeng et al., 2008). The roles of MisR/MisS and LptA in Gc pathogenesis remain to be examined. The gonococcal α-2,3-sialyltransferase Lst transfers sialyl groups from host-derived CMP-N-acetylneuraminic acid to the terminal galactose residue on the oligosaccharide of LOS (Gilbert et al., 1996). Sialylation contributes to Gc resistance to normal human serum as well as PMN-derived oxygen-independent antimicrobial factors (Shafer et al., 1986; Parsons et al., 1992). Importantly, sialylated Gc are more resistant to PMNs *in vitro*, and sialylation contributes to Gc survival in the murine female genital tract (Kim et al., 1992; Rest and Frangipane, 1992; Gill et al., 1996; Wu and Jerse, 2006). In addition to LOS, changes in other surface components may contribute to Gc resistance to non-oxidative antimicrobial factors. For instance, loss of Opa expression enhances Gc resistance to serine proteases (Blake et al., 1981; Cole et al., 2010), and *N. meningitidis* lacking pili (due to insertional mutagenesis of the *pilMNOPQ* operon) are more resistant to the model antimicrobial peptide polymyxin B (Tzeng et al., 2005).

Gc export of antimicrobial components

The multiple transferable resistance (*mtr*) locus is a key determinant of Gc resistance to antimicrobial agents (Shafer et al., 1998). Mtr, a member of the resistance-nodulation-cell division (RND) family of efflux pumps, is encoded by a three gene operon designated *mtrCDE*. MtrC spans the periplasm to link the inner membrane protein MtrD, the multidrug efflux transporter, with outer membrane protein MtrE, the channel for export of antimicrobials to the extracellular environment (Hagman et al., 1995). MtrCDE uses the proton motive force to export a variety of compounds from the Gc cytoplasm, including antibiotics, detergents, and antimicrobial peptides (Hagman et al., 1995; Veal et al., 2002). *mtrCDE* is negatively regulated by the MtrR transcriptional repressor (Hagman and Shafer, 1995) and positively regulated by the MtrA transcriptional activator (Rouquette et al., 1999). Mutations in *mtrR* and *mtrA* that modulate expression of MtrCDE affect Gc resistance to antimicrobial peptides (Hagman et al., 1995; Hagman and Shafer, 1995). MtrCDE expression promotes Gc survival in the murine female genital tract (Jerse et al., 2003) and enhances resistance to murine antimicrobial peptides (Warner et al., 2008), but its role in defense of Gc against PMNs is unclear. Gc also uses the FarAB efflux pump system to confer resistance to long-chain fatty acids, independent of Mtr activity (Lee and Shafer, 1999). The Far system is composed of the FarA membrane-spanning linker, the FarB cytoplasmic membrane transporter, and MtrE. Far expression is believed to be important for survival of isolates at the rectal mucosal surface, which is rich in diet-derived fatty acids, and does not contribute to Gc survival in the murine genital tract (Jerse et al., 2003). How the Far system contributes to defense of Gc against PMNs, which may release fatty acids (Huang et al., 2010), remains to be explored.

DISCUSSION

Despite the prevalence of gonorrhea in the human population and the abundance of PMNs during acute gonorrheal disease, we are just beginning to understand the molecular mechanisms underlying Gc interactions with and resistance to PMNs. There are three overarching questions which remain currently in the field. First, how does a subset of Gc survive PMN challenge? As we have described, Gc possesses gene products which protect against oxidative and non-oxidative components that are made by PMNs. Many of these gene products are necessary for *in vitro* protection against isolated antimicrobial components and some provide a selective advantage *in vivo*. However, in many cases, it has not been investigated whether these gene products also confer a survival advantage in the context of PMN challenge. Second, how does Gc persist over time inside PMNs, as is seen in PMNs isolated from gonorrheal exudates? Although virulence-associated Gc surface structures such as Opa proteins, pili, porin, and LOS have been highly investigated for their biochemistry and impact on Gc-epithelial interactions, their effects on Gc survival inside PMNs remain enigmatic. How complement or immunoglobulin opsonization affects Gc phagocytosis by and survival inside PMNs also needs to be examined. Finally, how and why does Gc stimulate PMN recruitment? That is, what is the benefit of recruiting professional antimicrobial cells to the site of Gc infection? Given the long history of Gc in the human population, Gc could have evolved mechanisms for inhibiting PMN recruitment; instead, Gc LOS and lipoproteins are strong initiators of the host innate immune response (Massari et al., 2002; Pridmore et al., 2003; Zughaier et al., 2004). The answer to this question remains enigmatic, but may be revealed once we have a better understanding of how Gc manipulates PMNs *in vitro* and *in vivo.*

Our current knowledge of Gc interactions with PMNs demonstrates the impressive ability of Gc to survive PMN challenge. Although we are just beginning to piece together the roles of many Gc surface structures and gene products in Gc survival after PMN exposure, we now have model systems in hand that will allow these issues to be directly addressed. We are optimistic that continuing to investigate the mechanisms used by Gc to defend against PMN antimicrobial responses will shed light on how Gc has remained a fixture in the human population for all of recorded history (Wain, 1947; Morton, 1977). This research also has the potential to reveal novel human and Gc targets that can be exploited for new therapeutics to treat the ever-growing threat of highly antibiotic-resistant gonorrhea.

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New concepts in immunity to *Neisseria gonorrhoeae*: innate responses and suppression of adaptive immunity favor the pathogen, not the host

Yingru Liu, Brandon Feinen† and Michael W. Russell*

Department of Microbiology and Immunology, Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, Buffalo, NY, USA

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Dennis Metzger, Albany Medical College, USA
Joseph Duncan, University of North Carolina at Chapel Hill, USA

***Correspondence:**
Michael W. Russell, Department of Microbiology and Immunology, University at Buffalo, Farber 138, University at Buffalo, 3435 Main Street, Buffalo, NY 14214, USA.
e-mail: russellm@buffalo.edu

†Current address:
Brandon Feinen, FDA/CIBER/OVER/DOPE/LISP, 29 Lincoln Drive, Bethesda, MD 20892, USA.
e-mail: brandon.feinen@fda.hhs.gov

It is well-known that gonorrhea can be acquired repeatedly with no apparent development of protective immunity arising from previous episodes of infection. Symptomatic infection is characterized by a purulent exudate, but the host response mechanisms are poorly understood. While the remarkable antigenic variability displayed by *Neisseria gonorrhoeae* and its capacity to inhibit complement activation allow it to evade destruction by the host's immune defenses, we propose that it also has the capacity to avoid inducing specific immune responses. In a mouse model of vaginal gonococcal infection, *N. gonorrhoeae* elicits Th17-driven inflammatory–immune responses, which recruit innate defense mechanisms including an influx of neutrophils. Concomitantly, *N. gonorrhoeae* suppresses Th1- and Th2-dependent adaptive immunity, including specific antibody responses, through a mechanism involving TGF-β and regulatory T cells. Blockade of TGF-β alleviates the suppression of specific anti-gonococcal responses and allows Th1 and Th2 responses to emerge with the generation of immune memory and protective immunity. Genital tract tissues are naturally rich in TGF-β, which fosters an immunosuppressive environment that is important in reproduction. In exploiting this niche, *N. gonorrhoeae* exemplifies a well-adapted pathogen that proactively elicits from its host innate responses that it can survive and concomitantly suppresses adaptive immunity. Comprehension of these mechanisms of gonococcal pathogenesis should allow the development of novel approaches to therapy and facilitate the development of an effective vaccine.

Keywords: Th17, IL-17, TGF-β, innate immunity, adaptive immunity, immunosuppression

INTRODUCTION

It is now well recognized that microbial pathogenesis involves a two-way interaction with the host immune system. Numerous examples are known of the ways in which bacteria resist or evade host defense mechanisms, and in which host responses attempt to counteract the efforts of pathogens to overcome them. This concept of the dynamic response–reaction paradigm of infectious disease has been held to underpin the emergence of the discipline of Cellular Microbiology. It is conventionally imagined that bacteria invade and attack the host, the host then mounts an immune response, the bacteria counterattack or deploy evasive strategies to which the host responds further, and so forth until one gains the upper hand such that either the pathogen is eliminated and the host is cured of the infection, or conversely that the host succumbs and ultimately dies. A third outcome can be a form of stalemate, in which both pathogen and host survive, in a form of chronic infection with ongoing pathology, or even an asymptomatic carrier state in which the host appears to suffer no ill effects. In the first instance it is supposed that the host response proves superior, in the second it is inadequate, while in the third some degree of balance is struck. In all three scenarios, vaccination aims to enhance the host immune response particularly by exploiting the phenomenon of immune memory in order to curtail the infection at an early point or shorten its course and mitigate disease severity. Numerous vaccines have been very successfully developed and deployed on the basis of the paradigm that vaccination mimics the natural infection without actually causing disease and thereby stimulates the host to mount adaptive immune responses generally in the form of specific antibodies against the pathogen.

Several aspects of gonorrhea, however, suggest that this is an oversimplistic view of infectious pathogenesis. In the first place, it has proven very difficult, despite efforts spanning a century, to develop an effective vaccine against *Neisseria gonorrhoeae* (Russell and Hook, 2009). Furthermore, it is clear that one of the essential preconditions for the standard vaccine paradigm is not met, namely that recovery from the disease confers a state of immunity against future infection. It is well-known that gonorrhea can be acquired repeatedly without any apparent diminution in probability of infection from exposure, or the severity or duration of the disease (Noble et al., 1977). Gonorrhea is not unique in this, as there are many examples of infectious diseases that can be acquired repeatedly including influenza, rubella, and even the common cold. The generally accepted explanation for this situation is the diversity of pathogens capable of causing the syndrome, or the antigenic variation of particular pathogens. This is the conventional view of the immune response to gonorrhea (**Table 1**).

Our recent findings, however, support an alternative or additional hypothesis. In the first place, quantitative studies of circulating and local mucosal antibody responses to naturally acquired uncomplicated gonorrhea (i.e., gonococcal cervicitis in women,

Table 1 | Conventional view of immunity to gonorrhea*.

Postulate	Evidence for	Evidence against
Neisseria gonorrhoeae induces immune responses in infected subjects	Infected (and many uninfected) humans have serum antibodies against gonococcal antigens	Little or no increase in antibody levels after infection, or in subjects with previous infection
Specific antibodies are rendered ineffective by antigenic variation	Most major gonococcal surface molecules undergo extensive variation through: • allelic polymorphism • genetic recombination • phase–variable expression • horizontal gene exchange	Partial serovar-specific immunity reported in one study Anti-Opa antibodies may be associated with resistance to salpingitis
N. gonorrhoeae avoids complement-mediated destruction	*N. gonorrhoeae* inhibits complement activation (C4BP, RMP, LOS sialylation, factor H binding) and resists bacteriolysis	Serum bactericidal assay taken as an index of immunity
N. gonorrhoeae resists phagocytic destruction	*N. gonorrhoeae* invades neutrophils and partially survives within vacuoles	Resistance to intracellular killing is partial
Hence *N. gonorrhoeae* can survive whatever the immune system develops against it		

**For discussion and references, see text.*

and urethritis in men, both confirmed by microbiological testing) led to the conclusion that the human adaptive immune response to the infection is minimal (Hedges et al., 1998, 1999). A modest increase in antibodies measured against the homologous clinical isolate was seen in some individuals, but responses were of short duration, and showed no relation to documented previous infections (Hedges et al., 1999). Furthermore, there was no substantially increased response in subjects who had rectal involvement, in a site where mucosal immune inductive tissues (lymphoid follicles) are abundant, in contrast to the genital tract. Yet some women, especially those having co-infection with *Chlamydia trachomatis* or *Trichomonas vaginalis*, showed considerably elevated inflammatory cytokine responses (IL-1, IL-6, IL-8, and IL-10), testifying to an acute inflammatory response (Hedges et al., 1998). Overall these findings led us to hypothesize that "gonococci avoid inducing humoral immune responses during uncomplicated natural infections … [and that] gonococci use an as yet undefined mechanism of protection which may subvert the natural immune response." (Hedges et al., 1999).

The discovery of the novel subset of helper T cells designated Th17, on account of their ability to produce the inflammatory cytokine IL-17 (Harrington et al., 2005; Park et al., 2005), provided the basis for a new concept in pathogenesis particularly for extracellular and mucosal bacterial pathogens such as *N. gonorrhoeae* (Curtis and Way, 2009; Khader et al., 2009). When IL-17 activates cells that carry its receptor, IL-17R, these cells (typically endothelial and stromal cells) secrete other inflammatory cytokines including TNF-α, as well as granulocyte colony-stimulating factor (GCSF), granulocyte-monocyte colony-stimulating factor (GMCSF), and CXC chemokines (IL-8 in humans, KC, LIX, and MIP-2α in mice) which mobilize neutrophils from bone marrow and recruit them to the inflammatory focus (Kolls and Linden, 2004; Kolls et al., 2008). In addition, epithelial cells at mucosal surfaces respond to stimulation with IL-17 and IL-22 (another cytokine typically produced by Th17 cells) by upregulating the secretion of antimicrobial defense peptides including defensins, S100 proteins, and lipocalin-2 (Ouyang and Valdez, 2008). Thus Th17 cells stand at the interface of the adaptive and innate immune systems, and activate potent innate defense mechanisms, both cellular (especially neutrophils) and molecular. IL-17 is also highly inflammatory in its mode of action, and has been heavily implicated in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease. Thus, like many potent immune mechanisms, Th17 cell activation has beneficial as well as harmful consequences, depending on the duration of responses, and the effectiveness of their control mechanisms (Onishi and Gaffen, 2010). Nevertheless, it is clear that Th17 responses play a major role in defense against bacterial infections. The evidence for this role in gonococcal infection is summarized below.

TH17 RESPONSE TO *N. GONORRHOEAE* IN THE MOUSE MODEL

We were initially inspired to investigate Th17 responses to gonococcal infection following findings that mice show an IL-17-dependent neutrophil response to the periodontal pathogen, *Porphyromonas gingivalis* (Yu et al., 2007). In these studies, neutrophils were recruited in response to CXC chemokines generated by cells responding to stimulation with IL-17. Mice lacking the principal receptor for IL-17 (IL-17RA-knockout or IL-17RA-ko mice) were more susceptible to oral infection with *P. gingivalis* and had increased periodontal bone loss as a result. Furthermore they showed reduced levels of CXC chemokines KC and LIX in response to *P. gingivalis* and had diminished infiltration of neutrophils into the gingival tissue. Given that a neutrophil-rich discharge is a classic diagnostic criterion of gonorrhea, we were impelled to determine whether IL-17 had a similar role in the response to gonococcal infection.

In order first to establish whether *N. gonorrhoeae* is capable of inducing cytokines characteristic of a Th17 response, *in vitro* cell culture studies were conducted in which mouse spleen mononuclear cells were incubated with *N. gonorrhoeae*. These showed that IL-17A, IL-22, and other cytokines typical of an innate and inflammatory response (IL-1β, IL-6, TNF-α) were released into

the supernatant in a time- and dose-dependent manner (Feinen et al., 2010). Notably, there was a lack of cytokines typical of Th1 or Th2 responses, i.e., IFN-γ, IL-12, IL-4, and the little IFN-γ that was secreted came not from CD4+ T cells but from innate NK cells. The ability of *N. gonorrhoeae* to induce secretion of IL-17, IL-22, and IL-6, but not IFN-γ in mouse spleen cell cultures, suggests that it is capable of eliciting Th17 responses. There were in fact two sources of IL-17: both CD4+ T cells, i.e., Th17 cells, and T cells bearing the alternative γδ T cell receptor which belong to the innate immune system and are abundant at mucosal surfaces, including the genital tract. The responses were not confined to one strain of *N. gonorrhoeae* and were not dependent on live gonococci, as similar results were seen with strains FA1090, MS11, and PID-2, and could be replicated with outer membrane vesicle (OMV) preparations which contain most of the surface components of the intact gonococci and are naturally shed by the live organisms (Feinen et al., 2010).

This raises the issue of which gonococcal surface components are responsible for eliciting the IL-17 response, and indeed whether it is "specific" to *N. gonorrhoeae*. With regard to the latter question, there are now numerous species of bacteria, fungi, protozoa, and even viruses that have been reported to induce Th17 responses in animal models and humans. Bacterial species include the aforementioned *P. gingivalis, Klebsiella pneumoniae, Escherichia coli, Citrobacter rodentium, Bordetella pertussis, Mycobacterium tuberculosis, Helicobacter pylori, Salmonella enterica,* and many others, as well as the yeast *Candida albicans* and the protozoan *Toxoplasma gondii* (Higgins et al., 2006; Shibata et al., 2007; Yu et al., 2007; Caruso et al., 2008; Conti et al., 2009; Godinez et al., 2009; Khader et al., 2009). Thus it is not a unique response specific to *N. gonorrhoeae*, although the consequences for different infections and the implications in particular for human disease have not been fully explored. While Th17 cells may be induced by nominal antigens, the key factors in Th17 differentiation are cytokines, especially TGF-β and IL-6 as discussed below. Among gonococcal surface components, we found that the effect of whole gonococci (or OMV) can be to a large extent replicated by gonococcal lipo-oligosaccharide (LOS), which induces a similar level of IL-17 production by murine spleen cells *in vitro* (Feinen et al., 2010). Signaling through TLR4 is important, since TLR4-deficient mouse cells did not respond to gonococcal LOS and showed diminished responses to gonococci, whereas TLR2-knockout mouse cells responded similarly to wild-type mouse cells. Interestingly, gonococcal LOS having different terminal glycan residues have been reported to induce Th1, Th2, or Th17 responses according to their interactions with different lectin receptors on dendritic cells (van Vliet et al., 2009). Heat-treatment of OMV did not affect their ability to elicit IL-17 responses *in vitro*, suggesting that proteins might not be integral to this response but consistent with the role of LOS. An Opa protein deficient mutant in which all *opa* genes have been eliminated (Jerse et al., 1994) was still capable of inducing IL-17 responses (Feinen et al., 2010).

As a first step in determining whether findings based on mouse spleen cells *in vitro* are applicable to the genital tract, we made use of genital tract tissue explants which can be cultured for up to 5 days *ex vivo* with *N. gonorrhoeae* (Feinen et al., 2010). These cultures showed that *N. gonorrhoeae* could induce IL-17 and IL-22, as well as other inflammatory cytokines such as IL-6, but not those typical of Th1 or Th2 responses (IFN-γ and IL-4, respectively), in genital tract tissue. Furthermore, the CXC chemokines KC (CXCL1), LIX (CXCL5), and MIP-2α (CXCL2) were also secreted in response to *N. gonorrhoeae*. These are the murine equivalent of IL-8 (CXCL8) which does not occur in mice, and are chemoattractant for neutrophils.

In order to determine whether a Th17 response occurs in response to gonococcal infection, the murine model (Jerse, 1999) was employed to examine the immune response to *N. gonorrhoeae in vivo*. In this model, gonococcal infection of the genital tract persists for 1–2 weeks, and it elicits a local influx of neutrophils and the production of cytokines (Jerse, 1999; Song et al., 2008; Packiam et al., 2010). In addition, the availability of IL-17RA-ko mice (Ye et al., 2001) allowed us to determine the role of IL-17 in the course of infection and the neutrophil influx. Whereas wild-type control mice started to eliminate *N. gonorrhoeae* from day 4 onwards and cleared the infection by about day 7, in IL-17RA-ko mice reduction of the gonococcal load was delayed until day 9 and it took about 12 days to clear the infection (Feinen et al., 2010). The neutrophil influx, which began on day 3 in control mice, was almost completely abrogated in IL-17RA-ko mice. These results indicate that IL-17RA is important in neutrophil recruitment and in clearance of *N. gonorrhoeae* in this mouse model. Similar findings were obtained when mice were treated with blocking antibody to IL-17A during gonococcal infection, although the neutrophil influx was diminished but not abrogated in these experiments (Feinen et al., 2010).

The local generation of IL-17 in the genital tract was first revealed by culturing cells from the draining iliac lymph nodes taken on 1–5 days after infection; the cultures released IL-17 into the supernatants (Feinen et al., 2010). Further studies of cells isolated from the genital tracts of infected mice showed that γδ T cells were abundant and accounted for a large proportion of the IL-17 production, in addition to CD4+ Th17 cells (Liu, Y. and Russell, M.W., in preparation). Gene-expression profiling of mouse genital tract tissue revealed upregulation of genes associated with Th17 and innate immunity in response to *N. gonorrhoeae*, but not those associated with Th1- or Th2-driven adaptive immunity (Liu, Y. and Russell, M.W., in preparation). Thus the local response of the genital tract to infection with *N. gonorrhoeae* parallels the response of spleen cells, and is dominated by IL-17-driven innate inflammatory responses, with consequent recruitment of innate defense proteins and phagocytic cells, predominantly neutrophils. These findings are in accord with other studies of the mouse genital tract infection model showing that neutrophils are the predominant infiltrating cell type, with smaller numbers of macrophages, and little or no induction of a specific antibody response (Song et al., 2008). They are also reminiscent of the response in humans where inflammatory cytokines such as IL-1, IL-6, TNF-α (Hedges et al., 1998) but little or no IL-12, IFN-γ, IL-4, or IL-5 have been reported (Ramsey et al., 1995; Naumann et al., 1997; Simpson et al., 1999; Fichorova et al., 2001; Makepeace et al., 2001). Moreover, elevation of IL-17 and IL-23, which is important for the functional development of Th17 cells (Weaver et al., 2007), has now been reported in humans with gonorrhea (Gagliardi et al., 2011).

It is important to note that, while the neutrophil influx in the murine gonococcal infection model is abrogated in IL-17RA-ko mice with concomitant prolongation of the infection, it is unlikely

that clearance is dependent on the neutrophils: the infection is still cleared in IL-17RA-ko mice (Feinen et al., 2010). Moreover, the extent of neutrophil influx varies considerably between mouse strains and bears no relation to susceptibility to gonococcal infection (Packiam et al., 2010). Numerous factors undoubtedly contribute to the inability of *N. gonorrhoeae* to persist in mice, in contrast to humans to which it has become specifically adapted.

ROLE OF TGF-β IN SUPPRESSING Th1/Th2 RESPONSES TO *N. GONORRHOEAE*

CD4+ T cells differentiate along at least four different pathways or lineages depending upon the cytokine milieu in which they are stimulated (Weaver et al., 2007). In the presence of IL-12, they differentiate as Th1 cells, whereas in the presence of IL-4 they become Th2 cells. Both of these are "classical" T-helper cells that are critical for adaptive immune responses whether cell-mediated (e.g., by CD8+ cytotoxic T cells) or antibody production by plasma cells. Th1 and Th2 cells are cross-regulatory, as IFN-γ produced by Th1 cells suppresses Th2 development, while IL-4 from Th2 cells suppresses Th1 development. Th17 cells differentiate in the presence of TGF-β and an inflammatory cytokine such as IL-6 or IL-1; IL-23 is further required for the maintenance and functional differentiation of Th17 cells. Both IFN-γ and IL-4 suppress Th17 development but there is no confirmed evidence for the suppression of Th1 or Th2 cells by products of Th17 cells. However, when precursor T cells are stimulated in the presence of TGF-β alone they become induced T regulatory (Treg) cells which regulate Th1 and Th2 cells (Bettelli et al., 2006). Thus TGF-β is critical for the development of both Th17 and Treg cells, the essential difference being whether or not inflammatory cytokines are also present. TGF-β is also well-known as a regulatory cytokine that is directly inhibitory for Th1 and Th2 cells. Furthermore, female genital tract tissues are known to be rich in TGF-β (Wira and Rossoll, 2003; Shen et al., 2007) which is involved in maintaining an immunosuppressive environment that is important for reproductive physiology, in particular, the admission of allogeneic sperm and the implantation of a semi-allogeneic fetus (Russell and Mestecky, 2010; Wira et al., 2010).

We therefore hypothesized that *N. gonorrhoeae* is capable of inducing the production of TGF-β, which in turn promotes the development of both Th17 and Treg cells. Indeed, the development of Treg cells in the mouse model of genital gonococcal infection has already been reported (Imarai et al., 2008). Studies of the interaction of *N. gonorrhoeae* with mouse spleen cells *in vitro* show that TGF-β is among the cytokines induced, and that it is produced by several different cell types, especially B cells, but also by T cells, macrophages, and dendritic cells (Liu et al., submitted). Further studies with genital tract tissue cultured *in vitro*, and in the mouse genital tract *in vivo*, showed that *N. gonorrhoeae* further elevated the generation of TGF-β in the genital tract. Moreover, *N. gonorrhoeae* inhibited the *in vitro* proliferation and differentiation of Th1 and Th2 cells with their characteristic cytokine production by a TGF-β-dependent mechanism. This effect was reversed in the presence of blocking antibodies to TGF-β, and under these conditions, Th1 and Th2 cells developed and secreted IFN-γ and IL-4, respectively.

The effect of TGF-β-blocking antibody could also be observed *in vivo* in the genital tract infection model. When mice were treated with anti-TGF-β antibody during infection with *N. gonorrhoeae*, the duration of infection was significantly shortened by about 4 days, and both Th1 and Th2 responses were generated in the genital tract or its draining lymph nodes (Liu, Y. and Russell, M.W., in preparation). Normally, when mice that have recovered from primary infection are later challenged with a secondary infection of *N. gonorrhoeae*, the duration of the infection follows the same kinetics as primary infection, and there is no significant antibody response resulting from either primary or secondary infection (Song et al., 2008). This implies that in mice, as in humans, there is no effective generation of immune memory to gonococcal infection, and consequently no protective immunity develops against genital tract infection. However, if mice that have been treated with anti-TGF-β antibody during primary infection are then re-challenged (without further treatment with anti-TGF-β antibody), the secondary infection is resisted and is cleared more rapidly than in control mice that have not received anti-TGF-β treatment during primary infection. Analysis of the immune responses developed in these mice showed that the anti-TGF-β-treated animals develop both Th1 and Th2 cell responses, as well as anti-gonococcal antibodies in the circulation (IgG) and vaginal fluid (IgG and IgA). Thus the alleviation of *N. gonorrhoeae*-induced immunosuppression by means of anti-TGF-β antibody treatment results in the generation of adaptive anti-gonococcal immune responses and protective immunity to *N. gonorrhoeae* (Liu, Y. and Russell, M.W., in preparation).

A NEW HYPOTHESIS FOR GONOCOCCAL–HOST INTERACTIONS

Neisseria gonorrhoeae has probably been associated with humans for several millennia, and through evolution has become extremely well-adapted to the human immune system. Thus it has evolved the capacity to cope with human innate defense mechanisms such as defensins and other secreted anti-microbial proteins as well as non-opsonic phagocytosis by neutrophils. There is evidence that multiple drug exporter mechanisms enable gonococci to resist defensins (Shafer et al., 1998). Gonococci are able to survive at least partially within neutrophils (Casey et al., 1979); indeed they utilize pathogen-directed endocytosis to invade neutrophils and other cells (Jerse and Rest, 1997). Recent findings indicate that *N. gonorrhoeae* can survive oxygen-dependent and non-oxidative intracellular killing mechanisms within neutrophils (Criss and Seifert, 2008; Criss et al., 2009). Notably in this context, *N. gonorrhoeae* has multiple mechanisms that inhibit the activation of human complement, especially by the alternate pathway, and that prevent lysis by the membrane-attack complex of complement (Ram et al., 1998, 2001; Lewis et al., 2010). This not only allows it to escape from direct complement-mediated bacteriolysis, but also helps it to evade C3b-mediated opsonization and phagocytosis by neutrophils. An old body of literature indicates that antibody- and complement-mediated opsonophagocytosis is more effective in killing ingested microbes than non-opsonic phagocytosis.

Neisseria gonorrhoeae has evolved potent mechanisms for avoiding destruction by adaptive immune responses, especially specific antibodies. An obvious immune evasion strategy is its extraordinary capacity for antigenic variation, in which most of its major surface components are subject to variable expression through multiple mechanisms. These include genetic polymorphism (porins, Opa proteins, transferrin-binding proteins), phase-variable on–off

switching of gene-expression either directly (Opa proteins, pili) or indirectly (LOS, through expression of enzymes involved in biosynthesis of the glycan chains), recombination of gene segments (pilin structural proteins), as well as natural competence for DNA uptake and DNA secretion that facilitate frequent horizontal gene exchange (Yang and Gotschlich, 1996; Dehio et al., 1998; Massari et al., 2003; Cornelissen, 2008; Maiden, 2008; Hill and Davies, 2009; Virji, 2009). Thus it can be argued that if the host mounts antibody responses to these components, their constantly shifting expression and antigenicity prevent recognition and binding of antibodies to the gonococcal surface. Some evidence in favor of this scenario can be seen in studies on highly exposed sex-workers in Nairobi, Kenya. Partial serovar (porin)-specific immunity to re-infection with the prevalent serovar, and reduced susceptibility to salpingitis associated with anti-Opa antibodies have been reported (Plummer et al., 1989, 1994). However, these findings were not replicated elsewhere (Fox et al., 1999), possibly because of a lower frequency of infection. Antibodies to porin or Opa proteins can mediate complement- or phagocyte-dependent killing of gonococci that express the homologous (or cross-reactive) antigens (Virji and Heckels, 1985; Heckels et al., 1989), but additional mechanisms of resistance are known. These include antibodies to the reduction-modifiable protein (RMP) which is closely associated with porin; these antibodies block bacteriolysis mediated by anti-porin antibody (Rice et al., 1986; Virji and Heckels, 1988). Sialylation of LOS also inhibits complement-mediated lysis (Wetzler et al., 1992a; Smith et al., 1995). Nevertheless, purified porin formed the basis for the development of a potential serovar-specific vaccine (Wetzler et al., 1992b). The outcome of the pilus vaccine effort also supports the concept of immune evasion by antigenic variation. This vaccine, based on the pilus structural protein (pilin) was successful in generating antibodies against homologous pilus protein, and antibodies to pilin could inhibit attachment of gonococci to epithelial cells (Tramont et al., 1981). However, the vaccine proved completely ineffective in a field trial because of the very high antigenic variability of pilin protein among naturally occurring gonococcal strains (Boslego et al., 1991).

As effective as antigenic variation may be in evading the consequences of adaptive immune responses, we further propose that *N. gonorrhoeae* avoids the generation of specific antibodies in the first place (**Table 2**). This was initially based on our observations of minimal antibody responses to uncomplicated gonorrhea in humans, despite symptomatic inflammatory disease and in some cases strong cytokine responses (Hedges et al., 1998, 1999). The human response appears to be quite well replicated in the mouse model of vaginal gonococcal infection, in that there is a neutrophil-dominated cellular infiltrate, inflammatory cytokines are induced through TLR4-dependent mechanisms, no antibody responses are detectable in the serum or genital secretions and tissues, and no protective immunity is generated against secondary infection with the same strain (Song et al., 2008; Packiam et al., 2010). We find that the LOS-TLR4-dependent inflammatory response involves IL-17 and Th17-driven innate responses, and that interference with these leads to diminished neutrophil infiltration and prolongation of the infection in mice (Feinen et al., 2010).

Initial support for the concept of gonococcal suppression of host immune responses was provided by the findings that CEACAM1-binding Opa proteins inhibit human T cell activation and B cell differentiation (Boulton and Gray-Owen, 2002; Pantelic et al., 2005), although this was not confirmed in another study (Youssef et al., 2009). The mouse homolog of human CEACAM1, however, does not possess the residues found to be critical for Opa recognition (Virji et al., 1999). Our own studies on mouse immune cells *in vitro*, and on genital tract tissues from infected mice, show that *N. gonorrhoeae* can exploit TGF-β to suppress adaptive immune responses driven by Th1 and Th2 cells. There is preliminary evidence that this involves Treg cells which have been reported to be induced in the mouse model of gonococcal

Table 2 | New hypothesis concerning immunity to gonorrhea*.

Postulate	Evidence for	Evidence against
Neisseria gonorrhoeae avoids inducing, interferes with, or suppresses adaptive immune responses	*N. gonorrhoeae* enhances TGF-β production and inhibits Th1/Th2 development in mice Antibodies not significantly enhanced in response to infection Opa-CEACAM1 interaction inhibits T and B cell activation	Induction of TGF-β and suppression of Th1/Th2 not yet shown in humans
N. gonorrhoeae preferentially elicits innate immune responses	*N. gonorrhoeae* induces Th17 development in mice	Not yet confirmed in humans
N. gonorrhoeae resists innate immune defense mechanisms	*N. gonorrhoeae* is resistant to complement, intracellular phagocytic killing, and innate defense peptides	Resistance is partial
N. gonorrhoeae is susceptible to adaptive (specific) immune defense mechanisms	Anti-TGF-β antibody reverses inhibition of Th1/Th2 responses and allows specific antibody development against infection in mice Anti-porin or anti-Opa antibody (plus complement, phagocytes) kills gonococci bearing homologous antigens	Not yet shown in humans Gonococci have multiple mechanisms for evading antibody recognition and avoiding complement- or phagocyte-mediated destruction
Hence *N. gonorrhoeae* suppresses the generation of potentially protective specific antibody responses, and proactively elicits a host response pattern that favors its own survival		

**For discussion and references, see text.*

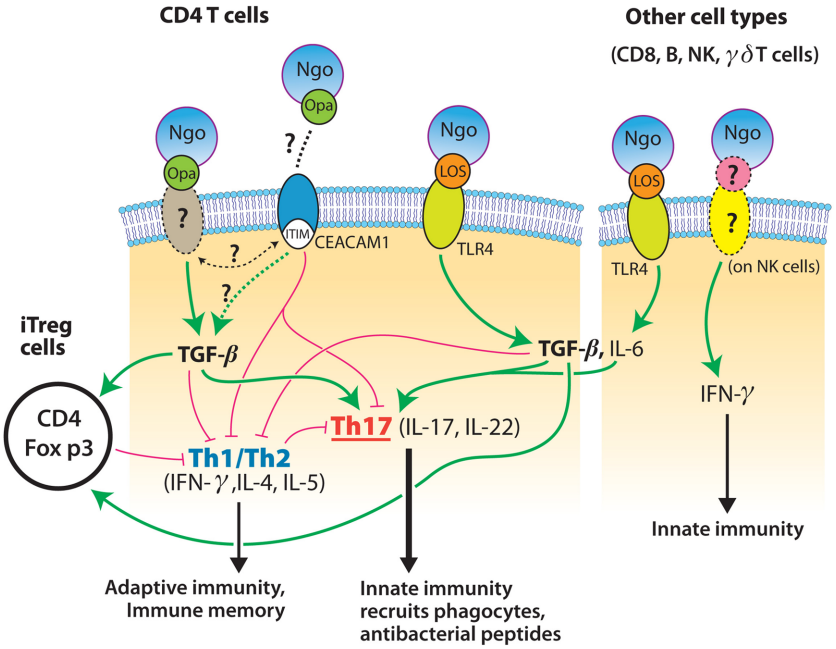

FIGURE 1 | Model for mechanisms of interaction of *N. gonorrhoeae* (Ngo) with cells of the immune system. Gonococcal LOS interacts with CD4+ T cells and other cells through TLR4 to induce the production of IL-6 and TGF-β which drive the development of Th17 cells; IFN-γ is also secreted by NK cells. Gonococcal Opa proteins interact with an unknown receptor on mouse cells (or with CEACAM1 on human cells), leading to the increased production of TGF-β, which directly suppresses Th1 and Th2 cells, and also enhances the development of Treg cells which regulate Th1 and Th2 cells. Blockade of TGF-β thus relieves the suppression of Th1 and Th2 cells, which then drive adaptive immune responses, leading to memory, and antibody production.

infection (Imarai et al., 2008), but this mechanism remains to be fully investigated. Blockade of TGF-β both *in vitro* and *in vivo* alleviates immunosuppression elicited by *N. gonorrhoeae* and permits the generation of Th1- and Th2-governed responses, with the development of immune memory and anti-gonococcal antibodies, and protection against re-infection. Thus we propose not only that *N. gonorrhoeae* elicits from its host Th17-driven innate responses that it can resist, but also that it suppresses adaptive responses that might be capable of eliminating it. In other words, *N. gonorrhoeae* does not merely *react* to the host's immune responses, but it *proactively* elicits a pattern of immune responses that is favorable to its own survival, not what is desirable for the host. Some possible mechanisms by which this is accomplished are illustrated in **Figure 1**.

How this relates to the human infection is unclear at present, because studies of Th17-dependent responses to gonococcal infection have not yet been conducted. We hypothesize that IL-17-driven innate responses are inadequate to eliminate gonococcal infection in humans and that potentially protective adaptive immune responses are suppressed, but this remains to be tested. However, increased serum levels of IL-17 and IL-23 have now been reported in men infected with gonorrhea (Gagliardi et al., 2011). It must be borne in mind that there are numerous differences between mouse and human systems, and that several of the known pathogenic mechanisms deployed by *N. gonorrhoeae* are specific to human cells or proteins. *N. gonorrhoeae* is not a natural pathogen of mice which do not develop signs of disease due to genital tract infection, the infection does not usually persist in mice for more than 1–2 weeks, and the mechanisms by which gonococci are eliminated from the genital tract in either mice or humans are not known. The murine studies cannot be directly replicated in humans for ethical and practical reasons, but if evidence can be obtained that human immune cells respond to *N. gonorrhoeae* in a similar way to murine cells, and that the natural infection of humans with gonorrhea elicits Th17 and/or TGF-β and Treg responses, then new approaches to therapy and vaccine development can be anticipated. For example, TGF-β or Treg cells might be targeted by novel therapeutics to alleviate gonococcal immunosuppression, although caution will be needed to avoid adverse consequences, such as autoimmunity, allergy, or other manifestations of undesirable immune responses that are held in check by immunoregulatory mechanisms. In vaccine development, it is clear that the standard paradigm, of seeking to mimic the natural infection without causing disease in order to elicit protective immunity, is inapplicable because the natural infection does not induce protective immunity, due to gonococcal antigenic variation and gonococcus-induced immunosuppression. Therefore, other strategies to induce effective immune responses against conserved gonococcal antigens and deliver them to the sites of infection will be needed.

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Vaccines for gonorrhea: can we rise to the challenge?

Weiyan Zhu[1], Ching-Ju Chen[2], Christopher E. Thomas[1], James E. Anderson[1], Ann E. Jerse[3] and P. Frederick Sparling[1]*

[1] *Department of Medicine, University of North Carolina, Chapel Hill, NC, USA*
[2] *Human Vaccine Institute, Duke University, Durham, NC, USA*
[3] *Department of Microbiology, Uniformed University of the Health Sciences, Bethesda, MD, USA*

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Magdalene So, University of Arizona, USA
Peter A. Rice, University of Massachusetts Medical School/UMass Memorial Medical Center, USA

****Correspondence:***
P. Frederick Sparling, Department of Medicine, University of North Carolina, 5113 Bioinformatics Building, 130 Mason Farm Road, Chapel Hill, NC 27599-7537, USA.
e-mail: zman@med.unc.edu

Immune responses to the gonococcus after natural infection ordinarily result in little immunity to reinfection, due to antigenic variation of the gonococcus, and redirection or suppression of immune responses. Brinton and colleagues demonstrated that parenteral immunization of male human volunteers with a purified pilus vaccine gave partial protection against infection by the homologous strain. However, the vaccine failed in a clinical trial. Recent vaccine development efforts have focused on the female mouse model of genital gonococcal infection. Here we discuss the state of the field, including our unpublished data regarding efficacy in the mouse model of either viral replicon particle (VRP) vaccines, or outer membrane vesicle (OMV) vaccines. The OMV vaccines failed, despite excellent serum and mucosal antibody responses. Protection after a regimen consisting of a PorB-VRP prime plus recombinant PorB boost was correlated with apparent Th1, but not with antibody, responses. Protection probably was due to powerful adjuvant effects of the VRP vector. New tools including novel transgenic mice expressing human genes required for gonococcal infection should enable future research. Surrogates for immunity are needed. Increasing antimicrobial resistance trends among gonococci makes development of a vaccine more urgent.

Keywords: *Neisseria gonorrhoeae*, vaccines, mouse models, immune responses, viral replicon particles, outer membranes, recombinant proteins

INTRODUCTION: NEED FOR A VACCINE FOR GONORRHEA

Neisseria gonorrhoeae (the gonococcus, or GC) remains an important disease. Still relatively common in the US, with over 300,000 reported cases annually, and probably as many that are not reported, it is much more common in Africa and in many other parts of the less-developed world. Untreated gonococcal infection in women may progress to pelvic inflammatory disease, increasing the risk of ectopic pregnancy and infertility. Calculations of attributable risk show that GC is one of the significant cofactors for HIV transmission (Fleming and Wasserheit, 1999), increasing risks of HIV transmission and acquisition about threefold. These factors alone should promote interest in a vaccine for this ancient disease.

Another reason to urge development of a gonococcal vaccine is emergence of antibiotic resistant GC. In many parts of the world, fluorinated quinolones are no longer recommended because of the prevalence of resistance (Newman et al., 2007; Schultz et al., 2001; Lewis, 2010). Resistance to cephalosporins also is emerging, rendering oral formulations such as cefixime less effective (Lo et al., 2008; Golparian et al., 2010; Lewis, 2010). A steady creep toward decreased *in vitro* susceptibility to ceftriaxone in SE Asia and elsewhere (Chisholm et al., 2010) will threaten utility of ceftriaxone, the principal remaining parenteral therapy for GC, if the trend continues. Current U.S. gonorrhea treatment guidelines increased the dose of parenteral ceftriaxone from 125 to 250 mg to attempt to counteract the slow increases in cephalosporin resistance (Centers for Disease Control, 2010). Resistance to quinolones and beta lactams is due to chromosomal mutations, including alterations of the target sites for these antimicrobials, and also alterations of porin entry channels and/or efflux pumps (reviewed in Lewis, 2010). Most of the resistant mutants appear to be fit, in the sense that they persist and spread in the natural world.

Although plasmid-mediated resistance to penicillins and tetracyclines has leveled off (Lewis, 2010), history teaches that continued selection of more resistant GC variants is to be expected, either by additional chromosomal mutations or acquisition of plasmids. We expect that in a decade, GC resistance to antimicrobials will increase, and therapy with existing cephalosporins will no longer be useful. There are few if any promising new antimicrobials for GC on the horizon. It takes at least a decade or more to create vaccines. Now is the time to be serious about the problem.

The increasing threat of difficult-to-treat GC should make a gonococcal vaccine an important objective, but there is relatively little work at present on such a vaccine. A PubMed search on 12.27/2010 under "gonococcal vaccine" yielded 247 entries, whereas a similar search under "meningococcal vaccine" yielded 3326 entries. The reasons for this glaring discrepancy are not obvious, but may include such commercial factors as estimated market size for vaccines, the probability that the public would accept and utilize the vaccine, and who would pay for the vaccine. It is possible that the shock of witnessing meningococcal (MC) disease and death in innocent infants and young adults helps to energize the MC vaccine programs. By contrast, GC disease is a silent killer of the unborn, due to salpingitis and ectopic pregnancy. Some might

view GC as just a minor infection, and one that is acquired by personal choice. If it can be treated, why bother with a vaccine? Another obvious consideration is the likelihood that a vaccine can be developed, based solely on the available science. In the absence of evidence for naturally acquired immunity after infection, and correlates for protective immunity, it is difficult for commercial operations to push forward with vaccine development. The result has been discontinuance of the former US military GC vaccine program, and in termination of related programs in virtually every large-scale manufacturers of vaccines in the US and Europe.

In this review, we examine the natural history of infection with emphasis on immune response and immunity to infection, and certain new evidence regarding the pathogenesis of infection. Other comprehensive reviews of GC pathogenesis and animal models are included elsewhere in this volume. Previous reviews of gonococcal vaccines covered pathogenesis and antigens that might be used for a vaccine (Blake and Wetzler, 1995; Sparling et al., 2003; Edwards and Apicella, 2004; Virji, 2009). We discuss previous vaccine attempts, as well as lessons learned from vaccines for comparable diseases, especially the close sibling of GC, the MC. We then present previously unpublished experiments from our laboratories, which were "negative" in the sense that they did not show evidence for protection in the female mouse model of genital GC infection. Nevertheless, they inform future investigations, and one surprising result in particular suggests a path forward for additional research.

IS THERE EVIDENCE FOR NATURALLY ACQUIRED IMMUNITY TO REINFECTION?

In the preantibiotic era, symptomatic infections resolved spontaneously over months, although the basis for apparent immune resolution was not studied (Hill, 1942). In the current era of readily available antibiotics, persons commonly acquire gonorrhea, are treated, but are soon reinfected. Mild or asymptomatic infection may be carried for months without treatment, both in men and women (Handsfield et al., 1974). A community outbreak due to a highly resistant strain demonstrated that reinfections with the same strain were common after treatment of uncomplicated genital infection (Faruki et al., 1985). There was no evidence for increased resistance to a second infection in studies of experimental GC of male human volunteers, even when the repeat infection was initiated only 2 weeks after treatment of the first infection by the same MS11 gonococcal strain (Schmidt et al., 2001) The infections of experimental subjects had to be terminated at onset of symptoms for ethical reasons, undoubtedly before a full immune response was initiated.

A study of highly experienced female commercial sex workers in Africa showed apparent immunity to recurrent infection; protection was specific to particular outer membrane porin protein B (PorB) serovars, and was not generally protective against other strains (Plummer et al., 1989). However, a similar study in a small US community, involving men and women, who certainly had fewer previous episodes of GC than the African female cohort, actually found an increased rate of reinfection by the same PorB serovar (Fox et al., 1999). Increased rates of second infections by the same strain could have been due to the greater likelihood of sexual reexposure to still-infected partners. Other data from this same study showed there was some antigenic variation in exposed PorB surface loops during repeated passage of this strain through the community (Hobbs et al., 1999). A small study claimed PorB serovar specific immunity to reinfection in women who had salpingitis (Buchanan et al., 1980). Conceivably, repeated infections elicited immune memory responses, providing the basis for protective immunity on reexposure.

A somewhat more optimistic view of immunity after local mucosal infection was provided by studies of experimental GC in chimpanzees. Certain GC strains were capable of infecting the chimp urethra, pharynx, and cervix; the infectious dose was high for the pharynx and cervix, but for the male urethra the required inoculum was about 1×10^4 colony forming units (CFU), essentially the same as for human urethral infection (Kraus et al., 1975). Infection persisted for weeks, but then an uncharacterized immune response cleared infection, as defined by two successive negative cultures. Initiation of a second infection by the same strain 1 week after termination of first infection required an infectious inoculum about 1000-fold greater. Repeat challenge 2 years after the first infection showed that immunity had completely waned (Kraus et al., 1975). These experiments also showed that bactericidal serum antibodies protected against urethral infection in the chimp: the only GC capable of infection were of the PorB1B serovar class, which were able to bind chimp complement four binding protein (C4bp), rendering them phenotypically serum resistant (Ngampasutadol et al., 2005). PorB1A strains were not able to bind C4bp and were unable to infect at the highest inocula tested. Chimps are expensive, difficult to work with, and are now virtually unavailable for similar studies. That is unfortunate, since they are the only non-human primate capable of mucosal infection by GC.

In sum, the evidence does not provide much support for naturally acquired immunity, and outside of chimps, almost none for immunity after uncomplicated infection. What evidence exists suggests immunity is strain specific, which is not helpful in the clinical arena. Regardless, failure of natural immunity after infection can have many causes, and does not in itself prove that a vaccine is not possible. A vaccine might induce a much greater and different immune response than occurs after mucosal infection.

HUMAN IMMUNE RESPONSE TO GC

Uncomplicated mucosal GC in humans results in an immune response, but in many studies the response was weak and brief. Serum antibodies have been documented against many different GC antigens (reviewed in Sparling et al., 2003). Men tend to have a poorer response than women (Tapchaisri and Srinisinha, 1976; Miettinen et al., 1989; Hedges et al., 1999). In almost all studies detectable serum antibodies were gone within a few months, or less. In cervical mucus, IgG antibodies were at least as prevalent as IgA (Tapchaisri and Srinisinha, 1976). In keeping with an overall modest immune response to GC, Hedges et al. (1998) found elevations only of lL-6 in serum of infected women; none of the measured cytokines was elevated in cervical mucus. Only transient elevations of IL-1β, IL-6, IL-8, and TNFα in urine and serum occurred in experimentally infected men (Ramsey et al., 1995).

Kasper et al. (1977) found bactericidal responses in about one-third of uncomplicated infections, but in only 5% of women with

salpingitis at the time of their presentation for care, suggesting that bactericidal antibodies might protect against salpingitis. Convalescent sera showed a fourfold rise in bactericidal titers in 70% of women with severe salpingitis, as compared to only 11% with mild disease (Kasper et al., 1977). Bactericidal antibodies were common in patients with mucosal infection, but did not prevent infection (Brooks and Ingwer, 1978).

Hedges et al. (1999) provided the best study in many ways of immune responses in uncomplicated GC infection of men and women. Sera and genital secretions were tested by ELISA for isotype-specific responses to whole formaldehyde-fixed GC strains, both their own infecting strain, and a standard MS11 strain. Strain-specific IgA1 but not IgA2 antibodies were observed in both serum and cervical mucus in some subjects. A weak secondary IgG response was seen in some individuals. Women had greater responses than men, but responses were brief and temporary, declining within weeks of treatment. Previous infection did not result in immunological memory; titers and duration of antibody responses were identical in first infections and in those with previous infections. The authors' suggested that GC might somehow suppress the immune response of the host, although no mechanism was offered.

The concept of immune suppression or redirection by commensal and pathogenic bacteria is now a very active area of research. There is evidence that GC are able to suppress either human CD4 T cell responses (Boulton and Gray-Owen, 2002; Lee et al., 2008) or human B cell antibody responses (Pantelic et al., 2005) *in vitro*. These effects of GC on human immune cells are regulated by binding of GC opacity (Opa) proteins to human carcinoembryonic antigen cellular adhesion molecule 1 (CEACAM1) on lymphocytes (Boulton and Gray-Owen, 2002; Pantelic et al., 2005). Binding CEACAM1 initiates a broad-range of inhibitory effects on T cells (Nagashi et al., 2008). In mice, GC infection stimulates a Th-17 response, with influx of mucosal polymorphonuclear leukocytes (PMNs; Feinen et al., 2010). This apparently is beneficial, since GC are able to substantially resist PMN-mediated killing (Simons et al., 2005, 2006; Criss and Seifert, 2008; Criss et al., 2009). From the view of a vaccine development, evidence that GC suppresses or redirects immune responses is good news, since it opens the possibility that a vaccine might circumvent such immune manipulation by GC.

The most promising approach has been to examine targets for biologically relevant immune responses. Much work has focused on a complex of three antigens that cluster together in the outer membrane (OM): lipooligosaccharide (LOS); Reduction modifiable protein (RMP), formerly designated PIII; and porin protein (PorB). The structures and biology of these molecules have been reviewed many times (Sparling et al., 2003; Virji, 2009). LOS undergoes frequent phase variation in the structure of its polysaccharide side chains, and also can be sialylated, rendering strains more serum bactericidal resistant but less invasive. Sialylated LOS also partially masks adjacent PorB trimers in the OM, reducing binding of monoclonal antibodies (mAbs) to PorB by about 50% (Elkins et al., 1992). RMP is a potent immunogen and elicits blocking antibodies that subvert the killing effects of bactericidal antibodies directed against PorB or LOS (Blake et al., 1989). PorB exists in two main classes, designated PorB1A and PorB1B, each the product of the *porB* gene. The PorB1A and PorB1B classes vary in certain domains of the eight exposed loops that project out from the OM, and within each class there are many minor variants that differ in sequence and antigenicity (serovars). PorB is the major protein in the outer membrane, and is essential; *porB* knockouts are lethal. PorB is crucial for entry of low molecular weight anions, and is involved with Pil and LOS in cooperative binding to complement receptor 3 (CR3) in primary cervical cells (Edwards et al., 2002). PorB also is capable of penetrating epithelial cells, facilitating invasion (Weel and Putten, 1991; see also reviews by Massari et al., 2003; Sparling et al., 2003; Edwards and Apicella, 2004; Virji, 2009), and leading to apoptosis, by means of entry into mitochondrial membranes within the cell (Massari et al., 2003; Kozjak-Pavlovic et al., 2009; Rudel et al., 2010). PorB does not undergo phase variation, unlike many important cell surface molecules involved in GC pathogenesis including LOS, pili (Pil), and opacity proteins (Opa). Thus, it is no surprise that PorB has evolved many mechanism for protection from host defenses, including binding the complement regulatory proteins C4bp and factor H (fH; Ram et al., 1998; Jarva et al., 2007; Madico et al., 2007; Lewis et al., 2008; Ngampasutadol et al., 2008), in addition to the protections offered by sialylation of LOS and the elicitation of blocking antibodies by RMP.

The immediate importance to this discussion is evidence that differences in immune responses to the RMP, LOS, and PorB triad affects susceptibility to infection. Data presented in summary form suggested that susceptibility to infection after sexual exposure to an infected partner was determined by an equation: antibodies against (PorB + LOS)/antibodies against Rmp equals susceptibility; the higher the ratio, the lower the risk (Rice et al., 1974; Blake and Wetzler, 1995). Unfortunately, complete details of these experiments have not been published, to our knowledge. Other efforts for a vaccine based on PorB or certain LOS epitopes are discussed below. Avoidance of Rmp antigen is one goal of current vaccine design.

GONOCOCCAL VACCINES FOR HUMANS

Only two vaccines for GC have entered into clinical trials. The first was a crude killed whole cell vaccine, which was studied in a controlled experiment in a population of Inuit in northern Canada with high incidence and prevalence of GC infection (Greenberg et al., 1974; Greenberg, 1975). There was no evidence for protection, even though the vaccine was said to be well tolerated and induced an antibody response in over 90% of vaccine recipients. Since it was a crude whole cell vaccine, the alleged good tolerance, with only mild reactions, was surprising, since the vaccine certainly contained highly inflammatory LOS. Similar efforts with MC vaccines have utilized either detergent delipidated OMs to remove toxic lipid A, or mutants of LOS that no longer produce toxic forms of lipid A (reviewed by Granoff, 2010). The whole cell GC vaccine was not developed further. An analogous vaccine made from a piliated GC strain was tested in chimps by investigators from the CDC in Atlanta, with good serum bactericidal and immunofluorescent antibody responses. This vaccine resulted in protection very similar to that observed after natural immune clearance of infection in chimps, requiring a 1000-fold larger inoculum to infect vaccinated animals (Arko et al., 1976).

The most significant effort focused on a purified Pil vaccine. Pil are crucial for initial attachment to a variety of human cells. Either the pilus fibril, the product of the pilE gene, or a minor pilus-associated protein designated PilC, or both, mediate attachment to a still uncertain receptor. On primary cervical epithelial cells, Pil mediate attachment to CR3 in conjunction with PorB (Edwards et al., 2002; Edwards and Apicella, 2004). A series of elegant experiments proved that isolated and purified Pil containing an uncertain amount, but probably not much, of contaminating PilC, was able to protect human volunteers from experimental urethral infection after parenteral immunization (Brinton et al., 1982). The vaccine elicited a broad antibody response in serum and in genital secretions, including secretory IgA. Mucosal antibodies blocked adherence *in vitro* of piliated GC to various cells (McChesney et al., 1982). A subsequent trial that was never published in detail showed no protection against a heterologous strain expressing antigenically variant Pil (Tramont and Boslego, 1985). Nevertheless, a large-scale filed trial of the single-antigen Pil vaccine was carried out in high-risk US military personnel stationed in Korea, using a smaller dose and a different route (intradermal) than that used in the initial proof-of-principle experiment. The result was not even a hint of protection (Boslego et al., 1991). The probable reason for failure was antigenic variation of expressed Pil in the naturally acquired infections (Criss et al., 2005). Since then, there have been relatively small efforts to discover and develop a common Pil epitope for use in a next-generation vaccine, but so far, no useful data have been forthcoming.

Other vaccine candidates have been and are being considered, as will be discussed below, but none has advanced to clinical trial.

WHAT CAN BE LEARNED FROM MENINGOCOCCAL VACCINES?

An examination of the state of MC vaccines may be instructive. Vaccines for MC disease traditionally used capsular polysaccharides, which induced bactericidal responses that were protective against invasive disease including bacteremia and meningitis. These developments built upon classic studies that showed that epidemic MC disease was the result of colonization of persons by epidemic strains to which the subjects had no pre-existing bactericidal antibodies (Goldschneider et al., 1969a,b). Serum bactericidal antibody (SBA) titers of at least 1:4 were strongly correlated with protection from disease, whether induced by naturally occurring infections with bacteria that share similar capsular antigens with encapsulated MC, or by the MC capsular antigen vaccines (Frasch et al., 2009; Granoff, 2009). SBA is a better measure of protection than ELISA titers against capsular antigen (Frasch et al., 2009). SBA also is well correlated with protection from invasive disease by several other encapsulated bacteria, including pneumococci and *Hemophilus influenzae* (Conference Report, 2010). A variety of efficacious capsular vaccines were developed, including capsular polysaccharides conjugated to protein antigens to increase immunogenicity in the young. Capsular vaccines against serogroup C MC result in decreased carriage as well as protection from invasive disease, yielding a significant effect on transmission within the community (Maiden et al., 2008). A conjugate vaccine for group A MC designated "MenAfriVac" is now being introduced in the meningitis belt in Africa, under the aegis of a consortium known as the Meningitis Vaccine Project (Butler, 2010), and conjugate vaccines for other MC capsular types are now widely deployed in practice.

MC disease differs from GC in two crucial respects: GC do not make capsular polysaccharides, and GC only occasionally invade the blood stream, typically restricting damage to the lower and upper genital tracts. Thus, correlates for protection against MC disease such as SBA may not predict correlates for protection against GC. Moreover, SBA is not the only predictor of protection for MC disease; Granoff argues that the SBA level of at least 1:4 is sufficient for protection, but is not a sensitive measure of protection, since many are protected with lower levels of measurable SBA (Granoff, 2009). Opsonophagoyctic activity (OPA) with human serum and PMNs appears to be a good predictor of efficacy for experimental group B MC vaccines (Granoff, 2009).

Progress in development of a vaccine for group B MC might help inform development of a GC vaccine. Non-capsulate GC and MC are not identical, but share many outer membrane proteins, and their LOS are similar. For decades, serogroup B MC have been an elusive target for a vaccine because the capsule of group B MC is identical to human central nervous system (CNS) antigens, causing fear of cross-reactive anti-CNS immune responses. For this reason, all efforts for a group B MC vaccine are now focused on non-capsular antigens. Detergent-extracted outer membrane vesicles (OMV) appear to be safe, and have been used successfully to control local epidemics. The LPS also can be modified genetically so as to express a lipid A bearing either four or five acyl residues instead of the usual six, reducing the reactogenicity of OMV vaccines significantly (Donnelly et al., 2010; Keiser et al., 2010). However, the immunodominant OMV protein PorA is antigenically variable (reviewed in Granoff, 2010). OMV vaccines prepared from multiple PorA type strains have been tested and are efficacious, but are not highly immunogenic in infants (Granoff, 2010). (GC contain a *porA* pseudogene, but do not express PorA).

By a variety of methods including whole genome sequencing, in silico predictions of which genes express novel outer membrane proteins, proteomics, genetic engineering, and immunology, a small set of candidate novel protein immunogens were selected for further study. SBA served as the surrogate for protection, and enabled more rapid development than would have been possible without such a reliable simple assay for protective immunity. Progress required a large investment of resources by industry, the FDA and academic laboratories. Two new vaccines are in late stages of clinical trials. In one, three protein immunogens, consisting of two fusion proteins and a third single protein, are coupled with an OMV vaccine expressing PorA (Keiser et al., 2010). The vaccine appears to be more effective when the recombinant proteins are coupled with OMV rather than being administered alone (Findlow et al., 2010). The key novel proteins are NadA, an adhesin; a heparin binding protein formerly designated 2132, now designated NHBP; and a protein that binds fH, designated fHbp (Beerink and Granoff, 2008; Donnelly et al., 2010). Antibodies against fHbp are directly bactericidal, and also prevent binding of fH, further promoting bactericidal activity. All of these proteins are widely expressed among MC strains, and are relatively conserved, although fHbp exists in three major antigenically distinct families (Beerink and Granoff, 2008; Donnelly et al., 2010). Clinical trials

have been conducted in adults and infants, and results are sufficiently promising to support large phase three trials, which are underway (Findlow et al., 2010; Snape et al., 2010). Meanwhile, others are investigating construction of additional novel immunogens (Lewis et al., 2010), including OMV expressing hybrids of the fHbp family that result in antibodies that cross react widely against MC strains (Beerink and Granoff, 2008; Koeberling et al., 2009). With any luck, we soon will witness the clinical deployment of efficacious vaccines in adults and infants for all MC strains, a triumph.

What are the lessons for a gonococcal vaccine? Many of the key protein antigens identified in MC are not present or are not functional in GC. PorA is not expressed in GC. NadA is absent from all of the sequenced GC genomes (Comanducci et al., 2002; and unpublished observations). GC has a fHbp homologue but it is not predicted to be localized to the surface as in MC, since it lacks a functional signal sequence. Deletion of this gene does not decrease factor H binding or alter serum resistance (Welsch and Ram, 2008). NHBP is present in several of the sequenced GC genomes and remains a viable target. Development of a vaccine similar to the MC group B vaccine should be possible, but will require a much larger effort than that currently underway. Use of OMVs with engineered LOS structures that are less reactogenic should be easy. OMVs can be constructed to express a variety of antigens, including hybrid PorB. Reliance on a single protein antigen is problematic. All the tools to do this are available, with one major exception: there is no reliable surrogate such as the SBA to guide development.

CHOICE OF THE MOUSE MODEL FOR DEVELOPMENT A GC VACCINE

Development of the female genital tract mouse model for studies of pathogenesis of GC (Jerse, 1999) opened up new possibilities for early phase studies of possible vaccines for GC. Infection in mice persists for about 2 weeks after the initial inoculation, and rates of decline can be used to measure resistance to infection. Mixed infections also can be used to test relative fitness of strains, and presumably could be used to test relative abilities to resist specific immune responses to particular antigens. The most attractive aspect of the mouse for vaccine development, apart from availability and cost, is the advanced state of development of mouse genetics, and the superb tools for monitoring immune responses in the mouse. Similar to observations in humans, mice develop an inflammatory immune response, but no resistance to repeat infection by the same strain, and no immune memory responses, after genital tract infection (Song et al., 2008).

The mouse model has many deficiencies, including (at the time our studies were performed, see below) lack of: receptors for binding Pil and Opa; human C4bp or fH; and iron binding proteins other than hemoglobin and heme that can be used by GC for growth. Each of these is a problem, and in aggregate, it is uncertain how findings in a mouse can be translated to human infection. Nevertheless, confidence that the mouse model is relevant to studies of GC biology is enhanced because of the correlations in roles of some virulence factors for both mice and humans. Sialylation of LOS appears to be requisite for maximal infectivity in mice, just as in humans (Wu and Jerse, 2006). Surprisingly, Opa expression is selected in the mouse, even in the absence of the CEACAM receptors for Opa (Cole et al., 2010). In humans, there also is strong selection for Opa expression in male volunteers (Jerse et al., 1994). Drug resistance efflux pumps involved in antibiotic resistance are required for maximal infectivity in the mouse, apparently by means of exporting cationic innate immune defense proteins (Warner et al., 2008). The mouse model, although imperfect, seemed the best choice for our efforts to investigate protective immune responses, aiming to discover which antigens might be useful in a vaccine, and correlates of protection.

But which antigens? Because of lack of their receptors, expression of phase-variable GC pili is not maintained in mice, making study of Pil vaccines impossible. Moreover, the mouse model is not particularly robust, making it very difficult to utilize a shotgun of antigens, either as genetic constructs or as proteins. We decided to focus on intranasal (IN) vaccination with outer membrane preparations, because IN immunization results in strong genital mucosal responses, and because of the success of the MC OMV vaccines. We also chose to closely examine a few outer membrane proteins. For initial studies of immunogenicity, we included the GC transferrin receptor protein TbpB, because it is an immunogenic lipoprotein, and is essential for gonococcal infection of male humans using strain FA1090 (Cornelissen et al., 1998; Cornelissen, 2008). The other candidate selected was PorB, because it plays many essential roles in infection, including binding to CR3 on female epithelial genital cells in a complex with Pil and LOS (Edwards et al., 2002); binding of PorB1A to Gp96 and SREC on epithelial cells, facilitating both adherence and invasion (Rechner et al., 2007); binding of both C4bp and fH to discrete and different loops on either PorB1A or PorB1B, enabling resistance to complement-mediated killing (summarized above); and evidence that some polyclonal and mAbs to PorB are bactericidal (Virji et al., 1986; Heckels et al., 1989; Butt et al., 1990). Moreover, PorB is an adjuvant, through binding to TLR2 (Wetzler, 2010).

OMV VACCINES NOT UNIFORMLY SUCCESSFUL IN MICE

Immunization with gonococcal OMV is an attractive vaccine strategy due to the potential of OMV to elicit an immune response against several different conformationally correct components on the bacterial surface. There was reason to believe OMV vaccines were likely to be effective, by analogy to success with MC OMV vaccines, and because of one report of a successful trial of a GC OMV vaccine in the mouse model. Plante et al. (2000) reported that IN immunization of female mice with gonococcal OMV reduced colonization following vaginal challenge with the homologous wild-type strain MS11. Significant protection was observed in the two experiments that were reported in this study, and in an unreported experiment that was performed prior to these experiments. However, no protection was observed in similar experiments testing either MS 11or FA1090 OMV that were performed subsequent to these published experiments. The OMV preparations that showed protection were prepared by shaking whole bacteria with glass beads for 2 h at 45°C, whereas the failed experiments used OMVs prepared by passing bacteria through a needle. It is possible that differences in the preservation of conformational epitopes might be responsible for inability to reproduce these data. Additional differences in the experiments included a

slightly higher dose of estradiol in the mice that did not show protection by OMV vaccine.

Failure to reproduce the initial OMV vaccine results could have been due to presence of Rmp in outer membranes from wild-type MS11. Anti-Rmp blocking antibodies could have reduced effectiveness of antibodies against either LOS or PorB. To test this, we immunized mice with OMV isolated from an *rmpA* insertion mutant of another PorB1B strain, strain FA1090, grown under iron-restricted conditions so as to express iron-repressed proteins. OMVs were prepared by passing bacteria through a small bore needle. Mice developed substantial anti-PorB serum IgG antibodies, a rise in SBAs, vaginal PorB-specific IgG, and very high levels of vaginal PorB-specific IgA antibodies (Zhu et al., 2005). Despite what one might have assumed were the types of responses that would correlate with protection, there was no protection after IN OMV immunization (**Table 1**). We concluded that anti-Rmp blocking antibodies were not responsible for the lack of effectiveness of OMV vaccine antigens *in vivo*. These results also showed that induction of mucosal IgG and IgA antibodies, and a bactericidal serum response, did not predict protection.

Another reason for failure of the vaccines could be *in vivo* sialylation of LOS, leading to resistance to mucosal antibodies and complement. Increased resistance to complement-mediated defenses in humans occurs via the covalent linkage of sialic acid to LOS molecules with a lactose-*N*-tetraose moiety, which reduces activation of the alternative pathway of complement activation via the binding of fH. Although mouse fH does not bind to sialylated LOS (Ngampasutadol et al., 2008), sialylation does reduce opsonophagocytosis following incubation of GC in mouse serum, and an *lst* mutant (which lacks sialyltransferase, and can not sialylate LOS) was attenuated for murine infection (Wu and Jerse, 2006). To test the effect of sialylation *in vivo* on possible escape from OMV-induced immune protection, we used wild-type F62 and an isogenic *lst* mutant as the challenge strains, after IN immunization with F62 OMVs. Ovariectomized BALB/c mice were used to circumvent the need for mice to be in anestrus or in the diestrus stage of the estrous cycle during the challenge phase of the experiment. High titers of OMV-specific serum IgG and vaginal IgG and IgA were detected following immunization. Mice were challenged 3 weeks after the final immunization. There was no difference in the duration of recovery or number of wild-type or *lst* mutant bacteria recovered (**Figure 1**). Similar results were obtained after IN immunization with the strain MS11 OMVs, followed by challenge with either MS11 or its isogenic MS11*lst* mutant. We concluded that sialylation was not responsible for gonococcal evasion of an OMV vaccine-induced host response in the mouse model.

Thus, three different outer membrane vaccines delivered IN resulted in high titers of serum antibodies, excellent serum bactericidal activity, and robust mucosal responses as assessed by analysis of vaginal wash and fecal pellet antibodies (Zhu et al., 2005; and data not shown), yet were uniformly completely ineffective in either accelerating clearance or preventing GC infection in the female mouse genital tract. OMV may still play some role as a component of a GC vaccine, but we shifted our attention to individual outer membrane protein antigens.

RECOMBINANT PROTEIN VACCINES

The first consideration was how to prepare purified protein antigens in a conformationally correct form, in sufficient amounts, and in the absence of contaminants that might be a problem, including LOS and the blocking antigen Rmp. Problems due to the blocking effects of antibodies to Rmp could be avoided by use of cloned recombinant preparations of PorB. Considerable effort by commercial partners went into preparing a refolded recombinant FA1090 PorB1B (rrPorB) from *E. coli*, in a conformation that closely mimicked that of native PorB extracted from GC (Matsuka et al., 1998). A phase 1 human trial was undertaken, but was not carried forward to test for protection of male volunteers, apparently because of problems with adverse rates of local inflammation due to the vaccine. The rrPorB served as an excellent immunogen for studies in mice. Preparing batches of the much less hydrophobic TbpB was less problematic (Thomas et al., 2006).

VRP AND DNA VACCINES

Another very attractive option was to use genetic vaccines expressing either PorB or TbpB. Such an approach would allow much greater flexibility in exploring combinations and variations in the antigens. DNA vaccines have the advantage of great simplicity. There has been a surge in interest in viral derivatives that deliver antigens either as a viral like particle (VLP) that do not replicate, or as viral replicon particles that have a single cycle

Table 1 | Immunization with OMV or rrPorB fails to protect female mice from genital infection by FA1090.

Vaccination regimen	Route	*N*	Days infected (Mean ± SD)	*p* value
Mock-PBS	Dorsal SQ	13	7.23 ± 2.68	
rmp OMV	Intranasal	8	7.50 ± 3.16	0.77
rrPorB	Dorsal SQ	10	7.10 ± 3.93	0.89
rrPorB	Footpad	8	5.50 ± 2.52	0.21

BALB/c mice were prepared in groups of 15 as described by Jerse (1999). Immunizations were conducted three times at intervals of 3 weeks, using either 20 μg protein of an Rmp mutant of FA1090 OMV (rmp OMV) in 20 μl of PBS; or 10 μg protein of recombinant renatured PorB from FA1090 (rrPorB) prepared as described by Matsuka et al. (1998) mixed 1:1 in 10 μl PBS with Ribi-700 adjuvant, delivered either by the dorsal or footpad route. Three weeks after the last boost, mice in diestrus phase were implanted with an estradiol pellet and treated with antibiotics to reduce the normal vaginal flora (Jerse, 1999). Two days later they were inoculated intravaginally with 1 × 10^6 CFU of FA1090, and followed with daily quantitative vaginal cultures. Days to last positive culture are shown. Log rank was used to compare the results between the groups. A weak but repeatable trend was observed for protection only in the rrPorB in Ribi delivered by footpad vaccine regimen.

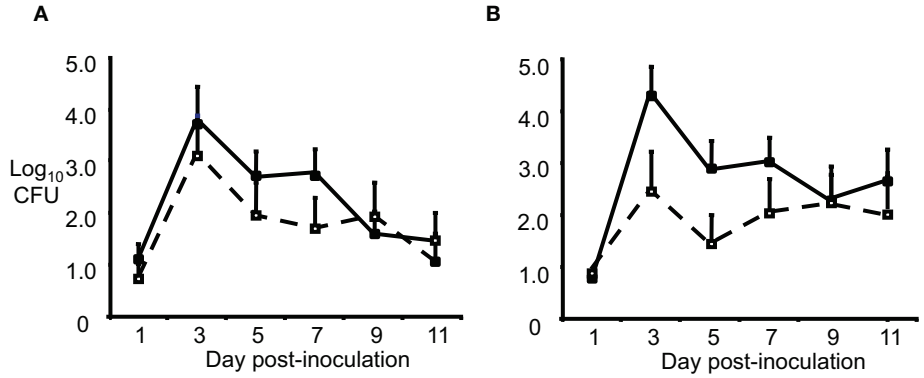

FIGURE 1 | Sialylation does not protect GC from OMV vaccine-induced immune responses. Groups of BALB/c mice ($n = 10$–12 per group) were immunized with 20 μg of OMV (■) from strain F62 via the IN, subcutaneous, and IN routes separated by 3-week intervals or given PBS (□) by the same routes. Three weeks after the final immunization, mice were challenged vaginally with **(A)** wild-type F62 bacteria or the **(B)** F62 *lst::aphA3* mutant (GP330). Vaginal mucus was cultured every other day for 11 days. The average $\log_{10}$CFU per 100 μl vaginal swab suspension over time is shown with standard error bars. There was no difference in the number of GC recovered (repeated measures ANOVA) or duration of colonization (Log rank test) for any experimental group. This experiment was repeated using three IN immunizations and another experiment was performed with strain MS11 and an MS11 *lst* mutant, and the results were similar.

of replication (VRP). One of these VRP systems, based on the alphavirus Venezuelan encephalitis virus (VEE), provides both systemic and mucosal immune responses, and is being used for a variety of novel vaccines Davis et al., 1996; Hubby et al., 2007). We prepared both DNA and VEE-VRP vaccines for each of FA1090 TbpB and PorB (Zhu et al., 2004, 2005; Thomas et al., 2006).

The PorB-DNA vaccine appeared promising in terms of immune responses (Zhu et al., 2004). PorB-DNA was particularly immunogenic in BALB/c mice when boosted either by rrPorB in Ribi-R700 adjuvant, or by PorB-VRP. Delivery of PorB-DNA by the IM route resulted in a polarized Th1 response, but delivery by a gene gun to the epidermis, resulted in a predominantly Th2 response (Zhu et al., 2004). Boosting with PorB-VRP drove the response to a more dominant Th1 response. Serum antibodies with opsonophagocytic activity against FA1090 were observed after DNA immunization. Ability to control the direction of the immune response might be useful for vaccine development in the mouse, but because of the relatively greater immune responses elicited by PorB-VRPs boosted by rrPorB (below), and by other practical considerations, our vaccine efficacy experiments were limited to the PorB-VRP and rrPorB antigens.

Based on the ratio of PorB-specific IgG1/IgG2 antibody responses, there was an apparent Th1 bias with either the PorB-VRP vaccine, delivered without additional adjuvants into a rear footpad (FP), or with the rrPorB vaccine delivered with Ribi-R700 adjuvant into a rear FP. In contrast, there was an apparent Th2 bias when the rrPorB vaccine with Ribi was delivered into the dorsal SQ region. ELISPOT assays of harvested splenocytes showed that there was a significant interferon-γ (IFN-γ) response to PorB peptides only after immunization with the PorB-VRP vaccine (Zhu et al., 2005). Highest serum IgG responses were observed with the rrPorB immunization, accompanied by vaginal mucosal IgG but not IgA responses. The best mucosal IgG responses were observed after rrPorB immunization, whereas the highest mucosal IgA responses were after IN OMV immunization (Zhu et al., 2005).

MICE WERE PROTECTED BY A VRP VACCINE

Initial vaccination/protection experiments utilized rrPorB with Ribi-R700 adjuvant. Delivery of the vaccine in the dorsal SQ route resulted in no protection, but delivery into the FP resulted in a weak, statistically insignificant, but repeatable trend toward protection, manifest by about a 2-day reduction in colonization (**Table 1** and data not shown). We designed additional experiments to test whether a prime-boost regimen utilizing the rrPorB and PorB-VRP vaccines might yield improved results. Four groups of 24 animals each were immunized three times at 2-week intervals with either mock control, rrPorB-R700 in the FP × 3, PorB-VRP in FP × 3, or PorB-VRP in FP × 2 plus a boost with rrPorB-R700 in FP (**Figure 2**). There was a trend toward reduced duration of infection in the PorB-VRP in FP × 3 group ($p = 0.11$), and in significant protection ($p < 0.01$) in the PorB-VRP plus rrPorB-R700 boost group.

These results were encouraging, even though the vaccines did not prevent infection. The experiment was repeated, adding an additional control consisting of an irrelevant influenza virus hemagglutinin antigen VRP construct (HA-VRP). Because the most effective vaccine in the previous experiment was PorB-VRP × 2 boosted with rrPorB, the control HA-VRP vaccine was given twice, and boosted with rrPorB. This tested for the possible effects of the VRP vector. The mock PBS control also was given twice and boosted once with rrPorB, to control for the effects of a single dose of rrPorB. The results (**Figure 3**) were interesting. Both the PorB-VRP vaccine, boosted once with rrPorB, and the HA-VRP vaccine boosted once with rrPorB resulted in significant ($p < 0.05$) reduction in duration of infection compared to the PBS control boosted once with rrPorB. The single booster dose of rrPorB given after PBS × 2 had no effect, compared to PBS without rrPorB. There was no difference in colonization between the HA-VRP × 2 + rrPorB boost and the PorB-VRP × 2 + rrPorB boost vaccines. We tentatively concluded that the VRP vector was the important component of the vaccine, and that PorB expressed

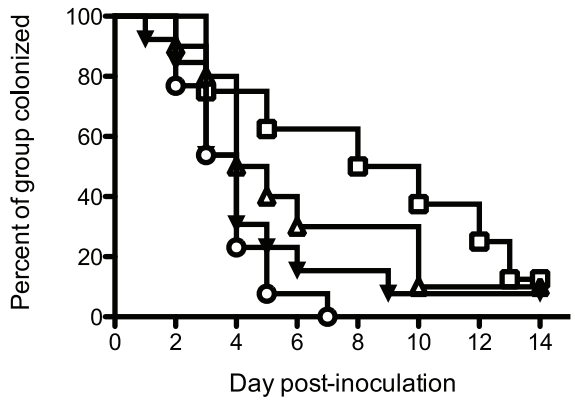

FIGURE 2 | PorB-VRP × 2 in FP plus boost with rrPorB in FP protects against vaginal infection in the mouse model. Three- to four-week old female BALB/c mice were divided randomly into four groups of 24 and vaccinated three times at 2 weeks interval. Mice were immunized with three doses of PBS (□, mock, $n = 8$), rrPorB (△, $n = 10$), PorB-VRP (▼, $n = 13$) or 2 doses of PorB-VRP with 1 boost of rrPorB (○, $n = 13$). All doses of immunogens, in a volume of 20 μl, were given through the left footpad after the animals had been anesthetized. Two weeks after the last boost, mice in the diestrus phase were implanted with estradiol pellets and treated with antibiotic. They were inoculated intravaginally with 1×10^6 CFU of FA1090 2 days later. Daily vaginal cultures were processed for total recovery from the infection. Days to last positive culture are shown. Log-rank test for equality to the Mock group yielded the following p values: PorB-VRP + rrPorB boost, $p = <0.01$; PorB-VRP without rrPorB boost, $p = 0.11$; rrPorB alone, $p = 0.40$.

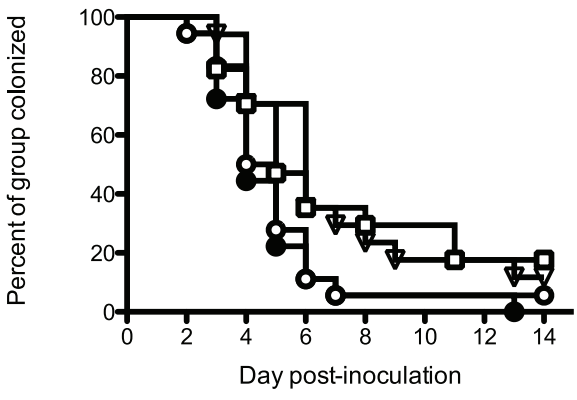

FIGURE 3 | A PorB-VRP vaccine for gonorrhea in the mouse is no more effective than a vaccine composed of the same vector expressing an irrelevant peptide. BALB/c female mice were immunized in FP with either PBS three times (□, mock, $n = 17$); PBS twice with a single boost of rrPorB (▽, $n = 17$); FA1090 PorB-VRP twice with a single boost of rrPorB (○, $n = 18$); or influenza HA-VRP twice, also boosted once with rrPorB (●, $n = 18$). The rrPorB boosts consisted of 10 μg of FA1090 rrPorB in 10 μl PBS mixed 1:1 with Ribi-700 adjuvant. Immunizations were at 2-week intervals. The VRP preparations were administered in a dose of about 1×10^6 particles without additional adjuvant. The volume of all immunizations was 20 μl. Two weeks after the final immunization, mice in diestrus were recruited for the challenge with FA1090, as in **Table 1**. Days to last positive culture are shown. Log rank was used to compare all four groups; results were significantly different among the groups ($p = 0.03$). The only pairwise comparisons which were significantly different ($p < 0.05$) were PBS alone vs HA-VRP + rrPorB ($p = 0.03$), PBS + rrPorB vs HA-VRP + rrPorB ($p = 0.01$), and PBS + rrPorB vs PorB-VRP + rrPorB ($p = 0.04$).

by the VRP vector might not be necessary for protection. Note that infectious challenge was initiated 4 weeks after the last dose of PorB-VRP in the PorB-VRP × 2 plus rrPorB boost group, so the effects of the VRP vector were durable.

How could a vector expressing an irrelevant peptide provide protection against GC infection? Protection was not correlated with serum PorB-specific IgG, since there was no more PorB IgG in sera after immunization with the effective HA-VRP + rrPorB boost vaccine than the ineffective control PBS + rrPorB boost vaccine (**Figure 4**). Serum levels of specific PorB IgG were at least 10-fold higher after immunization with the ineffective rrPorB × 3 vaccine (Zhu et al., 2005, and data not shown). Surface-bound antibodies to whole GC also were much higher after the rrPorB × 3 in FP vaccine than after any of the VRP-based vaccines (data not shown). Vaginal levels of PorB-specific IgA and IgG were much higher after immunization with the ineffective rrPorB and OMV vaccines than after the effective VRP-based vaccines (Zhu et al., 2005). The only bactericidal antibody responses were observed after the totally ineffective OMV vaccine. Thus, protection was not correlated with measured antibody responses. Since we did not measure specific IgG2b and IgG3 responses, conclusions about the roles of isotypes in protection were not possible.

Although quantities of serum PorB-specific IgG were the same in the ineffective PBS + rrPorB boost group and the effective HA-VRP + rrPorB boost group (**Figure 4**), there could have been unmeasured qualitative differences that accounted for the observed differences in protection. Responses in **Figure 4** were measured to synthetic peptides; PorB-conformation-specific antibodies were not measured, nor were antibody avidities. Moreover, there was a minimal serum IgG response to PorB peptides after HA-VRP immunization × 2, without the rrPorB boost (520 ng/ml PorB-reactive IgG as compared to 4135 ng/ml after PorB-VRP × 2 without rrrPorB boost, and no detectable response after immunization with PBS × 2, data not shown). Therefore, the rrPorB boost after the HA-VRP regimen could have resulted in a secondary immune response with different antibody characteristics as compared to a primary immunization with rrPorB after immunization with PBS. Reasons for the small serum IgG response to HA-VRP are not understood; no linear amino acid (aa) sequence identities longer than 4 aa were identified between FA1090 PorB, and either HA, the VEE capsid protein, or the VEE surface glycoproteins E1, E2, or E3 (data not shown). Since experiments were not conducted with an empty VRP vector, the observed protection could have had something to do with HA rather than the VRP vector.

We considered that an IFN-γ Th1 response might be the best correlate of protection, since PorB peptide-specific spenocyte IFN-γ responses were significantly greater after the VRP vaccines than after either the rrPorB or OMV vaccines (Zhu et al., 2005). However, the IFN-γ responses measured 4 weeks after the immunizations were completed, and 2 weeks after infection, were identical in the ineffective rrPorB × 3 and the PorB-VRP × 2 + rrPorB boost vaccines (Zhu et al., 2005, Figures 5A,B), suggesting that it might not have been the IFN-γ responses per se, but something

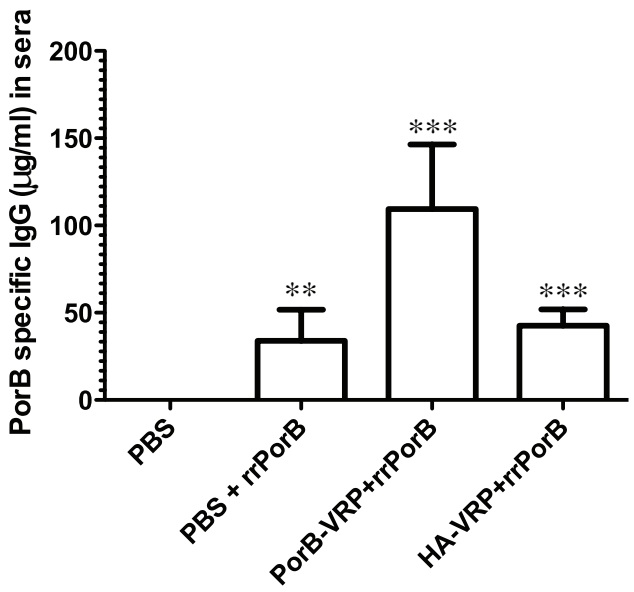

FIGURE 4 | PorB specific IgG levels in sera after different vaccination regimens. BALB/c mice were immunized with HA-VRP, PorB-VRP, or PBS for two times at 2 weeks intervals and then boosted with rrPorB as the last immunization. Another control group was immunized three times with PBS at 2 weeks intervals. Four weeks after the last immunization, sera were collected and IgG responses against a pool of synthetic PorB peptides were measured by quantitative ELISA. Each column represents mean ± SD. Data were analyzed by one-way ANOVA followed by unpaired Student's *t*-test. Symbols: $^{**}p < 0.01$; $^{***}p < 0.001$ as compared to PBS control. PBS + rrPorB is different from PorB-VRP + rrPorB ($p = 0.002$); PorB-VRP + rrPorB is different from HA-VRP + rrPorB ($p = 0.003$); but PBS + rrPorB is not different from HA-VRP + rrPorB ($p = 0.32$).

else triggered after vaccination with the HA-VRP vaccine that was most important. Although IFN-γ responses are important for immune responses to antigens expressed by VRP vectors, they are not important for the adjuvant effect of empty VRPs (Thompson et al., 2008a).

WHAT IS SPECIAL ABOUT VRPs?

The VEE VRP system has been shown to stimulate a strong adjuvant effect, especially when empty VRPs are delivered simultaneously with antigen (Thompson et al., 2006; Tonkin et al., 2010; Carroll et al., 2011). The effect is at least as great as that driven by other adjuvants such as CpG, and depends on VRP RNA replication (Thompson et al., 2006). Systemic and mucosal responses are enhanced, involving both B and T cells; a robust antigen-specific CD8+ T cell response is generated (Thompson et al., 2008b). The effect does not depend on delivery of VRP into the mouse FP, but is seen after IM injection as well (Tonkin et al., 2010). After VRP delivery, dendritic cells as well as macrophages and NK cells are stimulated to release a variety of cytokines and chemokines, with increased cellularity in involved lymph nodes. The list of cytokines and chemokines is interesting both by what is stimulated, and what is not: IL-12 and IL-17 are not stimulated, but IL-6, G-CSF, GM-CSF, IFN-γ, MIP-2, and MCP-1 are stimulated (Tonkin et al., 2010). We did not try to dissect the various components of innate immune responses after the VRP immunizations, and did not measure specific T cell responses. The purported effects of the VRP vector could have been mediated by a variety of mechanisms, including increased production of defensins or other innate responses, or by conformation or avidity differences in antibodies produced in the different vaccination regimens. Future exploration of the basis of the apparent protective effects of VRP-based immunization as a means to enhance GC vaccines should be a fertile field of study.

FUTURE DIRECTIONS

If there is to be a vaccine for GC, a renewed effort is needed in several areas. First is in the search for correlates of immune protection in humans. It is not obvious how to accomplish this in the absence of acquired immunity in humans. One way forward might be a prospective examination of sexual partners of infected subjects, inquiring as to the immune parameters (especial at mucosal surfaces, including innate responses) in those who, and those who do not, become infected. About two-thirds of exposed women become infected; there may be differences in immune responses at first exposure, or a week after exposure, that would provide leads. Similar studies in male volunteers are indicated as well; about 50% of volunteers exposed to 1×10^5 CFU of strain FA1090 develop urethral infection. There may be differences in early innate responses in those who do, and those who do not, become infected.

The mouse model also offers opportunities, especially as new transgenic mice become available, that express one or several human gene products essential for GC infection. Transgenic mice expressing receptors for Opa proteins including CEACAM1 (Gu et al., 2010), human fH (Ufret-Vincenty et al., 2010), and transferrin (Li et al., 2010) are available already. Humanized mouse models are being generated in multiple laboratories (Zhang et al., 2007; Denton and Garcia, 2009), and with further improvement, may be helpful in assessing human-like immune responses in mice. A mouse model that allowed substantial growth of the inoculum, and longer persistence of infection, would assist in developing correlates of protection in the mouse. Among other parameters, mice can be monitored for the quality of the immune response to experimental vaccines, focusing on generation of cells that are positive for each of IL-2, IFN-γ, and TNF. Substantial experience shows that such T cell responses are correlated with a variety of effective vaccines (Seder et al., 2008).

There are multiple candidate proteins that can be considered for a subunit vaccine, with or without lipid A-detoxified OMVs. These include PorB, which remains attractive even though extensive studies cited above have not been fruitful. An approach aimed specifically at PorB loop domains involved in binding fH, C4bp, and/or host receptor binding to CR3 might yield better results than whole PorB. Such a directed response might block binding to and invasion of host cells, and could be bactericidal either directly, or by blocking fH or C4b binding. One way to do this is being explored by one of us (Ann E. Jerse), utilizing circular loop peptides as the immmunogens, based on evidence that analagous circular Opa peptides are able to elicit potentially protective immune responses (Cole and Jerse, 2009). Intranasal immunization with the GC transferrin-binding proteins TbpA or TbpB, or both, elicited bactericidal immune responses; TbpA stimulated more broadly cross-reactive antibodies than did TbpB

(Price et al., 2005). Immunization of mice with genetic chimeras that fused parts of TbpA and TbpB stimulated production of vaginal antibodies that inhibited growth *in vitro* (Price et al., 2007). A novel adhesin designated OmpA is yet another candidate (Serino et al., 2007).

Extensive studies over the past 15 years have shown the potential of vaccines directed at a conserved LOS epitope, which is defined by binding the mAb 2C7 (Gulati et al., 1996). About 95% of GC strains express the 2C7 epitope, composed principally of a lactose residue attached to heptose-2 on GC LOS (Yamasaki et al., 1999). Synthetic peptides that mimic the 2C7 epitope are immunogenic, and result in bactericidal activity, even against strains that are resistant to killing by normal human serum (Ngampasutadol et al., 2006).

There may be other effective immunogens. Perhaps improved mouse models will permit a discovery approach that utilizes pools of antigens, delivered as DNA constructs. Priming with VRP-based vaccines, or a variety of other novel adjuvants (Gwinn et al., 2010; Pulendran et al., 2010), may enable more effective immune responses. The pace of discovery in mucosal immunology is rapid; efforts to develop more effective genital immune responses, including nasal immunization strategies (Gwinn et al., 2010), may pay dividends. Progress will require a substantial commitment of effort and resources. It is not too early to renew the effort.

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The pathobiology of *Neisseria gonorrhoeae* lower female genital tract infection

Jennifer L. Edwards[1,2]* and Emily K. Butler[1]

[1] *The Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, The Ohio State University, Columbus, OH, USA*
[2] *The Department of Pediatrics, The Ohio State University, Columbus, OH, USA*

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Margaret E. Bauer, Indiana University School of Medicine, USA
Daoguo Zhou, Purdue University, USA

****Correspondence:***
Jennifer L. Edwards, The Research Institute at Nationwide Children's Hospital, 700 Children's Drive, W503, Columbus, OH 43205, USA.
e-mail: edwardsj@ccri.net

Infection and disease associated with *Neisseria gonorrhoeae*, the gonococcus, continue to be a global health problem. Asymptomatic and subclinical gonococcal infections occur at a high frequency in females; thus, the true incidence of *N. gonorrhoeae* infections are presumed to be severely underestimated. Inherent to this asymptomatic/subclinical diseased state is the continued prevalence of this organism within the general population, as well as the medical, economic, and social burden equated with the observed chronic, disease sequelae. As infections of the lower female genital tract (i.e., the uterine cervix) commonly result in subclinical disease, it follows that the pathobiology of cervical gonorrhea would differ from that observed for other sites of infection. In this regard, the potential responses to infection that are generated by the female reproductive tract mucosa are unique in that they are governed, in part, by cyclic fluctuations in steroid hormone levels. The lower female genital tract has the further distinction of being able to functionally discriminate between resident commensal microbiota and transient pathogens. The expression of functionally active complement receptor 3 by the lower, but not the upper, female genital tract mucosa; together with data indicating that gonococcal adherence to and invasion of primary cervical epithelial cells and tissue are predominately aided by this surface-expressed host molecule; provide one explanation for asymptomatic/subclinical gonococcal cervicitis. However, co-evolution of the gonococcus with its sole human host has endowed this organism with variable survival strategies that not only aid these bacteria in successfully evasion of immune detection and function but also enhance cervical colonization and cellular invasion. To this end, we herein summarize current knowledge pertaining to the pathobiology of gonococcal infection of the human cervix.

Keywords: *Neisseria gonorrhoeae*, complement receptor 3, phospholipase, Akt, nitric oxide, uterine cervix, bacterial adherence and invasion, integrin signaling

INTRODUCTION

Health complications resulting from *Neisseria gonorrhoeae* (the gonococcus) disease occur mainly in women and are largely attributed to the predominately asymptomatic nature of lower genital tract, i.e., cervical, infection. Untreated, subclinical infection of the cervix can lead to upper genital tract involvement (e.g., salpingitis) and, potentially, to infertility. Consistent with the different clinical manifestations of disease observed between males (mostly acutely symptomatic) and females, the gonococcus uses variable mechanisms of pathogenesis that are dependent upon the host target cell, the specific microenvironment encountered within its (sole) human host, as well as strain-specific differences prevalent among *N. gonorrhoeae* strains. These include a repertoire of mechanisms to evade the host immune response and antimicrobial agents, to detoxify reactive oxidants, and to acquire iron during residence of the human host.

The gonococcus predominately infects and colonizes the mucosal epithelium of the human urogenital tract. Although gonococcal vaginitis develops in female children in which menarche has not yet occurred, keratinization occurring with menarche prevents gonococcal vaginitis in the adult female. Thus, after the onset of menarche, the clinical presentation of gonococcal disease is not varied. Gonococcal cervicitis results from urogenital gonococcal infection in females. Historically, conflicting models of gonococcal cervicitis have existed. Recent years have brought a resurgent interest in elucidating the molecular/cellular mechanisms contributing to cervical infection and its colonization, as well as in new technologies and model systems by which to examine many unanswered questions. It is now appreciated that not only are both the ecto- and the endocervix permissive for gonococcal infection/colonization but also that the gonococcus can no longer be considered a strictly extracellular pathogen (Evans, 1977; Edwards et al., 2001).

Although various models are commonly used to study *N. gonorrhoeae* disease, this pathobiology cannot be completely mirrored using any single model system. Each model is limited in its utility when attempting to extrapolate these data to infection and disease *in vivo*. For example: (1) Human-specific constituents implicated in gonococcal pathogenesis are lacking in animal models; (2) molecular and cellular expression patterns, as well as functional responses, become altered during the immortalization/malignancy of cells lines; (3) primary cells and organ culture do not incorporate exogenous host factors; and (4) only a fixed, often undefined, window or duration of infection may be represented by clinical (naturally acquired/infected human tissues and fluids) specimens. Further,

although studies have been performed using human volunteers, the asymptomatic nature of gonococcal cervicitis together with the risk for chronic disease sequelae in women, ethically restricts studies to males, and these investigations are limited to only those processes occurring early during the infection process as antibiotic therapy is given immediately with the onset of disease symptoms.

The present manuscript is not a global review of gonococcal pathogenesis; rather, data derived from the use of cervical cell models are reviewed in an effort to highlight those events potentially contributing to cervical infection and disease *in vivo*. Several comprehensive reviews of *N. gonorrhoeae* pathogenesis are published (Woods and McGee, 1986; Ram et al., 1999; Dehio et al., 2000; Kline et al., 2003; Edwards and Apicella, 2004; Hamilton and Dillard, 2006; Seib et al., 2006; Steichen et al., 2008; Virji, 2009; Sadarangani et al., 2010; Srikhanta et al., 2010). The reader is also directed to the accompanying papers comprising this current, *N. gonorrhoeae*-focused, volume of *Frontiers in Cellular and Immunity Microbiology* for more information.

SURFACE STRUCTURES MEDIATING ADHERENCE

Included among the better-studied neisserial adhesins are: (1) porin, the major outer membrane protein; (2) the opacity-associated (Opa) proteins; proteins represented by Opa_{50} adhere to heparin sulfate proteoglycans (HSPGs); whereas, proteins represented by Opa_{52} bind specific carcinoembryonic antigen-related cell adhesion molecules (CEACAMs); (3) lipooligosaccharide (LOS), a major glycolipid of the gonococcus outer membrane, which lacks the repeating O-antigen of lipopolysaccharide; and (4) type IV-A pili, long polymers of pilin proteins that extend from the gonococcus surface. Multiple gonococcal surface molecules, functioning independently or concertedly, participate in the varied mechanisms currently described as mediating adherence to human (cervical) epithelial cells. Additionally, an impressive number of gonococcal constituents randomly undergo phase (high frequency on/off expression) and/or antigenic (high frequency epitope switching) variation, providing great elasticity in the repertoire of surface antigens that are presented *in situ* during the course of gonococcal disease (Apicella et al., 1987; Schneider et al., 1991; Jerse et al., 1994; Seifert et al., 1994).

The importance of the above phenomena to *in vivo* infection is only beginning to be fully appreciated (Kline et al., 2003; Srikhanta et al., 2010; Jennings et al., 2011). Redundant adherence mechanisms have the potential to increase the overall success of the gonococcus in establishing disease, but there is greater evidence to support host–gonococcus interactions as discriminate cell-, site-, and gender-specific adhesion events (reviewed in Edwards and Apicella, 2004). Hence, it is likely that not all available adhesion mechanisms are actually used by this organism during the course of infection/colonization of any specific site. For example, whereas pilus-mediated adherence is critical to a gonococcus–epithelial cell interaction (Swanson, 1977), the Opa proteins appear to dictate adherence to neutrophils and other cells comprising the immune system (Sadarangani et al., 2010). Likewise, an interaction occurring between gonococcal ribosomal protein, L12, and the lutropin receptor (LHr; Spence et al., 1997, 2002) would be confined to the upper female genital tract because of its *in vivo* tissue distribution (Reshef et al., 1990). Additional/alternative surface constituents certainly contribute to adherence, colonization, and the pathogenesis of these highly human adapted bacterial pathogens *in vivo*, although their role in mediating a diseased state is less-well defined or has yet to be defined.

INFECTION IN MALES AND TRANSMISSION TO A FEMALE PARTNER

Neisseria gonorrhoeae infection in men presents as an acute urethritis in which epithelial cells lining the urethra produce pro-inflammatory mediators; e.g., TNF-α, IL-6, IL-8, and IL-1β (Ramsey et al., 1995; Harvey et al., 2002); in response to infection. Neutrophil chemotaxis to the site of infection perpetuates this inflammatory state as these cells generate additional pro-inflammatory mediators upon attempting to clear infection. It is this neutrophil influx, together with the shedding of damaged urethral epithelial cells, that accounts for the purulent urethral discharge, which is characteristic of gonococcal disease in men.

Using primary male urethral epithelial cells, urethral tissue, and clinical urethral exudates it is shown that urethral infection is likely established by a step-wise process involving two separate host cell surface molecules, i.e., I-domain-containing β_1-integrins (Edwards and Apicella, 2005) and the asialoglycoprotein receptor (ASGP-R; Harvey et al., 2001b). Adherence to the urethral mucosa is initially mediated by pilus binding to the I-domain region of $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrins. The gonococcus-bearing integrin then forms a transient interaction with the ASGP-R (Edwards and Apicella, 2005). This results in a tight association between the bacterial and urethral cell membranes (Harvey et al., 2001b), and, presumably, aids host cell invasion by poising the bacterium in such a way to enable binding of the terminal galactose of LOS to the ASGP-R and/or by refining the host cell signaling events required for bacterial uptake. A host-derived membrane pedestal is formed beneath the bound gonococcus (Apicella et al., 1996; Harvey et al., 1997). Bacteria are then internalized by an actin- (Giardina et al., 1998) and clathrin-dependent (Harvey et al., 1997) process. Although the intracellular fate of the gonococcus remains ill-defined, available data support gonococci-containing vacuoles as entering the endosomal recycling pathway as dictated by the ASGP-R (Harvey et al., 2001b).

Neisseria gonorrhoeae are observed intracellularly within the shed urethral epithelial cells and in the neutrophils comprising clinical exudates. Whereas Opa proteins are not required for an interaction with the urethral epithelium, they do play a role in the interaction of gonococci with (resident and recruited) neutrophils (Rest et al., 1982). Opaque gonococci are selected during male urethral infection (Schneider et al., 1995; Schmidt et al., 2000), as are those gonococci that specifically harbor a terminal lacto-*N*-neotetraose (LNnT) moiety on their LOS (Apicella et al., 1987; Schneider et al., 1991). LNnT mimics the paragloboside moiety prevalent on human cells (Harvey et al., 2001a). This form of molecular mimicry presumptively provides one means by which gonococci escape immune detection as (1) analysis of *N. gonorrhoeae* strains demonstrates the predominance of the LNnT epitope among gonococci (Campagnari et al., 1990; John et al., 1999) and (2) the LNnT epitope is selected in men during human volunteer studies as well as with naturally acquired gonococcal urethritis (Schneider et al., 1991, 1988).

The LNnT moiety terminates in a galactose residue that is capable of being sialylated by a sialytransferase present within the gonococcal outer membrane (Shell et al., 2002). LOS sialylation provides

another level of immune avoidance as well as playing a further role in the deactivation of some innate immune effectors (de la Paz et al., 1995; Gulati et al., 2005; Wu and Jerse, 2006). Therefore, it is not surprising that gonococci isolated from male urethral exudates do display sialic acid on their LOS (Apicella, et al., 1990; Parsons et al., 1992). However, LOS sialylation also impairs binding to the ASGP-R (Harvey et al., 2001b), the invasion of epithelial cell lines (van Putten, 1993; van Putten and Robertson, 1995), the ability to cause disease in human volunteers (Schneider et al., 1996), and the uptake of gonococci by neutrophils (Kim et al., 1992; Rest and Frangipane, 1992). Thus, if sialylation reduces gonococcal infectivity of the male urethra, how then is diseased established in men during the first days following exposure? This apparent conundrum is probably best resolved in view of disease progression and transmission.

Lacking the ability to synthesize cytosine 5′-monophosphate *N*-acetylneuraminic acid (CMP-NANA), the gonococcus must parasitize this sialic acid precursor from its human host. Gonococcal infectivity is restored upon sialic acid removal from LOS by the actions of local neuraminidase or by the replication of gonococci within the lumen of the urethra, an environment lacking host-derived CMP-NANA. Neuraminidase and the ASGP-R are present on human sperm as are identified [CD46 (Källström et al., 1997), I-domain-containing β1 integrins (Edwards and Apicella, 2005)] and potentially unidentified (Kirchner et al., 2005) receptors for pili.

Clinically isolated gonococci bear a predominance of pili on their surface (Seifert et al., 1994). Although piliated gonococci bind more readily to human sperm than do non-piliated bacteria (James-Holmquest et al., 1974), the role of any specific pili receptor in mediating an interaction with sperm has not been examined. Nevertheless, any receptor–pilus interaction would likely anchor gonococci in proximity to neuraminidase such that they are capable of becoming desialylated. Following the removal of sialic acid, gonococcal adherence to the spermatozoa-associated ASGP-R would be anticipated (Harvey et al., 2000), which in turn, facilitates disease transmission with copulation.

Neuraminidases are also associated with neutrophil and macrophage cells, and gonococci exhibit resistance to the cytotoxic activity of these immune cells (Shafer and Rest, 1989; Seib et al., 2005; Criss et al., 2009). In this scenario, desialylation of gonococci juxtaposed to neuraminidase could contribute to disease progression in that viable bacteria released from the hostile intracellular environment of the phagocyte would once again be free to invade the urethral epithelium via the ASGP-R. In this way, a diseased state would perpetuate. In summary, gonococci transmitted to the sexual partner of an infected male are likely opaque, piliated (Seifert et al., 1994), and may or may not bear a sialylated LOS. However, whether gonococci are sialylated does not appear to influence colonization of the lower female genital tract (Edwards and Apicella, 2002), but neuraminidases present within the female genital tract may prime the gonococcus for transmission and adherence to the urethral epithelium of a male partner.

THE CERVICAL MICROENVIRONMENT

The female genital tract presents a number of physical and immunological barriers to deter potential pathogens while simultaneously maintaining a diverse and abundant resident microflora. In this regard, the cervix plays a pivotal role in maintaining the health of the upper female genital tract (and, potentially, a developing fetus), and a healthy normal flora is associated with a decreased risk for sexually transmitted infections. *Gardnerella vaginalis* and *Lactobacilli sp.* are the most common residents of the female reproductive tract with *L. iners and L. gasseri* being the most predominant *Lactobacilli* species (Nikolaitchouk et al., 2008). Several studies suggest that *Lactobacilli* can inhibit an association with host cells and/or impair the *in vitro* growth of pathogenic organisms (Skarin and Sylwan, 1986; Klebanoff et al., 1991). However, there is also evidence to indicate that data obtained from *in vitro*-based assays may not accurately project how *Lactobacilli* affect the fate of a potential pathogen during the course of infection (Meunch et al., 2009; O'Hanlon et al., 2010). We are only beginning to understand how resident microbiota maintain a healthy mucosal environment within the lower female genital tract. Nevertheless, with respect to *Lactobacilli,* the production (by select strains) of lactic acid, bacteriocins, and hydrogen peroxide each likely contribute to a physiologically balanced status, but their effect on preventing or prohibiting infections requires further study.

N. GONORRHOEAE ASSOCIATION WITH CERVICAL EPITHELIA

The predominance of iC3b, the inactivated cleavage product of complement protein C3b, on the surface of clinically isolated gonococci suggests a role for the alternative complement pathway in modulating disease in women (Jarvis, 1994; McQuillen et al., 1999; Vogel and Frosch, 1999). CR3, the $\alpha_m\beta_2$ (or CD11b/CD18) integrin, is highly expressed on the human cervix (Edwards et al., 2001). iC3b is the primary natural ligand of CR3 and binds to the I-domain region of CD11b. Gonococcal infection studies using primary human cervical epithelial (pex) cells and quantitative analyses of clinical biopsies obtained from women with culture-documented cervical gonorrhea demonstrate that greater than 92% of gonococci co-localize with CR3 *in vivo* (Edwards et al., 2001). This implicates CR3 as the elemental receptor promoting infection of the cervix. Adherence to the cervical epithelium occurs in a sequential manner in which both host and gonococcal constituents cooperate (Edwards et al., 2002) (**Figure 1**). Pilus binding to the CR3 I-domain places the bacterium in proximity to the cervical cell surface where complement proteins (produced by the cervix) are in sufficient concentrations to allow efficient C3b/iC3b opsonization to occur, which is essential to the infection process (Edwards et al., 2002). However, pilus binding to the CR3 I-domain also plays an additional role in tempering the activation state of CR3 on the host cell surface (Jennings et al., 2011) that in turn could modulate the downstream cervical cell signaling events triggered with CR3 engagement.

Gonococcal pilus is covalently modified with an O-linked galactose (α1–3)-2,4-diacetimido-2,4,6-trideoxyhexose (Gal-DATDH) disaccharide (Hegge et al., 2004). As the (*pgl)* genes involved in the biosynthesis and presentation of this glycan are under phase-variable control, *in vivo* the mature gonococcal pilin possesses either a mono- (DATDH) or a disaccharide (Gal-DATDH). Integrins (e.g., CR3) rapidly oscillate in a dynamic equilibrium between active and inactive conformational states in which the inactive conformation is prevalent under normal physiological conditions. High-affinity receptor function is obtained upon ligand binding. We recently show that the pilin glycan is required to bind to the CR3 I-domain when it is in a closed, low-affinity, conformation, and to confer an

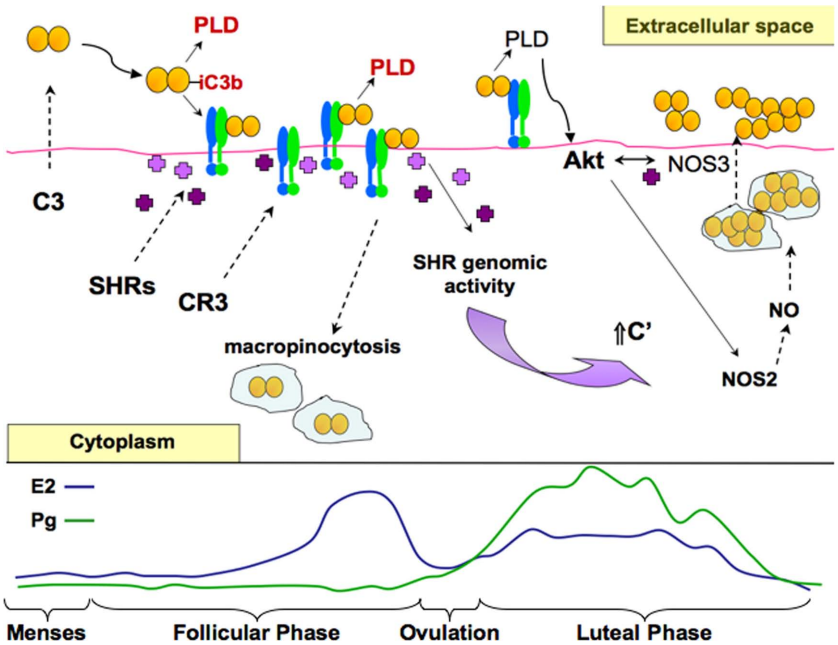

FIGURE 1 | A working model of cervical infection by gonococci. Based on published, as well as unpublished data, a putative, working model of the dynamic interactions mediating gonococcal disease of cervical epithelia is presented in context of the menstrual cycle. Estrogen (E2), predominant in the follicular phase is depicted by the blue line. Progesterone (Pg) is predominant in the luteal phase following ovulation (occurring around day 14) and is depicted in green. Menses occurs when both E2 and Pg levels are low. The actions of E2 and Pg are mediated by their cognate steroid hormone receptors (SHRs, shown as purple crosses). Upon stimulation, SHRs function as transcription factors mediating the expression of select cellular products (e.g., complement (C') protein C3) or as signaling mediators (e.g., in the Akt-dependent induction of nitric oxide, NO). Cervical epithelial cells produce and secret all of the proteins comprising the alternative pathway of the C' system. During the luteal phase and during menses, C3 is release to the extracellular milieu where it is activated to form C3b upon gonococcal infection. C3b binds to the gonococcus surface where it is rapidly cleaved to form inactive, iC3b, a necessary ligand for complement receptor 3 (CR3)-mediated cervical cell invasion. Deposition of C3b upon the gonococcus surface in conjunction with CR3 engagement promote the sustained release of secreted gonococcal products, including a phospholipase D (PLD) that aid colonization, invasion, and survival of gonococci during their residence within the lower female genital tract. As estrogen concentrations begin to rise, CR3 presence on the surface of pex cells is increased. More CR3 available to bind iC3b-opsonized gonococci correlates with the increased association of gonococci with pex cells. Engagement of CR3 triggers the recruitment of SHRs to the cytoplasmic domain of CR3 where they likely participate in the cervical cell signaling pathways triggered with gonococcal infection. The direct interaction of (Ng)PLD with (host) Akt, triggers ruffle formation and the internalization of gonococci within macropinosomes. Levels of CR3 on the surface of both uninfected and infected pex cells decrease with increasing E2. This might indicate that the internalization of gonococci is augmented by E2 in the mid-follicular phase. Gonococcus-containing vacuoles coalesce near the apical surface within pex cells, and CR3 is disengaged. Protein secretion by intracellular gonococci residing within the macropinosome under conditions of low Pg is likely down-regulated as a consequence of rapid C3 depletion. However, as progesterone levels begin to rise, C3 levels also would again increase because of transcription factor activity exerted upon the C3 gene by SHRs. It is then expected that Akt activity would be increasingly maintained as more PLD is released by gonococci in the presence of increased C3(b). The ability of Pg to promote the intracellular survival of gonococci occurs in part by augmenting Akt activity, which triggers nitric oxide (NO) production. NO supports (micro)aerobic respiration by intracellular (and extracellular) gonococci. Late in the luteal phase, Akt activity decreases with progressively decreasing Pg. Decreasing Akt, which also plays a role in cellular trafficking, in conjunction with other host and bacterial constituents, may modulate the release of C3 and gonococci from pex cells to the extracellular cell surface where ever increasing numbers of bacteria colonize the cell surface, eventually leading to the formation of a biofilm.

active state to CR3 (Jennings et al., 2011). We additionally show that mutant bacteria bearing only a monosaccharide, or no glycan, on their pili were dramatically impaired in their ability to invade and/or to survive within pex cells. Although these data are highly supportive of a role for the pilin glycan in providing a survival advantage to (pilin-)glycosylated gonococci during cervical infection, further analyses are required to determine if CR3 engagement by pilin glycan-deficient gonococci results in a host cell response that is substantively different from that observed for wildtype gonococci (capable of phase-variable pilin glycosylation). The activity state of CR3 is linked to its phagocytic function as well as to the cellular toxicity of those cells on which this receptor is expressed. Therefore, it is not unreasonable to speculate a role for CR3 in mediating the inflammatory response to infection and to propose that pilus engagement of the CR3 I-domain tempers this response.

iC3b and porin serve as secondary adhesins, also binding to the CR3 I-domain. In this way, a tight gonococcus–receptor interaction is likely achieved. Several studies demonstrate that iC3b-mediated adherence to the I-domain of CR3 does not result in a pro-inflammatory response in professional phagocytic cells. Consistent with data discussed above, we have proposed that the cooperative binding of pilus, iC3b, and porin to this region of CR3 may contribute to the asymptomatic nature of gonococcal cervicitis. Further support for this idea is that: (1) CR3 is not expressed by the urethral epithelium in men in which gonococcal infection is profusely symptomatic, (2)

the expression of CR3 decreases progressively from the ectocervix (very high expression, asymptomatic disease predominant) to the fallopian tubes (very low/limited expression, symptomatic disease predominant; Edwards et al., 2001),(3) infection of the anorectal mucosa, which potentially expresses CR3 protein (Hussain et al., 1995), also results in predominately asymptomatic disease, and (4) immortalization of primary cervical epithelial cells results in the loss of CR3 expression and the production of pro-inflammatory mediators in response to gonococcal infection (Fichorova et al., 2001). In contrast, the production of pro-inflammatory cytokines by pex cells is down-regulated during gonococcal challenge.

ALTERNATIVE MECHANISMS OF ADHERENCE AND INVASION

Data obtained from the use of established epithelial cell lines (which do not express CR3; Edwards et al., 2001), suggest that alternative mechanisms of adherence/invasion might occur during the course of gonococcal cervicitis or might promote progressive disease. Mechanisms involving LOS-, Opa-, and porin-mediated adherence and/or invasion are described for various cell lines.

As noted, adherence to primary male urethral epithelial cells is mediated by the concerted actions of pilus binding to I-domain-containing β1-integrins and of LOS adhesion to the ASGP-R (Harvey et al., 2001b). Although the ASGP-R is present within the female genital tract, it does not appear to mediate gonococcal infection of pex cells. However, the co-localization of gonococci with the ASGP-R on endometrial epithelium (Timmerman et al., 2005) could indicate that the ASGP-R plays a role in ascending infection in women. Challenge of ME-180 (omentum metastatic cervical epidermoid carcinoma) cells with a panel of LOS truncation mutants show adherence and invasion comparable to that of the wildtype strain (Minor et al., 2000). One exception was that the adherence and invasion of a *lgt*F mutant was significantly impaired. This suggests that under some conditions LOS might promote cervical infection.

The LOS core region is comprised of two L-glycero-D-manno-heptopyranose (heptose, Hep) and two 2-keto-3-deoxyoctulosonic acid (KDO) residues. The heptose molecules serve as docking sites for short (6–10 sugar moieties) oligosaccharide addition(s). During the biosynthesis of the LOS oligosaccharide, a single glucose is added to the Hep-KDO core structure. LgtF then functions to add a galactose (Gal) to this basal glucose (Glc) residue to yield a Galβ1-4Glc1-4Hep1-5KDO. A *lgt*F mutant can then be described as harboring a single glucose addition on the Hep-KDO core structure. Given that adherence to the ASGP-R is dependent upon galactose recognition, an interaction occurring between a host cell and a *lgt*F mutant would not be expected to involve the ASGP-R. Further, parallel studies performed using Opa-deficient gonococci showed a reduction in adherence and invasion for all of the mutant strains studied, indicating an Opa-dependence (under the conditions assayed) in the gonococcus-ME-180 cell association (Minor et al., 2000); whereas, the LOS–ASGP-R interaction (as described) occurs independently of Opa proteins.

Data presented by Virji et al. (1996) suggest that greater than 90% of clinical isolates are able to bind to CEACAM1, suggesting a role for Opa in modulating infection, *in vivo*. However, no mention of the anatomical site of isolation (e.g., the male urethra or the female cervix) for these bacteria is presented. Opa-expressing gonococci also predominate in isolates obtained from a mouse model of gonococcal infection. In that the structural differences observed between human and murine CEACAM1 likely prohibit an Opa-murine CEACAM1 interaction, alternative factors presumptively select for an Opa+ phenotype during gonococcal challenge (Simms and Jerse, 2006). In this regard, a correlation is made with the presence/absence of Opa proteins on clinically isolated *N. gonorrhoeae* and the site of isolation (Morse and Brooks, 1985). Isolates obtained from men tend to express Opa proteins (Jerse et al., 1994) as do cervical isolates obtained from women at the time of ovulation (i.e., midcycle). Opa− organisms predominate in asymptomatic men; in cervical isolates obtained during menses; in the fallopian tube; and in genital, blood, and joint fluid obtained from patients with disseminated gonococcal infection. These data have led to the suggestion that Opa− organisms are typically associated with asymptomatic, invasive, disease, whereas Opa+ gonococci are usually associated with symptomatic disease and rarely with complicated, invasive, disease (Morse and Brooks, 1985). Opa proteins mediate the interaction of gonococci with neutrophils (Virji and Heckels, 1986; Naids et al., 1991). Therefore, the predominance of Opa-expressing gonococci in isolates obtained from mice, men, and the (human) cervix at the time of ovulation may be related to an increased presence of neutrophils in proximity to gonococci. Neutrophil influx accompanies gonococcal disease in mice and in men, and immune cells normally (in the absence of infection) infiltrate vaginal tissue and secretions during (human) ovulation.

Although Opa proteins do not mediate the association of gonococci with primary epithelial cells (Harvey et al., 2001b; Swanson et al., 2001; Edwards et al., 2002), several groups demonstrate a role for Opa proteins in mediating gonococcal adherence and invasion of immortalized and/or transfected epithelial cell lines. Opa-mediated adherence can occur directly or indirectly. Bessen and Gotschlich (1986) show that the majority of gonococci tightly associated with HeLa cervical adenocarcinoma cells express Opa and, further, that continued adherence occurs independently of pili. The predominance of OpaA-expressing gonococci in this cell-associated population suggests that this interaction was mediated by HSPGs. Opa-mediated adherence to HSPGs is generally dependent upon vitronectin or fibronectin that function as bridging molecules mediating adherence with an integrin ($\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_v\beta_1$) co-receptor (Duensing and van Putten, 1997; Gómez-Duarte et al., 1997; van Putten et al., 1998b). A direct Opa–HSPG interaction is also demonstrated in HeLa and Chang, immortal conjunctiva epithelial cells (Grassmé et al., 1997). Similarly, the Opa–CEACAM interaction occurs as a direct protein–protein interaction (Virji et al., 1996, 1999; Popp et al., 1999).

An Opa–CEACAM interaction is also shown to mediate the association of gonococci with professional phagocytic cells (Virji and Heckels, 1986; Naids et al., 1991), B- and T-cells (Boulton and Gray-Owen, 2002; Pantelic et al., 2005), as well as endothelial cells (Muenzner et al., 2000). However, as aberrant expression of CEACAMs is frequently reported for immortal epithelial cell lines, it is currently not clear if an *epithelial* CEACAM–gonococcus interaction occurs during the course of *in vivo* gonococcal cervicitis. Invasion of pex cells is increased when challenged with Opa- mutant gonococci or in the presence of anti-CEACAM antibody (Edwards et al., 2002). These data might indicate that, although the majority of

bacteria associate and invade pex cells via CR3, a small subpopulation of gonococci potentially invade via an Opa–CEACAM interaction, which does not support their intracellular survival. Consistent with this idea is that CEACAM-mediated invasion of transfected HeLa cells results in the acidification of gonococci-containing vacuoles and the death of intracellular gonococci (Booth et al., 2003; McCaw et al., 2004). Co-localization of intracellular gonococci with CEACAMs (as well as CD46) is observed with extended infection of polarized pex cells (Edwards et al., 2000); perhaps suggesting a role for these molecules in the intracellular trafficking of gonococci or in promoting disease by an as yet undefined mechanism(s). The expression of CEACAMs on the mucosal surface of the female genital tract increases from the cervix to the fallopian tubes. Gonococci co-localize with CEACAMs on the luminal face of endometrial tissue (Timmerman et al., 2005). These data hint at a role for a CEACAM–gonococcus interaction in augment ascending infection in women; however, further studies to elucidate how ascending infection may progress in women are needed.

Porin of either a P.IA or a P.IB isotype can bind to the I-domain of CR3. However, under low-phosphate conditions, porin of a P.IA isotype mediates entry into HeLa and Chang cells (van Putten et al., 1998a; Kuhlewein et al., 2006). Rechner et al. (2007) have revealed that the heat shock protein, Gp96, functions in conjunction with the scavenger receptor, SREC, to promote invasive low-phosphate-dependent infection (LPDI). The significance of LPDI to *in vivo* gonococcal cervicitis is unclear. However, Kuhlewein et al. (2006) annunciate that LPDI of host cells may promote disseminated disease because: (1) LPDI is augmented by a heat-labile component of human serum, (2) P.IA-expressing gonococci are more frequently isolated from patients with disseminated gonococcal infection (Cannon et al., 1983), and (3) the physiological levels of phosphate in serum are conducive for such an interaction to occur.

INVASION OF CERVICAL EPITHELIA

In most immortalized cell lines, gonococcal adherence results in an intimate association between the host and bacterial cell membranes. In contrast, the gonococcus–CR3 interaction on pex cells triggers ruffling. Rho GTPases are activated with CR3 engagement (Edwards et al., 2001). Within minutes the actin-associated proteins, vinculin, and ezrin, form localized focal complexes beneath adherent gonococci (Edwards et al., 2000). Although the signaling pathway is not yet fully defined, the result is extensive rearrangement of the actin cytoskeleton promoting large protrusions of the cervical cell membrane, termed ruffles, which loosely engulf adherent gonococci (Edwards et al., 2000). Gonococci are internalized within macropinosome, i.e., large, spacious, vacuoles, which remain associated with actin and coalesce, without apparent fusion, at the apical surface within the cervical cell cytoplasm. Microscopy analyses of clinical cervical biopsies also demonstrate gonococci engulfed in membrane protrusions reflective of ruffles and within clustered, actin-lined, spacious, vacuoles. Gonococcus-induced ruffling is unique from that described for other pathogenic organisms in that it is specifically triggered by CR3 engagement (Edwards et al., 2001), and gonococci do not possess a type three-secretion system.

CR3 expression appears to be limited to primary cervical epithelia; it is not present on the immortal cell lines examined to date. Thereby, membrane ruffling and macropinocytosis do not mediate invasion of immortalized cell lines, although filopodia and lamellipodia are observed. Thus, among the various described mechanisms of gonococcal invasion, a requirement for actin-mediated cytoskeletal rearrangement appears to be a common factor. Differences exist in the events described as inducing actin rearrangement as well as in the kinetics in which actin is associated with gonococci or gonococcus-containing vacuoles. For example, there are data to indicate that pili, Opa, and/or porin are all capable of initiating the host cell signaling events mediating actin rearrangement. Similarly, actin involvement is transient in some cell lines, whereas in others it is more sustained. Collectively, these conflicting data are reflective of the variable mechanisms used by the gonococcus to invade human cells in conjunction with the highly variable nature of *N. gonorrhoeae*, even for a single gonococcal strain. In this regard, *in vitro* elucidation of the host cell signaling effectors mediating gonococcal invasion, *in vivo*, is extremely problematic and is further complicated when considering the continuum of variable steroid hormone concentrations that occur throughout the female menstrual cycle.

SIGNALING EVENTS AND INTRACELLULAR TRAFFICKING

Signaling events triggered upon CR3 engagement on pex cells are not fully defined; however, secreted gonococcal products, including a phospholipase D (NgPLD) homolog (Edwards et al., 2003; Edwards and Apicella, 2006), play a role in these events. NgPLD is present in every sequenced *N. gonorrhoeae* strain to-date (The Broad Institute, http://www.broadinstitute.org/annotation/genome/neisseria_gonorrhoeae/MultiHome.html). *N. meningitidis* and *N. lactima* also encode NgPLD; however, the *pld* homolog present in the genome of the commensal organism, *N. lactima*, is N-terminally truncated and lacks the predicted Sec signal peptide. Thus, NgPLD activity is presumably relegated to pathogenic *Neisseria sp.*

NgPLD appears to gain access to the cervical intracellular environment because NgPLD activity is observed in cervical cell cytosolic fractions, this activity is absent when pex cells are challenged with a *pld* mutant strain, and NgPLD co-immunoprecipitates with cervical cytosolic proteins in pull-down assays. Several bacterial and viral pathogens have developed mechanism to trigger Akt kinase (also known as protein kinase B) signaling pathways. The ability of Akt to inhibit apoptosis as well as to regulate the cell cycle, gene transcription, glucose/nutrient uptake and metabolism, and endocytosis make this kinase an attractive target by which pathogens could subvert normal host cell function. Further, Akt together with phosphatidylinositol phosphates (PtdIns P) play a critical role in regulating the actin cytoskeleton and membrane trafficking. Upon cell stimulation local levels of PtdIns(4,5)P_2 increase and are enriched at sites of bacterial contact as well as in large plasma membrane structures, e.g., lamellipodia and ruffles, which is of particular relevance to gonococcal pathobiology. In general, Akt activation is dependent upon the role of phosphatidylinositol 3-kinase (PI3-K) in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 formation. PI3-K-independent mechanisms of Akt activation, albeit limited, are also reported (Konishi et al., 1997; Moule et al., 1997; Sable et al., 1997; Filippa et al., 1999; Cenni et al., 2003; Perez-Garcia et al., 2004). Our data strongly suggest that *N. gonorrhoeae* subvert cervical cell signaling pathways by competing with PtdIns(3,4,5)P_3 (and possibly other phosphoinositides) for Akt binding (Edwards and

Apicella, 2006). We show NgPLD directly interacts with Akt kinase in a [presumptive, PtdIns(3,4)P_2-preserving] PI3-K-*independent* manner to promote membrane ruffling, CR3 recruitment to the cervical cell surface, and the survival of gonococci within pex cells (Edwards and Apicella, 2006). However, a similar study performed using A431 cells, a vulvular epidermal carcinoma cell line, suggest that the pilus retraction motor, PilT, triggers Akt activation in a PI3-K-*dependent* manner that results in gonococcus microcolony formation (Lee et al., 2005). Both mechanisms of Akt activation may occur *in vivo* and may differentially augment gonococcus infection during the course of disease.

One consequence of the Akt pathway is the production of nitric oxide (NO) through the actions of nitric oxide synthases (i.e., NOS). In this regard, it is noteworthy that progestin-based contraceptives are shown to increase the susceptibility of a woman to develop gonococcal disease (Louv et al., 1989; Fernandez et al., 2001; Morrison et al., 2004). Gonococci are able to use nitrite and/or nitric oxide (i.e., NOX, collectively) in an alternative respiratory mechanism under microaerobic–hypoxic conditions (Knapp and Clark, 1984). Although clinical data indicate that hormone-induced, cyclic changes occurring to the mucosal epithelium of the female genital tract modulate gonococcal disease; until recently how steroid hormones influence cervical gonorrhea has remained poorly defined. NOX are present in cervical secretions (Väisänen-Tommiska et al., 2003), which are capable of supporting *N. gonorrhoeae* growth. Overton et al. (2006) have proposed that host-initiated nitric oxide induction of *N. gonorrhoeae* nitric oxide reductase, NorB, could confer a survival advantage to these bacteria *in vivo* by serving as an energy source under conditions of oxygen limitation. Although NO is generally considered to exert a cytotoxic effect upon bacterial cells, consistent with this hypothesis, we show progesterone promotes gonococcal survival during pex cell infection by a NgPLD-dependent mechanism in which engagement of CR3 leads to Akt kinase activation and, in turn, to NO production (Edwards, 2010). Interestingly, although NO is believed to be a freely diffusible molecule, its spatial distribution during gonococcal infection plays an important role in mediating these processes (Edwards, 2010). Taken together, the above data might indicate that aerobic denitrification by gonococci might have evolved as an adaptation to promote survival during the transition to a microaerobic/anaerobic lifestyle while in residence within the female genital tract where local steroid hormones, in particular progestagens, would bind to the gonococcal membrane and impede oxygen uptake.

In addition to Rho GTPases; PtdIns(4,5)P_2 and myosin light chain kinase may also be required for successful gonococcal infection of pex cells (Edwards and Apicella, 2006). Although confirmatory studies have not been performed, these data are consistent with the PtdIns(4,5)P_2-enriched plasma membrane patches (PRPMPs) described by Huang et al. (2004) and the role of Rho GTPases in regulating membrane raft aggregation in response to integrin activation (LaCalle et al., 2002). PRPMPs define and are exclusively co-localized with regions of concentrated myosin, actin polymerization, ruffling, and endocytosis (Huang et al., 2004). Additionally, Akt resides within raft-like structures that coalesce into "super-rafts" (e.g., PRPMPs; Skaletz-Rorowski et al., 2003). Similarly, CR3 preferentially localizes to, and clusters within, membrane rafts in professional phagocytic cells (Peyron et al., 2000).

Opa–CEACAM-mediated host cell signaling was initially described to involve Src tyrosine kinases, triggering the Rho GTPases, which then resulted in epithelial cytoskeletal rearrangement and gonococci internalization by a PI3-kinase-dependent mechanism (Hauck et al., 1998). Using CEACAM-specific transfected cell lines it is now suggested that CEACAM3-mediated invasion (which is limited to granulocytes) is dependent upon Src. Conversely, CEACAM-mediated invasion of epithelial cells does not require Src (McCaw et al., 2004) and occurs through cholesterol-rich membrane microdomains (i.e., rafts; Muenzner et al., 2008; Schmitter et al., 2007). With regard to the Opa–HSPG interaction, phosphatidylcholine-dependent phospholipase C activation results in the subsequent generation of diacylglycerol, acidic spingomyelinase activation, and ceramide production (Grassmé et al., 1997). In this model, ceramide modulates the cytoskeletal rearrangements required for endocytosis of the cell-associated gonococcus.

Several human pathogens exploit ceramide-enriched microdomains to invade host cells. Although PRPMPs are larger than membrane microdomains, the structure and composition of membrane rafts are variable depending upon the specific cell type in which they reside as well as the particular pathogen that may induce their formation/aggregation (Zass et al., 2005). PRPMPs function to drive membrane ruffling (Huang et al., 2004); however, the ability of a cell to undergo ruffling is, in part, also dependent upon the surface to volume ratio of that cell. In our observation, cervical cells grown as primary cultures exhibit a more columnar appearance than do immortalized cell lines and as a result may be more permissive to PRPMP formation and ruffling. Collectively, the above data suggest that the gonococcus exploits and/or triggers (super-)raft formation within the host cell membrane as a common route by which to gain access to the intracellular environment, albeit by different mechanisms. Whether raft-mediated gonococcal internalization by variable receptor-mediated events results in their residence within a "common" vacuole is not known. However, McCaw et al. (2004) demonstrate that internalization of gonococci by different CEACAM receptors in transfected HeLa cells occurs by distinct endocytic mechanisms after which the gonococcus-containing vacuole appears similar.

INTRACELLULAR FATE

Generally, endocytosis mediated by membrane rafts does not result in fusion of the raft vesicle with lysosomes. Therefore, as is suggested for diverse microorganisms, the ability of gonococci to enter epithelial cells through raft-like structures could provide theses bacteria with an alternative or additional survival strategy, evading intracellular degradation or triggering signaling pathways within the host cell to produce an intracellular environment conducive to survival and/or enhanced uptake. However, studies using CEACAM-transfected murine fibroblast and/or HeLa cells suggest that gonococci-containing vacuoles acquire phagolysosomal characteristics (Booth et al., 2003; McCaw et al., 2004; Binker et al., 2007). Maturation and perinuclear trafficking of gonococci-containing vacuoles occurs in wildtype or lysosome-associated membrane proteins (LAMP)-1- or LAMP-2-deficient cells (Binker et al., 2007). In contrast, infection of LAMP-1 *and* LAMP-2 (double) deficient cells results in gonococci-containing vacuoles remaining at the cell periphery, which did not acquire

lysosomal characteristics. Collectively, these data indicate a critical role for LAMP-1 and LAMP-2 in maturation of the gonococcus-containing vacuole and, presumptively, in gonococcal killing.

Gonococci produce an IgA1 protease that is capable of cleaving LAMP-1 (Lin et al., 1997), but not LAMP-2 (Hauck and Meyer, 1997). Epithelial cell lines differ with respect to the level of expression and the glycosylation pattern of LAMP isoforms (Carlsson et al., 1988). The contribution of gonococcal IgA protease in promoting disease *in vivo* remains controversial. Whereas infection of male volunteers (Johannsen et al., 1999), fallopian tube tissue explants (Cooper et al., 1984), and Chang cells (Hauck and Meyer, 1997) using *N. gonorrhoeae* IgA protease mutants results in disease/adherence/invasion indistinguishable from that observed with the parental wildtype strain, studies performed using A431 cells result in decreased viability of mutant gonococci when compared to wildtype. However, gonococcal IgA protease either directly or indirectly, respectively, decreases LAMP-1 and LAMP-2 levels in A431 cells (Ayala et al., 1998). Therefore, IgA protease activity could potentially impair delivery of the gonococcus-containing vacuole to the lysosome during A431 cell challenge. Whether these conflicting data can be attributed to specific differences in LAMP expressed by each cell type, to the experimental protocols used, or to additional differences specific to each cell type or to gonococcal strains used, is currently unclear.

Gonococcal infection studies demonstrate that following endocytosis bacteria-containing vacuoles are transcytosed to the basolateral surface of fallopian tube epithelia where they are released to the extracellular space and, thus, are poised to invade the sub-epithelial mucosa (Ward et al., 1975; Johnson et al., 1977; Draper et al., 1980; McGee et al., 1981; Stephens et al., 1987). Adherence to the LHr by the L12 gonococcal protein is proposed to mediate the gonococcus–fallopian tube interaction as well as gonococcal transcytosis through HEC-1-B endometrium adenocarcinoma cells (Chen et al., 1991; Gorby et al., 1991; Spence et al., 1997). LHr-mediated gonococcal transcytosis is consistent with the normal function of this receptor in delivering human chorionic gonadotropin to target tissues. In contrast, internalization of leukocyte integrins (e.g., CR3) on transfected epithelial cells occurs via detergent-resistant membrane vacuoles that are sorted to an endocytic polarized recycling pathway (Fabbri et al., 1999, 2005). Although these events are not specifically described for CR3 on cervical epithelia, mutations in the cytoplasmic domain of CD18 (the β2 subunit shared among the leukocyte integrins, including CR3) result in aberrant sorting of β2-integrin-containing vacuoles from a recycling to a degradative pathway (Fabbri et al., 1999, 2005). Therefore, it is reasonable to propose that engagement of CR3 on cervical epithelia by gonococci results in their entrance into a recycling pathway.

Gonococci replicate within ME-180 (Hagen and Cornelissen, 2006) and pex cells (Wu et al., 2005, 2006; Seib et al., 2007) after which they are released to the extracellular milieu. Although some gonococci traverse the intracellular space and exit pex cells at the basolateral surface, the majority of gonococci-containing vacuoles remain clustered at the apical cell periphery (Evans, 1977; Edwards et al., 2000), resulting in the majority of gonococci being released from the apical surface without disruption of the cervical cell monolayer (Greiner et al., 2005). These data are consistent with the normal trafficking pathway described for the leukocyte integrins (Fabbri et al., 1999, 2005). Analyses of gonococcal transcytosis through polarized T84 lung metastasis colorectal carcinoma cells by Criss and Seifert (2006) show that, whereas more gonococci exit through the basolateral surface of T84 cells at earlier time points post-challenge, equivalent numbers of gonococci are released from both the apical and basolateral surfaces with extended infection. In this regard, internalized raft(-like) structures are commonly reported to be recycled back to the apical cell surface, although trafficking of rafts to the basolateral surface of epithelial cells is also reported. Others have reported the ability of gonococci to traverse through T84 and HEC-1-B cell monolayers; however, the ability of gonococci to exit apically from these cells was not examined in these studies (Merz et al., 1996; Ilver et al., 1998; Wang et al., 1998, 2007; Hopper et al., 2000). Although there are many striking differences between the immortalized and primary cells described above, collectively, these data indicate that multiple host and/or gonococcal constituents likely contribute to the intracellular fate of gonococci as well as the fate of the gonococcus-containing vacuole. Whereas transcytosis through the cervical epithelium may promote invasion of sub-mucosal tissues, recycling of gonococci-containing vacuoles to the apical cell surface may promote biofilm formation and ascension to the upper female genital tract.

CONCLUSION

Through co-evolution with their exclusive human hosts the pathogenic *Neisseria* have developed several mechanisms by which they successfully persist in the general population. Increasing multidrug-resistance among gonococcal strains serves as a reminder of a critical need to further our understanding of the different mechanisms used by *N. gonorrhoeae* to infect and to colonize the specific microenvironments of the female reproductive tract, at different phases of the menstrual cycle, to better understand the morbidity associated with such infections. To this end, a better appreciation of the molecular mechanisms dictating asymptomatic cervical disease will provide sound rationale on which to base identification of subclinical infections, treatment, and prevention of harmful sequelae in the future.

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Estradiol-treated female mice as surrogate hosts for *Neisseria gonorrhoeae* genital tract infections

Ann E. Jerse*, Hong Wu, Mathanraj Packiam, Rachel A. Vonck, Afrin A. Begum and Lotisha E. Garvin

Department of Microbiology and Immunology, F. Edward Hebert School of Medicine, Uniformed Services University, Bethesda, MD, USA

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Wenxia Song, University of Maryland, USA
Roger Rank, Arkansas Children's Hospital Research Institute, USA

****Correspondence:***
Ann E. Jerse, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799, USA.
e-mail: ajerse@usuhs.mil

Historically, animal modeling of gonorrhea has been hampered by the exclusive adaptation of *Neisseria gonorrhoeae* to humans. Genital tract infection can be established in female mice that are treated with 17β-estradiol, however, and many features of experimental murine infection mimic human infection. Here we review the colonization kinetics and host response to experimental murine gonococcal infection, including mouse strain differences and evidence that IL-17 responses, toll-like receptor 4, and T regulatory cells play a role in infection. We also discuss the strengths and limitations of the mouse system and the potential of transgenic mice to circumvent host restrictions. Additionally, we review studies with genetically defined mutants that demonstrated a role for sialyltransferase and the MtrC–MtrD–MtrE active efflux pump in evading innate defenses *in vivo*, but not for factors hypothesized to protect against the phagocytic respiratory burst and H_2O_2-producing lactobacilli. Studies using estradiol-treated mice have also revealed the existence of non-host-restricted iron sources in the female genital tract and the influence of hormonal factors on colonization kinetics and selection for opacity (Opa) protein expression. Recent work by others with estradiol-treated mice that are transgenic for human carcinoembryonic adhesion molecules (CEACAMs) supports a role for Opa proteins in enhancing cellular attachment and thus reduced shedding of *N. gonorrhoeae*. Finally we discuss the use of the mouse model in product testing and a recently developed gonorrhea chlamydia coinfection model.

Keywords: *Neisseria gonorrhoeae*, mouse, neutrophils, antimicrobial peptides, immune response, hormones, lactobacilli, Opa proteins

INTRODUCTION

The success of *Neisseria gonorrhoeae* (Gc) as a pathogen stems from the evolution of several sophisticated adaptation mechanisms that maintain its sole reservoir on the mucosae of infected humans. Some of these mechanisms are sex-specific and in the case of female infection, their evolution appears to be shaped by hormonal influences. Gc is also a genetically flexible pathogen that utilizes phase and antigenic variation to evade or capitalize upon host factors (Simms and Jerse, 2005) and the immunobiology of gonorrhea is both fascinating and puzzling due to the persistence of this organism during intense inflammation and the efficiency by which gonorrhea is transmitted to naïve and previously infected individuals (Sparling, 1999). Continued studies of the pathogenesis of this organism is therefore a rich field of investigation that can benefit from animal modeling to allow testing of hypotheses in the context of an intact host. Translational research is also needed to meet the pressing need for new prophylactic and therapeutic strategies against gonorrhea (Tapsall, 2009; Lewis, 2010).

Historically, animal modeling of Gc infections has been challenged by several host restrictions. However, the use of estradiol-treated mice as surrogate hosts for Gc has partially fulfilled the need for an animal model of Gc genital tract infection. Here we describe the characteristics and limitations of experimental murine infection, the potential of transgenic mice to improve the mouse system, and information gained thus far on Gc adaptation to the female genital tract using this model. We also briefly discuss the use of this model in developing vaccines and vaginal microbicides against gonorrhea and describe a newly developed female mouse model of gonorrhea chlamydia coinfection for pathogenesis studies and developing products against pelvic inflammatory disease (PID).

ANIMAL MODELING OF GONOCOCCAL INFECTIONS

In the 1970s and 1980s, much effort was invested toward developing animal models of Gc infection. Genital tract infection was only successful in chimpanzees (Arko, 1989), which are no longer used for gonorrhea research due to their enormous cost and limited availability. Fortunately, a clue as to the role of the murine estrous cycle in inhibiting Gc colonization came from the discovery that female mice can be colonized when challenged during the proestrus stage of the estrous cycle. Gc is cleared upon transition into the post-ovulatory stages, and because the estrous cycle lasts only 4–6 days, Gc is recovered for only a few days (Streeter and Corbeil, 1981; Braude, 1982; Johnson et al., 1989). In 1990, Taylor-Robinson et al. (1990) described the use of 17β-estradiol to promote long-term colonization of germ-free BALB/c mice with Gc. Ten years later we confirmed Taylor-Robinson's report and developed a protocol in which long-term Gc infection can be established in estradiol-treated BALB/c mice given antibiotics to suppress the overgrowth of commensal flora that occurs under the influence of estrogen (Jerse, 1999; Song et al., 2008; **Figure 1A**).

The susceptibility of rodents to human genital tract pathogens is often linked to the estrous cycle and the use of steroid hormones to promote susceptiblity to Gc is consistent with other mouse models of sexually transmitted infections (STIs; Furr et al., 1989; Zeitlin

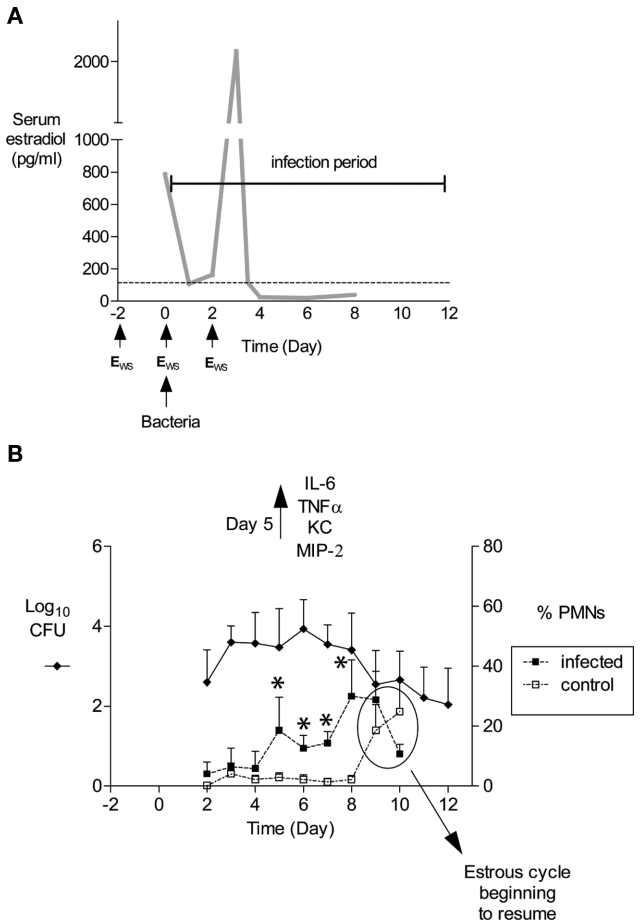

FIGURE 1 | Schematic of mouse infection protocol and characteristics of infection. (A) In our laboratory, mice are treated with 17β-estradiol to promote Gc infection by implantation of a slow-release pellet under their skin (Jerse, 1999; not shown) or subcutaneous administration of water-soluble estradiol ($estradiol_{ws}$; 0.5 mg) on days –2, 0, and 2 (Song et al., 2008). The benefit of using $estradiol_{ws}$ is that serum estradiol concentrations return to physiological levels within 24 h after administration, and in our treatment protocol, Gc is not exposed to abnormal levels by day 3 of infection as shown. The dotted line corresponds to estradiol levels in normal proestrus stage mice (Dalal et al., 2001) Mice are inoculated with bacteria on day 0. Not shown is the antibiotic treatment regimen, which has been adjusted over time due to changes in breeders and husbandry practices. Currently, streptomycin sulfate (Sm; 2.4 mg) and vancomycin hydrochloride (0.4 mg) are administered via intraperitoneal (i.p.) inoculation twice daily until day 2 of the infection period. Drinking water with trimethoprim sulfate (0.04 g/100 ml water) is used over the course of the experiment, with Sm (0.5 g/100 ml water) added on day 2 of the infection period. **(B)** An example of the average number of CFU recovered from a single vaginal swab suspended in 100 μl of PBS (left axis) and percent of PMNs in stained vaginal smears (right axis) is shown. Mice ($n = 6$) in this experiment were inoculated with 10^6 CFU of strain FA1090. In published (Packiam et al., 2010) and unpublished data from our laboratory, increased levels of proinflammatory cytokines and chemokines are detected on day 5 of infection as shown, which corresponds to the influx of PMNs in infected mice. Asterisks denote time points at which the percentage of PMNs in infected mice (dashed lines, solid squares) was significantly higher than that in mice given PBS (dashed lines, open squares). Increased PMNs were observed in control mice on days 9 and 10, which is due to the effects of the estradiol wearing off and the resumption of the estrous cycle. Mice were colonized for an average of 9.9 days in this 12-day experiment. This figure was constructed with combined data reported in (Song et al., 2008; Packiam et al., 2010).

et al., 2001; Darville et al., 2003; McGowin et al., 2009) and vaginal candidiasis (Fidel et al., 2000). The reason 17β-estradiol promotes susceptibility to Gc in mice is not known. The histology and physiology of the genital tracts of estradiol-treated mice mimic the most hospitable stages of the estrous cycle for Gc and estradiol suppresses the natural influx of polymorphonuclear leukocytes (PMNs) that occurs after ovulation. Estradiol is also likely to dampen the inflammatory response based on its immunosuppressive effect on cytokine production (Straub, 2007) or may alter the concentrations of innate receptors and effectors known to be influenced by reproductive hormones (Li et al., 2002; Yao et al., 2007).

CHARACTERISTICS OF MURINE GENITAL TRACT INFECTION

LOCALIZATION OF INFECTION AND COLONIZATION KINETICS

Using the protocols we have described, Gc is recovered from the lower genital tracts of estradiol-treated BALB/c mice for an average of 12 days in a 14-day period and as long as 40 days when slow-release estradiol pellets are used, and for an average of 10 days when three injections of water-soluble estradiol are given (Jerse, 1999; Jerse et al., 2002; Song et al., 2008). Gc is localized in the vaginal lumen and within vaginal and cervical tissue, including the lamina propria (Song et al., 2008). Endometrial cultures are positive in 17–20% of mice (Jerse, 1999), a rate that is similar to that reported for ascended cervical infections in women (Hook and Holmes, 1985). This rate may be dose-dependent and actually higher based on the detection of Gc in endometrial tissue by confocal fluorescent microscopy (Imarai et al., 2008). Mouse-passaged strains are not required for murine infection and in early experiments, did not show an enhanced capacity to colonize (Jerse, 1999).

Following inoculation of BALB/c mice with 10^6 CFU of Gc strains FA1090 or MS11, the average number of Gc recovered from a single vaginal swab ranges from 10^1 to 10^6 CFU with most cultures yielding 10^3–10^5 CFU (**Figure 1B**; Jerse, 1999; Jerse et al., 2002; Soler-Garcia and Jerse, 2007; Song et al., 2008). Periods of dramatically reduced recovery or negative cultures are observed followed by a marked increase in recovery in 60–80% of BALB/c mice inoculated with Gc strains FA1090 or 1291 (Jerse, 1999; Simms and Jerse, 2006; and O. Jones-Nelson and Jerse, unpublished observations). We refer to these periods of reduced recovery as culture negative windows or the mid-phase of infection. (Jerse, 1999; Simms and Jerse, 2006) and have evidence that this pattern is hormonally regulated (Cole et al., 2010). It is critical that researchers who use this model be aware of fluctuations in colonization levels, particularly when measuring the effect of a particular host response on clearance or testing the efficacy of vaccines or therapeutic products. We obtain negative cultures for five consecutive days from mice that appear to have resolved infection before concluding infection has cleared. It is also important to understand that the estrous cycle will resume as the effects of the estradiol wear off, and mice will re-gain their natural resistance to infection upon transition into the luteul phase (**Figure 1B**). Additional doses of estradiol can be administered to prolong infection further.

HOST RESPONSE TO INFECTION

Gonorrhea in women is characterized by a mucopurulent cervical discharge with numerous PMNs or it can be asymptomatic with over 50% of women reporting no symptoms (Hook and Holmes,

1985). Approximately 50% of infected BALB/c mice (range 30–80%) have a higher percentage of PMNs in stained vaginal smears on days 4–5 compared to uninfected control mice (Jerse, 1999; Jerse et al., 2002; Soler-Garcia and Jerse, 2007; Packiam et al., 2010), and significantly more PMNs are detected within vaginal and cervical tissue from infected mice on days 2 and 5 post-inoculation. (Song et al., 2008). Localized production of proinflammatory cytokines and chemokines IL-6, TNFα, KC, and MIP-2 is significantly increased on day 5 of infection, with MIP-2 expression positively correlating with PMN influx (Packiam et al., 2010; **Figure 1B**).

Mouse strain differences in susceptibility and host response have been documented. Like BALB/c mice, estradiol-treated CD1, SLC::ddY (Jerse, 1999), and C57BL/6 mice are susceptible to infection. Interestingly, Gc do not elicit an innate inflammatory response in C57BL/6 mice despite being colonized to similar levels as BALB/c mice (Packiam et al., 2010). C57BL/6 mice therefore appear to mimic asymptomatic infection and perhaps can be used to better define the pathways that lead to or suppress the inflammatory response to Gc. Known differences between C57BL/6 and BALB/c mice include the absence of phospholipase A2 in C57BL/6 mice (Kennedy et al., 1995), which may play a role in generating lipid mediators (Kudo and Murakami, 2002) and inducing proinflammatory cytokines and chemokines (Granata et al., 2005). Interestingly, estradiol-treated C3H/HeN mice are resistant to Gc (Packiam et al., 2010) and untreated C3H/HeN mice are also less susceptible to transient Gc colonization following intrauterine inoculation (Streeter and Corbeil, 1981). The natural resistance-associated macrophage protein (Nramp1) is responsible for the resistance of C3H/HeN mice against pathogens that are highly adapted for life within macrophages (Forbes and Gros, 2001). The impact of Nramp1 on susceptibility to Gc infection is not known.

A significant advance in understanding the immune response to Gc was made recently by Mike Russell's laboratory who showed Gc induces IL-17 responses and that secretion of IL-6, LIX, and MIP-2α is dependent on IL-17 receptor signaling. Importantly, inhibition of IL-17-induced responses in mice via antibody-mediated depletion of IL-17 or infection of IL-17 receptor knock-out mice resulted in increased recovery of bacteria and a significantly longer duration of infection compared to control mice (Feinen et al., 2010). The relevance of this finding to IL-17 responses in humans is supported by the recent report that levels of serum IL-17A and IL-23, which plays a role in the differentiation and proliferation of Th-17 cells, were elevated in patients with Gc urethritis or pharyngitis compared to healthy control subjects (Gagliardi et al., 2011). IL-17, along with IL-22, play a role in inducing antimicrobial peptides in epithelial cells (Kolls et al., 2008). Evidence that Gc induces IL-17 via endotoxin-mediated signaling through toll-like receptor 4 (TLR4; Feinen et al., 2010) may therefore provide a new angle for the design of preventive therapies.

There are many unresolved questions regarding the adaptive humoral response to Gc, which is not protective in humans and appears to be immunosuppressed. While there is some evidence of protective immunity in individuals with repeated exposure, humans can be reinfected with the same strain or serovar and antibody titers are not remarkable and decline over time (Sparling, 1999; Russell and Hook, 2008). Mice also develop a transient and insignificant humoral response to infection and there was no evidence of a humoral memory response or reduced infection in mice that were previously infected with the same strain compared to naïve age-matched, estradiol-treated control mice (Song et al., 2008). Imarai et al. (2008) investigated the basis for the lack of a humoral response to Gc using the mouse system. Gc were detected in endometrial tissue from estradiol-treated BALB/c mice for as long as 22 days and importantly, significantly higher numbers of TGF-β1$^+$ CD4$^+$ T cells and a subset of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells were detected in the regional lymph nodes of infected mice compared to control mice. Increased infiltration of TGF-β1$^+$ CD11b$^+$ macrophages into the genital tracts of infected mice also occurred, which could favor the differentiation of T regulatory cells, and thereby suppress immune responses against Gc (Imarai et al., 2008).

LIMITATIONS OF USING MICE TO STUDY GONOCOCCAL INFECTIONS

GENITAL TRACT PHYSIOLOGY

Several similarities and differences exist between the lower genital tracts of female mice and women. Similar factors include reduced O_2 tension, the presence of glucose and lactate (Exley et al., 2007), and cytidine monophosphate *N*-acetylneuraminic acid (CMP-NANA; Wu and Jerse, 2006) used to sialylate the Gc surface during infection. The average vaginal pH of estradiol-treated female mice is pH 6.6 (range 5.8–7.2; Muench et al., 2009). This pH is higher than human vaginal pH (average pH 3.5–4.5) but similar to human cervical pH [proliferative stage, average 6.8 (5.5–8.0); secretory stage, average 6.1 (range 5.1–8.4)] (Singer, 1975), which is the primary site of infection in women. Hormonally driven changes in mucus viscosity, commensal flora (Braude, 1982), and histology (Corbeil et al., 1985) are similar to that which occurs in the human reproductive cycle. A major difference is the fact that there is no period of menstrual bleeding in mice that brings proteases, hemoglobin, and other serum factors into the reproductive tract. Fewer anaerobes colonize the murine genital tract compared to the normal microbiota of most women of reproductive age (Noguchi et al., 2003), but Gram-positive and Gram-negative facultatively anaerobic flora are present. The use of antibiotics to reduce potentially inhibitory commensal flora in the protocols we have described eliminates bacteria of the *Enterobacteriaceae* and *Pseudomonas* familes and most Gram-positive flora. Some mice remain colonized with Gram-positive organisms, particularly when vancomycin is not used, and vaginal lactobacilli are frequently isolated (Jerse et al., 2002).

HOST RESTRICTIONS AND TRANSGENIC MICE

Colonization receptors

Gc expresses several ligands that bind to specific receptors to mediate adherence or uptake by epithelial cells. At least three of these receptors are host-restricted. Colonization pili are expressed by the vast majority of human isolates (Kellogg et al., 1963); in contrast, murine vaginal isolates lose the piliated colony morphology over time (Jerse, 1999). Mice probably lack the pilus colonization receptor, although its identity is unresolved. Human membrane cofactor protein (CD46) is hypothesized by some to serve as the neisserial pilus receptor (Kallstrom et al., 1997, 2001) and the demonstration that pilus-mediated interactions with CD46 trigger cellular responses through Src kinase-dependent phosphorylation (Lee et al., 2002; Weyand et al., 2009) suggests CD46 has an immunoregulatory role during infection. CD46$^+$ transgenic mice support

meningococcal dissemination to the brain following intranasal or intraperitoneal inoculation and induce higher levels of cytokines (Johansson et al., 2003, 2005). The impact of CD46 expression on Gc infection of mice has not been reported.

The human CR3 (hCR3) integrin is utilized by Gc to invade primary cervical cells via the binding of pili, LOS, and iC3b (Edwards et al., 2002). The hCR3 molecule has a high degree of similarity to murine CR3; however, it appears to be host-restricted based lack of staining with a hCR3-specific monoclonal antibody that blocks Gc invasion of human cells (unpublished data in collaboration with Drs. Jennifer Edwards and Michael Apicella). Additionally, Rice and colleagues recently showed human factor H (fH), which is host-restricted (Ngampasutadol et al., 2008a), bridges the interaction between the gonococcus and CR3 (Agarwal et al., 2010). Therefore a transgenic mouse model that reproduces this invasion pathway may require the expression of both hCR3 and fH.

Other host-restricted receptors known to be used by Gc to invade epithelial cells include the human carcinoembryonic antigen cellular adherence molecules (CEACAMs) -1, -5 and -6 to which the phase variable opacity (Opa) proteins bind and CEACAM-3, through which Opa-mediated uptake by PMNs occurs in the absence of opsonization. Opa–CEACAM-1 binding causes immunosuppression of T cells and killing of B cells (Sadarangani et al., 2011). CEACAM transgenic mice are available (Eades-Perner et al., 1994; Bhattacharya-Chatterjee et al., 2008) and CEACAM-5 transgenic mice were recently used by Christof Hauck's laboratory to demonstrate a novel role for Opa–CEACAM interactions in preventing detachment of infected epithelial cells via enhancement of integrin-mediated cell to cell adhesion and cellular adhesion to extracellular matix. CEACAM-5 transgenic mice had a higher colonization load than normal mice 1 day after inoculation. This finding supports the interesting hypothesis by this group that CEACAM-engaging bacteria subvert shedding from mucosal surfaces (Muenzner et al., 2005, 2010). Additional studies with CEACAM transgenic mice are needed to demonstrate the Opa–CEACAM invasion pathway during female genital tract infection and test the role of Opa proteins as immunosuppressive factors *in vivo*. Also, the importance of Opa–CEACAM-3 mediated uptake by PMNs relative to opsonic uptake during cervical infections, where complement is present in the absence of inflammation, is an interesting question that could potentially be addressed using complement depletion of CEACAM-3 transgenic mice.

Gc that express the lacto-*N*-neotetraose (LNT) species of LOS invade through the asialoglycoprotein receptor of primary urethral cells from men. This pathway is sex-specific (Edwards and Apicella, 2004) and thus unlikely relevant to animal modeling of female infections. Other less characterized adhesins and invasins have been described including OmpA, which mediates invasion of immortalized human endocervical and endometrial cells. Interestingly, an *ompA* mutant was attenuated for murine infection. OmpA was also required for uptake or survival within cultured murine macrophages and whether the observed attenuation *in vivo* was due to a colonization versus survival advantage is not known (Serino et al., 2007). P1A strains undergo porin-mediated invasion through Gp96 and scavenger receptor SREC. This pathway is phosphate-dependent and not restricted to human cells (van Putten et al., 1998; Kuhlewein et al., 2006; Rechner et al., 2007). Gc also invades human cervical cells that do not express CEACAMs (Swanson et al., 2001) and Opa^- variants are more invasive than Opa^+ variants in some systems (Song et al., 2000). The identification of bacterial ligands and receptors used during murine infection is handicapped by the lack of immortalized murine cervical or endometrial cell lines. Adherence and invasion pathway(s) exist for murine infection based on the visualization of Gc associated with genital tract cells (Jerse, 1999) and within mouse tissue (Song et al., 2008) and the detection of intracellular Gc in endometrial cells from murine uterine explant cultures (Imarai et al., 2008).

Iron-binding glycoproteins

The capacity of Gc to obtain iron from transferrin (Tf) and lactoferrin (Lf) through specific receptors is also host-restricted (Lee and Schryvers, 1988). Expression of either the Tf or Lf receptor was critical for urethral infection of male subjects (Cornelissen et al., 1998; Anderson et al., 2003), but the absence of these receptors or the hemoglobin receptor, which is not host-restricted (Stojiljkovic et al., 1996) does not confer a growth disadvantage in estradiol-treated mice (Jerse et al., 2002). Usable iron sources in the female genital tract include siderophores produced by commensal flora (Mickelsen et al., 1982; Carson et al., 1999; Strange et al., 2010), hemin, and ferritin from dying epithelial cells, which are rapidly turned over during the course of the reproductive cycle (Schryvers and Stojiljkovic, 1999), and iron complexed to citric acid, oxalic acetate, pyrophosphate, nitrilotriacetate, or other metabolites (Mickelsen et al., 1982). Additionally, iron is likely to be more soluble in the lower pH and reduced O_2 tension of the female genital tract and thus more available to Gc. A transgenic hTf mouse strain was recently used as an improved model of *N. meningitidis* septicemia from which meningococci were recovered for a longer period and at a 100-fold higher concentration compared to wild type mice (Zarantonelli et al., 2007). The use of hTf transgenic mice may also significantly increase Gc genital tract colonization and perhaps support Gc infection of other body sites. However, the capacity of Gc to replicate in normal mice is direct evidence that non-host-restricted iron stores exist in the female lower genital tract.

Complement regulatory proteins

Gc that show inherently high levels of porin-mediated resistance to normal human serum bind the soluble regulatory proteins C4b-binding protein (C4BP; Ram et al., 2001) and factor H (for strains of the P1A serotype; Ram et al., 1998) to down-regulate the classical and alternative pathways, respectively. Rice and colleagues showed that only human or chimpanzee fH and C4BP bind serum resistant strains, and the generation of hC4BP and/or hfH transgenic mice therefore holds promise for testing the importance of porin-mediated serum resistance during female genital tract infection (Ngampasutadol et al., 2008b). Due to the role that complement activation plays in inducing cytokines and chemokines and the adaptive response (Dunkelberger and Song, 2010) such mice would also be a valuable resource for studying the host response to infection.

IgA1

Like many human-specific mucosal pathogens, Gc produces an IgA1 protease that cleaves only primate IgA1 (Qiu et al., 1996). How important IgA1 protease is for infection is not known. Russell and

colleagues found no evidence of IgA cleavage products in cervical fluid from women with gonorrhea (Hedges et al., 1998), and IgA2 presumably can serve as a redundant protective factor. An IgA1 protease mutant was not attenuated for urethral infection of naïve male volunteers (Johannsen et al., 1999), although its importance in previously infected subjects cannot be ruled out. Attenuation of an IgA1 protease mutant of *Streptococcus pneumoniae* in a mouse septicemia model (Polissi et al., 1998) suggests non-host-restricted roles exist for this class of enzymes. Gc IgA1 protease cleaves a phagosomal maturation protein to promote increased survival and transit through polarized epithelial cells (Lin et al., 1997; Hopper et al., 2000) and may modulate the host response based on the reported inhibition of apoptosis of immune cells and induction of proinflammatory cytokines by purified IgA1 protease *in vitro* (Lorenzen et al., 1999; Beck and Meyer, 2000). Whether these interactions are host-restricted has not been reported.

PATHOGENESIS STUDIES

Gc encounters a variety of innate defenses in the female genital tract, some of which are illustrated in **Figure 2A**. The testing of bacterial mutants in factors that are hypothesized to promote Gc survival *in vivo* has confirmed predictions from *in vitro* studies and generated new information that may be relevant to lower genital tract infection in women, particularly with regard to evasion of phagocytes, antimicrobial peptides, and commensal bacteria and adaptation to hormonally regulated factors (**Figure 2B**).

SIALYLTRANSFERASE BUT NOT ANTI-OXIDANT FACTORS PROTECT AGAINST PMNS

Gc is superbly adapted for evasion PMNs in which they survive and perhaps replicate (Casey et al., 1979; Simons et al., 2005; Criss et al., 2009). Examination of PMN killing mechanisms in the mouse system has the advantage of reproducing natural pathways of PMN recruitment and activation and physiologically relevant concentrations of iron, oxygen, and glucose needed to fuel the oxidative burst (Storz et al., 1990) and CMP-NANA, which when added to gonococcal LNT LOS via the action of α-2,3-sialylatransferase (Lst), increases resistance to complement-mediated uptake by PMNs (Smith et al., 1995; Gill et al., 1996). Additionally, PMNs from estradiol-treated mice produce a dose-dependent oxidative burst when exposed to Gc that is primarily intracellular (Soler-Garcia and Jerse, 2007) as reported for human PMNs (Naids and Rest, 1991; Simons et al., 2005). Limitations include differences in the concentrations of myeloperoxidase and other granular enzymes

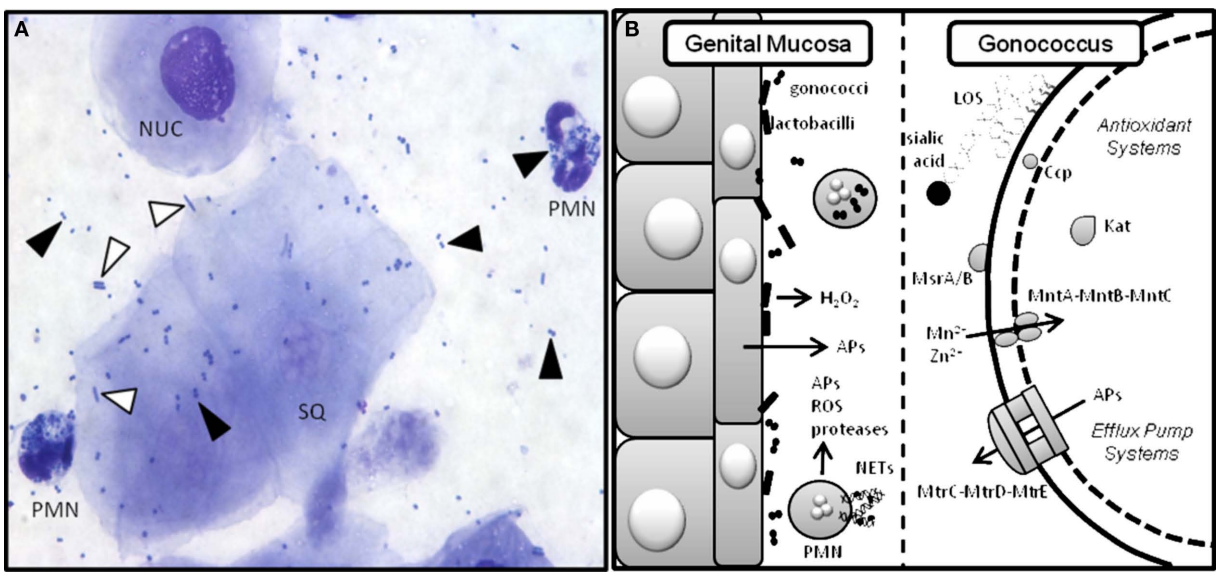

FIGURE 2 | Gc interactions with innate defenses in the murine genital tract. Gc encounters a variety of innate defenses in the female genital tract including hydrophobic antimicrobial substances that bathe the mucosal surface, complement, and commensal flora (Boris and Barbes, 2000). Pathogen-induced activation of innate receptors on epithelial and immune cells causes secretion of antimicrobial peptides and proinflammatory cytokines and chemokines (Kolls et al., 2008), which recruit phagocytes to the infection site. PMNs can take up bacteria that are opsonized with complement deposition products. Phagocyte-produced reactive oxygen species (ROS) kill bacteria by damaging DNA, protein, and other macromolecular structures (Storz et al., 1990). PMNs also kill microbes via the release of pre-formed enzymes and antimicrobial peptides and entrapment in neutrophil extracellular traps (NETs) where they are exposed to toxic molecules produced by both pathways (Kobayashi et al., 2005; Papayannopoulos and Zychlinsky, 2009). **(A)** Stained vaginal smear from a Gc-infected mouse reveals the presence of nucleated (NUC) and squamous (SQ) epithelial cells and PMNs with intracellular diplococci. Black arrows denote Gc and white arrows denote lactobacilli. Human strains of H_2O_2-producing lactobacilli can be added to this ecosystem as described (Muench et al., 2009). **(B)** Cartoon depicting the murine genital mucosa on the left and some of the bacterial factors that have been tested to measure their role in protection from host innate defenses on the right. Gc has many redundant anti-oxidant systems including Ccp (cytochrome c peroxidase), Kat (catalase), MsrA/B (a methionine sulfoxide reductase), and the MntA–MntB–MntC manganese transporter, which do not protect Gc from phagocyte-derived (Kat, Ccp, MntC; Wu et al., 2009), or *Lactobacillus*-derived (Kat, Ccp) ROS *in vivo* (Muench et al., 2009). Gc add host sialic acid to their LOS via the action of sialyltransferase. This modification reduces uptake of the bacteria by PMNs and increases survival *in vivo* (Wu and Jerse, 2006). Antimicrobial peptides (APs) are actively expelled from the cell via the MtrC–MtrD–MtrE efflux pump and inactivation of this system is highly attenuating for murine infection (Jerse et al., 2003).

in murine and human PMNs (Rausch and Moore, 1975), and the absence of Opa–CEACAM-mediated uptake by PMNs. Although it is possible that estradiol impairs PMN killing, we detect no difference in the killing capacity of PMNs from estradiol-treated versus untreated mice (Soler-Garcia and Jerse, 2007).

There is now much evidence that the oxidative burst of phagocytes does not challenge Gc, including studies with PMNs from humans with chronic granulomatous disease and the use of pharmacological inhibitors of the respiratory burst (Rest et al., 1982; Criss et al., 2009). Infection studies in C57BL/6 mice that have a defective NADPH oxidase (Phox) provided *in vivo* evidence that phagocytic-derived ROS do not challenge Gc during genital tract infection (Wu and Jerse, 2006) with the caveat that C57BL/6 mice do not produce a signficant PMN response to Gc infection (Packiam et al., 2010). Whether this host defense is more potent in body sites where oxygen levels are higher such as the pharynx, is not known, but seems unlikely based on PMN killing assays performed under anaerobic versus aerobic conditions (Casey et al., 1986; Frangipane and Rest, 1992).

Perhaps a bigger question is how Gc evades phagocyte-derived ROS. Gc is equipped with a battery of factors that protect it from *in vitro* exposure to H_2O_2 and inducers of intracellular and extracellular ROS, including the detoxifying enzymes catalase (Kat) and cytochrome C peroxidase (Ccp), non-enzymatic quenching of O_2^- by Mn^{2+}, which is taken up by the manganese transporter MntABC, methionine sulfoxide reductase (MsrA/B), which repairs oxidatively damaged proteins, a thiol-disulfide oxidoreductase (Sco), azurin, bacterioferritin and novel peroxidase-induced genes of unknown function (Seib et al., 2006). This abundance of anti-oxidant mechanisms is impressive, yet there if no evidence that these factors protect Gc from the vigorous PMN response encountered *in vivo*. For example, a *kat* mutant and *kat ccp* and *kat sco* mutants of strain 1291 were not more susceptible to killing by human PMNs (Seib et al., 2005). Similarly, single, double, and triple mutants in the *kat*, *ccp*, *msrAB*, and *mntC* genes in strain MS11 were not more susceptibile to killing by murine PMNs. These mutants were also not attenuated during infection of normal BALB/c mice or Phox-deficient and Phox-sufficient C57BL/6 mice (Wu et al., 2009). Further genetic stripping of the factors known to protect Gc from exposure to H_2O_2 or inducers of ROS may debilitate Gc against O_2-dependent PMN killing. The answer may, however, reside in novel mechanisms that do not involve direct protection from ROS (Lorenzen et al., 2000; Criss and Seifert, 2008; Criss et al., 2009).

Possible new functions for MsrA and MntC were revealed using murine infection. The *msrA* mutant showed delayed attenutation *in vivo* in BALB/c mice and because macrophages appear later than PMNs during infection of BALB/c mice (Song et al., 2008) reduced recovery of *msrA* mutant may be due to increased sensitivity to macrophage killing as described for other bacteria (St John et al., 2001; Douglas et al., 2004). Additionally, inactivation of *mntC* resulted in an attenutated phenotype in normal BALB/c and Phox-deficient and Phox-sufficient C57BL/6 mice. MntC-deficient Gc may be more susceptible to ROS produced by epithelial cells. Inactivation of *mntC* may also reduce biofilms, which may be important in stabilizing colonization (Lim et al., 2008).

In contrast to the anti-oxidant factors tested above, Lst plays a detectable role in defending Gc from PMN killing *in vitro* and in the mouse model. Sialylation of Gc occurs within the first day of murine infection and inactivation of the *lst* gene confers a survival disadvantage in BALB/c mice. Decreased resistance to PMN killing is the most likely explanation for the observed attenuation based on the greater susceptibility of the *lst* mutant to opsonophagocytic uptake and killing by murine PMNs *in vitro* and significantly faster clearance of the *lst* mutant following intraperitoneal injection compared to sialylated, wildtype bacteria (Wu and Jerse, 2006). LOS also increases resistance to complement-mediated bacteriolysis by enhancing the binding of fH to P1B porin (Madico et al., 2007). The importance of sialyltransferase in Gc survival is therefore likely underestimated in studies with normal mice due the host restriction for fH. While the host restriction for fH did not affect opsonophagocytosis at a functionally detectable level in our study, the *lst* mutant was equally attenuated in normal and C5-deficient mice (Wu and Jerse, 2006). These findings are consistent with the requirement for higher numbers of membrane attack complexes for bacteriolysis than opsonins for phagocytosis. Again, studies in fH transgenic mice could further illustrate the importance of sialyation in infection.

EVIDENCE OF COMPLEX INTERACTIONS BETWEEN GC AND LACTOBACILLI

Epidemiological data show an increased risk for gonorrhea in women that lack vaginal lactobacilli, with some studies implicating H_2O_2-producing lactobacilli as a defense in particular (Antonio et al., 1999; Wiesenfeld et al., 2003) and others showing the same association regardless of the H_2O_2 production phenotype (Saigh et al., 1978; Hillier et al., 1992; Martin et al., 1999). It is not clear whether lactobacilli are directly responsible for the inverse correlation between gonorrhea and vaginal lactobacilli because an absence of lactobacilli is associated with bacterial vaginosis in which an imbalance of other bacteria also occurs (Hillier et al., 1992). H_2O_2-producing *Lactobacillus sp.* inhibit Gc *in vitro* (Saigh et al., 1978; St Amant et al., 2002), and Gc *kat* and *ccp* mutants are more susceptible than wild type Gc. However, surprisingly, there was no difference in the recovery of wild type, *kat*, or *kat ccp* mutant Gc from mice that were pre-colonized with H_2O_2-producing *L. crispatus* compared to mice that were not colonized with this human commensal (Muench et al., 2009). *L. crispatus* makes several factors that could enhance Gc growth or survival, one of which is lactate (Smith et al., 2007). As inactivation of the gonococcal lactate permease (*lctP*) gene was attenuating for murine infection (Exley et al., 2007), we hypothesized that utilization of *Lactobacillus*-produced lactate might balance the detrimental effects caused by *Lactobacillus*-produced H_2O_2 *in vivo*. However, a *kat lctP* Gc mutant also colonized mice with *L. crispatus* as well as mice without *L. crispatus* (Muench et al., 2009).

The murine infection data predict that vaginal H_2O_2-producing lactobacilli do protect against Gc, possibly because the O_2 tension may not be sufficient for adequate H_2O_2 production. However, it is possible that H_2O_2 is more stable in the lower pH of the human vagina or the colonization density of *L. crispatus* in mice is not sufficiently high. A recent report that human cervical secretions block H_2O_2-mediated bactericidal activity however, also suggests *Lactobacillus*-derived H_2O_2 is not a formidable defense against STI pathogens (O'Hanlon et al., 2010). These studies do not rule out a role for other *Lactobacillus* factors in challenging *N. gonorrhoeae* (Spurbeck and Arvidson, 2010). Such factors may have also been produced by non-H_2O_2 producing murine lactobacilli in control

mice in the mouse study discussed above. Nonetheless, interesting observations have been made while working with the mouse model in our laboratory that suggest Gc has evolved mechanisms to coexist with these commensals. For example, *L. murinus*, a mouse *Lactobacillus sp*., supports growth of Gc on solid agar (Jerse et al., 2002) as do H_2O_2-producing human *Lactobacillus* strains if catalase is added, and mice with *L. murinus* usually have a very high Gc colonization load (A. E. Jerse, unpublished observation).

THE MTRC–MTRD–MTRE ACTIVE EFFLUX PUMP PLAYS AN IMPORTANT ROLE IN EVASION OF HOST DEFENSES

Gc produces several active efflux pump systems that expel antimicrobial substances from the periplasm. Host-derived substrates expelled by the MtrC–MtrD–MtrE efflux pump system include human cathelicidin LL37, the mouse homolog cathelicidin-related antimicrobial peptide (CRAMP), progesterone, and fatty acids (Shafer et al., 2001; Jerse et al., 2003; Warner et al., 2008). MtrCDE-deficient mutants are the most attenuated of all mutants tested thus far in the mouse model, which is consistent with a role for this efflux system in protecting against host defenses (Jerse et al., 2003). Inactivation of MtrR and MtrA, which negatively and positively regulate *mtrCDE* expression, respectively causes a phenotype in mice that is consistent with the importance of the MtrC–MtrD–MtrE efflux pump in infection (Warner et al., 2007). In contrast, the FarA–FarB–MtrE efflux pump system does not have a detectable role in murine infection (Jerse et al., 2003). This system primarily protects Gc from long chain fecal lipids, and thus may be more important during rectal infections (Lee and Shafer, 1999). Infection studies in mice that are deficient in CRAMP (Nizet et al., 2001) may facilitate further investigation of the role of the MtrC–MtrD–MtrE pump in infection; however, the presence of more than one pump substrate may complicate definitive identification of host factors that challenge MtrCDE-deficient Gc *in vivo*. *mtrE* mutants are also more sensitive to bactericidal/permeability-increasing (BPI) protein (A. A. Begum and A. E. Jerse, unpublished data), which is a lipid A-binding protein that is present in both the human and murine genital tracts (Canny et al., 2006; Eckert et al., 2006). Also, increased susceptibility of MtrCDE-deficient Gc to progesterone *in vitro* and the faster clearance of MtrCDE-deficient Gc from normal mice versus ovariectomized mice suggests progesterone or progesterone-regulated factors challenge these mutants during infection.

Derepression of the *mtrCDE* operon through a single base pair deletion in the *mtrR* promoter region or point mutations in the MtrR structural gene is a mechanism by which Gc strains become resistant to macrolide antibiotics and high levels of penicillin (Shafer et al., 2001). Antibiotic resistance mutations often confer reduced fitness *in vitro* due to the effect these mutations have on growth. An exception to this paradigm are *mtrR* locus mutations that confer increased fitness in the mouse model. Commonly isolated *mtrR* locus mutations confer different levels *mtrCDE* derepression, which is mirrored by different levels of fitness advantage *in vivo* (Warner et al., 2008). One might predict that the percentage of *mtrR* locus mutants would be higher among clinical isolates should the observed fitness benefit in mice hold true for human infection. It may be that the mouse data are not predictive of events during human infection or there are sex-based or anatomical differences in the types of innate effectors that select for *mtrR* locus mutations. Alternatively, *mtrR* locus mutants frequently carry more than one antibiotic resistance mutation, which may confer an overall fitness cost (Komp Lindgren et al., 2005).

ADAPTATION TO HORMONALLY REGULATED FACTORS THROUGH *OPA* GENE PHASE VARIATION

The neisserial Opa proteins are a family of phase variable outer membrane proteins that are encoded by 10–12 unlinked genes (Simms and Jerse, 2005). Selection for Opa expression during urethral infection occurs in naturally (James and Swanson, 1978) and experimentally infected male volunteers (Swanson et al., 1988; Jerse et al., 1994). Selection for Opa variants in the female genital tract appears more complex, with predominantly opaque (Opa^+) colonies isolated during the proliferative stage of the menstrual cycle and transparent (Opa^-) colonies isolated during or shortly after menses. Based on this intriguing observation, James and Swanson (1978) hypothesized that subpopulations of bacteria are better adapted for different stages of the reproductive cycle. This hypothesis is consistent with reported higher rates of positive cultures from women with gonorrhea during the proliferative stage than the middle secretory stage (Koch, 1947; Johnson et al., 1969; James and Swanson, 1978; McCormack and Reynolds, 1982; **Figure 3A**).

Interestingly, Opa^+ variants are isolated from BALB/c mice in a cyclical recovery pattern that consists of early (Opa^+), mid (Opa^-) and late (Opa^+) phases. Consistent with subpopulations of bacteria being cleared or having an advantage, fluctuations in the predominant Opa phenotype recovered are paralleled by changes in the total number of Gc recovered (Simms and Jerse, 2006; **Figure 3B**). A cyclical recovery pattern also occurs during short-term colonization of mice that are not treated with estradiol. The importance of Opa proteins during the early and late phases was confirmed by the demonstration that an Opa-deficient mutant colonized mice less well than the mutant expressing one functional *opa* allele during the first day of infection, and unlike the Opa-deficient strain, the Opa^+ strain was recovered following the mid-phase (Cole et al., 2010). A link between the reproductive cycle and the cyclical recovery pattern was revealed by studies with ovariectomized mice, which select for Opa^+ variants early in infection and maintain this selection over time. Also, unlike intact (normal) mice, fluctuations in the number of Gc recovered from ovariectomized mice were not observed (Cole et al., 2010). The selective forces responsible for the cyclical changes in Opa phenotype in normal mice must be due to a CEACAM-independent Opa function. Complement was shown to preferentially kill Opa^+ variants of strain MS11 (Bos et al., 1997) and complement levels in the female human and murine genital tracts are hormonally regulated (Hasty et al., 1994; Li et al., 2002). However, experiments with complement-depleted mice did not support complement as the selective factor (Cole et al., 2010).

The factors that select for Opa^+ variants during human infections are also not known. In women, CEACAM-mediated adherence to and invasion of cervical cells may select for Opa-expressing variants during infection, as may increased attachment of epithelial cells with adherent bacteria (Muenzner et al., 2005, 2010) as discussed. Based on data from BALB/c mice, we hypothesize that CEACAM-independent factors also exist. CEACAMs were not detected on

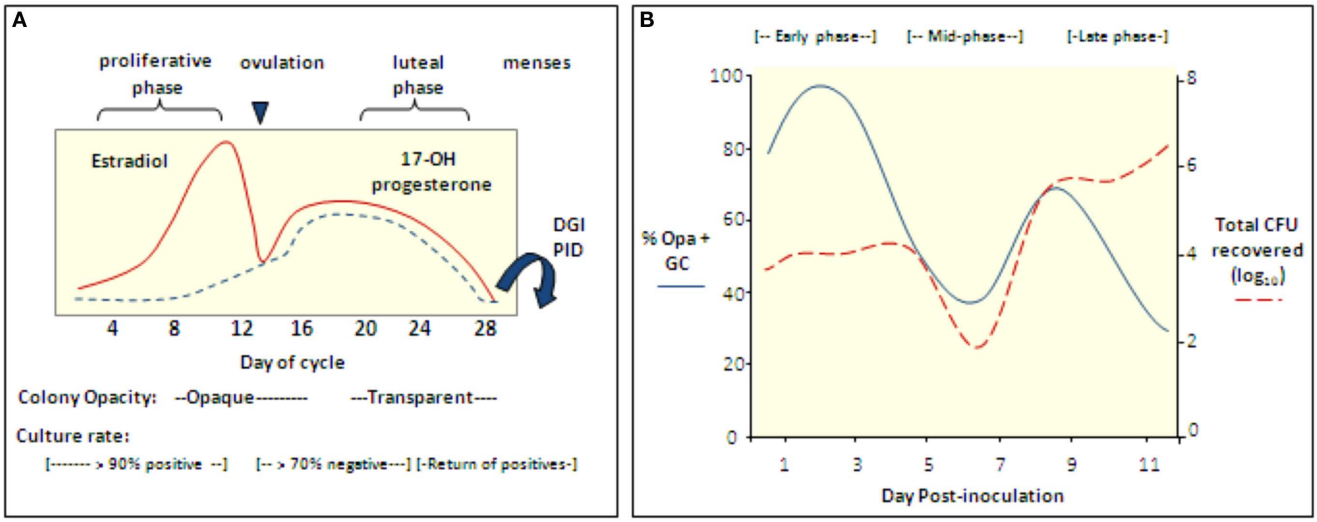

FIGURE 3 | The reproductive cycle influences Gc infection of women and mice. (A) Culture rates from women with gonorrhea and the opacity phenotype of cervical isolates from infected women with respect to stages of the menstrual cycle (Koch, 1947; Johnson et al., 1969; James and Swanson, 1978; McCormack and Reynolds, 1982). In one study, women with gonorrhea were hospitalized without treatment and in four of four women, positive cultures were followed by five to six consecutive negative cultures during the secretory phase; cultures became positive again at menses (Koch, 1947). Gonococcal PID and disseminated gonococcal infection most frequently occur at or shortly after menses (Holmes et al., 1971) and Gc from fallopian tubes from women with salpingitis were reported to be Opa^- (Draper et al., 1980). **(B)** Cartoon depicting the cyclical recovery pattern seen in intact mice following inoculation with mostly Opa^- variants of strain FA1090. Within a day after inoculation with mostly Opa^- Gc, Opa^+ variants predominate (early phase). This phase is followed by a period in which mostly Opa^- variants are isolated (mid-phase) and then a second Opa^+ phase (late phase). A high percentage of isolates express multiple Opa proteins in the late phase. In mice that remain infected for more than 8 days, a second mid-phase is observed. The rise and fall of the Opa^+ population corresponds to fluctuations in the total number of Gc recovered. This pattern is not seen in Ov^- mice. Hostile factors may reduce colonization during the mid-phase or perhaps Opa^+ variants are less accessible for culture due to tissue invasion (Simms and Jerse, 2006; Cole et al., 2010).

primary urethral cells from men (Edwards and Apicella, 2004) and therefore, the selection of Opa^+ Gc during natural or experimental urethral infection of men may also be due to another factor(s).

The reason for periods of reduced recovery of Gc from female mice and women is also not understood. In women, the capacity of Gc to invade and survive within cervical cells via the host-restricted CR3 pathway is affected by reproductive hormones and therefore, the presence of Gc within intracellular niches may contribute to cyclical culture rates from women (Edwards, 2010). Hormonally driven changes in metalloprotease and cathepsin expression, which function to remodel tissue (Afonso et al., 1997; Jokimaa et al., 2001), could also open avenues for invasion into tissue in mice or women as described for other pathogens (Azghani et al., 2000; Katz et al., 2000) or alter the host response as demonstrated for chlamydial infection using matrix metalloprotease nine knock-out mice (Imtiaz et al., 2007). Up-regulation of anti-Gc effectors during the luteal phase of the cycle may also contribute to selection patterns via hormonal regulation of TLRs (Yao et al., 2007).

FUTURE DIRECTIONS

PRODUCT DEVELOPMENT

Safe and effective therapeutic and prophylactic products against Gc are greatly needed to reduce the incidence of gonorrhea and protect women's reproductive health. Infertility treatment is a significant and hidden cost of ascended infections and ectopic pregnancy causes 4.1% of maternal-related deaths in industrialized countries (Khan et al., 2006). Gonorrhea is also a cofactor for HIV transmission (Cohen et al., 1997). Alarmingly, the reliance on antibiotic treatment as a control measure is seriously threatened by the rapid emergence of antibiotic-resistant strains (Tapsall et al., 2005). The need for an animal model to facilitate pre-clinical testing of products against gonorrhea was reflected by the numerous academic and industrial collaborators who approached our laboratory upon the first publication of the mouse model. Continued research in this area is critical. Here we briefly describe our experience with testing vaccines and vaginal microbicides in the mouse model with an emphasis on practical considerations and challenges for product development.

Vaccine development

Successful development of a gonorrhea vaccine faces many challenges as recently reviewed (Zhu et al., 2011). The availability of an animal model for systematic testing of different antigens, immunization strategies, and defining correlates of protection should facilitate vaccine development as should growing information on protective immunological pathways in mice and humans (Imarai et al., 2008; Feinen et al., 2010; Gagliardi et al., 2011). An OMV-based vaccine demonstrated protection against strain MS11 (Plante et al., 2000) but was not successful in subsequent studies or with other strains (Zhu et al., 2011). As of yet, vaccine-induced protection of mice with other antigens has not been reported, although data we have obtained thus far are useful for evaluating the effectiveness of different immunization strategies in inducing local and systemic immune responses. A practical obstacle for vaccine studies

in the estradiol-treated mouse model is the need to immunize more mice than are actually challenged since only diestrus or anestrus stage mice can be used in the challenge phase of the experiment. Ovariectomized mice can be used to circumvent this problem since they do not need to be staged prior to estradiol treatment. Ovariectomized mice are more expensive, however, and we speculate that their innate responses may be compromised based on the absence of the cyclical recovery pattern in ovariectomized mice as discussed (Cole et al., 2010). Certain host restrictions should especially be considered when testing vaccines for humans. IgA1 protease and restrictions in the complement cascade may most significantly challenge the power of murine infection to predict vaccine efficiacy, particularly against serum resistant strains. The development of hC4BP and fH transgenic mice should therefore be a useful tool for vaccine testing (Ngampasutadol et al., 2008b). Passive delivery of purified fH or hC4BP could also be used as was recently shown to improve experimental infection of rats by *N. meningitidis* (Granoff et al., 2009).

Vaginal microbicides

The development of topically applied products for reducing the risk of STIs in women has been a public health priority in recent years. Topical agents that have been tested against Gc in the estradiol-treated mouse model include porphyrin binding proteins (Bozja et al., 2004), formulated natural, and synthetic sulfated and sulfonated polymers [CarraGuard™, Ushercell, T-PSS, PRO 2000™], acid-buffering agents [ACIDFORM, BufferGel™], and cellulose acetate phthalate (CAP; Spencer et al., 2004). With the exception of Carraguard, there was good correlation between the *in vitro* activity of each agent and effectiveness *in vivo*. Interestingly, CarraGuard prevented infection of mice but did not inhibit Gc *in vitro* (Spencer et al., 2004). This finding underscores the importance of testing products in an animal model system. Several of these products have since undergone safety and acceptiblity testing in humans (Carraguard, 2010; von Mollendorf et al., 2010).

Many challenges face the assessment of vaginal microbicide effectiveness in humans (Ramjee et al., 2010), but animal modeling can continue to play useful role in the development of these products for screening novel compounds and expanding testing protocols to include the assessment of repeated application of agents, which can affect susceptiblity (Cone et al., 2006). A limitation of the gonorrhea mouse model, like most STI models, is that it is not a transmission model and the bacterial suspension used to inoculate mice does not simulate the bodily fluids that transmit the microbe in terms of pH, biochemical make-up, and the presence of immune effectors and inhibitors of host defenses.

ANIMAL MODELING OF COINFECTIONS

An important charactersitic of STIs is the frequency by which more than one pathogen is present. The availability of well characterized models of gonorrhea (Song et al., 2008), chlamydia (Darville et al., 2003), and *M. genitalium* (McGowin et al., 2009) infection affords the opportunity to develop coinfection models for these three pathogens, which are major causes of PID. As many as 70% of individuals with gonorrhea have chlamydia (Miller et al., 2004) and we recently developed a gonorrhea chlamydia coinfection model for use as a research tool (Vonck et al., 2011). Significantly more vaginal PMNs were detected in coinfected mice compared to mice infected with either pathogen alone. This result is consistent with the reported higher levels of symptoms in individuals coinfected with Gc and *C. trachomatis* (Nsuami et al., 2004; Rosenvinge and Lau, 2009). Interestingly, higher numbers of Gc were recovered from coinfected mice compared to mice infected with Gc alone (Vonck et al., 2011). Continued use of the gonorrhea chlamydia coinfection model combined with human-based systems should illuminate the basis for these observations. The development of dually active therapeutic agents should also be accelerated by this model. Such agents are needed to simplify treatment regimens and potentially reduce the costs associated with therapy.

SUMMARY

The development of the estradiol-treated mouse model of Gc infection has expanded the research tools available for studying Gc genital tract infections. Researchers can now experimentally manipulate the host response and utilize mice that are genetically defective in immunological pathways and effector molecules to inform our understanding of the host response against gonorrhea, as has benefitted the study of many other infectious diseases. The growing availability of transgenic mice should improve the relevance of mice as surrogate hosts for Gc and provide an opportunity to test the biological significance of host-restricted bacterial–host cell interactions observed *in vitro*. The mouse model is also a useful system for studying hormonal influences on bacterial–host cell interactions, which is an exciting but understudied area. Finally, the mouse model has accelerated product testing, which is greatly needed for gonorrhea, and can be adapted to develop STI coinfection models.

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Experimental gonococcal infection in male volunteers: cumulative experience with *Neisseria gonorrhoeae* strains FA1090 and MS11mkC

Marcia M. Hobbs[1,2]*, P. Frederick Sparling[1,2], Myron S. Cohen[1,2], William M. Shafer[3,4], Carolyn D. Deal[5] and Ann E. Jerse[6]

[1] *Departments of Medicine, University of North Carolina, Chapel Hill, NC, USA*
[2] *Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, USA*
[3] *Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA*
[4] *Laboratories of Bacterial Pathogenesis, Veterans Affairs Medical Center (Atlanta), Decatur, GA, USA*
[5] *National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA*
[6] *Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA*

Edited by:
Cynthia N. Cornelissen, Virginia Commonwealth University School of Medicine, USA

Reviewed by:
Stanley Spinola, Indiana University–Purdue University at Indianapolis, USA
Catherine Ann Ison, Health Protection Agency, UK

****Correspondence:***
Marcia M. Hobbs, Department of Medicine, University of North Carolina, CB# 7031, Chapel Hill, NC 27599, USA.
e-mail: mmhobbs@med.unc.edu

Experimental infection of male volunteers with *Neisseria gonorrhoeae* is safe and reproduces the clinical features of naturally acquired gonococcal urethritis. Human inoculation studies have helped define the natural history of experimental infection with two well-characterized strains of *N. gonorrhoeae,* FA1090 and MS11mkC. The human model has proved useful for testing the importance of putative gonococcal virulence factors for urethral infection in men. Studies with isogenic mutants have improved our understanding of the requirements for gonococcal LOS structures, pili, opacity proteins, IgA1 protease, and the ability of infecting organisms to obtain iron from human transferrin and lactoferrin during uncomplicated urethritis. The model also presents opportunities to examine innate host immune responses that may be exploited or improved in development and testing of gonococcal vaccines. Here we review results to date with human experimental gonorrhea.

Keywords: gonorrhea, pathogenesis, infection, urethritis

INTRODUCTION

In nature, gonococcal infection is strictly limited to the human host. Uncomplicated infection can be asymptomatic or manifest as urethritis in men and cervicitis in women. Complications resulting from tissue invasion include orchitis, epididymitis and pelvic inflammatory disease, and systemic dissemination can lead to arthritis, tenosynovitis, or dermatitis. Like other inflammatory mucosal infections, gonorrhea also facilitates HIV transmission and acquisition. Treatment options for gonococcal infection are dwindling as worldwide, *Neisseria gonorrhoeae* is becoming increasing resistant to currently available antibiotics (Lewis, 2010). Experimental human urethral infection offers the potential to better understand the contributions of microbial factors and host immune responses to infection that may eventually translate into the development of an effective gonococcal vaccine and provides an appropriate model for vaccine testing.

The use of live *N. gonorrhoeae* to cause experimental infection has a long and sometimes sordid history dating back to the late eighteenth century when British surgeon John Hunter repeatedly inoculated patients with "venereal matter" to test whether venereal disease was infectious (Hunter, 1835). Recent reports of experimental gonorrhea studies conducted during the 1940s in vulnerable populations who were often deceived and infected without informed consent highlight the ethical issues that surround clinical research with human subjects (Frieden and Collins, 2010); the issues are particularly sensitive for experimental human infections with sexually transmitted pathogens. All of the studies described herein were subject to rigorous safety and ethical review by appropriate Institutional Review Boards. Written informed consent was obtained from all subjects in accordance with the ethical principles set forth in the Declaration of Helsinki and the US Code of Federal Regulations for the Protection of Human Subjects. Several hundred subjects have participated in experimental infection studies without reported complications or adverse events.

EXPERIMENTAL MODEL

Experimental gonorrhea is restricted to male subjects; women cannot safely be included due to potential complications from ascendant gonococcal infection. Inoculation is accomplished by the instillation of 0.2–0.3 mL of a gonococcal suspension through a pediatric catheter inserted approximately 5 cm into the urethra. There are slight variations in the preparation and delivery of experimental inocula used in previously published work detailed by Cohen and Cannon (1999), Cohen et al. (1994), and Schmidt et al. (2001). Following inoculation, infection can be monitored by bacterial recovery from urogenital specimens including urine, urethral swabs and semen. Gonococcal urethritis is defined by the development of urethral discharge containing gram negative diplococci or recovery of gonococci from a urethral swab culture. Host responses can be monitored by enumeration of white blood cells in urine and urethral exudates and measurement of soluble immune mediators including antibodies and cytokines in urine

and serum. Clinical disease is monitored by daily physical examination. Subjects receive prompt effective antibiotic treatment as soon as infection is clinically apparent, when requested because of intolerance for symptoms or at the end of the experimental trial, up to 7 days after inoculation, regardless of whether or not the subject was judged to be infected during the trial.

Two gonococcal strains, *N. gonorrhoeae* FA1090 and MS11mkC, have been used in the majority of experimental infection studies conducted over the past 20 years. FA1090 is a porin serotype PIB-3 strain originally isolated in the 1970s from the endocervix of a woman with probable disseminated gonococcal infection (Nachamkin et al., 1981) and has been used extensively in experimental infection studies conducted at the University of North Carolina at Chapel Hill (Cohen et al., 1994; Jerse et al., 1994; Seifert et al., 1994; Cornelissen et al., 1998; Cohen and Cannon, 1999; Johannsen et al., 1999). MS11 is a porin serotype PIB-9 strain originally isolated in 1970 from a patient with uncomplicated anterior urethritis (Edwards et al., 1984; Swanson et al., 1985). MS11mk is descended from MS11, and MS11mkC is a lipooligosaccharide (LOS) variant isolated from an experimentally infected male volunteer in the late 1980s (Schneider et al., 1991). MS11mkC was used in subsequent volunteer studies conducted at the Walter Reed Army Institute of Research (Ramsey et al., 1995; Schneider et al., 1995, 1996; Schmidt et al., 2000, 2001).

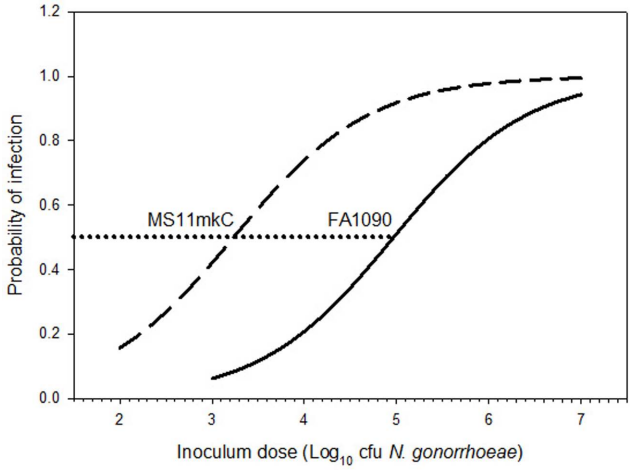

FIGURE 1 | *Neisseria gonorrhoeae* strain MS11mkC is more infectious than strain FA1090 in experimental infection of the male urethra. Multiple logistic regression analysis was used to generate dose response models with data from 55 men inoculated with MS11mkC (dashed line) and 43 men inoculated with FA1090 (solid line). All inocula contained predominantly piliated, Opa-negative, wild-type gonococci. The estimated ID_{50} (indicated by the horizontal dotted line) is 1.8×10^3 cfu for MS11mkC and 1.0×10^5 cfu for FA1090.

N. GONORRHOEAE MS11mkC IS MORE INFECTIOUS THAN FA1090 IN THE MALE URETHRA

Too few subjects can be included in individual experimental trials to reliably evaluate the infectivity of a given inoculum. However, the cumulative experience to date includes sufficient numbers of subjects and a wide enough range of doses to estimate the relative infectivities of the two strains. We analyzed available data from 55 men inoculated with wild-type MS11mkC at doses from 2.5×10^2 to 5.7×10^4 colony-forming units (cfu; Schneider et al., 1995; Schmidt et al., 2001 and unpublished data from J. G. Cannon) and 43 men inoculated with wild-type FA1090 at doses from 1.0×10^4 to 3.5×10^6 cfu (Cohen et al., 1994 and unpublished data from J. G. Cannon and M. M. Hobbs). We conducted multiple logistic regression analyses using SigmaStat version 3.5 for Windows (Systat Software, Inc., Point Richmond, CA, USA) to predict the probability of infection over a range of doses for each strain (**Figure 1**). The quality of the fit for each dose response curve was tested using the Hosmer–Lemeshow statistic (Hosmer and Lemeshow, 2000). A non-significant P value (>0.05) indicates a good fit between the logistic equation and the raw data; $P = 0.369$ for MS11mkC, and $P = 0.878$ for FA1090. The estimated dose resulting in infection of 50% of inoculated subjects (ID_{50}) is 1.8×10^3 cfu for MS11mkC and 1.0×10^5 cfu for FA1090. Similar differences in infectivity for the two strains have been observed in the murine model of female genital tract infection (Jerse, 1999).

The reasons for the apparent nearly 2 $\log_{10}$ difference in ID_{50} between FA1090 and MS11mkC for urethral infection are not clear. Great care has been taken to describe and control (as much as possible) the expression of recognized phase-variable determinants in experimental inocula. Inoculum variants of both strains used in the experimental infections included in the dose response analyses contained predominantly piliated (P+), Opacity protein-negative (Opa−) gonococci expressing the lacto-*N*-neotetraose LOS epitope recognized by monoclonal antibody (Mab) 3F11, which is associated with the development of urethral discharge in naturally acquired and experimental gonococcal infection in men (Schneider et al., 1991). Strains FA1090 and MS11 have been extensively characterized in numerous laboratories, and there are many recognized differences. Though not an exhaustive list, **Table 1** summarizes some of the features that differ between FA1090 and MS11mkC, potentially influencing infectivity in the experimental model of urethral infection.

The relatively recent human passage of MS11mkC compared to FA1090 may also have resulted in unrecognized differences between the two strains. Though inocula prepared from both strains have been described as piliated, it is possible that MS11mkC is hyperpiliated compared to FA1090.

NATURAL HISTORY OF EXPERIMENTAL INFECTION WITH WILD-TYPE GONOCOCCI

Despite the apparent difference in infectivity, the course of experimental infection and development of clinical signs and symptoms caused by wild-type piliated, Opa-negative inocula of *N. gonorrhoeae* strains FA1090, and MS11mkC are very similar. The clinical and microbiological courses of infection in individual infected subjects have been described previously (Schneider et al., 1991, 1995, 1996; Cohen et al., 1994; Jerse et al., 1994; Cohen and Cannon, 1999; Schmidt et al., 2001) and are summarized here. Gonococci are recovered at low levels in urine from most subjects 2 h after inoculation. A so-called eclipse period of variable length ensues during which few or no viable gonococci are recovered from urine. Then, infected individuals begin to

Table 1 | Recognized differences between *N. gonorrhoeae* strains FA1090 and MS11mkC.

Characteristic	FA1090	MS11mkC	References
Serum susceptibility	Resistant	Sensitive (characterized as Intermediate by some)	Cohen et al. (1994), Ram et al. (2001)
Gonococcal genetic island	Absent	Present	Dillard and Seifert, (2001)
Lactoferrin utilization (expression of lactoferrin-binding proteins B and A)	Lf− (LbpB-A−)	Lf+ (LbpB-A+)	Anderson et al. (2003)
Mtr efflux pump expression	Not inducible due to 11bp deletion in the coding region of activator *mtrA*; wt *mtrCDE* promoter	Inducible with wt *mtrA*; higher expression of pump due to novel *mtrCDE* promoter and missense mutation in repressor *mtrR*	Rouquette et al. (1999), Warner et al. (2008), and W. M. Shafer (unpublished data)

Lf, lactoferrin; Lbp, lactoferrin-binding protein; Mtr, multiple transferable resistance; bp, base pair; wt, wild-type.

shed increasing numbers of organisms in the urine, though the number of gonococci recovered from infected subjects does not correlate with the severity of infection. Bacteriuria persists until signs and symptoms of infection including dysuria and urethritis develop. Most men experimentally infected with wild-type gonococci develop gonococcal disease characterized by a urethral discharge and dysuria, consistent with the clinical presentation of naturally acquired gonorrhea. The incubation period between inoculation and development of signs or symptoms of infection is variable, ranging from 1 to 6 days for both FA1090 and MS11mkC and is independent of the number of gonococci in the inoculum. Though rare, asymptomatic experimental infection with wild-type FA1090 variants occurs and is characterized by recovery of gonococci from urine in the absence of signs or symptoms during the 5- to 6-day trial period (J. G. Cannon and A. E. Jerse, unpublished data); asymptomatic infection with MS11mkC has not been reported.

Cellular and soluble mediators of the host response to experimental infection follow a similar pattern. Pyuria, as determined by the presence of white blood cells in urine sediment (Cohen et al., 1994; Ramsey et al., 1995), and elevated urinary interleukin (IL)-6 and IL-8 (Ramsey et al., 1995) are frequently observed within the first 24 h after inoculation. Whether the early appearance of these inflammatory mediators is the result of mild irritation caused by the insertion of the catheter during inoculation or a direct response to gonococci is not clear. In subjects who remain uninfected, pyuria resolves (Cohen et al., 1994; Ramsey et al., 1995), and cytokines quickly return to pre-challenge levels (Ramsey et al., 1995). Pyuria persists in infected subjects, and the numbers of white blood cells in urine generally increase until men develop symptoms and require treatment (Cohen et al., 1994; Ramsey et al., 1995). Elevated levels of inflammatory cytokines IL-6, IL-8, TNFα and IL-1β are consistently detected in urine in response to experimental infection with MS11mkC or MS11mkA, (an LOS variant lacking the 3F11 epitope; Schneider et al., 1991; Ramsey et al., 1995); similar cytokine studies have not been conducted with strain FA1090. The cytokine responses detected in urine during experimental gonorrhea are comparable to those seen during bacterial urinary tract infections (Sheu et al., 2006, 2007). During experimental gonococcal urethritis, inflammatory host response indicators generally peak at the onset of symptoms and return to pre-inoculation levels within 2 days after treatment (Ramsey et al., 1995). Elevated inflammatory cytokines in peripheral circulation are also detected during experimental infection, returning to baseline after treatment. Cytokines in urine are uniformly detectable in infected subjects with elevated plasma cytokines. However, elevated urinary cytokine levels in the absence of concomitant increases in plasma in some infected subjects suggest local involvement in inflammatory responses to experimental urethral infection (Ramsey et al., 1995).

Because experimentally infected subjects are treated at the onset of signs or symptoms of infection, assessment of immune responses is limited to those that occur during the early stages of infection such as the innate immune responses described above. Acquired immune responses including production of specific antibodies are generally thought to require longer exposure than occurs during experimental infection. However, Schneider and colleagues demonstrated increases in serum IgG antibodies recognizing gonococcal LOS in 8 of 14 subjects (57%) experimentally infected for 3–7 days with an ID_{90} of MS11mkC (Schmidt et al., 2001). Interestingly, increased anti-LOS antibody titers were significantly associated with resistance to homologous reinfection, though equivalent proportions of previously infected and naïve subjects were infected with an ID_{50} challenge (Schmidt et al., 2001). Men who experienced a fourfold or greater increase in anti-LOS IgG titer during initial infection were more likely to resist reinfection than men with lower and static LOS antibody titers during initial infection. The mechanisms by which anti-LOS antibodies in serum could protect from urethral gonococcal infection are not clear. Increases in these circulating antibodies may be a surrogate for protective mucosal immune responses, or transudation to the urethral mucosa could result from early inflammation in experimental infection. Apicella and colleagues have shown that gonococcal entry into primary human urethral epithelial cells is dependent on LOS interactions with the asialoglycoprotein receptor (Harvey et al., 2001), and van Kooyk and colleagues demonstrated that gonococcal LOS influences dendritic cell function and subsequent immune responses that promote bacterial survival (van Vliet et al., 2009). Mucosal anti-LOS antibodies could interfere with these processes at very early stages of infection and

potentially result in host immune responses that favor bacterial clearance.

EXPRESSION AND ANTIGENIC VARIATION OF GONOCOCCAL SURFACE STRUCTURES *IN VIVO*

The remarkable variation in surface structures expressed by different *N. gonorrhoeae* strains and the extraordinary plasticity of the bacterial surface that an individual strain can present to its infected host are hallmarks of the gonococcus that likely contribute to high rates of recurrent gonococcal infection. The frequent modulation of bacterial surface determinants results from a combination of phase variation (the switch between on and off states of gene expression) and antigenic variation (the expression of different antigenic versions of a structure) that result in differential expression of outer membrane components including pili, Opa proteins, and LOS (reviewed in Edwards and Apicella, 2004). The genetic mechanisms that result in phase and antigenic variation have been extensively characterized (reviewed in Simms and Jerse, 2005), and *in vitro* models of gonococcal interactions with host cells indicate that different antigenic versions of pilin (the major pilus subunit), Opa proteins, and LOS recognize different host cell receptors, consistent with differential tissue tropism and pathogenesis of naturally occurring gonococcal variants (Virji, 2009). Phenotypic variation of these surface determinants occurs during experimental infection in the male urethra.

LOS VARIATION AND MODIFICATION DURING EXPERIMENTAL URETHRAL INFECTION

Production of multiple LOS structures occurs within a gonococcal strain as well as within individual colonies of a given strain. Alterations in LOS structures result from phase-variable production of enzymes involved in extending carbohydrate structures from the α- and β-chains of the core oligosaccharide. Variation in α-chain residues is due to changes in polynucleotide repeat elements in the *lgtA*, *lgtC,* and *lgtD* genes, encoding glycosyl transferases, organized in an operon that also includes *lgtB* and *lgtE*, whereas β-chain variation is due to alterations in a polynucleotide repeat in *lgtG* (Banerjee et al., 1998). The different LOS structures resulting from "phase-on" or "phase-off" expression of these genes, as well as variable modifications of the oligosaccharide core (e.g., attachment of phosphoethanolamine [PEA] to heptose I and II), can impact the biologic properties of gonococci, altering bacterial susceptibility to mediators of innate and acquired immune host defense and attachment to host cells. A critical question is whether different structures provide an advantage for gonococci during infection and at different stages (e.g., before or after development of inflammation). The importance of the lacto-*N*-neotetraose paraglobosyl epitope of gonococcal LOS was recognized early in the history of experimental infection with strain MS11mk. Following inoculation of male volunteers with MS11mkA, which only produced a 3.6-kD lactosyl LOS and lacked higher molecular weight gangliosyl and paraglobosyl structures, gonococci isolated at the onset of dysuria expressed gangliosyl LOS moieties, and bacteria isolated at the onset of urethral discharge (including the MS11mkC variant) expressed paraglobosyl structures (Schneider et al., 1991; John et al., 1999). Gonococci isolated from symptomatic men with naturally acquired gonorrhea also widely express gangliosyl or paraglobosyl LOS, but rarely express the lactosyl moiety (Schneider et al., 1991). In the MS11 background, expression of paraglobosyl LOS increased the apparent infectivity of the mkC variant compared to the mkA variant (5/5 vs. 2/5 inoculated volunteers infected with 4×10^4 cfu, Schneider et al., 1995), suggesting that this structure is important in the pathogenesis of gonococcal urethritis. However, *N. gonorrhoeae* strain FA1090, which is less infectious than MS11mkC in the human model, also expresses a paraglogosyl LOS. Thus, the relative infectivity of the two different strains cannot be attributed to expression of this structure.

Taken together, the observations described above suggest that gonococci producing gangliosyl or paraglobosyl LOS species are at a selective advantage during infection of the male urethra. To directly test this idea, Shafer and colleagues are constructing a variant of *N. gonorrhoeae* FA1090 that should not express gangliosyl or paraglobosyl LOS due to an internal deletion that removes most of *lgtA* and *lgtC* and all of *lgtB*. This mutant should only express the 3.6-kD lactosyl LOS structure. Competitive experimental infections with inocula containing this non-variable LOS mutant and wild-type strain FA1090, which can freely phase vary its LOS structures, could determine whether phase-variable production of LOS structures is important during infection. If the ability to alter LOS structures during urethral infection is advantageous, the "locked-in" mutant is predicted to have a fitness defect or disadvantage *in vivo*.

The lipid A core of gonococcal LOS has endotoxic activity, and recent evidence indicates that PEA modification at the 4′ position is an important determinant in the capacity of strains to resist complement-mediated killing by normal human serum (Lewis et al., 2009; Balthazar et al., 2011). The presence of PEA can also enhance resistance to cationic antimicrobial peptides. Interestingly, PEA modification at heptose II does not impart such resistance. Shafer and co-workers have constructed an *lptA* deletion mutant of *N. gonorrhoeae* strain FA1090 (W. M. Shafer, unpublished data). Mixed experimental infections with this mutant, which cannot modify lipid A with PEA, and wild-type FA1090 will test whether PEA-containing lipid A provides a fitness advantage to gonococci during urethral infection.

The terminal paraglobosyl oligosaccharide recognized by Mab 3F11 and expressed by MS11mkC and FA1090 variants used in experimental infection studies to date can be modified by gonococcal sialyltransferase, resulting in the addition of host-derived sialic acid to the bacterial surface, mimicking human cell surface antigens (Mandrell et al., 1988, 1990; Apicella et al., 1990; Parsons et al., 1994). Sialylation of gonococcal LOS *in vitro* increases serum resistance of intrinsically serum sensitive strains (Griffiss et al., 1991), promotes gonococcal resistance to killing by human neutrophils (Kim et al., 1992; Rest and Frangipane, 1992; Wetzler et al., 1992; Gill et al., 1996), and protects *N. gonorrhoeae* from the bactericidal activity of porin and LOS-specific antibodies (Parsons et al., 1989; Elkins et al., 1992; Wetzler et al., 1992; Gill et al., 1996). Furthermore, immunoelectron microscopic analysis of gonococci in urethral secretions from men with naturally acquired gonorrhea indicates that sialylation occurs during infection (Apicella et al., 1990). However, sialylation of MS11mkC prior to urethral inoculation appears to reduce infectivity of this strain and lengthen

the incubation period for development of symptomatic urethritis (Schneider et al., 1996). Sialylation also reduces gonococcal invasion of primary urethral epithelial cells *in vitro* (Harvey et al., 2001). These observations suggest that gonococcal sialylation in the male urethra may occur after the initial stages of infection. The presence or absence of sialylation of LOS may also be important for bacterial survival during different phases of infection or in different compartments during infection. For example, non-sialylated gonococci may more easily enter urethral epithelial cells, whereas sialylation maybe provide protection from the bactericidal effects of transudated serum that could affect extracellular bacteria on the urethral mucosa. *N. gonorrhoeae* likely alternates between intracellular and extracellular compartments during infection, and variable LOS sialylation may facilitate transitions between these compartments.

Sialyltransferase (*lst*) mutants of *N. gonorrhoeae* MS11mkC and FA1090 have been tested in the experimental human model, and *lst*-deficient gonococci from both backgrounds are able to cause infection in the male urethra (J. G. Cannon, unpublished data). After inoculation with an *lst* null mutant in the serum resistant FA1090 background at an approximate ID_{80} for wild-type FA1090, three out of four subjects became infected. FA1090*lst* gonococci were recovered from the urine of all three infected subjects; two developed urethral discharge during the 5-days after inoculation, and the third remained asymptomatic during the experimental trial. In the relatively serum sensitive MS11mkC background at an approximate ID_{80} for the wild-type strain, four out of eight subjects became infected after inoculation with an *lst* null mutant. MS11mkC*lst* gonococci were recovered from the urine of all four infected subjects; two developed urethral discharge during the 5-days after inoculation, and the other two infected subjects remained asymptomatic during the experimental trial.

Because asymptomatic experimental infection with wild-type MS11mkC is rare, the lack of sialyltransferase activity may have altered the pathogenesis of this strain in experimental urethral infection. However, experimental human infection trials with small numbers of subjects receiving individual inocula containing only mutant or wild-type gonococci often does not provide sufficient power to demonstrate reduced infectivity or pathogenesis with statistical confidence. In experimental infection of female mice inoculated with wild-type MS11 or an isogenic *lst* mutant, Wu and Jerse saw no significant difference in infectivity, consistent with the results of experimental human infection. Using more sensitive competitive infections in mice inoculated with mixtures of wild-type gonococci and similar numbers of the *lst* mutant, bacteria lacking *lst* were significantly attenuated in the capacity to colonize the lower female murine genital tract (Wu and Jerse, 2006). Competitive infections with mixed inocula in experimental human infection may reveal an advantage for expression of sialyltransferase in MS11 and/or FA1090 in the male urethra that was not apparent in previous infections with pure inocula.

The effects of differences in LOS expression on experimental *N. gonorrhoeae* infection of male volunteers are summarized in **Table 2**.

OPACITY (OPA) PROTEIN EXPRESSION DURING EXPERIMENTAL URETHRAL INFECTION

Individual *N. gonorrhoeae* strains possess 11 or 12 *opa* genes, each of which is subject to reversible, high frequency changes in expression state. As a result, individual gonococci can express one or multiple different Opa proteins, or none at all. Sequence diversity in regions of *opa* genes encoding antigenic determinants exposed on the bacterial surface confers specificities for different receptors on human cells. Most Opa variants bind to human carcinoembryonic antigen cell adhesion molecule (CEACAM) family receptors, however a small number of Opa proteins bind heparin sulfate proteoglycans (HSPGs). Opa-mediated interactions between gonococci and host cells can activate neutrophils or suppress activation and proliferation of CD4+ T lymphocytes, depending on which receptors are engaged (reviewed in Sadarangani et al., 2011). Opa protein repertoires of different *N. gonorrhoeae* strains differ in primary amino acid sequences, and the nomenclature in the literature describing individual Opa proteins in different strains is ambiguous. The *opa* genes of strains FA1090 and MS11 show little homology in the variable regions encoding antigenic portions of the proteins (Connell et al., 1990; Bhat et al., 1991), and proteins referred to as OpaA, OpaB, etc., are not the same in the two strains.

Early observations by James and Swanson (1978) documented that gonococci isolated from men with urethritis predominantly formed the characteristically opaque colonies associated with Opa protein expression. There is also strong selection for expression of Opa proteins in the male urethra following inoculation with wild-type variants of FA1090 or MS11mkC that are predominantly

Table 2 | Effects of lipoologosaccharide (LOS) features on experimental gonorrhea.

Phenotype tested	Strain background	Experimental outcome	References
Natural phase variation resulting in changes in LOS structures	MS11mkC	Expression of paraglobosyl structures favored during infection	Schneider et al. (1995)
Ability to sialylate paraglobosyl LOS structures	FA1090 (serum resistant)	Sialyltransferase (*lst*) mutant as infectious as wild-type with pure inocula	J. G. Cannon (unpublished data)
	MS11mkC (serum sensitive)	Trend toward lower infectivity for *lst* mutant compared to wild-type with pure inocula	J. G. Cannon, (unpublished data)

LOS, lipooligosaccharide; lst, gene encoding gonococcal sialyltransferase.

Opa-negative (Jerse et al., 1994; Schmidt et al., 2000). Schneider and colleagues showed that transition from Opa-negative to Opa-positive MS11mkC gonococci shed by experimentally infected men is associated with the onset of symptoms (Schneider et al., 1995, 1996; Schmidt et al., 2000). Using strain FA1090, Jerse et al. (1994) showed that not only are Opa+ variants uniformly isolated from urine and urethral swab cultures from experimentally infected men, the proportion of isolates expressing multiple Opa proteins increases over time. No single Opa protein in either strain's repertoire is selectively expressed during experimental infection; different Opas predominated among colonies isolated from individual subjects. However, there are Opa proteins in each strain's repertoire that appear to be under-represented among isolates from experimentally infected subjects (Jerse et al., 1994; Schmidt et al., 2000).

Following inoculation with predominantly Opa-negative *N. gonorrhoeae* FA1090, variants expressing this strain's OpaA alone were not isolated from any of the subjects, and only a small number of reisolates expressed OpaA in conjunction with one or more other Opa proteins (Jerse et al., 1994). Opa-expressing FA1090 have also been used to initiate experimental urethral infections. Inoculation with 10^4 cfu of FA1090 expressing predominantly OpaA (along with the 3F11 LOS epitope and 98.8% identical pilin amino acid sequence compared to the Opa-negative inoculum variant) resulted in infection in four out of five subjects, and three of the four infected subjects developed acute urethritis within 1–3 days after inoculation. The fourth subject shed gonococci in his urine, but remained asymptomatic during the 4-day trial. Gonococci isolated at the onset of signs of acute urethritis resulting from the predominantly OpaA inoculum continued to express OpaA, but in conjunction with one or more other Opa proteins. Gonococci expressing multiple Opa proteins represented a markedly higher proportion of isolates recovered from subjects inoculated with FA1090 OpaA (mean 96.7%, range 92–100%; $n = 3$ subjects) than was observed with predominantly Opa-negative inocula (mean 47.8%; range 18–100%; $n = 9$ subjects) or with an FA1090 variant expressing predominantly OpaF (mean 28.3%; range 7–75%; $n = 3$ subjects; J. G. Cannon and A. E. Jerse, unpublished data). In contrast to FA1090 OpaA, the OpaF protein was highly represented among reisolates from subjects inoculated with Opa-negative FA1090 (Jerse et al., 1994). Thus, gonococci expressing OpaA alone appeared to be at a disadvantage during experimental urethral infection.

Rest and colleagues demonstrated that, in contrast to other Opa variants of FA1090, OpaA variants are resistant to neutrophil killing *in vitro* (Fischer and Rest, 1988; Elkins and Rest, 1990). The relevance of *in vitro* Opa-mediated stimulation of neutrophils to urethral pathogenesis is not clear. One might predict that resistance to neutrophil killing would provide an advantage for OpaA expression during infection. However, the rarity of OpaA expression following inoculation with Opa-negative gonococci, and the additional expression of other Opa proteins following inoculation with OpaA expressers confound this hypothesis, as presumably multiple expressers would be killed by neutrophils. These results suggest that FA1090 OpaA may not mediate key function(s) required for gonococcal survival in the male urethra.

The rapid and uniform shift from Opa-negative to Opa protein expression by gonococci in the male urethra exhibited by both wild-type strains FA1090 and MS11mkC suggested that the capacity to express opacity proteins may be required during urethral infection, and an Opa-deficient FA1090 mutant was predicted to be attenuated in the human experimental model. FA1090*opaA-K* is a genetically defined mutant in which all 11 *opa* genes of this strain have been inactivated (Cole et al., 2010). Inoculation with FA1090*opaA-K* at doses ranging from 5.5 to 6.4 Log_{10} cfu resulted in infection in four out of eight subjects (J. G. Cannon, unpublished data). FA1090*opaA-K* gonococci were recovered from the urine of all four infected subjects; three of the four developed urethral discharge within 4 days after inoculation, the fourth remained asymptomatic during the 5-day experimental trial. Thus, Opa protein expression is not required for gonococcal infection in the male urethra.

Opa protein expression does confer an advantage in the late stages of female murine genital tract using FA1090*opaA-K* and a complemented strain that constitutively expresses OpaB, with statistically significant differences in colonization load observed on days 9–14 of infection (Cole et al., 2010). Whether a similar advantage for Opa expression could be demonstrated in the human male urethra is not clear. Experimental infection in men is limited to earlier stages of infection than can be studied using the murine model; an advantage for Opa protein expression that did not manifest until later stages of urethral infection could not be observed in the human model. The murine and human genital tracts differ in expression of receptors for Opa proteins, and the selective pressures influencing Opa protein expression in the male and female human genital tracts likely differ. Thus, the apparent advantage conferred by Opa expression in the female murine genital tract may not apply to urethral infection in men.

PILIN EXPRESSION AND VARIATION *IN VIVO*

Gonococcal type 4 pili are important for attachment to host cells, and phase and antigenic variation of PilE, the major pilin protein, are thought to provide the gonococcus with mechanisms of immune evasion and tissue tropism (reviewed in Virji, 2009). Gonococci recovered from men and women with symptomatic, naturally acquired gonorrhea are uniformly piliated (Kellogg et al., 1963, 1968). Early experimental infections of male volunteers (with *N. gonorrhoeae* strain F62) showed that piliated gonococci were infectious and produced purulent urethral exudates, and virulence was retained after extensive selective passage *in vitro* (Kellogg et al., 1963). In contrast, though non-piliated inocula were infectious, infected men developed a watery urethral discharge or none at all (Kellogg et al., 1968). Furthermore, after extensive passage *in vitro*, non-piliated F62 gonococci were non-infectious (Kellogg et al., 1963). Regardless of whether piliated or non-piliated inocula were used, gonococci recovered from infected subjects were uniformly piliated (Kellogg et al., 1963, 1968).

Rapid and extensive antigenic variation of PilE has been shown to occur during experimental urethral infection with *N. gonorrhoeae* FA1090 (Seifert et al., 1994; Wright et al., 1994; Hamrick et al., 2001) and MS11mk (Swanson et al., 1987). These

observations suggested that type 4 pilus expression may be required for urethral gonococcal infection, and a non-reverting, non-piliated FA1090 mutant was predicted to be non-infectious in the human experimental model. The promoter and 5′ end of the single pilin expression locus was deleted in FA1090*pilE* (Cannon et al., 1996). Inoculation with approximately 10^6 cfu (∼ID_{80} for piliated wild-type FA1090) of Opa-negative FA1090*pilE* expressing the paraglobosyl LOS epitope recognized by Mab 3F11 resulted in infection in six out of eight subjects (J. G. Cannon, unpublished data). Non-piliated gonococci were cultured from urine from all infected subjects. Three of the six subjects infected with the *pilE* mutant developed a watery urethral discharge, and the remaining subjects remained asymptomatic throughout the 5-day experimental trial, consistent with Kellogg et al. (1963, 1968) earlier observations with phase-variable, non-piliated inocula that switched to pilus expression during infection. These results suggest that host responses to piliated and non-piliated gonococci may differ during early stages of urethral infection. The fact that pilus expression is not required for infection in men suggests there may be a role for alternative adhesins in urethral colonization.

Although FA1090*pilE* does not elaborate a pilus fiber, this mutant does express the pilus-associated PilC protein, which can be present both in pilus fibers and in the gonococcal outer membrane (Rudel et al., 1995; Rahman et al., 1997). PilC is important for adherence of piliated gonococci to human epithelial cells (Rudel et al., 1992; Nassif et al., 1994; Kirchner and Meyer, 2005), and in the absence of PilE, PilC has also been shown to increase adherence of FA1090 to epithelial cells *in vitro*, though the effect of PilC expression on non-piliated gonococci is modest compared to adherence of piliated bacteria (C. E. Thomas, unpublished data). Mixed experimental infections with FA1090*pilE* expressing PilC and a mutant that expresses neither PilE nor PilC may help elucidate the roles of these two proteins in gonococcal pathogenesis in the male urethra.

The effects of opacity protein and pilin expression on experimental *N. gonorrhoeae* infection of male volunteers are summarized in **Table 3**.

IgA PROTEASE IS NOT REQUIRED DURING EXPERIMENTAL GONOCOCCAL URETHRITIS

IgA1 protease production is associated with mucosal pathogens including *N. gonorrhoeae*, *N. meningitidis*, and *Haemophilus influenzae* (Kilian et al., 1996). In addition to its ability to cleave IgA1 at mucosal surfaces, gonococcal IgA1 protease also cleaves the lysosome-associated membrane protein 1 (Lamp1) promoting intracellular bacterial survival in epithelial cells *in vitro* (Lin et al., 1997; Ayala et al., 2002). These observations suggested that the enzyme may contribute to gonococcal pathogenesis. Cannon and colleagues showed that a *N. gonorrhoeae* FA1090 *iga* mutant (Opa−, P+, and expressing the 3F11 LOS epitope) that lacks IgA1 protease activity is fully capable of causing urethritis in male volunteers (Johannsen et al., 1999).

IgA1 protease could be more important in later stages of infection than can be assessed in the human model of uncomplicated urethritis in naïve volunteers. Protease function could contribute to later-stage invasive complications, or may play a role in reinfection of previously exposed individuals by inactivating pre-existing mucosal antibodies (Johannsen et al., 1999). The role of IgA1 protease in gonococcal infection in women has not been tested.

IMPORTANCE OF TRANSFERRIN AND LACTOFERRIN RECEPTORS IN GONOCOCCAL PATHOGENESIS

Like all *N. gonorrhoeae* strains, FA1090, and MS11mkC can use iron from human transferrin (Tf) for growth. One of the notable differences between these two strains (**Table 1**) is their differential ability to obtain iron from human lactoferrin (Lf). MS11mkC expresses lactoferrin-binding protein A (LbpA) and is phenotypically Tf+Lf+, whereas FA1090 has a naturally occurring deletion that eliminates expression of LbpA and is unable to grow on lactoferrin as a sole source of iron (Anderson et al., 2003). Thus, wild-type FA1090, like approximately half of clinical isolates of *N. gonorrhoeae* that have been examined (Mickelsen et al., 1982; Fox et al., 1998; Anderson et al., 2001), is phenotypically Tf+Lf−. Cornelissen et al. (1998) showed that a transferrin-receptor mutant of FA1090, which cannot obtain iron from either Tf or Lf, is incapable of causing urethritis in the experimental human gonorrhea model.

Table 3 | Effects of opacity protein (Opa) and pilin (PilE) expression on experimental gonorrhea.

Phenotype tested	Strain background	Experimental outcome	References
Natural phase variation resulting in changes in Opa expression	MS11mkC and FA1090	Opa expression favored during infection after inoculation with Opa-negative variant	Jerse et al. (1994), Schmidt et al. (2000)
Requirement for Opa expression	FA1090	Mutant unable to express Opa proteins as infectious as wild-type	J. G. Cannon (unpublished data)
Natural antigenic variation in PilE protein	MS11mkC and FA1090	Extensive variation observed during infection with piliated variant	Seifert et al. (1994), Wright et al. (1994), Hamrick et al. (2001), Swanson et al. (1987)
Requirement for PilE expression	FA1090	Non-piliated mutant unable to express PilE as infectious as wild-type	J. G. Cannon (unpublished data)

Opa, opacity protein; PilE, pilin protein.

Sparling and colleagues showed that a *N. gonorrhoeae* FA1090 engineered mutant capable of using Lf but not Tf, a combination not seen in nature, causes experimental urethral infection in male volunteers that is similar to infections caused by wild-type FA1090 (Anderson et al., 2003). Thus iron from either Tf or Lf enables gonococcal growth and pathogenesis in the male urethra.

To determine whether the ability to use human Lf provides an advantage to gonococci that also can use human Tf, Sparling and colleagues conducted competitive infections with wild-type FA1090 (Tf+Lf−) and a Tf+Lf+ mutant. Inoculation with an approximate ID_{50} for wild-type FA1090 containing a mixture of equivalent numbers of both strains resulted in infection in nearly half of subjects. In each of five infected subjects, the Tf+Lf+ strain exhibited a significant competitive advantage over wild-type FA1090; urine and urethral swab cultures from all subjects contained 100% mutant gonococci at the end of the experimental trial (Anderson et al., 2003). Thus, the experimental model using a competitive infection study design has the potential to demonstrate differences in fitness between gonococcal strains with statistical confidence, even when the overall infectivity of the two strains is not demonstrably different.

The requirements for IgA protease and iron acquisition from human iron-binding proteins during experimental *N. gonorrhoeae* infection of male volunteers are summarized in **Table 4**.

POPULATION DYNAMICS IN EXPERIMENTAL INFECTION

Tracking the variation of opacity and pilin proteins during experimental infection with wild-type *N. gonorrhoeae* reveals that the phenotype of infecting bacteria changes multiple times in the interval between inoculation and the development of urethral discharge (Jerse et al., 1994; Seifert et al., 1994; Wright et al., 1994; Schmidt et al., 2000; Hamrick et al., 2001). As shown schematically in **Figure 2**, inoculum variants disappear rapidly and are replaced by a series of new variants expressing different combinations of surface antigens. The gonococcal population becomes more complex as infection proceeds, consisting of a mixture of multiple variants at the time a urethral discharge is present. These changes are consistent with sequential outgrowth and disappearance of clonal populations expressing combinations of Opa and PilE and possibly other traits. The eclipse period that occurs immediately after inoculation could result in a population bottleneck, with the subsequent gonococcal population expanding from one or a few organisms present in the inoculum. New variants generated through phase and antigenic variation may descend from a single organism; these new variants proliferate if they express advantageous combinations of traits and eventually replace the previous population.

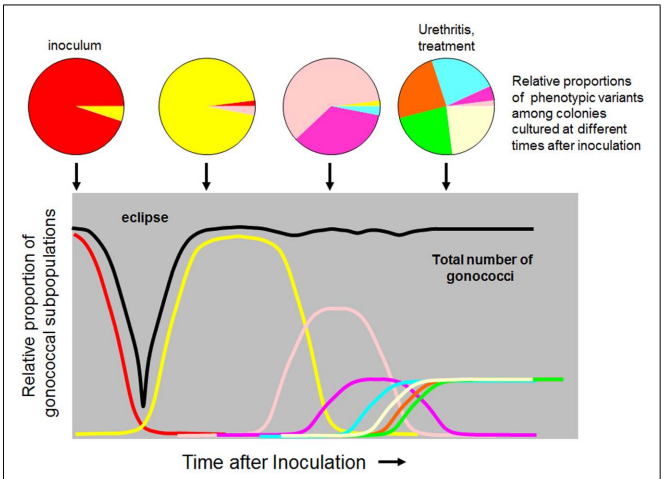

FIGURE 2 | Population dynamics during experimental gonococcal infections. The schematic representation depicts an inoculating population that is predominantly composed of one combination of variable traits, represented by red in the first pie chart and the red trace in the graph below. Following the eclipse period during which few gonococci are recovered, new variants, indicated by different colors, emerge. The population of infecting gonococci becomes increasingly complex over time. The colors represent different phenotypic variants that could arise by phase and/or antigenic variation of opacity and pilin proteins and are based on previously reported observations (Jerse et al., 1994; Seifert et al., 1994; Wright et al., 1994; Hamrick et al., 2001) but are not intended to represent specific characteristics of actual reisolates from an experimental infection.

The selective pressures that could result in the sequential outgrowth and disappearance of gonococcal variants are not known. However, the possibility that very few bacteria emerge from a

Table 4 | Requirements for IgA protease and iron acquisition from human iron-binding proteins during experimental gonorrhea.

Phenotype tested	Strain background	Experimental outcome	References
Requirement for IgA protease activity	FA1090	Mutant unable to express IgA protease as infectious as wild-type	Johannsen et al. (1999)
Requirement for lactoferrin (Lf) utilization in the absence of transferrin (Tf) utilization	FA1090	Mutant unable to use either Lf or Tf is non-infectious	Cornelissen et al. (1998)
Ability to use Lf in the absence of Tf utilization	FA1090	Mutant able to use Lf only as infectious as wild-type that uses Tf only	Anderson et al. (2003)
Ability to use both Lf and Tf	FA1090	Mutant able to use both Lf and Tf showed competitive advantage over wild-type in mixed infections	Anderson et al. (2003)

IgA, immunoglobulin A; Lf, lactoferrin; Tf, transferrin.

selective bottleneck to initiate infection following the initial eclipse period could confound interpretation of results from mixed experimental infections with isogenic strains differing in expression of a postulated virulence factor. **Figure 3A** shows theoretical outcomes of mixed infection initiated under conditions with and without the hypothetical bottleneck. In the absence of a dramatic population restriction, gonococcal populations reisolated from infected subjects would be expected to contain mixtures of the two strains in similar proportions to their representation in the inoculum, if they have equal fitness during infection (Pattern A). If one strain has a competitive advantage, this strain would eventually predominate among reisolated bacteria (Pattern B). If the bottleneck does reduce the infecting gonococcal population to very few bacteria, and one strain has a competitive advantage, that strain would be expected to predominate early among reisolates and be recovered exclusively from all infected subjects (Pattern C). If both input strains have equivalent fitness, each would be equally likely to survive the bottleneck, and each should predominate among reisolates from roughly half of infected subjects (Pattern D).

Figure 3B shows the actual results of mixed infections with wild-type Tf+Lf− and mutant Tf+Lf+ *N. gonorrhoeae* FA1090 strains. Perhaps not surprisingly, the data do not fit clearly into one single predicted outcome pattern. From over half of the infected subjects (3/5), only Tf+Lf+ gonococci were recovered following a noticeable eclipse. From the other two subjects, Tf+Lf+ gonococci eventually overtook the population after mixtures of both inoculating strains were recovered. The persistence of substantial numbers of both input strains with gradual out competition by the Tf+Lf+ strain in some subjects suggests that there is not a universal bottleneck limiting the establishment of experimental urethral infection to one or a few gonococci in the infecting population. The fact that cultures from all infected subjects ultimately contained 100% Tf+Lf+ gonococci suggests that this strain had a selective advantage over the Tf+Lf− wild-type strain during experimental infection of the male urethra. However, the population dynamics appeared to be different in different subjects. It is possible that there are individual differences in the effectiveness of innate host defenses, and these could affect the number of organisms in the original inoculum that survive and replicate to generate the infecting population.

Interpretation of mixed experimental infections could be further confounded by the potential for *in vivo* transformation between co-infecting gonococcal cells. *In vitro* models suggest that transformation during infection is possible, and the formation of mosaic genes is indirect evidence that it occurs in the human host. Gonococcal isolates that have been recovered to date from subjects inoculated with mixtures of isogenic strains showed no evidence of transformation during the relatively short experimental infection period (Anderson et al., 2003). However, only genetic exchanges that resulted in mosaic structures involving the engineered gene would have been detected. Recent improvements in whole genome sequencing technologies may enable a more comprehensive and sensitive approach to explore whether horizontal genetic exchange occurs during experimental urethral infection.

SAFETY AND UTILITY OF THE EXPERIMENTAL HUMAN GONORRHEA MODEL

Experimental infection of male volunteers with *N. gonorrhoeae* is safe, and subjects infected with wild-type gonococci exhibit the signs and symptoms of natural infection. The risks of serious complications from urethral gonococcal infection are extremely rare, even in natural infection. Hundreds of subjects have participated in experimental infection trials without severe adverse events. Using a competitive infection design with mixed inocula, the model can discriminate among gonococcal strains with different degrees of fitness for urethral colonization and infection. Experience with the FA1090 transferrin-receptor mutant showed that it is possible to render *N. gonorrhoeae* non-infectious by inactivation of a single virulence factor (in a strain that does not express the lactoferrin receptor). Thus the urethral infection model

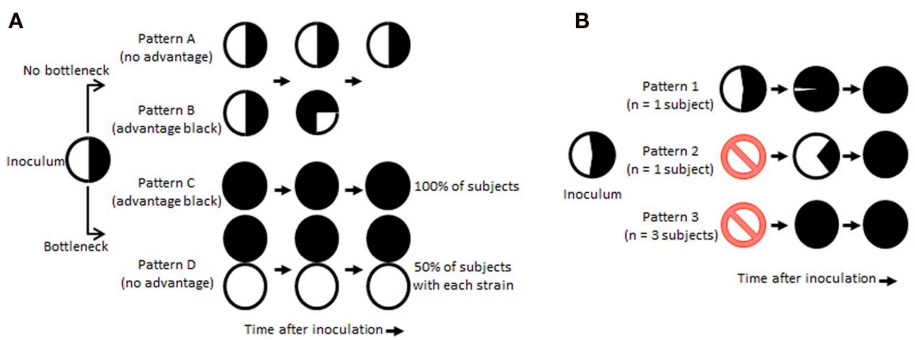

FIGURE 3 | Potential influence of population dynamics on outcomes of mixed experimental infections. In **(A)**, theoretical outcomes are shown for infections initiated by a 50:50 mixture of two isogenic strains differing in expression of a predicted virulence factor, depicted as black and white, under conditions with (bottom) and without (top) a population-restricting bottleneck early after inoculation. Potential outcomes consistent with and without a competitive advantage for the black strain are indicated as four patterns of pie charts representing populations of gonococci recovered from infected subjects. In **(B)**, actual outcomes are shown for infections initiated by a 45:55 mixture of isogenic Tf+Lf− (white) and Tf+Lf+ (black) strains of *N. gonorrhoeae* FA1090 (Anderson et al., 2003). In patterns 1–3, each pie chart represents the mixture of gonococcal strains recovered from infected subjects over the 1- to 5-day period after inoculation. The ⊘ symbol indicates no gonococci were recovered at early times (1–3 days in different volunteers) after inoculation of four subjects.

is well-suited to provide insights into the mechanisms of pathogenesis of *N. gonorrhoeae* and eventually may prove useful as a component in the clinical development plan for the evaluation of a gonococcal vaccine.

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