α -2,3-Sialyltransferase Enhances *Neisseria gonorrhoeae* Survival during Experimental Murine Genital Tract Infection

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The addition of host-derived sialic acid to *Neisseria gonorrhoeae* lipooligosaccharide is hypothesized to be an important mechanism by which gonococci evade host innate defenses. This hypothesis is based primarily on in vitro assays of complement-mediated and phagocytic killing. Here we report that a nonpolar α -2,3-sialyltransferase (*lst*) mutant of *N. gonorrhoeae* was significantly attenuated in its capacity to colonize the lower genital tract of 17- β estradiol-treated female BALB/c mice during competitive infection with the wild-type strain. Genetic complementation of the *lst* mutation restored recovery of the mutant to wild-type levels. Studies with B10.D2-HC^aH2^dH-T18c/OSN (C5-deficient) mice showed that attenuation of the *lst* mutant was not due to increased sensitivity to complement-mediated bacteriolysis, a result that is consistent with recently reported host restrictions in the complement cascade. However, *Lst*-deficient gonococci were killed more rapidly than sialylated wild-type gonococci following intraperitoneal injection into normal mice, which is consistent with sialylation conferring protection against killing by polymorphonuclear leukocytes (PMNs). As reported for human PMNs, sialylated gonococci were more resistant to killing by murine PMNs, and sialylation led to reduced association with and induction of a weaker respiratory burst in PMNs from estradiol-treated mice. In summary, these studies suggest sialylation confers a survival advantage to *N. gonorrhoeae* in mice by increasing resistance to PMN killing. This report is the first direct demonstration that α -2,3-sialyltransferase contributes to *N. gonorrhoeae* pathogenesis in an in vivo model. This study also validates the use of experimental murine infection to study certain aspects of gonococcal pathogenesis.

Neisseria gonorrhoeae is responsible for a large number of uncomplicated infections of the lower urogenital tract and serious upper reproductive tract infections in the United States (7) and the developing world (14). The gonococcus exclusively infects humans and has no outside animal or environmental reservoir. Accordingly, this organism has evolved many elegant adaptation mechanisms to maintain its existence in the human population, despite numerous hostile physiological and immunological factors that constitute the host mucosal defense. Phagocytes and complement are two critical antimicrobial components of the innate immune system; the existence of a sophisticated interplay between *N. gonorrhoeae* and these two factors during genital tract infection is supported by the persistence of the organism amid an intense influx of polymorphonuclear leukocytes (PMNs) during acute infection (22) and the detection of complement deposition products on gonococci isolated from endocervical lavage (32).

The gonococcus has evolved several mechanisms to evade host complement and phagocytes, including the covalent transfer of sialic acid to the terminal galactose of the lacto-*N*-tetraose moiety of the lipooligosaccharide (LOS) α -chain. This modification, which is catalyzed by gonococcal α -2,3-sialyltransferase (29, 30), is the basis of the unstable serum resistance phenotype exhibited by gonococci within patient exudates (41, 42) in which resistance to the bactericidal activity of normal human serum (NHS) is rapidly lost upon subculture in the absence of cytidine monophospho-*N*-acetylneuraminic acid

(CMP-NANA). Unlike *N. meningitidis*, this substrate must be supplied to *N. gonorrhoeae* by the host (31, 41, 59).

LOS sialylation is predicted to have a significant impact on gonococcal pathogenesis based on in vitro assays of complement and phagocytic function (reviewed in reference 52). In addition to protection from cross-reacting bactericidal antibodies present in serum from naïve hosts (16), LOS sialylation increases gonococcal resistance to opsonic (15, 28, 61) and nonopsonic (45) killing by human PMNs. LOS sialylation also protects *N. gonorrhoeae* from the bactericidal activity of porin and LOS-specific antibodies produced via immunization (10, 15, 40, 61), and therefore it may contribute to the ease with which *N. gonorrhoeae* causes repeated infections. At the molecular level, sialylated LOS down-regulates complement activation by binding factor H (fH), a negative regulatory protein of the alternative pathway of the complement cascade (44). Factor H inactivates the alternative pathway convertase (38, 39, 60) and acts as a cofactor in the cleavage of C3b to C3bi (39). The result of fH binding to the bacterial surface is reduced surface deposition of C3b; C4 deposition is also reduced, a step that is needed for activation of the classical pathway (15, 32). Fully sialylated gonococci also do not bind mannan-binding lectin, a key activator of the lectin complement pathway (17).

The apparent selection for the lacto-*N*-neotetraose LOS species during experimental urethral infection of volunteers (47) indirectly supports a role for LOS sialylation during genital tract infection, as does immunochemical evidence that a majority of gonococci with the lacto-*N*-neotetraose structure in urethral exudates is sialylated (1). However, direct testing of the importance of LOS sialylation in pathogenesis using an in vivo infection model has not yet been reported. As the con-

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centration of CMP-NANA is limiting with respect to its effect on serum resistance and PMN killing in vitro (13, 45, 61), it is critical that the role of LOS sialylation in pathogenesis be tested in models with physiologically relevant levels of CMP-NANA and complement. Such models should also reproduce the O_2 tension of mucosal surfaces due to the reported influence of sialylation on the induction of the oxidative burst in PMNs (45); the importance of O_2 tension is also indicated by the demonstration that anaerobic growth increases CMP-NANA-dependent serum resistance (13) and reports that LOS biosynthesis genes may be oxygen regulated (5, 13). Although several host restrictions challenge animal modeling for gonorrhea, female mice in the proestrus stage of the estrous cycle can be transiently colonized with *N. gonorrhoeae* (9). Long-term infection can be accomplished by administering 17- β estradiol, which stabilizes the estrous cycle in an estrus-like state (23, 56). An inflammatory response occurs in 50 to 80% of infected mice (23, 24), which, together with similarities in O_2 tension, iron limitation, and pH between the human endocervix and the mouse lower genital tract, supports the use of female mice as a surrogate host for studying the role of gonococcal α -2,3-sialyltransferase in infection.

Here we tested the importance of LOS sialylation in *N. gonorrhoeae* adaptation to the genital tract by studying the survival of an isogenic nonpolar *lst* mutant during experimental murine infection. We also used in vitro assays of phagocytic function to investigate our in vivo results and to further define the potential of using estradiol-treated mice to study gonococcal evasion of innate host defenses.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* MS11 (PorB.1B, Sm^r) is a serum-resistant strain from a patient with uncomplicated endocervical infection. Strain MS11 has been tested extensively in male volunteers (47, 48, 55). *N. gonorrhoeae* was cultured on GC agar (Difco) with Kellogg's supplements (27) at 37°C under 7% CO_2 or in supplemented GC broth (GCB) with 5 mM $NaHCO_3$ in an air incubator with rotation as described previously (25). For experiments in which sialylated gonococci were tested, bacteria were cultured overnight in GCB containing saturating concentrations (50 μ g/ml) of CMP-NANA. GC agar containing kanamycin (Km) (50 μ g/ml) or erythromycin (Em) (3 μ g/ml) was used to select *lst* mutant GP300 and the complemented mutant GP322, respectively. GC-vancomycin, colistin, nystatin, trimethoprim, streptomycin (GC-VCNTS) agar was used in single-strain mouse infection experiments as described previously (23). For competitive infections, GC agar containing streptomycin (Sm) (100 μ g/ml) and Sm plus Km (50 μ g/ml) was used. *Escherichia coli* TOP10 (Invitrogen) was the host strain for all recombinant plasmids and was cultured on Luria-Bertani (LB) agar or in LB broth at 37°C. Km (50 μ g/ml), ampicillin (Amp) (200 μ g/ml), tetracycline (Tet) (30 μ g/ml), and Em (300 μ g/ml) were used to maintain plasmids in *E. coli* when indicated. CMP-NANA and antibiotics were from Sigma Biochemical Corporation.

Construction of an *lst*-deficient mutant (GP300). The *lst* gene and a 10-bp neisserial DNA uptake sequence (11) located 122 bp downstream of the translational stop site were amplified from chromosomal DNA of strain MS11 via PCR using primers F1-*lst* (5' CGTCGCGAATGGAGTTTTAGGATATGGG) and R1-*lst* (GCGTCGACCTGCCACGACAGTTTCCG). An NruI or SalI site (underlined) was introduced into each primer, respectively, to facilitate cloning. F1-*lst* corresponds to the 17 nucleotides directly upstream of the *lst* translational start codon, and R1-*lst* corresponds to a 20-bp region 181 nucleotides downstream of the *lst* open reading frame. The resultant 1,343-kb PCR product was cloned into PCR-Blunt vector (Invitrogen) to generate plasmid pHW501, and the insert was sequenced to confirm its identity. The *lst* gene and uptake sequence were subcloned from pHW501 into the SalI and NruI sites of pBR322 to create pHW501a, thereby changing the selectable marker from Km^r to Amp^r. A nonpolar *aphA-3* (Km^r) cassette was obtained from plasmid pUC18Km on an 840-bp SmaI fragment (34) and inserted into a unique EagI site at nucleotide position 672 of the *lst* gene to create pHW501a-Km. Restriction

endonuclease analysis and nucleotide sequencing were used to confirm that the transcriptional orientation of the *aphA-3* cassette was the same as that of the *lst* gene. The inactivated *lst* gene was PCR amplified from pHW501a-Km using primers F1-*lst* and R1-*lst*, and the purified PCR fragment was introduced into MS11 by spot transformation (18). The occurrence of allelic exchange in a Km^r transformant (GP300) was verified by PCR using primers F45 (5'ACAGCCGGTATAAAGGGACCAC) and R46 (5'ACGCAGAAGGCAATGTCATACC) to detect the *aphA-3* cassette and by PCR using primers F2-*lst* (5'AACCTGATACGGGAGAGCAGCT) and R2-*lst* (5'ATGCGTAAACGTGCACATTGG), which anneal within the *lst* gene to sequences on each side of the EagI site into which the *aphA-3* gene was inserted. Primers F1-*lst* and R3 (5'GTTTGGTTCGATGGTGCAA) were used to PCR amplify the mutated *lst* gene in mutant GP300, and the mutation was confirmed by nucleotide sequence analysis. To complement the *lst* mutation, we took advantage of the Neisserial Insertional Complementation System (NICS) (provided by H.S. Seifert, Northwestern University), which allows for genetic complementation by targeting a chromosomal intergenic region of no known function between the lactate permease (*lctP*) and aspartate transaminase (*aspC*) genes (33, 50). We were unable to successfully clone the wild-type *lst* gene with its own promoter. Therefore, the *lst* structural gene was obtained on a SalI, NruI fragment from pHW501 and cloned into the PmeI site of the NICS vector pGCC4, which has an inducible *lac* promoter to allow regulated expression of genes cloned downstream to create pGCC4.Lst. An 8-kb ClaI fragment that contained the *lst* gene, *lac* promoter, and an Em^r gene flanked by gonococcal DNA corresponding to the *lctP* *aspC* locus was purified from pGCC4.Lst, and it was transformed into mutant GP300 to create strain GP322 (Em^r, Km^r). Confirmation of the desired allelic exchange in strain GP322 was by PCR and sequence analysis. Isopropyl- β -D-thiogalactopyranoside (IPTG) (1 mM) (Sigma) was used to maximally induce *lst* transcription in strain GP322 in serum resistance studies; IPTG induction was not used when testing interactions between GP322 and PMNs (i.e., opsonophagocytic killing, PMN uptake, and chemiluminescence assays). Ligations, electroporation, and PCR were performed using standard methods. Restriction enzymes, DeepVent polymerase, and DNA ligase were from New England Biolabs. Plasmid isolation and purification of PCR products were performed with the QIAprep Spin miniprep and gel extraction kits (QIAGEN), respectively. Synthesis of oligonucleotide primers and automated nucleotide sequencing using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems) were by the Biomedical Instrumentation Center of the Uniformed Services University of the Health Sciences (USUHS). Primers were designed based on sequence information from the *N. gonorrhoeae* FA1090 genome database (<http://www.genome.ou.edu/gono.html>).

Serum bactericidal assays. Sensitivity of gonococci to NHS or heat-inactivated (HI) NHS was tested in a microtiter plate assay as described previously (23). NHS was obtained from venous blood collected from a healthy female volunteer under a protocol approved by the USUHS Institutional Review Board. HI-NHS was obtained by incubating serum at 56°C for 30 min. Bactericidal assays were performed using bacteria cultured in GCB in the presence or absence of CMP-NANA. Broth cultures were centrifuged at 10,000 rpm for 30 s, and the bacterial pellet was suspended in PBS containing 0.1% gelatin, 0.01% $CaCl_2$, and 0.01% $MgCl_2$ (PBS-GCM) to a concentration of 10^5 CFU/ml. To directly test gonococci in mouse vaginal mucus, samples were collected on a sterile swab and suspended in 1 ml PBS-GCM. Bacterial aggregates were removed by centrifugation in a microfuge at 3,000 rpm for 10 s. The supernatant was centrifuged at 10,000 rpm for 30 s, and the pellet was resuspended in PBS-GCM. In some experiments, bacteria (10^6 CFU) were incubated with neuraminidase (NANase) (*Clostridium perfringens* type V) (Sigma) at a concentration of 8.3 U/ml for 1 h at 37°C or in a similar volume of distilled H_2O (control). The bactericidal₅₀ titer was defined as the percentage of NHS from which the number of CFU was 50% of that recovered from the assay medium without serum.

Opsonophagocytic killing assay. Mouse PMNs were elicited via intraperitoneal (i.p.) injection of 2.5 ml of 3% thioglycollate broth (Difco) into estradiol-treated (3 to 14 days posttreatment) or untreated female BALB/c mice. Mice were humanely sacrificed 4 to 5 h postinjection, and PMNs were obtained by peritoneal lavage with 10 ml cold Hanks' balanced salt solution (HBSS) and centrifuged for 5 min at setting 3 of an IEC Model CL centrifuge (International Equipment Co., Needham Heights, MA). The cell pellet was washed with an equal volume of HBSS and centrifuged as described above. The washed cells were resuspended in HBSS containing 10 mM glucose, 0.1% gelatin, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (HBSS II) and adjusted to 2×10^7 cells per ml using a hemacytometer. Trypan blue exclusion staining was used to confirm that >95% of the PMNs were viable. Normal mouse serum (NMS) was used for opsonization and separated from blood obtained by cardiac puncture; serum was placed on ice and used within an hour after collection. The PMN killing assay was performed essentially as described previously (12). Briefly, 10^5 to 10^6 CFU of

gonococci were suspended in 500 μ l of HBSS II containing 10% NMS (opsonized) or 10% HI-NMS (control) for 10 to 15 min at 37°C. Mouse PMNs were added (10^6 PMNs in a 50- μ l volume), and the final suspension was incubated on a rotary shaker at 37°C. Samples (20 μ l) were taken in quadruplicate at 45 and 90 min of incubation, serially diluted in GCB with 0.05% saponin, and cultured on GC agar with Sm for 24 to 36 h. The mean number of CFU recovered from tubes in which opsonized bacteria were tested was compared to that recovered from tubes in which gonococci were preincubated with HI-NMS (viability control) for each time point.

Acridine orange staining. The number of PMNs with associated gonococci was assessed using acridine orange staining. Briefly, 5×10^5 PMNs suspended in 250 μ l HBSS II were inoculated onto coverslips (12 mm diameter) within a 24-well tissue culture dish. Gonococci were added at a ratio of 10 GC to 1 PMN. Coverslips were gently rinsed after 90 and 135 min of incubation, stained with acridine orange (2), and examined with an Olympus BX60 fluorescent microscope using an excitation filter of 488 nm. One-hundred PMNs were examined on each of three coverslips, and the number of PMNs associated with no gonococci or with 1 to 2, 3 to 10, or >10 gonococci was recorded.

Experimental murine infection. Infection of estradiol-treated female BALB/c mice (National Cancer Institute, Bethesda, MD) or congenic female B10.D2-HC^oH2^dH2-T18c/OSN (C5⁻) and B10.D2-HC^oH2^dH2-T18c/NSN (C5⁺) mice (Jackson Laboratories, Bar Harbor, Maine) (8) was essentially as described previously (24). For single-strain infections, mice were inoculated intravaginally with 10^4 , 10^5 , or 10^6 CFU of piliated MS11 or GP300 gonococci suspended in 20 μ l of PBS-GCM ($n = 8$ mice per group). Vaginal mucus was cultured quantitatively for *N. gonorrhoeae* every other day for 14 days. Variants of wild-type and GP300 bacteria that expressed a single Opa protein of the same apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels were used for all experiments. For competitive infections, groups of 8 to 10 mice were inoculated with mixed suspensions of approximately equal numbers of wild-type MS11 and GP300 or GP322 gonococci (total dose, 10^6 CFU per 20- μ l inoculum). Nonpiliated variants were used to minimize the transfer of the antibiotic resistance marker in vivo. Equal volumes of undiluted and diluted vaginal swab suspensions were cultured on GC agar with Sm (total) and Sm plus Km (GP300 or GP322) on days 2, 4, 6, and 8 postinoculation. The ratio of GP300 or GP322 CFU to the calculated number of wild-type CFU was determined for inoculum (input) and vaginal cultures (output), and the competitive indices (CI; output ratio over input ratio) were calculated as described previously (3). For i.p. challenge studies, overnight cultures of MS11 and GP300 bacteria grown in GCB with CMP-NANA were centrifuged at 10,000 rpm at room temperature. Bacterial pellets were resuspended in sterile PBS and adjusted to an A_{600} of 0.08. Groups of 12 female BALB/c mice treated 4 days previously with 17- β estradiol per the infection protocol were injected i.p. with approximately 250 μ l of the bacterial suspensions (2×10^7 CFU), and three mice in each group were killed at 0 h, 3 h, 4 h, and 5 h postinjection. The peritoneal cavities were rinsed with 5 ml of PBS, and the lavage fluid was diluted at least 1:10 in 0.1% tryptic soy broth to lyse the phagocytes. The number of CFU recovered from 250 μ l of lavage fluid was determined by quantitative culture for each time point. Animal experiments were conducted in the laboratory animal facility at USUHS, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the USUHS Institutional Animal Care and Use Committee.

Chemiluminescence assay. The NADPH-oxidase activity in PMNs isolated by peritoneal lavage was measured using isoluminol- or luminol-dependent chemiluminescence (CL) assays that allow separate determinations of extracellularly released and intracellularly generated reactive oxygen species (ROS), respectively (6). CL activity was measured in an MLX microtiter plate luminometer (Dinex Technologies, Inc., Chantilly, VA) using white luminescence 96-well microtiter plates and a 350- μ l reaction mixture containing 10^6 PMNs isolated from mice 4 to 11 days after initiating estradiol treatment. For measuring extracellular ROS release, assays were conducted with 4 U horseradish peroxidase (a cell-impermeable peroxidase) and 3.75×10^{-5} M isoluminol (a cell-impermeable CL substrate). To measure intracellular ROS, assays contained 50 U superoxide dismutase (SOD; a cell-impermeable scavenger of $O_2^{\cdot-}$), 2,000 U catalase (a cell-impermeable scavenger of H_2O_2), and 3.75×10^{-5} M luminol (a cell-permeable CL substrate). The plate was equilibrated for 15 min at 37°C, after which 10^7 CFU of opsonized bacteria were added (ratio of bacteria to PMN, 10:1). Light emission was recorded continuously starting 5 min after the addition of bacteria. Experiments were performed on three separate occasions with similar results.

Data analysis. An unpaired *t* test was used to evaluate differences in the average duration of recovery from mice, susceptibility to PMN killing, the number of viable gonococci recovered following i.p. challenge, and the number of

TABLE 1. Sensitivity of wild-type (MS11), *Lst*-deficient (GP300), or complemented mutant (GP322) gonococci cultured in vitro with or without CMP-NANA or cultured in vivo

Culture type	% NHS required to kill 50% of gonococci of strain:		
	MS11	GP300	GP322
GCB	3	3	3
GCB + CMP-NANA	>16	3	$>16^c$
GCB + CMP-NANA + NANase	4	3	4
Vaginal swab suspension ^a	>16	3	10
Vaginal swab suspension + NANase	3	4	4
Vaginal culture ^b	3	3	2
Vaginal culture + CMP-NANA	>16	3	10^d
Vaginal culture + CMP-NANA + NANase	3	2	2

^a Gonococci within vaginal swab suspensions tested directly without culture.

^b Gonococci tested after one in vitro passage from infected mice.

^c IPTG was used to induce the *lst* gene in GP322.

^d GP322 cultured in vitro in the absence of IPTG.

PMNs with associated gonococci in the acridine orange assay. All statistical analyses were performed with SPSS software (Chicago, IL). All experiments were performed at least twice to test reproducibility.

RESULTS

In vitro characterization of an α -2,3-sialyltransferase-deficient mutant. The *lst* mutant GP300 was constructed via allelic exchange with an insertionally inactivated *lst* gene (*lst::aphA-3*) as described in Materials and Methods. Mutant GP300 was functionally deficient for sialyltransferase activity, as evidenced indirectly by the absence of a serum-resistant phenotype following growth in the presence of CMP-NANA (Table 1). To confirm that the observed increase in serum sensitivity was due to an inactivated *lst* gene, we showed serum resistance was restored via complementation in *trans* using strain GP322, a derivative of mutant GP300, which carries an inducible copy of the *lst* gene in a nonessential region of the chromosome. Growth of GP322 in the presence of CMP-NANA and IPTG to maximally induce expression of the intact *lst* gene resulted in serum resistance at a level similar to that of wild-type strain MS11. A slightly lower level of serum resistance was displayed by GP322 when cultured in the presence of CMP-NANA but not IPTG. NANase treatment resulted in a fully serum-sensitive phenotype (data not shown); therefore, the serum resistance shown by GP322 in the absence of IPTG induction is likely due to leakage from the *lac* promoter in the complementation vector at levels sufficient for conferring serum resistance.

Gonococci in the mouse genital tract exhibit unstable serum resistance. High concentrations of CMP-NANA (2 to 500 μ g/ml) are often used to demonstrate the effect of sialylation on gonococcal resistance to bactericidal antibodies (13, 15, 61) and to PMN uptake and killing (28, 45). These concentrations are 100- to 1,000-fold higher than those in physiological conditions. To test if LOS sialylation occurs during murine infection at a level that is functionally similar to that exhibited by gonococci cultured under saturating amounts of CMP-NANA, we first determined if wild-type gonococci within vaginal swab suspensions from infected mice exhibited an unstable serum resistance phenotype. Mice were inoculated intravaginally with

FIG. 1. Serum resistance of in vivo- and in vitro-grown wild-type *N. gonorrhoeae*. The number of viable gonococci recovered following incubation in increasing concentrations of NHS is shown for wild-type MS11 gonococci tested directly in vaginal swab suspensions or following in vitro subculture in the presence or absence of CMP-NANA. Of all conditions tested, only gonococci within vaginal swab suspensions and/or isolated from in vitro culture in the presence of CMP-NANA exhibited high levels of serum resistance. NANase was used to confirm the basis of the serum resistance.

10^6 CFU of wild-type strain MS11 or *lst* mutant GP300 gonococci, and the serum sensitivity of gonococci within vaginal mucus (without in vitro passage) was determined over the course of infection. Wild-type gonococci within vaginal swab suspensions were highly resistant to NHS as early as 1 day after inoculation and on every day tested (through day 9). Incubation of vaginal swab suspensions with NANase resulted in loss of the serum-resistant phenotype, as did a single in vitro passage of the wild-type strain from infected mice in the absence of CMP-NANA (Fig. 1). In contrast, GP300 gonococci were sensitive to NHS throughout the course of the experiment. Vaginal washes from mice infected with the complemented mutant GP322 also contained serum-resistant gonococci, although the bactericidal₅₀ titer was slightly less than that of the wild-type strain. NANase treatment dropped the bactericidal₅₀ titer of in vivo-grown GP322 to the serum-sensitive range, however, which confirmed that the addition of sialic acid was responsible (Table 1). The slightly lower serum resistance of in vivo-grown GP322 gonococci compared to that of MS11 bacteria is most likely due to the absence of IPTG induction during infection.

We found it necessary to partially fractionate vaginal mucus from infected mice before we could detect serum resistance of gonococci within the suspensions, and therefore we hypothesize sialidases or inhibitors of sialyltransferase may be present in mouse vaginal fluid. A low-speed centrifugation was used to remove epithelial cells and debris; a higher speed was required to remove a more soluble inhibitor from the supernatant. These findings are consistent with a protocol used by others to process human urethral exudate samples before performing bactericidal assays (42, 59). Martin et al. (31) postulated that concentrations of CMP-NANA in biological samples are lim-

FIG. 2. Recovery of the *lst* mutant (GP300) or complemented mutant (GP322) relative to the wild-type strain. Results are expressed as competitive indices (CI) from individual mice at each time point. The CI was calculated as described in Materials and Methods. The ratios of mutant to wild-type gonococci in the inoculum for all experiments were 0.47 to 0.50. Horizontal bars represent the geometric mean; a CI of <1.0 indicates a decrease in the ratio of mutant to wild-type gonococci with respect to that of the inoculum. Open symbols represent mice from which at least 100 wild-type CFU, but no GP300 or GP322 CFU, were recovered; the limit of detection (0.04 CFU per 100 μ l of swab suspension) was used as the number of mutant CFU recovered in these cases.

iting, based on variability in the capacity of human seminal plasma, cervical scrapings, and vaginal exudates to confer serum resistance when added to growth media. Consistent with this proposal, we were not successful in inducing serum resistance by culturing wild-type gonococci in broth containing 20% vaginal wash material from mice.

Attenuation of the *lst* mutant in vivo. We first tested the capacity of *Lst*-deficient gonococci to successfully colonize estradiol-treated BALB/c mice by comparing the infectivity of wild-type and *Lst*-deficient gonococci in mice inoculated with either MS11 or *lst* mutant GP300 gonococci. We detected no difference in the duration of colonization over time using doses of 10^6 , 10^5 , and 10^4 CFU, which include and flank the 80% infectious dose (10^5 CFU) for strain MS11 (data not shown). To further examine the importance of α -2,3-sialyltransferase in murine genital tract infection, we next utilized the more sensitive technique of competitive infection (3). For these experiments, mice were inoculated with a suspension containing wild-type gonococci mixed with similar numbers of the *lst* mutant GP300 or the complemented strain GP322 bacteria. A dramatic decrease in the recovery of the *lst* mutant relative to the wild-type strain occurred early in infection, as shown in Fig. 2. Based on a value of 1.0 as indicative of no change in the recovery of mutant relative to that of wild-type bacteria, the mean competitive index (CI) for mice inoculated with wild-

FIG. 3. Susceptibility of wild-type (MS11), Lst-deficient (GP300), or complemented *lst* mutant (GP322) to opsonophagocytic killing by murine PMNs. Gonococci cultured in the presence or absence of CMP-NANA or with CMP-NANA followed by NANase treatment were preincubated with NMS (opsonized) or HI-NMS (control) and then incubated with murine PMNs. GP322 was cultured without IPTG for these experiments. Results are expressed as the mean number of CFU recovered after 90 min of incubation; standard error bars are based on samples cultured in quadruplicate. *, $P < 0.05$ compared to HI-NMS control; **, $P < 0.005$ compared to HI-NMS control.

type and GP300 gonococci was greater than 10-fold reduced within 2 days postinoculation; 10^2 - to 10^5 -fold decreases in the CI were detected in several mice. The mutant was no longer recovered after 2 to 6 days from 12 out of 15 mice (Fig. 2, open symbols), and the mean length of time that mutant and wild-type gonococci were detected in vaginal washes was 4.7 versus 9.5 days, respectively (two experiments combined; $P = 0.001$). In contrast, recovery of the complemented mutant GP322 was similar to that of the wild-type strain, and the mean CI was statistically different from that obtained from mice inoculated with GP300 and wild-type gonococci on days 2 and 4 postinoculation ($P = 0.003$ and 0.006 , respectively). No significant difference between the mutant and complemented control groups was seen on day 6. However, unlike the GP300 mutant, which cleared in most mice by day 6, the complemented strain was recovered from all mice but one at the later time points, and there was no significant difference in the average length of time that GP322 and wild-type bacteria were recovered from mice (9.1 and 11.5 days, respectively). These *in vivo* complementation data rule out any potential difference in the *in vivo* growth rate between the mutant and wild-type strain that is unrelated to the *lst* gene; we also detected no difference in the growth rate of MS11, GP300, or GP322 gonococci when cultured separately in broth or in mixed broth cultures (data not shown). We conclude from these experiments that possession of a functional *lst* gene confers a detectable survival advantage in the lower genital tract of mice.

Increased susceptibility of Lst-deficient gonococci to uptake and killing by mouse PMNs. Based on the demonstration that LOS sialylation promotes resistance to opsonophagocytic killing by human PMNs (15, 61), we assessed the role of sialyltransferase in protecting *N. gonorrhoeae* from PMNs isolated from estradiol-treated mice. Human phagocytes can take up gonococci via the C3b receptor or, in the absence of opsonins, via the binding of certain gonococcal opacity (Opa) proteins to carcinoembryonic antigen cellular adhesion molecules on the PMN (21). The latter pathway is host restricted, and therefore we opsonized bacteria with NMS for these assays. Following preincubation with NMS, nonsialylated but not sialylated wild-

type MS11 gonococci were significantly killed after 45 and 90 min of exposure to PMNs, based on comparison with the number of CFU recovered when HI-NMS was used. In contrast, opsonized, Lst-deficient GP300 gonococci were significantly killed by murine PMNs regardless of whether the bacteria were cultured in the presence or absence of CMP-NANA. CMP-NANA-dependent resistance to PMN killing was restored to wild-type levels through complementation of the *lst* mutation; IPTG induction of the complemented gene was not needed to restore resistance (Fig. 3). No appreciable decrease in viable counts occurred in the HI-NMS controls over the course of the assay; the lack of PMN killing when using HI-NMS supports the need for complement opsonization in the mouse system. Additionally, the killing capacity of PMNs from untreated or estradiol-treated mice against nonsialylated and sialylated bacteria was comparable (data not shown).

The observed protection against opsonophagocytic killing that is afforded by sialylation may be explained by reduced bacterial adherence to and uptake by PMNs due to decreased deposition of complement opsonins. We therefore compared the degree to which sialylated versus nonsialylated gonococci associated with PMNs following opsonization. More PMNs with associated gonococci were detected when nonsialylated bacteria were tested compared to that observed for wild-type bacteria cultured in the presence of CMP-NANA (Fig. 4A). As expected, preincubation of wild-type gonococci in HI-NMS did not promote efficient association with PMNs, regardless of whether the bacteria were sialylated or nonsialylated. The number of gonococci associated with PMNs was also higher for samples that contained opsonized, nonsialylated wild-type or *lst* mutant gonococci compared to those with opsonized, sialylated wild-type bacteria. Complementation of the *lst* mutation restored association to wild-type levels (Fig. 4B).

Sialylation reduced but did not abolish opsonic interactions between *N. gonorrhoeae* and murine PMNs (Fig. 4); this result is similar to observations regarding nonopsonic (Opa-mediated) uptake of gonococci by human PMNs as reported by Rest and Frangipane (45). This study also showed nonopsonized, sialylated gonococci induce a weaker phagocytic respira-

FIG. 4. Association of sialylated or nonsialylated gonococci with murine PMNs. Results were quantitated by examining 100 PMNs for each of three coverslips stained with acridine orange and determining the average number of PMNs associated with gonococci and the number of gonococci per PMN. A. Average number of PMNs associated with wild-type MS11 gonococci preincubated with NMS (opsonized) or HI-NMS. Standard error bars are shown. Significantly more PMNs were associated with opsonized gonococci ($P < 0.05$; unpaired t test) regardless of whether MS11 was cultured with or without CMP-NANA. B. Average number of PMNs associated with 0, 1 to 2, 3 to 10, or >10 MS11 or GP322 gonococci cultured in the presence or absence of CMP-NANA or GP300 gonococci cultured with CMP-NANA. All gonococci were preincubated in NMS. The average total number of PMNs associated with MS11 or GP322 gonococci cultured in CMP-NANA (and without IPTG) was statistically different from that detected for nonsialylated MS11, GP322, or GP300 ($P < 0.05$; unpaired t test).

tory burst than that induced by nonsialylated gonococci when incubated with human PMNs. We therefore tested the effect of sialylation on the capacity of *N. gonorrhoeae* to induce an oxidative burst in murine PMNs under opsonizing conditions. We found that sialylated wild-type gonococci induced a markedly reduced luminol-dependent chemiluminescence response in mouse PMNs compared to that induced by *lst* mutant GP300 cultured in the same concentration of CMP-NANA; this phenotype was restored by genetic complementation, as shown by the luminol-dependent chemiluminescence response induced by strain GP322 cultured in CMP-NANA. By measuring the extracellular and intracellular CL responses as described in Materials and Methods, we determined that the majority of the ROS were produced intracellularly following incubation with any of the three strains. The level of extracellular ROS produced was virtually undetectable (Fig. 5).

Role of complement-mediated bacteriolysis in attenuation of the *lst* mutant. The ability to test the effect of LOS sialyla-

FIG. 5. Oxidative response of mouse PMNs to sialylated and nonsialylated gonococci. Isoluminol- or luminol-enhanced CL assays were utilized to measure the total (top panel), intracellular (middle panel), and extracellular (bottom panel) release of ROS by PMNs from estradiol-treated mice following exposure to wild-type MS11, *lst* mutant GP300, and complemented strain GP322, as described in Materials and Methods. Bacteria were cultured in CMP-NANA; IPTG was not used to maximally induce the complementing *lst* gene in GP322.

tion on resistance to mouse serum is technically challenging, since mouse serum does not kill *N. gonorrhoeae* in vitro (23) due to the hyperlability of terminal components of the mouse complement cascade (Sanjay Ram, personal communication). We therefore performed competitive infections with the wild-type strain and *lst* mutant in C5-deficient mice to test the hypothesis that LOS sialylation protects gonococci from complement-mediated bacteriolysis in vivo. We predicted that if evasion of complement-mediated killing contributed to the observed attenuation of the *lst* mutant in normal mice, the mutant would be less attenuated in mice that were unable to form the membrane attack complex. Contrary to this predicted outcome, however, *lst* mutant GP300 was attenuated in C5⁻ mice at a level that was comparable to the attenuation observed in the C5⁺ congenic strain (Fig. 6). The decrease in CI was similar to that observed with BALB/c mice, and the mutant was cleared in most mice by day 4 (Fig. 6, open symbols). We

FIG. 6. Recovery of the *lst* mutant (GP300) from the C5-deficient and C5-sufficient mice relative to the wild-type strain. C5⁻ results are expressed as competitive indices (CI) for individual C5⁻ ($n = 4$) and C5⁺ ($n = 6$) mice as described in the Fig. 2 legend. Horizontal bars represent the mean CI. The ratio of mutant to wild-type gonococci in the inoculum was 0.5. Cultures from which no GP300 gonococci were recovered are represented by open symbols.

conclude this arm of the complement system is not responsible for the observed attenuation of the *lst* mutant in the mouse model. This result may be due to recently reported host restrictions in the complement activation pathway (37), as is discussed further below.

Increased survival of sialylated gonococci following i.p. challenge. The data presented thus far suggest that the survival disadvantage of the *lst* mutant during experimental genital tract infection occurs at the level of phagocytic killing. To further test this hypothesis, we compared the survival of the *lst* mutant following i.p. injection of mice with that of sialylated wild-type gonococci. This approach was used by others to study the resistance of *Yersinia enterocolitica* to phagocytes (19), and it has the advantage of bypassing any known or undescribed effects that sialylation may have on the interaction of gonococci with nonprofessional phagocytic cells in the genital mucosa (i.e., epithelial cells). For these experiments, wild-type MS11 and *lst* mutant GP300 bacteria were cultured in the presence of CMP-NANA and injected into separate groups of estradiol-treated mice; the survival of bacteria over time was assessed by quantitative culture of peritoneal lavage fluid. Both sialylated wild-type and GP300 gonococci elicited a rapid and large influx of PMNs into the peritoneal cavity, and a decrease in the number of viable gonococci recovered occurred over time for both strains. However, significantly fewer *lst* mutant gonococci were recovered at 4 and 5 h postinoculation than were recovered from wild-type bacteria ($P < 0.0001$ and 0.02 , respectively) (Fig. 7). The outcome of these experiments is consistent with our hypothesis that the *lst* mutant is more

FIG. 7. Survival of sialylated and nonsialylated gonococci following i.p. injection of estradiol-treated mice. Wild-type MS11 and *lst* mutant GP300 gonococci cultured in CMP-NANA were used in these assays. The mutant was significantly killed compared to results for the wild-type strain at 4 and 5 h ($P < 0.0001$ and $P = 0.02$, respectively).

susceptible to phagocytic killing in vivo. We did not compare sialylated versus nonsialylated MS11 gonococci in this assay, since sialylation of the bacteria might occur within the peritoneal cavity. Such an event would impede our ability to perform a clean comparison and might lead to a false conclusion as to the effect of sialylation on survival in this body site.

DISCUSSION

N. gonorrhoeae is a highly successful pathogen that is exquisitely well-adapted to evade host innate defenses. Persistence of this organism amid a vigorous PMN inflammatory response is likely due to the influence of several factors which have been hypothesized to protect *N. gonorrhoeae* phagocytic killing based on in vitro studies. One or more antioxidant factors may protect against toxic oxygen species produced by phagocytes (26, 51, 53, 57, 58); phase variation of Opa protein expression may protect against nonopsonic uptake by PMNs, and as discussed previously, sialylation of LOS may reduce nonopsonic and opsonophagocytic uptake (45) and killing by PMNs (15, 28, 45). Considerable debate as to the role of LOS sialylation in infection has stemmed from the fact that functional advantages conferred by sialylation are dose dependent, with both CMP-NANA and complement levels limiting (15, 46, 61). Additionally, the degree to which O₂-dependent mechanisms of phagocytic killing challenge the gonococcus in vivo is not known. Studies designed to test the resistance of gonococci to PMN killing in vitro are also usually performed under conditions that may falsely represent the microenvironment of mucosal sites inhabited by this pathogen.

Here we utilized a female mouse model of gonococcal gen-

ital tract infection to show that the *lst* gene conferred a strong survival advantage in vivo, as evidenced by the rapid loss of the *lst* mutant compared to the parent strain within 2 days after intravaginal inoculation. Recovery of the mutant from mice was restored through genetic complementation, which rules out polar effects of the mutation or the influence of unrelated mutations that may have occurred during mutant construction that might affect growth or survival in vivo. The *lst* mutant was also more susceptible to killing following i.p. inoculation; this result points to increased susceptibility to phagocytic killing being responsible for reduced survival of the mutant rather than differences in the interaction of Lst-sufficient and -deficient gonococci with the genital mucosa. Our results are consistent with other bacterial sialyltransferases; specifically, sialyltransferase mutants of *Haemophilus influenzae* were unable to cause acute otitis media and were less infectious in a chinchilla model (4).

In contrast to the sialyltransferase of *H. influenzae*, gonococcal sialyltransferase was not required in strain MS11 for experimental murine infection in that no difference in duration of infection or colonization load was detected in mice inoculated with wild-type or *lst* mutant gonococci at doses 10-fold lower than that required to infect 80% of mice. Competitive infection, which minimizes variability between animals and is the basis of signature-tagged mutagenesis (54), is a proven strategy for detecting the importance of virulence factors in pathogens that utilize functionally redundant colonization or survival mechanisms. The fact that we observed attenuation of the gonococcal *lst* mutant by competitive infection but not in comparisons of animals infected with either wild-type or mutant gonococci could be explained by the numerous mechanisms by which the gonococcus can evade complement-mediated and phagocytic killing. Mutating one of several functionally redundant factors may not cause a detectable effect unless doses lower than the 50% infectious dose are used; the number of mice required to generate a statistically significant difference in such an experiment is difficult to justify, however, when the more sensitive technique of competitive infection can be used. We also must consider the possibility that perhaps the wild-type strain causes a more productive infection than the Lst-deficient strain, which would result in a greater host challenge to the mutant than that which occurs during infection by the mutant alone. We currently have no evidence to support this latter theory, however, since we saw no difference in the degree of PMN influx or the presence of gonococci within PMNs upon examination of stained smears from mice infected with wild-type or *lst* mutant gonococci (data not shown).

Another explanation for the reduced recovery of the *lst* mutant relative to the wild-type strain during competitive infection is that lack of sialyltransferase, which is associated with the outer membrane (49), may make gonococci less viable. Functional advantages conferred by sialylation were abolished via treatment with NANase in vitro, which is consistent with the addition of sialic acid being important for increased survival and not the physical presence of the enzyme. However, we cannot rule out that absence of this protein in the membrane might alter survival in vivo. We do not favor this hypothesis, however, since sialyltransferase is a minor membrane component (49), and we saw no difference in the in vitro growth characteristics of the mutant cultured with the wild-

type strain, even in the stationary phase. Another Lst-associated phenotype that might explain our results is the possibility that nonsialylated gonococci may retreat into an intracellular niche via binding of nonsialylated LOS to the murine homologue of the human asialoglycoprotein receptor (ASGP-R), which is expressed on primary urethral cells from men (20, 43). In this case, mutant gonococci might be less accessible for culture. Whether or not the rodent ASGP-R molecule can function as an adherence receptor for *N. gonorrhoeae* is not known, and we have not yet detected intracellular bacteria in mouse tissue (W. Song and A. E. Jerse, unpublished data). We also believe this explanation to be unlikely, since no difference in the colonization load was detected in mice inoculated with either wild-type or Lst-deficient gonococci.

Our investigation of the effect of sialylation on gonococcal interactions with mouse PMNs revealed several similarities with what has been reported for human PMNs. We demonstrated here that sialylation (i) dramatically increased resistance to killing by murine PMNs, (ii) dramatically reduced bacterial association with murine PMNs, and (iii) led to a reduced oxidative burst, all of which is consistent with studies using human PMNs (15, 45). We also found that phagocytes from estradiol-treated mice undergo a respiratory burst in response to *N. gonorrhoeae* that is primarily intracellular, as was reported with human PMNs (35). Together, these data support the usefulness of the mouse model as an experimental system for examining interactions between *N. gonorrhoeae* and PMNs during mucosal infection. As with all experimental models, however, differences from natural infection can confound the interpretation of data. As discussed, Opa-mediated (nonopsonic) uptake does not occur with mouse PMNs. The relative contribution of the opsonic and nonopsonic pathways during infection is not known, however, and certainly in the presence of inflammation, uptake of bacteria via the opsonic pathway occurs. Perhaps more importantly, during preparation of this work, Ram and colleagues reported that the binding of C4bp (36) and fH (37) to *N. gonorrhoeae* is human specific. Down-regulation of the alternative pathway via the binding of fH to sialic acid residues on gonococcal LOS leads to reduced deposition of opsonins as well as reduced bacteriolysis due to the amplification loop that links the alternative and classical pathways (44). The fact that the *lst* mutant was also attenuated in C5-deficient mice is consistent with a host restriction for fH. The impact of the fH host restriction on opsonic interactions with phagocytes is likely to be less pronounced, since relatively few opsonin molecules are needed for effective opsonization compared to the number of membrane attack complexes required for lysis. In this regard, the mouse model probably underdetects the impact of sialylation during genital tract infection. We are currently developing mice that are transgenic for human complement regulatory proteins in hopes that they will provide a better background for studying factors, such as sialyltransferase, which are known to play a role in evasion of complement-mediated bacteriolysis.

In summary, our study demonstrates a protective role for *N. gonorrhoeae* sialyltransferase early during infection, when serum factors and PMNs are introduced as part of the inflammatory response. We present in vitro and in vivo evidence to support the hypothesis that sialylation of gonococcal LOS confers a survival advantage in experimentally infected mice by

increasing resistance to opsonophagocytic killing. This advantage was detectable despite newly recognized host restrictions in the complement cascade. The development of transgenic mice that express human fH and C4bp should allow us to more fully test the role of sialylation *in vivo*; similarly, the testing of mutants in mice that are defective in phagocytic function may help define the relative importance of different gonococcal defense mechanisms as proposed from *in vitro* studies.

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REFERENCES

1. Apicella, M. A., R. E. Mandrell, M. Shero, M. E. Wilson, J. M. Griffiss, G. F. Brooks, C. Lammell, J. F. Breen, and P. A. Rice. 1990. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: an immunoelectron microscopic analysis. *J. Infect. Dis.* **162**:506–512.
2. Belland, R. J., T. Chen, J. Swanson, and S. H. Fischer. 1992. Human neutrophil response to recombinant *Neisseria* Opa proteins. *Mol. Microbiol.* **6**:1729–1737.
3. Beuzon, C., and D. Holden. 2001. Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions *in vivo*. *Microbes Infect.* **3**:1345–1352.
4. Bouchet, V., D. W. Hood, J. Li, J. R. Brisson, G. A. Randle, A. Martin, Z. Li, R. Goldstein, E. K. Schweda, S. I. Pelton, J. C. Richards, and E. R. Moxon. 2003. Host-derived sialic acid is incorporated into *Haemophilus influenzae* lipopolysaccharide and is a major virulence factor in experimental otitis media. *Proc. Natl. Acad. Sci. USA* **100**:8898–8903.
5. Braun, D. C., and D. C. Stein. 2004. The *lgtABCDE* gene cluster, involved in lipooligosaccharide biosynthesis in *Neisseria gonorrhoeae*, contains multiple promoter sequences. *J. Bacteriol.* **186**:1038–1049.
6. Bylund, J., M. Samuelsson, L. V. Collins, and A. Karlsson. 2003. NADPH-oxidase activation in murine neutrophils via formyl peptide receptors. *Exp. Cell Res.* **282**:70–77.
7. Centers for Disease Control and Prevention. 2005. Summary of notifiable diseases—United States, 2002. *Morb. Mortal. Wkly. Rep.* **53**:1204–1212.
8. Cooper, M. D., and S. A. Floyd. 1982. *In vitro* kinetics of phagocytosis and intracellular killing of gonococci by peritoneal macrophages from mice deficient in complement component 5. *Infect. Immun.* **36**:363–370.
9. Dalal, S. J., J. S. Estep, I. E. Valentin-Bon, and A. E. Jerse. 2001. Standardization of the Whitten Effect to induce susceptibility to *Neisseria gonorrhoeae* in female mice. *Contemp. Top. Lab. Anim. Sci.* **40**:13–17.
10. Elkins, C., N. H. Carbonetti, V. A. Varela, D. Stirewalt, D. G. Klapper, and P. F. Sparling. 1992. Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. *Mol. Microbiol.* **6**:2617–2628.
11. Elkins, C., C. E. Thomas, H. S. Seifert, and P. F. Sparling. 1991. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. *J. Bacteriol.* **173**:3911–3913.
12. Fischer, S. H., and R. F. Rest. 1988. Gonococci possessing only certain P.II outer membrane proteins interact with human neutrophils. *Infect. Immun.* **56**:1574–1579.
13. Frangipane, J. V., and R. F. Rest. 1993. Anaerobic growth and cytidine 5'-monophospho-N-acetylneuraminic acid act synergistically to induce high-level serum resistance in *Neisseria gonorrhoeae*. *Infect. Immun.* **61**:1657–1666.
14. Gerbase, A. C., J. T. Rowley, D. H. Heymann, S. F. Berkley, and P. Piot. 1998. Global prevalence and incidence estimates of selected curable STDs. *Sex. Transm. Infect.* **74**(Suppl. 1):S12–S16.
15. Gill, M. J., D. P. McQuillen, J. P. van Putten, L. M. Wetzler, J. Bramley, H. Crooke, N. J. Parsons, J. A. Cole, and H. Smith. 1996. Functional characterization of a sialyltransferase-deficient mutant of *Neisseria gonorrhoeae*. *Infect. Immun.* **64**:3374–3378.
16. Griffiss, J. M., G. A. Jarvis, J. P. O'Brien, M. M. Eads, and H. Schneider. 1991. Lysis of *Neisseria gonorrhoeae* initiated by binding of normal human IgM to a hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain-specific, properdin-binding-dependent alternative complement pathway activation. *J. Immunol.* **147**:298–305.
17. Gulati, S., K. Sastry, J. C. Jensenius, P. A. Rice, and S. Ram. 2002. Regulation of the mannan-binding lectin pathway of complement on *Neisseria gonorrhoeae* by C1-inhibitor and alpha 2-macroglobulin. *J. Immunol.* **168**:4078–4086.
18. Gunn, J. S., and D. C. Stein. 1996. Use of a non-selective transformation technique to construct a multiply restriction/modification-deficient mutant of *Neisseria gonorrhoeae*. *Mol. Gen. Genet.* **251**:509–517.
19. Hanski, C., M. Naumann, A. Grutzkau, G. Pluschke, B. Friedrich, H. Hahn, and E. O. Riecken. 1991. Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. *Infect. Immun.* **59**:1106–1111.
20. Harvey, H. A., M. P. Jennings, C. A. Campbell, R. Williams, and M. A. Apicella. 2001. Receptor-mediated endocytosis of *Neisseria gonorrhoeae* into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor. *Mol. Microbiol.* **42**:659–672.
21. Hauck, C. R., and T. F. Meyer. 2003. 'Small' talk: Opa proteins as mediators of *Neisseria*-host-cell communication. *Curr. Opin. Microbiol.* **6**:43–49.
22. Hook, E. a. H. H. 1999. Sexually transmitted diseases, 3rd ed. McGraw-Hill Companies, Inc., New York, N.Y.
23. Jerse, A. E. 1999. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. *Infect. Immun.* **67**:5699–5708.
24. Jerse, A. E., E. T. Crow, A. N. Bordner, I. Rahman, C. N. Cornelissen, T. R. Moench, and K. Mehrazar. 2002. Growth of *Neisseria gonorrhoeae* in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. *Infect. Immun.* **70**:2549–2558.
25. Jerse, A. E., N. D. Sharma, A. N. Simms, E. T. Crow, L. A. Snyder, and W. M. Shafer. 2003. A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. *Infect. Immun.* **71**:5576–5582.
26. Johnson, S. R., B. M. Steiner, D. D. Cruce, G. H. Perkins, and R. J. Arko. 1993. Characterization of a catalase-deficient strain of *Neisseria gonorrhoeae*: evidence for the significance of catalase in the biology of *N. gonorrhoeae*. *Infect. Immun.* **61**:1232–1238.
27. Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and D. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* **85**:1274–1279.
28. Kim, J. J., D. Zhou, R. E. Mandrell, and J. M. Griffiss. 1992. Effect of exogenous sialylation of the lipooligosaccharide of *Neisseria gonorrhoeae* on opsonophagocytosis. *Infect. Immun.* **60**:4439–4442.
29. Mandrell, R. E., J. M. Griffiss, H. Smith, and J. A. Cole. 1993. Distribution of a lipooligosaccharide-specific sialyltransferase in pathogenic and non-pathogenic *Neisseria*. *Microb. Pathog.* **14**:315–327.
30. Mandrell, R. E., H. Smith, G. A. Jarvis, J. M. Griffiss, and J. A. Cole. 1993. Detection and some properties of the sialyltransferase implicated in the sialylation of lipopolysaccharide of *Neisseria gonorrhoeae*. *Microb. Pathog.* **14**:307–313.
31. Martin, P. M., P. V. Patel, N. J. Parsons, and H. Smith. 1982. Induction in gonococci of phenotypic resistance to killing by human serum by human genital secretions. *Br. J. Vener. Dis.* **58**:363–365.
32. McQuillen, D. P., S. Gulati, S. Ram, A. K. Turner, D. B. Jani, T. C. Heeren, and P. A. Rice. 1999. Complement processing and immunoglobulin binding to *Neisseria gonorrhoeae* determined *in vitro* simulates *in vivo* effects. *J. Infect. Dis.* **179**:124–135.
33. Mehr, I. J., and H. S. Seifert. 1998. Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol. Microbiol.* **30**:697–710.
34. Menard, R., P. J. Sansonetti, and C. Parsot. 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.* **175**:5899–5906.
35. Naidu, F. L., and R. F. Rest. 1991. Stimulation of human neutrophil oxidative metabolism by nonopsonized *Neisseria gonorrhoeae*. *Infect. Immun.* **59**:4383–4390.
36. Ngampasutadol, J., S. Ram, A. M. Blom, H. Jarva, A. E. Jerse, E. Lien, J. Goguen, S. Gulati, and P. A. Rice. 2005. Human C4b-binding protein selectively interacts with *Neisseria gonorrhoeae* and results in species-specific infection. *Proc. Natl. Acad. Sci. USA* **102**:17142–17147.
37. Ngampasutadol, J., S. R., A. M. Blom, S. Gulati, and P. A. Rice. 2004. Serum resistance of *Neisseria gonorrhoeae* is restricted to humans; a possible explanation for the species specificity of gonococcal infections. *Mol. Immunol.* **41**:285.
38. Pangburn, M. K., and H. J. Muller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of beta1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci. USA* **75**:2416–2420.
39. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. *J. Exp. Med.* **146**:257–270.
40. Parsons, N. J., J. R. Andrade, P. V. Patel, J. A. Cole, and H. Smith. 1989. Sialylation of lipopolysaccharide and loss of absorption of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5'-monophospho-N-acetyl neuraminic acid. *Microb. Pathog.* **7**:63–72.

41. Parsons, N. J., P. R. Ashton, C. Constantinidou, J. A. Cole, and H. Smith. 1993. Identification by mass spectrometry of CMP-NANA in diffusible material released from high M(r) blood cell fractions that confers serum resistance on gonococci. *Microb. Pathog.* **14**:329–335.
42. Parsons, N. J., J. A. Cole, and H. Smith. 1990. Resistance to human serum of gonococci in urethral exudates is reduced by neuraminidase. *Proc. R. Soc. Lond. B Biol. Sci.* **241**:3–5.
43. Porat, N., M. A. Apicella, and M. S. Blake. 1995. *Neisseria gonorrhoeae* utilizes and enhances the biosynthesis of the asialoglycoprotein receptor expressed on the surface of the hepatic HepG2 cell line. *Infect. Immun.* **63**:1498–1506.
44. Ram, S., A. K. Sharma, S. D. Simpson, S. Gulati, D. P. McQuillen, M. K. Pangburn, and P. A. Rice. 1998. A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. *J. Exp. Med.* **187**:743–752.
45. Rest, R. F., and J. V. Frangipane. 1992. Growth of *Neisseria gonorrhoeae* in CMP-*N*-acetylneuraminic acid inhibits nonopsonic (opacity-associated outer membrane protein-mediated) interactions with human neutrophils. *Infect. Immun.* **60**:989–997.
46. Rest, R. F., and R. E. Mandrell. 1995. *Neisseria* sialyltransferases and their role in pathogenesis. *Microb. Pathog.* **19**:379–390.
47. Schneider, H., J. M. Griffiss, J. W. Boslego, P. J. Hitchcock, K. M. Zahos, and M. A. Apicella. 1991. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. *J. Exp. Med.* **174**:1601–1605.
48. Schneider, H., K. A. Schmidt, D. R. Skillman, L. Van De Verg, R. L. Warren, H. J. Wylie, J. C. Sadoff, C. D. Deal, and A. S. Cross. 1996. Sialylation lessens the infectivity of *Neisseria gonorrhoeae* MS11mkC. *J. Infect. Dis.* **173**:1422–1427.
49. Shell, D. M., L. Chiles, R. C. Judd, S. Seal, and R. F. Rest. 2002. The *Neisseria* lipooligosaccharide-specific alpha-2,3-sialyltransferase is a surface-exposed outer membrane protein. *Infect. Immun.* **70**:3744–3751.
50. Skaar, E. P., M. P. Lazio, and H. S. Seifert. 2002. Roles of the *recJ* and *recN* genes in homologous recombination and DNA repair pathways of *Neisseria gonorrhoeae*. *J. Bacteriol.* **184**:919–927.
51. Skaar, E. P., D. M. Tobiason, J. Quick, R. C. Judd, H. Weissbach, F. Etienne, N. Brot, and H. S. Seifert. 2002. The outer membrane localization of the *Neisseria gonorrhoeae* MsrA/B is involved in survival against reactive oxygen species. *Proc. Natl. Acad. Sci. USA* **99**:10108–10113.
52. Smith, H., N. J. Parsons, and J. A. Cole. 1995. Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. *Microb. Pathog.* **19**:365–377.
53. Soler-Garcia, A. A., and A. E. Jerse. 2004. A *Neisseria gonorrhoeae* catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. *Microb. Pathog.* **37**:55–63.
54. Sun, Y. H., S. Bakshi, R. Chalmers, and C. M. Tang. 2000. Functional genomics of *Neisseria meningitidis* pathogenesis. *Nat. Med.* **6**:1269–1273.
55. Swanson, J., K. Robbins, O. Barrera, D. Corwin, J. Boslego, J. Ciak, M. Blake, and J. M. Koomey. 1987. Gonococcal pilin variants in experimental gonorrhea. *J. Exp. Med.* **165**:1344–1357.
56. Taylor-Robinson, D., P. M. Furr, and C. M. Hetherington. 1990. *Neisseria gonorrhoeae* colonises the genital tract of oestradiol-treated germ-free female mice. *Microb. Pathog.* **9**:369–373.
57. Tseng, H. J., Y. Srihanta, A. G. McEwan, and M. P. Jennings. 2001. Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. *Mol. Microbiol.* **40**:1175–1186.
58. Turner, S., E. Reid, H. Smith, and J. Cole. 2003. A novel cytochrome c peroxidase from *Neisseria gonorrhoeae*: a lipoprotein from a gram-negative bacterium. *Biochem. J.* **373**:865–873.
59. Ward, M. E., P. J. Watt, and A. A. Glynn. 1970. Gonococci in urethral exudates possess a virulence factor lost on subculture. *Nature* **227**:382–384.
60. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein beta1H. *Proc. Natl. Acad. Sci. USA* **73**:3268–3272.
61. Wetzler, L. M., K. Barry, M. S. Blake, and E. C. Gotschlich. 1992. Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. *Infect. Immun.* **60**:39–43.

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