RESEARCH ARTICLE

α-2,3-Sialyltransferase Expression Level Impacts the Kinetics of Lipooligosaccharide Sialylation, Complement Resistance, and the Ability of *Neisseria gonorrhoeae* to Colonize the Murine Genital Tract

Lisa A. Lewis, Sunita Gulati, Elizabeth Burrowes, Bo Zheng, Sanjay Ram, Peter A. Rice

Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts, USA

ABSTRACT *Neisseria meningitidis* and *Neisseria gonorrhoeae* modify the terminal lacto-*N*-neotetraose moiety of their lipooligosaccharide (LOS) with sialic acid. *N. gonorrhoeae* LOS sialylation blocks killing by complement, which is mediated at least in part by enhanced binding of the complement inhibitor factor H (FH). The role of LOS sialylation in resistance of *N. meningitidis* to serum killing is less well defined. Sialylation in each species is catalyzed by the enzyme LOS α-2,3-sialyltransferase (Lst). Previous studies have shown increased Lst activity in *N. gonorrhoeae* compared to *N. meningitidis* due to an ~5-fold increase in *lst* transcription. Using isogenic *N. gonorrhoeae* strains engineered to express gonococcal *lst* from either the *N. gonorrhoeae* or *N. meningitidis* *lst* promoter, we show that decreased expression of *lst* (driven by the *N. meningitidis* promoter) reduced LOS sialylation as determined by less incorporation of tritium-labeled cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA; the donor molecule for sialic acid). Diminished LOS sialylation resulted in reduced rates of FH binding and increased pathway activation compared to *N. gonorrhoeae* promoter-driven *lst* expression. The *N. meningitidis* *lst* promoter generated sufficient Lst to sialylate *N. gonorrhoeae* LOS *in vivo*, and the level of sialylation after 24 h in the mouse genital tract was sufficient to mediate resistance to human serum *ex vivo*. Despite demonstrable LOS sialylation *in vivo*, gonococci harboring the *N. meningitidis* *lst* promoter were outcompeted by those with the *N. gonorrhoeae* *lst* promoter during coinfection of the vaginal tract of estradiol-treated mice. These data highlight the importance of high *lst* expression levels for gonococcal pathogenesis.

IMPORTANCE *Neisseria gonorrhoeae* has become resistant to nearly every therapeutic antibiotic used and is listed as an "urgent threat" by the Centers for Disease Control and Prevention. Novel therapies are needed to combat drug-resistant *N. gonorrhoeae*. Gonococci express an α-2,3-sialyltransferase (Lst) that can scavenge sialic acid from the host and use it to modify lipooligosaccharide (LOS). Sialylation of gonococcal LOS converts serum-sensitive strains to serum resistance, decreases antibody binding, and combats killing by neutrophils and antimicrobial peptides. Mutant *N. gonorrhoeae* that lack Lst (cannot sialylate LOS) are attenuated in a mouse model. Lst expression levels differ among *N. gonorrhoeae* strains, and *N. gonorrhoeae* typically expresses more Lst than *Neisseria meningitidis*. Here we examined the significance of differential *lst* expression levels and determined that the level of LOS sialylation is critical to the ability of *N. gonorrhoeae* to combat the immune system and survive in an animal model. LOS sialylation may be an ideal target for novel therapies.

Address correspondence to Lisa A. Lewis, lisa.lewis@umassmed.edu.

This article is a direct contribution from a Fellow of the American Academy of Microbiology.

The pathogenic *Neisseria* species, *Neisseria meningitidis* and *N. gonorrhoeae* are obligate human pathogens. As a result, these organisms are uniquely adapted to evade killing by the human host. Complement, a component of the innate immune system, is an important line of defense against infections caused by both *N. meningitidis* and *N. gonorrhoeae*. The classical pathway mediates complement-dependent killing of *Neisseria* (1, 2); however, the alternative pathway (AP) plays a key role in amplifying complement component 3 (C3) deposited on the organism. Inhibition of either pathway is associated with increased serum resistance (3, 4). *N. meningitidis* and *N. gonorrhoeae* have evolved numerous mechanisms to evade

host complement, including strategies that specifically target the AP. The distinct mechanisms used by these highly related organisms may reflect optimal strategies to best combat defenses in their specific niches.

Gonococci scavenge cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA; the donor molecule for sialic acid) from the human host and sialylate the terminal galactose of the lacto-*N*-neotetraose (LNT) moiety of lipooligosaccharide (LOS). Sialylation of gonococcal LOS converts serum-sensitive strains to serum resistance, a phenomenon that has been termed "unstable" serum resistance to reflect its dependence on exogenously supplied CMP-NANA. Mechanisms of unstable serum resistance include

decreased binding of select antibodies (Abs) (5) and increased binding of the host AP inhibitor, factor H (FH) (6). Increased FH binding requires expression of gonococcal PorB (7).

N. meningitidis inhibit the AP using several redundant mechanisms, including expression of capsular polysaccharide (serogroups B and C) (8–10), expression of three FH binding proteins, neisserial surface protein a (NspA) (11), FH binding protein (FHbp) (12), and PorB2 (13), and LOS sialylation (14–18). Although meningococcal LOS sialylation plays a role in downregulating the AP, sialylation of meningococcal LNT LOS does not enhance binding of FH to PorB (13, 57); rather, it increases the association of the C terminus of FH to C3 fragments deposited on the bacterial surface.

Sialylation of pathogenic *Neisseria* LOS is catalyzed by the enzyme α-2,3-sialyltransferase (Lst) (19, 20). Lst is located in the outer membrane (21), and strains *N. gonorrhoeae* F62 and *N. meningitidis* MC58 express Lst proteins that share ~95% amino acid identity. Triton X-100 extracts prepared from strains of *N. gonorrhoeae* contain, on average, 2.2-fold more Lst activity than those prepared from strains of *N. meningitidis* (20, 22), and a study by Packiam et al. (22) revealed that differences in expression are due, in part, to differential levels of *lst* transcription. DNA sequence analysis of the *lst* promoters of *N. gonorrhoeae* F62 and *N. meningitidis* MC58 revealed a 105-bp Correia repeat-enclosed element (CREE) in the 5′ region of the *N. meningitidis lst* promoter which is absent in *N. gonorrhoeae*. CREE displaces the σ^{70} promoter used to transcribe the gonococcal *lst* (22) and downregulates expression of *N. meningitidis lst* mRNA approximately 5-fold. Additional factors that may govern the rate of LOS sialylation include the efficiency of the Lst, CMP-NANA levels, and expression levels of a sialylatable LOS epitope.

The role of LOS sialylation in protecting *N. gonorrhoeae* from complement-mediated killing is clear, and *N. gonorrhoeae* may require higher levels of Lst activity to combat host defenses than *N. meningitidis*. Wu and Jerse demonstrated that a mutant of gonococcal strain MS11 that lacked *lst* was attenuated relative to wild-type MS11 in a mouse model of colonization (23). However, differences in *lst* expression levels have not been examined in the context of evasion of host immune defenses. In this study, we sought to examine the biological significance of differential *lst* expression in *N. gonorrhoeae*.

RESULTS

Lst expression level correlates with incorporation of radiolabeled CMP-NANA by live, intact *N. gonorrhoeae*. Triton X-100 extracts from *N. gonorrhoeae* F62 contained 4.5-fold more Lst activity than extracts from *N. meningitidis* MC58 *in vitro* (22), attributable to the 105-bp CREE element in the *N. meningitidis lst* promoter as described above (22). To determine if higher rates of *lst* transcription correlated with increased incorporation of sialic acid onto LNT LOS of live, intact gonococci, we compared the levels of incorporation of tritiated CMP-NANA by isogenic strains of *N. gonorrhoeae* F62ΔD (*lgtD* inactivated, LNT LOS predominant [24]) that express the native F62 Lst (Lst$_{-Ng}$) from either the F62 *lst* promoter (P$_{lst-Ng}$) or the *N. meningitidis* 126E *lst* promoter (P$_{lst-Nm}$) (strains F62ΔD P$_{lst-Ng}$ and F62ΔD P$_{lst-Nm}$, respectively). An *lst* mutant that cannot sialylate LOS was also constructed in the strain F62ΔD background (F62ΔD Δlst). All *N. gonorrhoeae* strains presented are in the background of strain F62ΔD, and these strains are referred to here as P$_{lst-Ng}$, P$_{lst-Nm}$, and strain Δlst for

simplicity. The 126E *lst* promoter is 99% identical to that of MC58 and also contains the downregulating CREE. In accordance with the observations of Packiam et al. (22), real-time quantitative reverse transcription-PCR (qRT-PCR) demonstrated that the 126E *lst* promoter in F62 decreased *lst* transcript levels (data not shown). CMP-NANA spiked with CMP-sialic-6-³H (total CMP-NANA concentration, 2 μg/ml) was added to exponential-phase cultures of P$_{lst-Ng}$ and P$_{lst-Nm}$, and radioactivity incorporation was measured over time (Fig. 1A). After 15, 60, and 120 min of growth, P$_{lst-Nm}$ incorporated 8.3-, 3.7-, and 2-fold less radiolabeled CMP-NANA than P$_{lst-Ng}$, respectively (Fig. 1A). As expected, radiolabeled NANA was not detected on the negative controls, P$_{lst-Ng}$ grown without CMP-NANA, and Δlst (Fig. 1A). Incorporation of NANA by a *siaA* mutant of *N. meningitidis* strain NMB (which cannot synthesize CMP-NANA but can sialylate its LOS with exogenously supplied CMP-NANA) is shown for comparison. NMB *siaA*, with its native *lst* promoter (containing the 105-bp CREE) and *lst* coding sequence, incorporated 5.7-, 3.7-, and 2.9-fold less radiolabeled NANA than P$_{lst-Ng}$ (Fig. 1A). These values are similar to what we observed with P$_{lst-Nm}$. Overall, these data are consistent with the *in vitro* results obtained by Packiam et al. using Triton X-100 extracts and establish that decreased transcription of *lst* associated with the CREE in the *lst* promoter results in less incorporation of NANA onto live bacteria.

Maximal binding of FH to *N. gonorrhoeae* occurs prior to maximal LOS sialylation. Sialylation of LNT LOS enhances binding of FH (6, 7, 57). The correlation between FH binding to live gonococci and the extent of LOS sialylation has not been determined. To address this, we used flow cytometry to examine binding of FH and monoclonal antibody (MAb) 3F11 in parallel at 0, 15, 60, and 120 min following the addition of CMP-NANA (final concentration of 2 μg/ml) to an early log culture of strain F62ΔD. MAb 3F11 recognizes the unsialylated LNT LOS epitope, and sialylation of LNT LOS results in loss of 3F11 binding (25–27). Maximal binding of FH occurred approximately 15 min after the addition of CMP-NANA (Fig. 1B), while binding of MAb 3F11 had decreased only marginally after 15 min and continued to decline over the duration of the assay (120 min) (Fig. 1B). These data are consistent with the gradual uptake of tritiated CMP-NANA by LNT LOS over time (Fig. 1A). These data establish that maximal FH binding occurs prior to and does not require maximal LOS sialylation.

Reduced expression of *lst* is associated with reduced binding of human FH. Maximal binding of FH to strain F62ΔD occurred prior to full sialylation (Fig. 1B). Accordingly, we examined whether decreased sialylation associated with expression of *lst* from the meningococcal *lst* promoter (Fig. 1A) also diminished FH binding to gonococci. Levels of binding of FH to P$_{lst-Ng}$ and P$_{lst-Nm}$ were compared over time in the presence of CMP-NANA (0.5 μg/ml); physiological concentrations are ~0.02 to ~0.5 μg/ml (23) using flow cytometry. Binding of FH to P$_{lst-Ng}$, expressing wild-type levels of *lst*, was detectable almost immediately after adding CMP-NANA and reached near maximal levels by 5 min (Fig. 2A and B). Binding of FH to P$_{lst-Nm}$ was also enhanced following incubation with CMP-NANA; however, the level of FH binding achieved was significantly lower than that seen with wild-type *lst* expression, and the level of binding seen after a 30-min incubation with either a low (0.5 μg/ml) or high (25 μg/ml) concentration of CMP-NANA was statistically lower (Fig. 2B). In the absence of CMP-NANA (Fig. 2A and B) or of *lst* expression (Δlst;
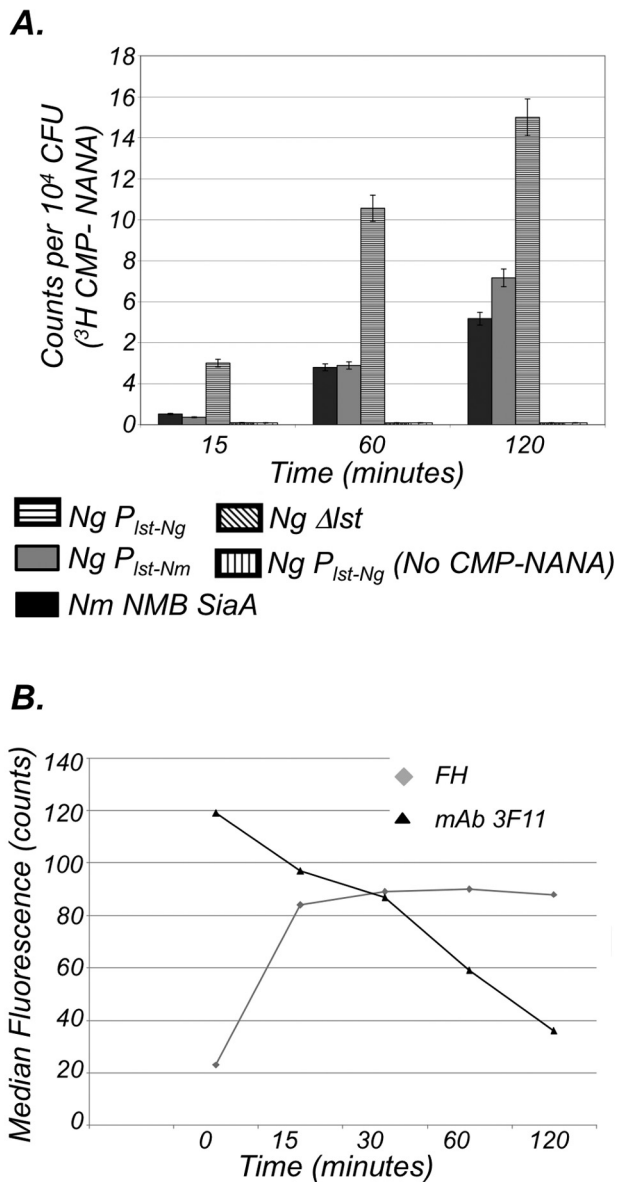
FIG 1 Incorporation of CMP-NANA onto LNT-LOS by Lst and the relationship of sialylation kinetics to binding of FH and MAb 3F11. (A) Incorporation of CMP-NANA by *N. gonorrhoeae* F62ΔD expressing *lst* either from its native promoter (*Ng* P_{lst-Ng}) or from the *N. meningitidis* 126E *lst* promoter (*Ng* P_{lst-Nm}) and *N. meningitidis* NMB SiaA (*Nm NMB SiaA*). Strains were grown in the presence of 2 μg/ml CMP-NANA (50:1 [wt/wt] unlabeled CMP-NANA/labeled CMP sialic acid [sialic-6-³H]), and incorporation was measured over time. Negative controls included *N. gonorrhoeae* Δlst lacking the sialyltransferase enzyme, Lst (*Ng* Δlst), and *N. gonorrhoeae* P_{lst-Ng} grown in the absence of CMP-NANA [*Ng* P_{lst-Ng} (*No CMP-NANA*)]. Values are the means and standard deviations of the results of triplicate measurements from three independent experiments. (B) Kinetics of FH binding in relation to LOS sialylation. Flow cytometry was used to measure binding of human FH (10 μg/ml) and MAb 3F11 (recognizes the unsialylated LNT-LOS epitope) to *N. gonorrhoeae* F62ΔD over time following the addition of CMP-NANA (2 μg/ml) to log-phase cultures. Bound human FH was detected with MAb 90X, and sialylation of the LNT LOS epitope was detected by loss of reactivity with MAb 3F11. The *x* axis represents time in minutes, and the *y* axis represents the median fluorescence. The analysis was conducted three times, and results from a representative experiment are shown.

data not shown), strain F62ΔD did not bind FH. These data indicate that decreased expression of *lst* impacts both the level of sialylation and the level of binding of FH.

Differences in *N. gonorrhoeae* and *N. meningitidis* Lst protein sequences do not affect FH binding. The gonococcal and meningococcal Lst proteins differ by as many as 21 amino acids (28). To determine if the primary Lst sequence played a role in Lst activity, we constructed a mutant of F62ΔD that expressed the *N. meningitidis* MC58 *lst* coding sequence driven by the *N. gonorrhoeae* F62 *lst* promoter. When sialylated, this strain (F62ΔD Lst$_{Nm}$), bound FH with kinetics and amounts similar to those observed with the gonococcal *lst* coding sequence driven from the *N. gonorrhoeae* *lst* promoter (Fig. 2C), indicating that amino acid sequence differences between *N. gonorrhoeae* Lst and *N. meningitidis* Lst do not significantly influence Lst activity insofar as FH binding is concerned.

Reduced expression of *lst* is associated with increased AP activation. FH limits deposition of C3 by the AP by (i) acting as a cofactor in the factor I-mediated cleavage of C3b to iC3b (inactive C3b), (ii) irreversibly dissociating the AP C3 convertase (C3bBb; decay-accelerating activity), and (iii) competing with factor B for C3b binding (29). To determine whether differences in the amounts of FH bound by strains harboring the *N. meningitidis* or *N. gonorrhoeae* *lst* promoter translated to differences in AP regulation, we measured, by enzyme-linked immunosorbent assay (ELISA), deposition of C3 fragments and binding of factor B to gonococci following exposure to pooled normal human serum (PNHS). In the absence of CMP-NANA or *lst* expression, strain F62ΔD does not bind FH (see above) and activation of the AP proceeds unimpeded, as evidenced by high binding of C3 and factor B (Fig. 3; gray bars (− CMP-NANA) and Δlst). As expected, AP activation was unaffected by CMP-NANA in the *lst* mutant strain (Fig. 3; Δlst). CMP-NANA decreased deposition of C3 and binding of factor B to strain F62ΔD that harbored either the *N. gonorrhoeae* or the *N. meningitidis* *lst* promoter (P_{lst-Ng} or P_{lst-Nm}, respectively) compared to the unsialylated controls (Fig. 3). In accordance with increased FH binding, activation of the AP (measured by C3 fragment deposition and FB binding) on P_{lst-Ng} was significantly lower than that on P_{lst-Nm} (Fig. 3).

Increased *lst* transcription confers increased resistance to killing by human serum. The role of *lst* expression levels in serum resistance was determined by comparing the abilities of strains P_{lst-Nm} and P_{lst-Ng} to resist killing by PNHS following growth in various concentrations of CMP-NANA. Concordant with its greater FH binding and lower AP activation, P_{lst-Ng} was more resistant than P_{lst-Nm} to killing by PNHS (Fig. 4A).

Low expression of *lst* facilitates unstable serum resistance of *N. gonorrhoeae* in vivo. LOS sialylation occurs in the murine genital tract as early as 24 h postinfection and is sufficient to mediate resistance of organisms *ex vivo* to human serum *in vitro* (23). To determine whether serum resistance conferred by LOS sialylation *in vivo* is compromised by decreased *lst* expression, we assessed the ability of P_{lst-Ng} and P_{lst-Nm} to resist killing by PNHS *ex vivo*, following their recovery from the murine genital tract. Estradiol-treated mice were inoculated intravaginally with either P_{lst-Ng} or P_{lst-Nm}, and bacteria harvested on vaginal swabs 24 and 48 h postinfection were tested, without subculturing, for their ability to resist killing by PNHS *ex vivo*. Both gonococcal strains that were recovered from the genital tract 24 h postinfection were highly resistant to killing by 30% PNHS (Fig. 4B; − [without neur-
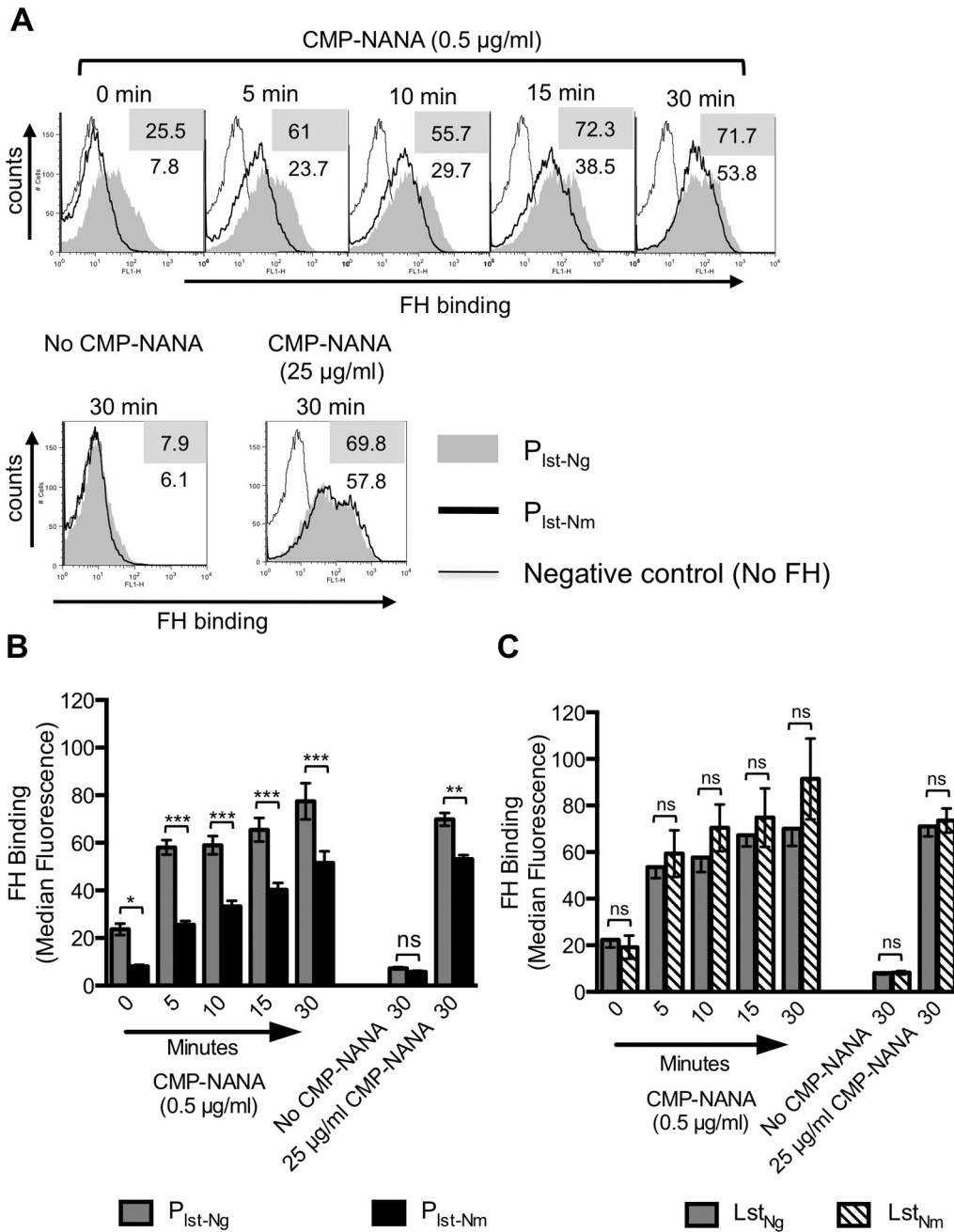
FIG 2 FH binding to *N. gonorrhoeae* correlates with *lst* expression level. Binding of FH to *N. gonorrhoeae* F62ΔD expressing the F62 Lst amino acid sequence driven by either the F62 *lst* promoter (P_{lst-Ng}) or the *N. meningitidis* 126E *lst* promoter (P_{lst-Nm}) (A and B) or binding of FH to strain F62ΔD expressing either the *N. gonorrhoeae* Lst or the *N. meningitidis* Lst amino acid sequence (C) was measured by FACS analysis using human FH and affinity-purified polyclonal goat anti-human FH. Binding was measured over time (0 to 30 min as indicated) following the addition of CMP-NANA (0.5 μg/ml) to bacteria. Control reactions included (i) bacteria incubated with high concentrations of CMP-NANA (25 mg/ml) for 30 min, (ii) bacteria incubated without CMP-NANA, and (iii) bacteria that were not incubated with FH. An *lst* mutant (Δ*lst*) also served as a negative control (data not shown). (A) Histograms from a representative experiment comparing P_{lst-Ng} (grey shaded) and P_{lst-Nm} (bold black line) are shown. The *y* axis represents the number of events (counts), and the *x* axis represents fluorescence on a $\log_{10}$ scale. FH was omitted in the negative control (thin black line). Numbers alongside the histograms (e.g., the shaded box corresponds to the shaded histograms) are the median fluorescence values. Results showing binding of FH to both strains following incubation with 25 μg/ml CMP-NANA for 30 min are shown (lower left histograms). (B) Median fluorescence with standard errors of the means (SEM) of the results from 6 independent experiments comparing FH binding to P_{lst-Ng} (grey bars) and P_{lst-Nm} (black bars) are shown on the *y* axis. Each bar represents the mean of the median fluorescence values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ (Bonferroni's posttest 2-way analysis of variance [ANOVA]). (C) Median fluorescence values with SEM from 5 independent experiments comparing levels of FH binding to strain F62ΔD expressing either the *N. gonorrhoeae* Lst (Lst_{Ng}) or *N. meningitidis* Lst (Lst_{Nm}) amino acid sequence are shown on the *y* axis. Each bar represents the mean of the median fluorescence values. ns, not significant ($P > 0.05$; Bonferroni's posttest 2-way ANOVA).
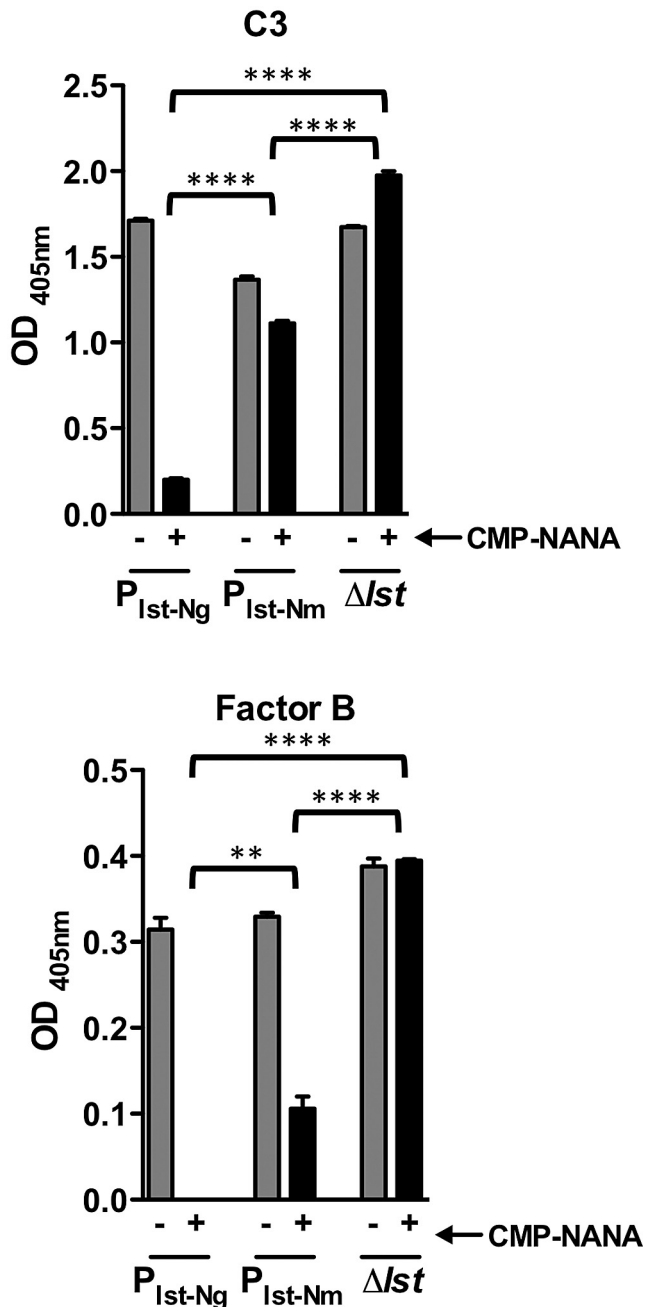
FIG 3 Complement deposition (C3 and factor B) on strain F62ΔD expressing high (P_{lst-Ng}) and low (P_{lst-Nm}) levels of *lst*. Data represent levels of binding of total C3 and factor B to strain F62ΔD expressing *lst* using either the F62 *lst* promoter (P_{lst-Ng}) or the *N. meningitidis* 126E *lst* promoter (P_{lst-Nm}) measured by ELISA. Gonococcal strains grown in the presence or absence ("+" or "-") of CMP-NANA (1 μg/ml) were incubated with 33.3% PNHS for 10 min, washed, and dispensed onto microtiter wells. Bound C3 and factor B were detected using goat anti-human C3 (Complement Technology, Inc.) and goat anti-human factor B (Quidel), respectively. Binding to strain Δ*lst* is shown as a negative control. The mean OD_{405} values with SEM from two independent experiments performed in duplicate are plotted on the *y* axis. **, $P < 0.01$; ****, $P < 0.0001$ (1-way ANOVA using Tukey's multiple comparison test).
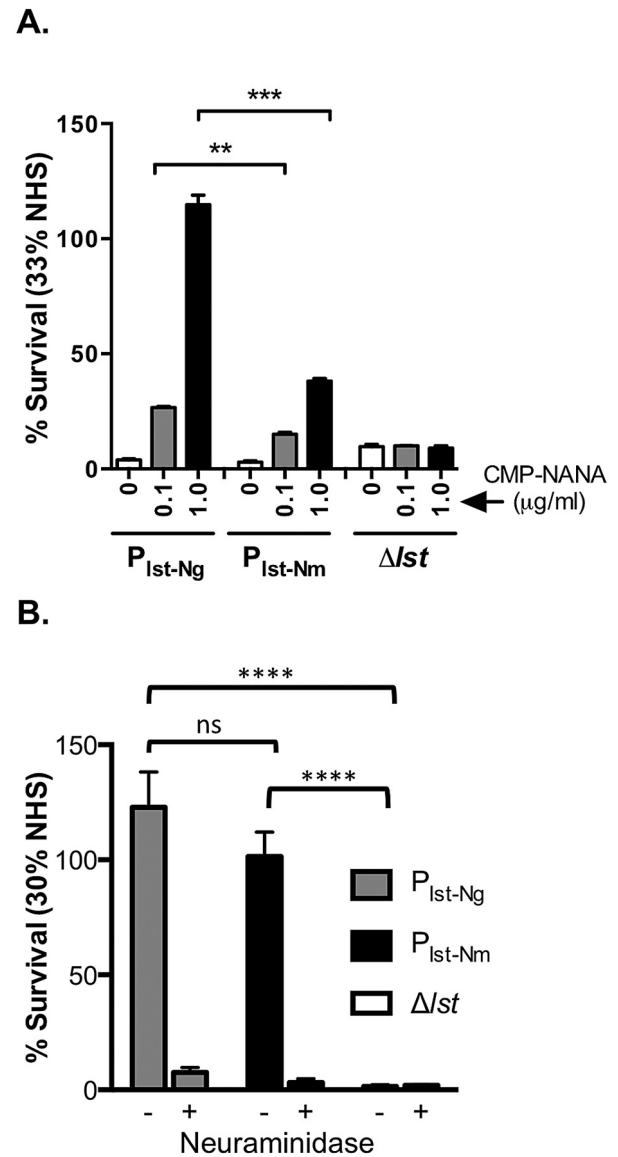
FIG 4 Serum resistance as a function of Lst expression level. (A) *In vitro* sialylation and serum resistance of gonococci expressing low and high levels of *lst*. Data represent survival of isogenic F62ΔD expressing gonococcal Lst from either the gonococcal F62 *lst* promoter (P_{lst-Ng}) or the meningococcal 126E *lst* promoter (P_{lst-Nm}) in 33.3% PNHS following growth in the absence (0 μg/ml) or presence (0.1 or 1.0 μg/ml) of CMP-NANA. The percentages of survival at 30 min relative to time zero are plotted on the *y* axis. Each bar represents the mean and SEM of the results of 2 independent observations. **, $P < 0.01$; ***, $P < 0.001$ (Tukey's posttest 1-way ANOVA). (B) *In vivo* sialylation and serum resistance of gonococci expressing low and high levels of *lst*. Bacteria harvested from the genital tract of mice infected with *N. gonorrhoeae* expressing lst_{Ng} using either the gonococcal *lst* promoter (P_{lst-Ng}) or the meningococcal *lst* promoter (P_{lst-Nm}) were tested for their ability to resist killing by 30% PNHS *ex vivo*. Samples from 3 infected mice in each group were pooled and tested on 2 consecutive days, 24 and 48 h postinfection. Neuraminidase removes sialic acid from LOS and was used to determine the *in vivo* role of LOS sialylation (23) in the serum resistance of bacteria harvested from infected mice. Bacteria harvested from mouse vaginal samples were treated with either neuraminidase (+) or buffer (−) prior to bactericidal testing. Mice infected with an *lst* mutant (Δ*lst*) that lacks the enzyme required for LOS sialylation were used as a control. The percentages of survival relative to time zero are plotted on the *y* axis. Each bar represents the mean and SEM of 2 independent observations. ns, not significant; ****, $P < 0.0001$ (1-way ANOVA using Tukey's multiple comparison test).

aminidase]). Treatment of bacteria harvested from the murine genital tract with neuraminidase to desialylate the LOS restored serum sensitivity to both strains and confirmed that the observed

serum resistance was the result of LOS sialylation (Fig. 4B; + [with neuraminidase]). F62ΔD Δlst harvested from infected mice were fully sensitive to killing by PNHS, with or without neuraminidase treatment (Fig. 4B; white bars). These data indicate that the level of lst produced by either the N. gonorrhoeae lst promoter or the N. meningitidis lst promoter mediates LOS sialylation in vivo and that the level of LOS sialylation attained at 24 h is sufficient for resistance to PNHS ex vivo.

N. gonorrhoeae F62ΔD expressing Lst from the *N. meningitidis lst* promoter is attenuated in the murine infection model. Using a competitive infection model, a mutant of N. gonorrhoeae MS11 that lacked lst was reported to be significantly attenuated in its ability to colonize the lower genital tract of BALB/c mice compared to its lst-expressing parent strain (23). We sought to determine if low levels of lst similarly affected the ability of N. gonorrhoeae to colonize mice. Using the same model, estradiol-treated wild-type BALB/c mice (6 per group) were inoculated with a suspension containing similar numbers of P_{lst-Ng} ("wild type") and P_{lst-Nm} ("mutant"). As a control, a competitive infection was performed in a separate group of mice using P_{lst-Ng} (wild type) and Δlst (mutant). Vaginal samples were obtained daily, and recovery of the mutant strain (either P_{lst-Nm} or Δlst) relative to the wild-type strain (P_{lst-Ng}) was expressed as a competitive index (CI); a CI of <1.0 indicates a decrease in the ratio of mutant to wild type with respect to that of the inoculum. Consistent with prior data (23), recovery of Δlst (mutant) relative to P_{lst-Ng} (wild type) in wild-type BALB/c mice was dramatically decreased early in infection (Fig. 5, left). Interestingly, despite evidence of LOS sialylation in vivo (Fig. 4B), P_{lst-Nm} was also significantly attenuated in wild-type mice relative to P_{lst-Ng} (Fig. 5; middle). By day 3, P_{lst-Nm} was no longer detected in 5 of 6 infected animals (Fig. 5; middle open symbols). All strains showed similar growth rates in vitro when cultured separately or in mixed-broth cultures (data not shown). Overall, these data indicate that, despite the full resistance (>100% survival) to human complement conferred by LOS sialylation in vivo by the N. meningitidis lst promoter ("low-level" lst expression) (Fig. 4B), the ability to colonize the genital tract of female mice relative to that of the N. gonorrhoeae lst promoter ("high-level" lst expression) in a competitive infection experiment was significantly hampered by decreased lst expression.

N. gonorrhoeae bind specifically to human FH; we have developed transgenic BALB/c mice that express human FH in concentrations similar to those seen in humans (30). Estradiol-treated human FH-transgenic BALB/c mice (n = 9) were inoculated with a suspension containing similar numbers of P_{lst-Ng} (wild type) and P_{lst-Nm} (mutant), and daily recovery of the mutant (P_{lst-Nm}) relative to the wild type (P_{lst-Ng}) was expressed as a competitive index (CI). As in wild-type BALB/c mice, P_{lst-Nm} was significantly attenuated in FH-transgenic mice relative to P_{lst-Ng} (Fig. 5; right side). We hypothesize that, as in wild-type BALB/c mice, downregulation of lst may render N. gonorrhoeae sensitive to killing by polymorphonuclear leukocytes (PMNs) and cathelicidin-related antimicrobial peptides (CRAMPS) and that this masks human FH-mediated protection against the AP.

DISCUSSION

Sialylation of gonococcal LNT LOS renders serum-sensitive strains resistant to killing by NHS (31). In contrast, sialylation of meningococcal LNT LOS appears to play a role subsidiary to that of the capsule in resistance to serum (32–34). In this study, we
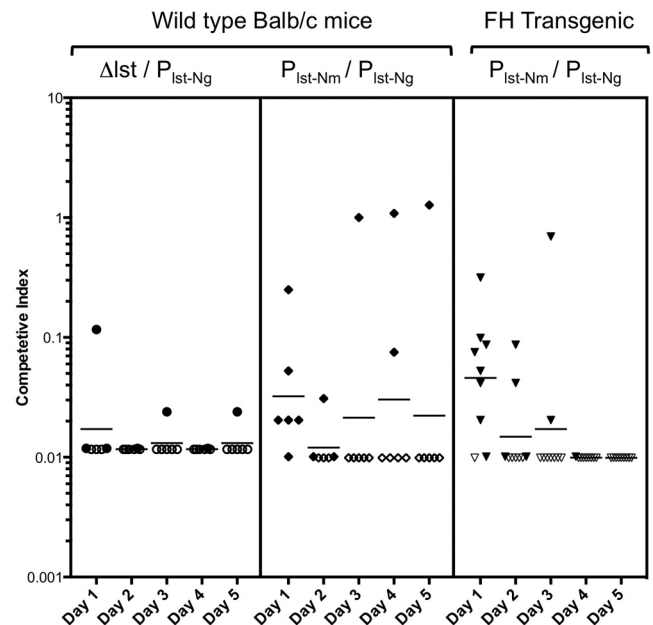

FIG 5 Attenuation of N. gonorrhoeae expressing Lst from the meningococcal lst promoter in the mouse vaginal colonization model. Data representing recovery of strain F62ΔD expressing lst from the N. meningitidis promoter (P_{lst-Nm}) relative to recovery of strain F62ΔD expressing lst from its native gonococcal promoter (P_{lst-Ng}) from estradiol-treated BALB/c mice (wild type or human FH transgenic) coinfected with both strains are expressed as competitive indices (CI); each point represents the CI calculated from an individual mouse. Data representing recovery of an lst mutant (Δlst) relative to that of strain F62ΔD expressing lst from its native gonococcal promoter (P_{lst-Ng}) are also shown. A CI of <1.0 indicates a decrease in the ratio of mutant to wild type with respect to that of the inoculum. The ratios of strain P_{lst-Nm} to strain P_{lst-Ng} and of strain Δlst to strain P_{lst-Ng} in the inocula were 1 and 0.85, respectively. Horizontal bars represent the geometric mean CI values; open symbols represent mice from whom at least 50 wild-type (P_{lst-Ng}) CFU/ml, but no mutant (P_{lst-Nm} or Δlst) CFU, were recovered. The limit of detection (40 CFU/ml) was used as the number of mutant CFU recovered in these cases.

have defined the functional role of increased Lst expression in N. gonorrhoeae relative to Lst expression in N. meningitidis as it relates to human AP regulation and the ability to colonize the mouse genital tract. Our studies expand the observations of differential levels of Lst activity (22) in live N. gonorrhoeae and provide both in vitro and in vivo evidence for the biological significance of its higher lst expression. Decreased LOS sialylation may offer advantages such as facilitating adhesion of N. meningitidis to host cells (35–37) and the central role of capsule in N. meningitidis serum resistance (33, 34, 38–41) likely compensates for any loss of complement inhibition the organism might suffer as a consequence of lst downregulation.

We have demonstrated that maximal binding of FH to gonococci occurs well before saturation of LNT LOS with NANA and this suggests that the threshold amount of sialylation necessary for maximal FH binding is rapidly achieved by gonococci. The ability to bind large amounts of FH in the face of low levels of LOS sialylation may be critical for the bacteria to evade complement-dependent killing and yet permit adhesion and invasion through the asialoglycoprotein receptor (ASGP-R) that may otherwise be impaired by the presence of high levels of LOS sialylation (35–37). Further, unlike N. meningitidis, N. gonorrhoeae cannot synthesize CMP-NANA and may require a highly efficient system to

scavenge sufficient amounts of CMP-NANA from the host, where availability of this substrate may be limited (physiological concentration, ~0.02 to ~0.5 µg/ml). Using live, intact *N. gonorrhoeae*, we demonstrated that decreased expression of *lst*, associated with transcription from the *N. meningitidis lst* promoter, decreases LOS sialylation and alters the binding kinetics of FH *in vitro*, which translates to impaired inhibition of the human AP and a decreased ability of CMP-NANA to mediate complement resistance *in vitro*.

LOS sialylation is an important virulence determinant that contributes to the ability of *N. gonorrhoeae* to colonize the genital tract of estradiol-treated mice (23). Our studies indicate that, in addition, high-level *lst* expression is important for colonizing the genital tract of female BALB/c mice; *N. gonorrhoeae* strains expressing low levels of *lst* were outcompeted by *N. gonorrhoeae* strains expressing normal levels of *lst* following intravaginal inoculation. The *lst* mutant of *N. gonorrhoeae* MS11 was as attenuated in C5-deficient mice as in C5-sufficient mice (23), suggesting that complement-mediated killing (involving the terminal complement pathway) did not contribute measurably to the attenuation of the *lst* mutant in BALB/c mice (23). That study proposed that LOS sialylation conferred a survival advantage to *N. gonorrhoeae* in mice by increasing resistance to opsonophagocytic killing by murine PMNs. *N. gonorrhoeae* with sialylated LOS induced a weaker respiratory burst in and were more resistant to killing by PMNs isolated from estradiol-treated mice *in vitro*. Consistent with increased susceptibility to phagocytic killing, the *lst* mutant was also more susceptible to killing following intraperitoneal (i.p.) inoculation.

Cationic antimicrobial peptides (CAMPs), found in the granules of phagocytic cells and expressed by epithelial cells in response to stimuli, including pathogens, are another important component of the innate immune system that *N. gonorrhoeae* must combat to successfully cause infection. *N. gonorrhoeae* use a variety of mechanisms to combat killing by CAMPS; the most important of these are excretion by the MtrCDE efflux pump (42–44) and, to a lesser extent, repulsion by LOS sialic acid, which reduces binding of CAMPS to *N. gonorrhoeae* (H. Wu and A. Jerse, presented at the 17th International Pathogenic Neisseria Conference, Banff, Alberta, Canada, September 11 to 16, 2010; H. Wu, W. Shafer, and A. Jerse, presented at the 17th International Pathogenic Neisseria Conference, Wurtzburg, Germany, September 9 to 14, 2012). Cathelicidin-related antimicrobial peptide (CRAMP) is the murine homologue of the human cathelicidin, LL-37. Wu et al. recently reported that an *lst* mutant of *N. gonorrhoeae* F62 was significantly less attenuated in CRAMP-deficient mice than in wild-type BALB/c mice (Wu et al., presented at the 17th International Pathogenic Neisseria Conference, Wurtzburg, Germany). These studies highlight the significance of CRAMP-mediated killing in the mouse model and may support a key role for LOS sialylation in protection against CRAMPs.

Our studies revealed that, despite decreased LOS sialylation *in vitro*, expression of *lst* from the *N. meningitidis lst* promoter was adequate for LOS sialylation *in vivo* and that the level of sialylation obtained after 24 h in the mouse vagina was sufficient to mediate full resistance to killing by human sera *ex vivo*. One explanation is that the mouse vagina provides a continuous, albeit low, level of CMP-sialic acid, which after 24 h, was sufficient to mediate full resistance to killing by human sera *in vitro*. Based on this outcome, one could hypothesize that the *N. meningitidis lst* promoter likely

drives sufficient LOS sialylation *in vivo* to effectively combat killing by the AP.

The sensitivity of *lst* mutants to killing by PMNs and CRAMPs in the murine model confounds evaluation of human FH-mediated resistance to complement-dependent killing *in vivo*. *N. gonorrhoeae* bind specifically to human FH, and the results of a coinfection experiment using human FH-transgenic mice (30) showed attenuation of P$_{lst-Nm}$ relative to P$_{lst-Ng}$ (Fig. 5, right) similar to that seen in wild-type mice. We hypothesize that, as in wild-type BALB/c mice, downregulation of *lst* may render *N. gonorrhoeae* sensitive to killing by PMNs and CRAMPS and that this masks human FH-mediated protection against the AP.

Lst activity also varies among *N. gonorrhoeae* strains; Lst activity in 16 clinical isolates of *N. gonorrhoeae* ranged from ~685 to 5,367 cpm/µl, representing differences of as much as ~8-fold (22). Packiam et al. found that the regions 5′ to *lst* in 28 *N. gonorrhoeae* strains varied considerably but that none contained the downregulating CREE found in *N. meningitidis* and that *lst* mRNA levels among *N. gonorrhoeae* strains differed by less than 2-fold (22). At this time, the mechanism(s) associated with variation in Lst activity among *N. gonorrhoeae* strains remains unknown. We noted that the *N. gonorrhoeae* and *N. meningitidis* Lst proteins differ by as many as 21 amino acids (28) but did not detect significant differences in the activity of the *N. meningitidis* Lst and *N. gonorrhoeae* Lst proteins when FH binding was used as a functional readout (Fig. 2C). In addition, Matthias and Rest recently reported repression of the *N. gonorrhoeae* F62 *lst* by CrgA, a LysR-type transcriptional regulator; deletion of CrgA resulted in overexpression of *lst* and enhanced serum resistance (45). In conclusion, our findings demonstrate that the level of Lst activity is crucial for pathogenesis and impacts the ability of *N. gonorrhoeae* to resist killing by human serum and to colonize the genital tract of experimentally infected mice.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School, All subjects who donated blood for this study provided written informed consent. Use of animals in this study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School.

Bacterial strains and culture conditions. The neisserial strains used in this study are described in Table 1. *N. gonorrhoeae* F62ΔD (a gift from Daniel C. Stein, University of Maryland) is an *lgtD* mutant of *N. gonorrhoeae* F62 (24) that expresses only the LNT LOS epitope on HepI. Neisserial strains were routinely cultured on chocolate agar or in GC broth supplemented with IsoVitaleX equivalent at 37°C in an atmosphere of 5% CO$_2$. Antibiotics were added to GC agar plates as required at the following concentrations: kanamycin (Kan), 100 µg/ml; erythromycin (Erm), 2 µg/ml; streptomycin (Sm), 10 mg/ml; and chloramphenicol (Cm), 7 µg/ml. *Escherichia coli* strains Top10 and InvaF′ (Invitrogen) were cultured on LB agar with ampicillin (125 µg/ml), Kan (50 µg/ml), Erm (400 µg/ml), or Cm (50 µg/ml) as appropriate. InvaF′ was used for propagation of all plasmids containing the Sm sensitivity cassette.

Construction of strains and mutagenesis. Plasmids and primer sequences used in this study are described in Tables S1 and S2 in the supplemental material, respectively. All *N. gonorrhoeae* strains were rendered resistant to Sm to facilitate use in the murine model of infection by transformation with *rpsL* from Sm-resistant *N. gonorrhoeae* FA1090. In-

TABLE 1 Bacterial strains used in this study

Strain	Description	Reference or source
N. gonorrhoeae		
F62	Por1B	54
F62ΔD	F62 *lgtD* inactivated; LNT LOS predominates	24
F62ΔD P$_{lst-Ng}$	F62ΔD with F62 *lst* promoter, F62 *lst* coding sequence, *aphA3* downstream of *lst*, *rpsL*$_{FA1090}$, *ErmC′* inserted in intergenic region between *lctP* and *aspC* (Kan^r, Sm^r, Erm^r)	This study
F62ΔD P$_{lst-Nm}$	Strain F62ΔD with *N. meningitidis* 126E *lst* promoter, *N. gonorrhoeae* F62 *lst* coding sequence, *aphA3* downstream of *lst*, *rpsL*$_{FA1090}$ (Kan^r, Sm^r)	This study
F62ΔD Lst$_{Nm}$	Strain F62ΔD with F62 *lst* promoter, *N. meningitidis* MC58 *lst* coding sequence, *aphA3* downstream of *lst*, *rpsL*$_{FA1090}$ (Kan^r, Sm^r)	This study
F62ΔD Δ*lst*	Strain F62ΔD *lst::kan*, *rpsL*$_{FA1090}$ (Kan^r, Sm^r)	This study
N. meningitidis		
MC58	L3 immunotype, serogroup B (B:15:P1.7,16)	55
126E	L1 immunotype, serogroup C	56
NMB	Serogroup B (B:2b:P1.5,2)	48
NMB *siaA*	*siaA::cat* (Cm^r)	This study

sertional inactivation of *lst* in strain F62ΔD was achieved using pGH7 as described previously (31).

Exchange of the F62 *lst* coding sequence with the *N. meningitidis* MC58 *lst* coding sequence in strain F62ΔD Lst$_{Nm}$ was achieved by transforming strain F62ΔD with plasmid plst$_{MC58}$KanCytC, which contains (5′ to 3′) the MC58 *lst* coding sequence, a kan resistance gene, and DNA homologous to the region downstream of *lst* in F62.

Exchange of the F62ΔD *lst* promoter with the *N. meningitidis* 126E *lst* promoter in strain F62ΔD P$_{lst-Nm}$ was achieved using a two-step mutagenesis system (46) and plasmids pTOPO*lst*prom and pTOPO*lst*prom-Erm/Sm. pTOPO*lst*prom contains (5′ to 3′) the region upstream of the F62 *lst* promoter, the *N. meningitidis* 126E *lst* promoter, and the F62 *lst* coding and downstream sequences. pTOPO*lst*prom-Erm/Sm is pTOPO*lst*prom with a cassette that codes for resistance to Erm and sensitivity to Sm inserted in the HincII site. Strain F62ΔD was transformed with pTOPO*lst*prom-Erm/Sm, and Erm-resistant but Sm-sensitive colonies were selected and transformed with pTOPO*lst*prom. Sm-resistant transformants were selected and screened for sensitivity to Erm.

The introduction of the *N. meningitidis* MC58 *lst* gene into F62ΔD Lst$_{Nm}$ introduced a Kan resistance gene downstream of *lst*. For this reason, plst$_{F62}$KanCytC, which contains (5′ to 3′) the F62 *lst* coding sequence, *aphA3*, and *lst* F62 downstream region, was constructed and used to introduce a Kan resistance gene downstream of *lst* in strains F62ΔD P$_{lst-Ng}$ and F62ΔD P$_{lst-Nm}$. Lastly, an Erm resistance gene was inserted into the region between the *lctP* and *aspC* genes of P$_{lst-Ng}$ to allow for differentiation in competitive infections in BALB/c mice (47).

The DNA sequence of the *lst* locus and flanking regions was verified in all strains. All strains expressed similar LOS structures (see Fig. S1 in the supplemental material) as determined by silver staining and Western blot analysis performed with MAbs 3F11 (HepI LNT-LOS), 2C7 (HepII lactose), and L8 (HepI lactose, HepII 3-PEA).

LOS sialylation and group B capsule expression were abrogated simultaneously in *N. meningitidis* strain NMB (48) by inactivation of *siaA*. Cm-resistant colonies were screened for inactivation of *siaA* by PCR.

Incorporation of radiolabeled ^{3}H-CMP-NANA into gonococcal LNT-LOS. Neisserial strains grown overnight on chocolate agar plates were inoculated to an optical density at 600 nm (OD$_{600}$) of 0.1 in GC liquid media and grown to an OD$_{600}$ of 0.2 (early logarithmic phase). At this point, a 50:1 (wt/wt) mixture of unlabeled and ^{3}H-labeled CMP-NANA (CMP sialic acid [sialic-6-^{3}H]; American Radiolabeled Chemicals, Inc.) was added (final concentration, 2 μg/ml), and triplicate cell samples (100 μl each) were collected at 0, 15, 60, and 120 min of incubation; OD$_{600}$ readings were recorded at each time point. Cells were pelleted (3 min at 13,000 × *g*), washed twice in 1 ml Hanks' balanced salt solution (HBSS^{++}), and suspended in a final volume of 100 μl of HBSS^{++}. The cell sample was spotted onto Whatman paper, placed in a scintillation vial, and dried, and then 5 ml of Betaplate Scint fluid (PerkinElmer) was added. Radioactivity (cpm) was measured with a United Technologies liquid scintillation counter. A standard curve correlating CFU/ml to OD$_{600}$ was generated and used to determine the CFU present at each time point. Incorporation of ^{3}H-labeled CMP-NANA per CFU was calculated by dividing the total counts detected per 100 μl sample by the calculated CFU/100 μl.

Flow cytometry. Binding of human FH (Complement Technology, Inc.) (10 μg/ml) to log-phase bacteria incubated with CMP-NANA (concentrations and incubation times are indicated for each experiment) or buffer (negative control) was measured by fluorescence-activated cell sorting (FACS) (FACSCalibur; Becton, Dickinson), and data were analyzed (FlowJo version 9.2; Tree Star, Inc.) as described previously (6). Bound FH was detected either with anti-human FH monoclonal Ab 90X (anti-human factor H#2; Quidel) or with affinity-purified polyclonal goat anti-human FH (16). Each experiment was performed at least three times.

The sialylation state of the LNT LOS epitope was monitored by FACS analysis using monoclonal antibody (MAb) 3F11 (provided by Michael A. Apicella, University of Iowa, Iowa City), which recognizes the unsialylated LNT epitope of neisserial LOS (25). Bound 3F11 was disclosed using anti-mouse IgM fluorescein isothiocyanate (FITC; Sigma).

Detection of bound complement components. The binding of complement components in PNHS to gonococci was measured by ELISA as described previously (49). In brief, bacteria in the early log phase of growth were incubated for 2 h with 1 μg/ml CMP-NANA or without CMP-NANA and then opsonized with either 33.3% PNHS or heat-inactivated PNHS for 10 min. Treated cells were washed with HBSS^{++} and used to coat ELISA plates. Bound C3 and factor B were detected using goat anti-human C3 (Complement Technology, Inc.) or goat anti-human factor B (Quidel) with anti-goat IgG alkaline phosphatase (Sigma). Murine MAb 2C3 (50) recognizes the conserved gonococcal H.8 lipoprotein (Lip) and was used to measure total bacteria in all samples and verify equivalent amounts of coating of ELISA wells (data not shown). ELISAs were performed in duplicate, and at least 2 independent experiments were performed on separate days.

Sera. Normal human sera (NHS) obtained from 10 healthy adult volunteers with no history of neisserial infection were pooled (PNHS), divided into aliquots, and stored at −80°C.

Bacterial killing by PNHS. The susceptibility of log-phase gonococci grown in liquid culture with CMP-NANA (at the concentrations indicated) to complement-mediated killing by PNHS was determined using a serum bactericidal assay as described previously (11, 51). Survival was

calculated as the number of viable colonies at time 30 min relative to time zero.

Susceptibility of gonococci isolated directly from the murine genital tract to killing by PNHS was determined using vaginal mucus samples collected from infected mice as described previously (23). Estradiol-treated female BALB/c mice (Jackson Laboratories) (8 weeks old) were infected (3 mice per group; 9 mice total), separately, with strain P_{lst-Ng} (6.04×10^5 CFU/mouse), strain P_{lst-Nm} (4.02×10^5 CFU/mouse), or strain Δlst (4.36×10^5 CFU/mouse) as described below and previously (23, 52). Vaginal mucus samples collected 24 and 48 h postinfection were suspended in HBSS^{++}–0.1% bovine serum albumin (BSA) and spun (10 s at 3,000 rpm) to remove cell debris and large bacterial aggregates. The supernatant containing gonococci was collected and spun (30 s, 10,000 rpm) to pellet bacteria. Bacteria from 3 infected mice were pooled (in HBSS^{++}–0.1% BSA); half of the sample was treated with neuraminidase (New England Biolabs) (final concentration, 100 U/ml), and the remainder was incubated in HBSS^{++} (1 h at 37°). The organisms were used in a standard bactericidal assay with 15% or 30% PNHS, and survival was calculated as described above following incubation on GC plates containing vancomycin, colistin, nystatin, and trimethoprim (VCNT inhibitor; Becton, Dickinson) and streptomycin (100 μg/ml).

Mouse infection studies. To determine whether increased expression of *lst* provides gonococci with a survival advantage in the mouse model of infection, a competitive infection experiment was performed using a mixture of strains P_{lst-Ng} and P_{lst-Nm} or, as a control, strains P_{lst-Ng} and Δlst. Female BALB/c mice (Jackson Laboratories) (10 weeks old) or female BALB/c human FH-transgenic mice (30) in the diestrus phase of the estrus cycle were treated with water-soluble 17β-estradiol (Sigma) (1.5 mg) and antibiotics as described previously (52). In competitive infections, mice were inoculated intravaginally (on day 0) with a suspension of bacteria (total dose, ~8×10^5 CFU) containing similar numbers of all strains (strains P_{lst-Ng} [4.68×10^5] and P_{lst-Nm} [4.68×10^5] or strains P_{lst-Ng} [3.92×10^5] and Δlst [3.34×10^5 CFU]). Mice in each group colonized with *N. gonorrhoeae* (6 wild-type BALB/c mice/group or 9 FH-transgenic mice/group) were used, and CFU levels were determined daily by plating bacterial dilutions prepared from vaginal swabs onto GC agar supplemented with VCNT inhibitor and 100 μg/ml Sm. The level of detectable infection was 50 CFU per ml (the lowest dilution plated was 20 μl from a 100-μl undiluted sample), and mice from which no CFU were recovered were assigned a value of 40 CFU per ml. The ratio of mutant (strain P_{lst-Nm} or strain Δlst) to wild type (strain P_{lst-Ng}) was determined in each mouse daily, using erythromycin resistance to differentiate between strains. Individual colonies (100 colonies per mouse) from each mouse were plated in replicate experiments on a nonselective GC plate and a GC plate containing erythromycin; the latter permits the growth of strain P_{lst-Ng} but not the growth of strain P_{lst-Nm} or strain Δlst. Using this method, the level of detection for the wild-type strain relative to the mutant is 0.0099 (<1%). Recovery of the mutant strain relative to the wild-type strain was expressed as a competitive index (CI) as described previously (23, 53). The CI was calculated daily for each mouse by dividing the ratio of the mutant to the wild type in the output by the ratio of the mutant to the wild type in the input as follows: CI = (mutant/wild-type CFU from vaginal cultures on each day)/(mutant/wild-type CFU in the inoculum on day 0). A CI of <1.0 indicates a decrease in the ratio of mutant to wild type compared to the ratio in the inoculum.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.02465-14/-/DCSupplemental.

Figure S1, TIF file, 0.4 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

REFERENCES

1. **Joiner KA, Warren KA, Brown EJ, Swanson J, Frank MM.** 1983. Studies on the mechanism of bacterial resistance to complement-mediated killing. IV. C5b-9 forms high molecular weight complexes with bacterial outer membrane constituents on serum-resistant but not on serum-sensitive *Neisseria gonorrhoeae*. J Immunol **131**:1443–1451.

2. **Shafer WM, Guymon LF, Sparling PF.** 1982. Identification of a new genetic site (sac-3+) in *Neisseria gonorrhoeae* that affects sensitivity to normal human serum. Infect Immun **35**:764–769.

3. **Gulati S, Agarwal S, Vasudhev S, Rice PA, Ram S.** 2012. Properdin is critical for antibody-dependent bactericidal activity against Neisseria gonorrhoeae that recruit C4b-binding protein. J Immunol **188**:3416–3425. http://dx.doi.org/10.4049/jimmunol.1102746.

4. **Giuntini S, Reason DC, Granoff DM.** 2012. Combined roles of human IgG subclass, alternative complement pathway activation, and epitope density in the bactericidal activity of antibodies to meningococcal factor H binding protein. Infect Immun **80**:187–194. http://dx.doi.org/10.1128/IAI.05956-11.

5. **Elkins C, Carbonetti NH, Varela VA, Stirewalt D, Klapper DG, Sparling PF.** 1992. Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. Mol Microbiol **6**:2617–2628. http://dx.doi.org/10.1111/j.1365-2958.1992.tb01439.x.

6. **Ram S, Sharma AK, Simpson SD, Gulati S, McQuillen DP, Pangburn MK, Rice PA.** 1998. A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. J Exp Med **187**:743–752. http://dx.doi.org/10.1084/jem.187.5.743.

7. **Ram S, McQuillen DP, Gulati S, Elkins C, Pangburn MK, Rice PA.** 1998. Binding of complement factor H to loop 5 of porin protein 1A: a molecular mechanism of serum resistance of nonsialylated *Neisseria gonorrhoeae*. J Exp Med **188**:671–680. http://dx.doi.org/10.1084/jem.188.4.671.

8. **Jarvis GA, Vedros NA.** 1987. Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. Infect Immun **55**:174–180.

9. **Ram S, Lewis LA, Agarwal S.** 2011. Meningococcal group W-135 and Y capsular polysaccharides paradoxically enhance activation of the alternative pathway of complement. J Biol Chem **286**:8297–8307. http://dx.doi.org/10.1074/jbc.M110.184838.

10. **Uria MJ, Zhang Q, Li Y, Chan A, Exley RM, Gollan B, Chan H, Feavers I, Yarwood A, Abad R, Borrow R, Fleck RA, Mulloy B, Vazquez JA, Tang CM.** 2008. A generic mechanism in *Neisseria meningitidis* for enhanced resistance against bactericidal antibodies. J Exp Med **205**:1423–1434. http://dx.doi.org/10.1084/jem.20072577.

11. **Lewis LA, Ngampasutadol J, Wallace R, Reid JE, Vogel U, Ram S.** 2010. The meningococcal vaccine candidate neisserial surface protein A (NspA) binds to factor H and enhances meningococcal resistance to complement. PLoS Pathog. **6**:e1001027. http://dx.doi.org/10.1371/journal.ppat.1001027.

12. **Madico G, Welsch JA, Lewis LA, McNaughton A, Perlman DH, Costello CE, Ngampasutadol J, Vogel U, Granoff DM, Ram S.** 2006. The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. J Immunol **177**:501–510. http://dx.doi.org/10.4049/jimmunol.177.1.501.

13. **Lewis LA, Vu DM, Vasudhev S, Shaughnessy J, Granoff DM, Ram S.** 2013. Factor H-dependent alternative pathway inhibition mediated by porin B contributes to virulence of Neisseria meningitidis. mBio **4**:e00339-13. http://dx.doi.org/10.1128/mBio.00339-13.

14. **Estabrook MM, Griffiss JM, Jarvis GA.** 1997. Sialylation of *Neisseria meningitidis* lipooligosaccharide inhibits serum bactericidal activity by masking lacto-*N*-neotetraose. Infect Immun **65**:4436–4444.

15. **Fox AJ, Jones DM, Scotland SM, Rowe B, Smith A, Brown MR, Fitzgeorge RG, Baskerville A, Parsons NJ, Cole JA, et al.** 1989. Serum killing of meningococci and several other gram-negative bacterial species is not decreased by incubating them with cytidine 5′-monophospho-N-acetyl neuraminic acid. Microb Pathog **7**:317–318.

16. **Lewis LA, Carter M, Ram S.** 2012. The relative roles of factor H binding protein, neisserial surface protein A, and lipooligosaccharide sialylation in

regulation of the alternative pathway of complement on meningococci. J Immunol 188:5063–5072. http://dx.doi.org/10.4049/jimmunol.1103748.

17. Vu DM, Shaughnessy J, Lewis LA, Ram S, Rice PA, Granoff DM. 2012. Enhanced bacteremia in human factor H transgenic rats infected by Neisseria meningitidis. Infect Immun 80:643–650. http://dx.doi.org/10.1128/IAI.05604-11.

18. Geoffroy MC, Floquet S, Métais A, Nassif X, Pelicic V. 2003. Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. Genome Res 13:391–398. http://dx.doi.org/10.1101/gr.664303.

19. Gilbert M, Watson DC, Cunningham AM, Jennings MP, Young NM, Wakarchuk WW. 1996. Cloning of the lipooligosaccharide alpha-2,3-sialyltransferase from the bacterial pathogens Neisseria meningitidis and Neisseria gonorrhoeae. J Biol Chem 271:28271–28276. http://dx.doi.org/10.1074/jbc.271.45.28271.

20. Mandrell RE, Smith H, Jarvis GA, Griffiss JM, Cole JA. 1993. Detection and some properties of the sialyltransferase implicated in the sialylation of lipopolysaccharide of Neisseria gonorrhoeae. Microb Pathog 14:307–313. http://dx.doi.org/10.1006/mpat.1993.1030.

21. Shell DM, Chiles L, Judd RC, Seal S, Rest RF. 2002. The Neisseria lipooligosaccharide-specific alpha-2,3-sialyltransferase is a surface-exposed outer membrane protein. Infect Immun 70:3744–3751. http://dx.doi.org/10.1128/IAI.70.7.3744-3751.2002.

22. Packiam M, Shell DML, Liu SV, Liu YB, McGee DJ, Srivastava R, Seal S, Rest RF. 2006. Differential expression and transcriptional analysis of the alpha-2,3-sialyltransferase gene in pathogenic Neisseria spp. Infect Immun 74:2637–2650. http://dx.doi.org/10.1128/IAI.74.5.2637-2650.2006. PubMed.

23. Wu H, Jerse AE. 2006. Alpha-2,3-sialyltransferase enhances Neisseria gonorrhoeae survival during experimental murine genital tract infection. Infect Immun 74:4094–4103. http://dx.doi.org/10.1128/IAI.00433-06.

24. Song W, Ma L, Chen R, Stein DC. 2000. Role of lipooligosaccharide in Opa-independent invasion of Neisseria gonorrhoeae into human epithelial cells. J Exp Med 191:949–960. http://dx.doi.org/10.1084/jem.191.6.949.

25. Apicella MA, Bennett KM, Hermerath CA, Roberts DE. 1981. Monoclonal antibody analysis of lipopolysaccharide from Neisseria gonorrhoeae and Neisseria meningitidis. Infect Immun 34:751–756.

26. Apicella MA, Shero M, Jarvis GA, Griffiss JM, Mandrell RE, Schneider H. 1987. Phenotypic variation in epitope expression of the Neisseria gonorrhoeae lipooligosaccharide. Infect Immun 55:1755–1761.

27. Mandrell RE, Kim JJ, John CM, Gibson BW, Sugai JV, Apicella MA, Griffiss JM, Yamasaki R. 1991. Endogenous sialylation of the lipooligosaccharides of Neisseria meningitidis. J Bacteriol 173:2823–2832.

28. Gulati S, Cox A, Lewis LA, Michael FS, Li J, Boden R, Ram S, Rice PA. 2005. Enhanced factor H binding to sialylated gonococci is restricted to the sialylated lacto-N-neotetraose lipooligosaccharide species: implications for serum resistance and evidence for a bifunctional lipooligosaccharide sialyltransferase in gonococci. Infect Immun 73:7390–7397. http://dx.doi.org/10.1128/IAI.73.11.7390-7397.2005.

29. Ferreira VP, Pangburn MK, Cortés C. 2010. Complement control protein factor H: the good, the bad, and the inadequate. Mol Immunol 47:2187–2197. http://dx.doi.org/10.1016/j.molimm.2010.05.007.

30. Beernink PT, Shaughnessy J, Braga EM, Liu Q, Rice PA, Ram S, Granoff DM. 2011. A meningococcal factor H binding protein mutant that eliminates factor H binding enhances protective antibody responses to vaccination. J Immunol 186:3606–3614. http://dx.doi.org/10.4049/jimmunol.1003470.

31. Parsons NJ, Patel PV, Tan EL, Andrade JR, Nairn CA, Goldner M, Cole JA, Smith H. 1988. Cytidine 5′-monophospho-N-acetyl neuraminic acid and a low molecular weight factor from human blood cells induce lipopolysaccharide alteration in gonococci when conferring resistance to killing by human serum. Microb Pathog 5:303–309.

32. Vogel U, Claus H, Heinze G, Frosch M. 1997. Functional characterization of an isogenic meningococcal alpha-2,3-sialyltransferase mutant: the role of lipooligosaccharide sialylation for serum resistance in serogroup B meningococci. Med Microbiol Immunol 186:159–166. http://dx.doi.org/10.1007/s004300050059.

33. Vogel U, Claus H, Heinze G, Frosch M. 1999. Role of lipopolysaccharide sialylation in serum resistance of serogroup B and C meningococcal disease isolates. Infect Immun 67:954–957.

34. Vogel U, Weinberger A, Frank R, Müller A, Köhl J, Atkinson JP, Frosch M. 1997. Complement factor C3 deposition and serum resistance in iso-

genic capsule and lipooligosaccharide sialic acid mutants of serogroup B Neisseria meningitidis. Infect Immun 65:4022–4029.

35. Apicella MA, Ketterer M, Lee FK, Zhou D, Rice PA, Blake MS. 1996. The pathogenesis of gonococcal urethritis in men: confocal and immuno-electron microscopic analysis of urethral exudates from men infected with Neisseria gonorrhoeae. J Infect Dis 173:636–646. http://dx.doi.org/10.1093/infdis/173.3.636.

36. Harvey HA, Ketterer MR, Preston A, Lubaroff D, Williams R, Apicella MA. 1997. Ultrastructural analysis of primary human urethral epithelial cell cultures infected with Neisseria gonorrhoeae. Infect Immun 65:2420–2427.

37. Zenni MK, Giardina PC, Harvey HA, Shao J, Ketterer MR, Lubaroff DM, Williams RD, Apicella MA. 2000. Macropinocytosis as a mechanism of entry into primary human urethral epithelial cells by Neisseria gonorrhoeae. Infect Immun 68:1696–1699. http://dx.doi.org/10.1128/IAI.68.3.1696-1699.2000.

38. Kahler CM, Martin LE, Shih GC, Rahman MM, Carlson RW, Stephens DS. 1998. The (alpha2—>8)-linked polysialic acid capsule and lipooligo-saccharide structure both contribute to the ability of serogroup B Neisseria meningitidis to resist the bactericidal activity of normal human serum. Infect Immun 66:5939–5947.

39. Lewis LA, Ram S. 2014. Meningococcal disease and the complement system. Virulence 5:98–126. http://dx.doi.org/10.4161/viru.26515.

40. Vogel U, Hammerschmidt S, Frosch M. 1996. Sialic acids of both the capsule and the sialylated lipooligosaccharide of Neisseria meningitis serogroup B are prerequisites for virulence of meningococci in the infant rat. Med Microbiol Immunol 185:81–87. http://dx.doi.org/10.1007/s004300050018.

41. Mackinnon FG, Borrow R, Gorringe AR, Fox AJ, Jones DM, Robinson A. 1993. Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for Neisseria meningitidis using an infant mouse intranasal infection model. Microb Pathog 15:359–366. http://dx.doi.org/10.1006/mpat.1993.1085.

42. Shafer WM, Qu X, Waring AJ, Lehrer RI. 1998. Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc Natl Acad Sci U S A 95:1829–1833. http://dx.doi.org/10.1073/pnas.95.4.1829.

43. Warner DM, Folster JP, Shafer WM, Jerse AE. 2007. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. J Infect Dis 196:1804–1812. http://dx.doi.org/10.1086/522964.

44. Warner DM, Shafer WM, Jerse AE. 2008. Clinically relevant mutations that cause derepression of the Neisseria gonorrhoeae MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol 70:462–478. http://dx.doi.org/10.1111/j.1365-2958.2008.06424.x.

45. Matthias KA, Rest RF. 2014. Control of pili and sialyltransferase expression in Neisseria gonorrhoeae is mediated by the transcriptional regulator CrgA. Mol Microbiol 91:1120–1135. http://dx.doi.org/10.1111/mmi.12522.

46. Johnston DM, Cannon JG. 1999. Construction of mutant strains of Neisseria gonorrhoeae lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection. Gene 236:179–184. http://dx.doi.org/10.1016/S0378-1119(99)00238-3.

47. Mehr IJ, Seifert HS. 1998. Differential roles of homologous recombination pathways in Neisseria gonorrhoeae pilin antigenic variation, DNA transformation and DNA repair. Mol Microbiol 30:697–710. http://dx.doi.org/10.1046/j.1365-2958.1998.01089.x.

48. Stephens DS, Swartley JS, Kathariou S, Morse SA. 1991. Insertion of Tn916 in Neisseria meningitidis resulting in loss of group B capsular polysaccharide. Infect Immun 59:4097–4102.

49. McQuillen DP, Gulati S, Ram S, Turner AK, Jani DB, Heeren TC, Rice PA. 1999. Complement processing and immunoglobulin binding to Neisseria gonorrhoeae determined in vitro simulates in vivo effects. J Infect Dis 179:124–135. http://dx.doi.org/10.1086/314545.

50. Apicella MA, Mandrell RE, Shero M, Wilson ME, Griffiss JM, Brooks GF, Lammel C, Breen JF, Rice PA. 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. http://dx.doi.org/10.1093/infdis/162.2.506.

51. McQuillen DP, Gulati S, Rice PA. 1994. Complement-mediated bacterial

killing assays. Methods Enzymol **236:**137–147. http://dx.doi.org/10.1016/0076-6879(94)36013-8.

52. **Jerse AE, Crow ET, Bordner AN, Rahman I, Cornelissen CN, Moench TR, Mehrazar K.** 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. http://dx.doi.org/10.1128/IAI.70.5.2549-2558.2002.

53. **Beuzón CR, Holden DW.** 2001. Use of mixed infections with salmonella strains to study virulence genes and their interactions *in vivo*. Microbes Infect **3:**1345–1352. http://dx.doi.org/10.1016/S1286-4579(01)01496-4.

54. **Schneider H, Griffiss JM, Williams GD, Pier GB.** 1982. Immunological basis of serum resistance of *Neisseria gonorrhoeae*. J Gen Microbiol **128:**13–22. http://dx.doi.org/10.1099/00221287-128-1-13.

55. **McGuinness BT, Clarke IN, Lambden PR, Barlow AK, Poolman JT, Jones DM, Heckels JE.** 1991. Point mutation in meningococcal por A gene associated with increased endemic disease. Lancet **337:**514–517. http://dx.doi.org/10.1016/0140-6736(91)91297-8.

56. **Wakarchuk WW, Watson D, St Michael F, Li J, Wu Y, Brisson JR, Young NM, Gilbert M.** 2001. Dependence of the bi-functional nature of a sialyltransferase from Neisseria meningitidis on a single amino acid substitution. J Biol Chem **276:**12785–12790. http://dx.doi.org/10.1074/jbc.M011293200.

57. **Madico G, Ngampasutadol J, Gulati S, Vogel U, Rice PA, Ram S.** 2007. Factor H binding and function in sialylated pathogenic neisseriae is influenced by gonococcal, but not meningococcal, porin. J Immunol **178:**4489–4497.