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

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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 monophosphate-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.
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 (sero-groups 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 α70 promoter used to transcribe the gonococcal *lst* (22) and down-regulates 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* F62AD (lgtD inactivated, LNT LOS predominant [24]) that express the native F62 Lst (LstNg) 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 ∆lst). 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 ∆lst 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 ∆lst (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;
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 onto LNT-LOS by Lst and the relation-

**FIG 1**

Counts per 10⁶ CFU (FH CMP-NANA)

- Ng Pₙₙ-Ng
- Ng Δₙₙ
- Ng Pₙₙ-Nm
- Ng Pₙₙ-Ng (No CMP-NANA)
- Nm NMB SiaA

(B.)

Median Fluorescence (counts)

- FH
- mAb 3F11

**FIG** 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 F62ΔΔ expressing Lst either from its native promoter (Ng Pₙₙ-Ng) or from the N. meningitidis 123E Lst promoter (Ng Pₙₙ-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 Δₙₙ lacking the sialyltras-

ferase enzyme, Lst (Ng Δₙₙ), and N. gonorrhoeae Pₙₙ-Ng grown in the absence of CMP-NANA [Ng Pₙₙ-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 Lstₙₙₙₙ), 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 Δₙₙ). As expected, AP activation was unaffected by CMP-NANA in the lst mutant strain (Fig. 3; Δₙₙ). 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 (Pₙₙ-Ng or Pₙₙ-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 Pₙₙ-Ng was significantly lower than that on Pₙₙ-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 Pₙₙ-Nm and Pₙₙ-Ng to resist killing by PNHS following growth in various concentrations of CMP-NANA. Concordant with its greater FH binding and lower AP activation, Plst-Ng was more resistant than Plst-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 Pₙₙ-Ng and Pₙₙ-Nm to resist killing by PNHS ex vivo, following their recovery from the murine genital tract. Estradiol-treated mice were inoculated intravaginally with either Pₙₙ-Ng or Pₙₙ-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 (Plst-Ng) or the N. meningitidis 126E lst promoter (Plst-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 μg/ml) for 30 min, (ii) bacteria incubated without CMP-NANA, and (iii) bacteria that were not incubated with FH. An lst mutant (Δlst) also served as a negative control (data not shown). (A) Histograms from a representative experiment comparing Plst-Ng (grey shaded) and Plst-Nm (bold black line) are shown. The y axis represents the number of events (counts), and the x axis represents fluorescence on a log10 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 Plst-Ng (grey bars) and Plst-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 < 0.001 (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 (LstNg) or N. meningitidis Lst (LstNm) 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).
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

![FIG 3](image1.png) Complement deposition (C3 and factor B) on strain F62ΔD expressing high (P<sub>lst-Ng</sub>) and low (P<sub>lst-Nm</sub>) 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<sub>lst-Ng</sub>) or the N. meningitidis 126E lst promoter (P<sub>lst-Nm</sub>) 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 Δlst is shown as a negative control. The mean OD<sub>405nm</sub> 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](image2.png) 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<sub>lst-Ng</sub>) or the meningococcal 126E lst promoter (P<sub>lst-Nm</sub>) 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<sub>Ng</sub> using either the gonococcal lst promoter (P<sub>lst-Ng</sub>) or the meningococcal lst promoter (P<sub>lst-Nm</sub>) 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 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 (Δlst) 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).
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.

**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 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 impared 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 uses 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 in vitro. 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 Pst-Sm relative to Pst-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 5,567 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 down-regulating 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 bst 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 F62ΔΔ (a gift from Daniel C. Stein, University of Maryland) is an lst mutant of N. gonorrhoeae F62 (24) that expresses only the LNT LOS epitope on HepII. Neisserial strains were routinely cultured on chocolate agar or in GC broth supplemented with IsoVitalX equivalent at 37°C in an atmosphere of 5% CO2. 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 InvA’ (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. InvA’ 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-
sertional inactivation of \textit{lst} in strain \textit{F62}\textDelta did achieve using pGH7 as described previously (31).

Exchange of the \textit{F62} \textit{lst} coding sequence with the \textit{N. meningitidis} MC58 \textit{lst} coding sequence in strain \textit{F62}\textDelta \textit{Lst} was achieved by transforming strain \textit{F62}\textDelta with plasmid \textit{pLst}\textit{MC58KanCytC}, which contains (5' to 3') the MC58 \textit{lst} coding sequence, a kan resistance gene, and DNA homologous to the region downstream of \textit{lst} in \textit{F62}.

Exchange of the \textit{F62}\textDelta \textit{lst} promoter with the \textit{N. meningitidis} 126E \textit{lst} promoter in strain \textit{F62}\textDelta \textit{Plst-Nm} was achieved using a two-step mutagenesis system (46) and plasmids \textit{pTOPO} \textit{lstF62KanCytC}, which contains (5' to 3') the region upstream of the \textit{F62} \textit{lst} promoter, the \textit{N. meningitidis} 126E \textit{lst} promoter, and the \textit{F62} \textit{lst} coding and downstream sequences. \textit{pTOPO} \textit{lstPprom-Erm/Sm} is \textit{pTOPO} \textit{lst} with a cassette that codes for resistance to Erm and sensitivity to Sm inserted in the HincII site. Strain \textit{F62}\textDelta was transformed with \textit{pTOPO} \textit{lstPprom-Erm/Sm}, and Erm-resistant but Sm-sensitive colonies were selected and transformed with \textit{pTOPO} \textit{lstPprom-Erm/Sm}. Sm-resistant transformants were selected and screened for sensitivity to Erm.

The introduction of the \textit{N. meningitidis} MC58 \textit{lst} gene into strain \textit{F62}\textDelta \textit{Lst} introduced a Kan resistance downstream of \textit{lst} in strains \textit{F62}\textDelta \textit{Plst-Ng} and \textit{F62}\textDelta \textit{Plst-Nm}. Lastly, an Erm resistance gene was inserted into the region between the \textit{lst} and \textit{aspC} genes of \textit{MC58} to allow for differentiation in competitive infections in BALB/c mice (47).

The DNA sequence of the \textit{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 MAb 3F11 (Hepl LNT-LOS), 2C7 (Hepl lactose), and L8 (Hepl lactose, HeplP 3-PEA).

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

\textbf{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\textsubscript{600}) of 0.1 in GC liquid media and grown to an OD\textsubscript{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--3H]); American Radiolabeled Chemicals, Inc.) was added (final concentration, 2 $\mu$g/mL), and triplicate cell samples (100 $\mu$L each) were collected at 0, 15, 60, and 120 min of incubation; OD\textsubscript{600} readings were recorded at each time point. Cells were pelleted (3 min at 13,000 $\times$ g), washed twice in 1 ml Hanks' balanced salt solution (HBSS++), and suspended in a final volume of 100 $\mu$L 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\textsubscript{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 $\mu$L sample by the calculated CFU/100 $\mu$L.

\textbf{Flow cytometry.} Binding of human FH (Complement Technology, Inc.) (10 $\mu$g/mL) to log-phase bacteria incubated with CPM-NANA (concentrations and incubation times are indicated for each experiment) or buffer (negative control) was measured by fluorescence-activated cell sorting (FACS) (FACS Calibur; 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; Quidel) or with affinity-purified polyclonal goat anti-human FH (16). Each experiment was performed at least three times.

The sialylation data 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 antimouse IgM fluorescein isothiocyanate (FITC; Sigma).

\textbf{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 $\mu$L/mL CPM-NANA or without CPM-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.

\textbf{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 $-80^\circ$C.

\textbf{Bacterial killing by PNHS.} The susceptibility of log-phase gonococci grown in liquid culture with CPM-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

\begin{table}
\centering
\begin{tabular}{lll}
\hline
\textbf{Strain} & \textbf{Description} & \textbf{Reference or source} \\
\hline
\textit{N. gonorrhoeae} & & \\
F62 & Por1B & 54 \\
F62\textDelta & \textit{F62} \textit{lst} inactivated; LNT LOS predominates & 24 \\
\textit{F62}\textDelta \textit{Plst-F62KanCytC} & inserted in intergenic region between \textit{lst} and \textit{aspC} (\textit{Kan'}, \textit{Sm'}, \textit{Erm'}) & This study \\
\hline
\textbf{\textit{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 \textit{siaA} & \textit{siaA} (\textit{Cm'}) & This study \\
\hline
\end{tabular}
\caption{Bacterial strains used in this study}
\end{table}
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 PHNS 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 Pst-Ng (6.04 x 10^5 CFU/mouse), strain Pst-Nm (4.02 x 10^5 CFU/mouse), or strain Δgst (4.36 x 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 x 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–0.1% (1 h at 37°C). The organisms were used in a standard bacteriical assay with 15% or 30% PHNS, 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 Ist provides gonococci with a survival advantage in the mouse model of infection, a competitive infection experiment was performed using a mixture of strains Pst-Ng and Pst-Nm or, as a control, strains Pst-Ng and Δgst. Female BALB/c mice (Jackson Laboratories) (10 weeks old) or female BALB/c human FH-transgenic mice (30) in the diestrus phase of the estrous 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 (strains Pst-Ng [4.68 x 10^5] and Pst-Nm [4.68 x 10^5] or strains Pst-Ng [4.39 x 10^5] and Δgst [3.34 x 10^5]) at 3 x 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 Pst-Nm or strain ΔIst) to wild type (strain Pst-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 Pst-Ng but not the growth of strain Pst-Nm or strain Δgst. 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.

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