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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

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We isolated serologically identical (by serovar determination and porin variable region [VR] typing) strains of *Neisseria gonorrhoeae* from an infected male and two of his monogamous female sex partners. One strain (termed 398078) expressed the L1 (Galα1 → 3Galβ1 → 4Glcβ1 → 4HepI) lipooligosaccharide (LOS) structure exclusively; the other (termed 398079) expressed the lacto-*N*-neotetraose (LNT; Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4HepI) LOS structure. The strain from the male index case expressed both glycoforms and exhibited both immunotypes. Nuclear magnetic resonance analysis revealed that sialic acid linked to the terminal Gal of L1 LOS via an α2 → 6 linkage and, as expected, to the terminal Gal of LNT LOS via an α2 → 3 linkage. Insertional inactivation of the sialyltransferase gene (known to sialylate LNT LOS) abrogated both L1 LOS sialylation and LNT LOS sialylation, suggesting a bifunctional nature of this enzyme in gonococci. Akin to our previous observations, sialylation of the LNT LOS of strain 398079 enhanced the binding of the complement regulatory molecule, factor H. Rather surprisingly, factor H did not bind to sialylated strain 398078. LOS sialylation conferred the LNT LOS-bearing strain complete (100%) resistance to killing by even 50% nonimmune normal human serum (NHS), whereas sialylation of L1 LOS conferred resistance only to 10% NHS. The ability of gonococcal sialylated LNT to bind factor H confers high-level serum resistance, which is not seen with sialylated L1 LOS. Thus, serum resistance mediated by sialylation of gonococcal L1 and LNT LOS occurs by different mechanisms, and specificity of factor H binding to sialylated gonococci is restricted to the LNT LOS species.

The lacto-*N*-neotetraose (LNT) lipooligosaccharide (LOS) of *Neisseria gonorrhoeae* can become sialylated when grown in medium supplemented with 5′-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA) (20). LOS sialylation renders strains that are otherwise susceptible to complement-mediated killing, resistant to killing by nonimmune normal human serum (NHS) (26, 31). One explanation is the ability of gonococci bearing the sialylated LNT LOS to bind factor H (37), an important fluid-phase regulatory protein of the alternative pathway of complement (9, 29, 53, 56). Gonococcal LOS undergoes significant phase variability in vivo, which may result in a shift of expression away from the "conventional" (terminal lactosamine of LNT) sialylation site (3, 12). LOS phase variation that results in the loss of the ability of an otherwise serum-sensitive gonococcal strain to sialylate its LOS may render such a strain highly susceptible to complement-mediated killing and therefore may be disadvantageous to an organism, necessitating a redundant strategy to evade killing by complement. The terminal Gal on meningococcal L1 LOS (Galα1 → 3Galβ1 → 4Glcβ1 → 4HepI) can also be sialylated (51). The HepI hexose substitution of L1 LOS is structurally identical to the P^K blood group antigen (19) and is commonly found in the LOS of *Haemophilus* and *Moraxella* spp. (5, 21, 48). Preserving the ability to express L1 LOS may enable *N. gonorrhoeae* that has lost the ability to express the LNT LOS to retain the ability to sialylate LOS at an alternative site.

The interaction of the complement system (and in particular, regulatory molecules such as factor H) with sialylated L1 LOS has never been studied. The identification of four strains of *N. gonorrhoeae* isolated from four different sexual partners of a single index case, that were found to be identical by serotyping but different in their LOS structure (the strains expressed either the LNT LOS, the L1 LOS, or both), provided us a unique opportunity to compare complement regulatory events mediated by sialic acid in the context of the two LOS immunotypes. In this study, we show that sialylation of only the LNT LOS species, but not the L1 LOS, results in enhanced binding of the alternative pathway regulatory molecule, factor H. We also show that the LOS sialyltransferase elaborated by gonococci is "bifunctional" in nature (i.e., capable of adding sialic acid via two distinct linkages to LNT and L1 LOS

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species). Sequence differences between gonococcal and bifunctional meningococcal sialyltransferases are detailed.

MATERIALS AND METHODS

Bacterial strains and growth. *N. gonorrhoeae* strain 398 was isolated from a male index case, and four additional strains (termed 398078 to 398081) were obtained from four of his female sex contacts. Strain 1291b is a mutant derivative of strain 1291 described previously (15), which had been selected under pyocin pressure and expresses L1 LOS. Bacteria were grown in standard gonococcal liquid medium supplemented with IsoVitaleX equivalent. In order to sialylate LOS, CMP-NANA (Sigma Chemical Corporation, St. Louis, MO) was added to growth media (concentration specified for each experiment).

In addition to the strains indicated above, *N. gonorrhoeae* strains F62 (40), FA1090 (54), 24-1 (6), WG (28), and 179008, 255034, 269041, 374073, 339063, 256036, 274045, and 252035 (36) were used for sequence analysis of the LOS sialyltransferase (*lst*).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting for LOS visualization and immunochemical characterization. Bacteria were digested with proteinase K (Sigma), and lysates were treated with NuPAGE LDS sample buffer (Invitrogen Life Technologies, Carlsbad, CA) and resolved on 12% Bis-Tris NuPAGE gels (Invitrogen) using MES (morpholineethanesulfonic acid) running buffer, according to the manufacturer's instructions. LOS was visualized using a silver staining kit (Bio-Rad Laboratories, Hercules, CA). In some experiments, LOS was transferred to polyvinylidene difluoride membranes (Millipore) by Western blotting and probed with monoclonal antibodies (MAbs) 3F11 (2) and L1 (42) for immunochemical characterization of LOS.

LOS purification and analytical techniques. To sialylate LOS, bacteria were grown in 2 liters of gonococcal liquid medium supplemented with IsoVitaleX equivalent (1% [vol/vol]) and CMP-NANA (10 μg/ml). Bacteria were harvested, and LOS was purified by the hot water-phenol extraction method (55). Sugars were determined as their alditol acetate derivatives by gas-liquid chromatography–mass spectrometry, and methylation analysis was carried out by the NaOH-dimethy sulfoxide-methyl iodide procedure and analyzed by gas-liquid chromatography–mass spectrometry as described previously (25). LOS was de-O-acylated with anhydrous hydrazine to yield LOS-OH and examined by capillary electrophoresis-electrospray mass spectroscopy as described previously (25). Nuclear magnetic resonance (NMR) experiments were acquired on a Varian Inova 400-MHz spectrometer using a 5-mm triple-resonance (^{1}H, ^{13}C, ^{31}P) probe. The lyophilized sugar sample was dissolved in 600 μl of 99% D_2O with deuterated sodium dodecyl sulfate (10 mg/ml), and EDTA (1 mg/ml) was added to improve the resolution of the spectrum. The experiments were performed at 25°C with suppression of the HOD (deuterated H_2O) signal at 4.78 ppm. The methyl resonance of acetone was used as an internal reference at 2.225 ppm for ^{1}H spectra and 31.07 ppm for ^{13}C spectra. Standard homo- and heteronuclear correlated two-dimensional pulse sequences from Varian, correlation spectroscopy, total correlated spectroscopy, nuclear Overhauser effect spectroscopy, and ^{13}C-^{1}H heteronuclear single quantum correlation were used for general assignments.

Construction of *lst* mutants. *lst* was amplified from chromosomal DNA prepared from strain 398078 using a modification of primers SIALM-5F and SIALM-16R (11), to incorporate PstI and HindIII restriction sites in the forward and reverse primers, respectively: primer Lst-F (5′ AAAA**CTGCAG**TTCAATTT GTCGGAATGGAGTTTTAGG 3′; PstI site in boldface) and primer Lst-R (5′ CCC**AAGCTT**CTCATTAATTTTTATCGTCAAATGTCAAAATC 3′; HindIII site in boldface). PCR was carried out by denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min for 30 cycles. The PCR product was digested with PstI and HindIII, gel purified using the QIAquick gel extraction kit (QIAGEN), and cloned into plasmid pUC18 (Invitrogen; GenBank accession number L09136) (27) that had been digested with the same enzymes, to yield plasmid pUC18-lst. The kanamycin resistance marker was amplified by PCR from pUC4K (GenBank accession no. X06404; GE Healthcare [formerly Amersham Biosciences], Piscataway, NJ) using primers 5′ CC**CGGCCG**GGGGGATCCGTCGACCTGCAG 3′ and 5′ CCCG**GCCGG C**CCCCGGATCCGTCGACCTGC 3′ (introduced EagI sites indicated in boldface), digested with EagI, and cloned into a unique EagI restriction site at nucleotide 672 of the *lst* in pUC18-lst, to yield a plasmid designated pUC18-lst-Km containing insertionally inactivated *lst*. Gonococcal strains 398078 and 398079 were transformed with 1 μg pUC18-lst-Km by previously described methods (4), and transformants were selected on GCB agar plates supplemented with 100 μg/ml kanamycin. Insertional inactivation of *lst* was confirmed by PCR using primers lst-F and lst-R, which yielded the expected ~2.4-kb product. We also amplified and sequenced the ~5-kb region surrounding *lst* in strains 398, 398078, and 398079 using primers based on the FA1090 genome sequence (GenBank accession no. AE004969). The genetic organization in all of these strains was identical to that of strain FA1090 and indicates that *lst* is monocistronic.

***lst* sequencing.** Several isolated colonies from an overnight growth of *N. gonorrhoeae* on chocolate agar plates were suspended in 100 μl double-distilled water and heated to 90°C for 10 min. *lst* was amplified from the lysate (forward primer 5′-GGACACTCGGGGCGTATGTTCAA-3′ and reverse primer 5′-ATCCTGCCACGACAGTTTCCGC-3′), and the resultant PCR product was purified (Qiaquick) and sequenced (Tufts University Core Facility; http://www.tucf.org/index.html) using the internal primers 5′-TCAGCGGTGCGGTGT TGACGATG-3′ and 5′-TACTTACTCCGCCATTTTCTATTT-3′. DNA sequences were assembled using SeqMan (DNASTAR, Inc.) (45), and open reading frames were identified using Gene Construction Kit 2 (Textco BioSoftware, Inc., West Lebanon, NH). Translated *lst* sequences were aligned using ClustalW (http://clustalw.genome.jp/) (47), and the alignments were analyzed using SeqVu (http://www.imtech.res.in/pub/mmbm/seqvu/).

Sera and complement reagents. Sera obtained fresh from 11 healthy adults who had no history of neisserial infection were pooled and stored at −80°C until used. Purified factor H was purchased from Advanced Research Technologies (San Diego, CA).

Antibodies. Anti-LOS MAb 3F11 (2) was provided by Michael A. Apicella, University of Iowa, Iowa City, and MAb L1 (13) was provided by Wendell Zollinger, Walter Reed Army Institute, Washington, D.C. Factor H that bound to bacterial surfaces was detected by flow cytometry using affinity-purified goat anti-human factor H at a concentration of 10 μg/ml (kind gift of Michael K. Pangburn, University of Texas Health Sciences Center, Tyler), and disclosed using fluorescein isothiocyanate-conjugated anti-goat immunoglobulin G (Sigma) at a dilution of 1:50.

Flow cytometry. Factor H bound to the bacterial surface was quantified by flow cytometry using a Becton Dickinson FACScan cytometer, as described previously (37).

Bactericidal assays. Serum bactericidal testing was performed as described previously (23). Briefly ~2,000 CFU of bacteria grown to the mid-log phase were incubated with NHS (concentration of NHS specified for each experiment) in a final reaction mixture volume of 150 μl. Duplicate aliquots of 25 μl were inoculated onto chocolate agar plates at 0 and 30 min. Survival was calculated as the percentage of the number of colonies that survived at 30 min relative to the baseline colony counts at 0 min.

Nucleotide sequence accession numbers. The *lst* sequences have been submitted to GenBank under accession numbers AY953446 to AY953459.

RESULTS

Identification of epidemiologically related gonococcal strains bearing two different LOS sialylation sites and immunochemical characterization of LOS. Previously we have screened several strains of *N. gonorrhoeae* that were sensitive to the bactericidal action of 10% NHS in the native state but became serum resistant upon growth in media supplemented with CMP-NANA (22). We examined these strains initially by Western blotting for expression of the LNT LOS species (MAb 3F11 reactive) in the unsialylated state to identify 3F11-negative strains (i.e., strains that could become serum resistant upon sialylation, but lacked the well-characterized LNT LOS acceptor site). Using Western blotting with MAb 3F11 as the probe, we identified one such strain, 398078, isolated from a female contact of an index male with uncomplicated gonococcal infection (infected with strain 398). Three additional female sex partners were infected with strains 398079, 398080, and 398081. The five strains belonged to the Por1B-32 serovar (17) and were also identical by Por variable region (VR) typing (Por VR type B2,2,nt,1,3) (46), but showed dissimilar LOS migration patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and varied with respect to MAb 3F11 binding. Prior studies have shown that the L1 LOS in *N. meningitidis* can be sialylated (51), and thus we hypothesized that the sialylatable non-3F11 LOS species in strain 398078 was likely

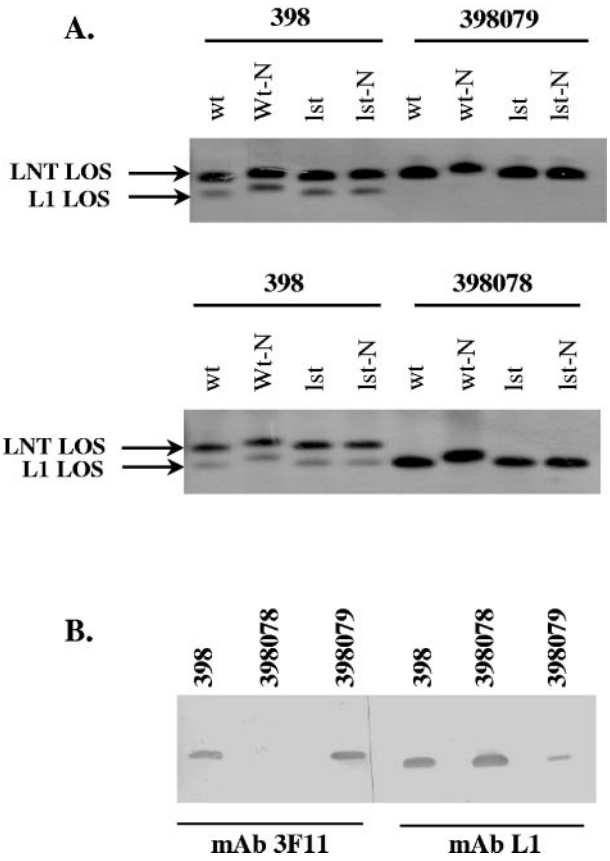

FIG. 1. Effects of sialylation on LOS migration of wild-type strains and sialyltransferase (*lst*) mutants. Proteinase K-digested bacterial lysates were resolved on a 12% Bis-Tris gel using MES running buffer, and LOS was visualized by silver staining (A) and Western blotting (B). Strain 398 expressed both the 3F11 (4.5 kDa) and L1 (a lower-molecular-mass LOS species) epitopes. Strain 398079 expressed predominantly the 3F11 LOS species, while 398078 expressed only the L1 LOS. In all cases, migration of the 3F11 and L1 LOS species was retarded upon growth in CMP-NANA-containing media. Inactivation of *lst* resulted in loss of the ability of all strains to sialylate either LOS species.

to be L1 LOS (Galα1 $\rightarrow$ 3Galβ1 $\rightarrow$ 4Glcβ1 $\rightarrow$ 4HepI). Strain 398 expressed two distinct LOS species (Fig. 1A) and was found to react with both MAb 3F11 and a second MAb, L1, specific for the L1 LOS type that also defines the P^K blood group antigen (19) (Fig. 1B), Strain 398078 expressed only the lower-M_r LOS species and reacted with MAb L1, while isolate 398079 expressed only the higher-M_r LOS species, as seen on silver stain that reacted with 3F11 (Fig. 1A and B), and weak reactivity with MAb L1 by Western blotting (no corresponding band seen on silver stain).

Strains 398080 and 398081 had LOS migration patterns similar to those of the three strains examined (Fig. 1) and were not studied further. As seen in Fig. 1A, both LOS species migrated with slower velocities when strains were grown in media supplemented with CMP-NANA, suggesting that a sialic acid residue was added to both LOS species. Also shown in Fig. 1A are the migration patterns of the LOS sialyltransferase (*lst*) deletion mutants in the native (unsialylated) state and when grown in the presence of CMP-NANA (discussed in detail below). Collectively, these data suggested that the lower-M_r LOS species that could be sialylated was similar to the L1 LOS of *N. meningitidis*, and this was confirmed by mass spectrometry analysis (see below).

Mass spectrometric analysis of the LOS of strain 398078, and characterization of the nature of the sialic acid linkage. The MS spectra of LOS-OH isolated from strains 398, 398078, 398078-NANA, 398079, 398079-NANA and a control strain, 1291b-NANA, were determined. As summarized in Table 1, it can be seen that 398078 LOS-OH elaborated only the L1-like extension, as evidenced by a single HexNAc known to be substituted on the distal HepII residue and three hexoses which comprise the Galα1 $\rightarrow$ 3Galβ1 $\rightarrow$ 4Glcβ1 $\rightarrow$ extension from HepI, whereas 398079 LOS-OH elaborated only the 3F11-like (Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 3Galβ1 $\rightarrow$ 4Glcβ1 $\rightarrow$) extension from HepI plus another HexNAc known to be substituted on the distal HepII residue. Strain 398 expressed both the L1- and 3F11-like extensions. It is worth noting that the epidemiologically linked strains (398, 398078, and 398079) display two

TABLE 1. Negative-ion MS data and proposed compositions of O-deacylated LPS from *N. gonorrhoeae* strains 398-NANA, 398078-NANA, 398079-NANA, and 1291b-NANA[a]

Strain	Observed ions (m/z) $(M-3H)^{3-}$	Molecular mass (Da)		Relative intensity	Molecular mass (Da)		Proposed composition
		Observed	Calculated		Lipid A[b]	Core OS	
398-NANA	903.3	2,712.3	2,712.4	1.0	952	1,761	3Hex, HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	1,000.3	3,004.1	3,003.4	0.5	952	2,052	Sial, 3Hex, HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	971.0	2,916.2	2,915.6	0.2	952	1,964	3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	1,068.3	3,207.9	3,206.6	trace	952	2,255	Sial, 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
398078-NANA	903.3	2,712.1	2,712.4	1.0	952	1,761	3Hex, HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	1,000.4	3,004.3	3,003.4	0.5	952	2,052	Sial, 3Hex, HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
398079-NANA	971.3	2,916.9	2,915.6	1.0	952	1,967	3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	1,068.8	3,209.4	3,206.6	0.4	952	2,257	Sial, 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
1291b-NANA	862.3	2,589.9	2,589.4	1.0	952	1,641	3Hex, HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
	959.8	2,882.4	2,880.4	0.5	952	1,932	Sial, 3Hex, HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH

[a] Average mass units were used for calculation of molecular mass based on proposed composition as follows: Sial, 291.00; Hex, 162.15; Hep, 192.17; HexNAc, 203.19; PEtn, 123.05; Kdo, 220.18. The average molecular weight of the O-deacylated lipid A (lipid A-OH) is as indicated. Relative intensity is expressed as relative to the largest peak.

[b] As determined by MS-MS analyses.

phosphoethanolamine (PEtn) molecules in their core OS, and both of these PEtn moieties were localized to the distal heptose residue by NMR studies (HepII) of the inner core OS (data not shown). Control strain 1291b only elaborates one PEtn residue in the core OS also located at the HepII sugar (data not shown). 1291b also exhibits the L1-like extension. In order to elucidate the position of the sialic acid linkage in these strains, two approaches were adopted. For strain 398078-NANA, methylation analysis revealed the presence of 6-linked galactose residues, the amount being consistent with the level of sialylation in the molecule. Strain 1291b-NANA was also shown to elaborate sialic acid attached to the 6-position of a galactose residue by NMR methods, whereby the inter-nuclear Overhauser effect connectivities from the equatorial proton of the sialic acid residue identified signals for the 6-position of galactose as observed previously for *N. meningitidis* L1 LOS (51). Finally, strain 398079-NANA was shown to elaborate sialic acid at the 3-position of a galactose residue by virtue of inter-nuclear Overhauser effect connectivities from the equatorial proton of the sialic acid residue that identified a signal for the 3-position of galactose as observed previously for *N. meningitidis* L3 LOS (32).

As detailed above, strains 398078 and 1291b differed in their PEtn substitutions on HepII; the former strain had PEtn simultaneously at the 3- and 6-positions, while the latter had only a 3-PEtn. Therefore, these data suggest that PEtn substitutions on HepII did not influence the nature of the linkage of sialic acid to LOS.

Interruption of *lst* results in the loss of the ability of gonococcal L1 LOS to sialylate. To determine if the well-characterized lipooligosaccharide transferase, *lst*, was responsible for sialylation of the L1 LOS in gonococci, we constructed mutants using strains 398, 398078, and 398079 in which *lst* was interrupted. The LOS migration patterns of the *lst* mutants in the native (unsialylated) state and when grown in media supplemented with 100 μg/ml CMP-NANA are shown in Fig. 1A (lanes lst and lst-N, respectively). As expected, interruption of *lst* in 398079 (expresses predominantly the 3F11-like LOS) abrogated LOS sialylation in media containing CMP-NANA (Fig. 1A, upper panel, lane marked lst-N). Insertional inactivation of the same *lst* in 398078 resulted in loss of sialylated L1 LOS upon growth in CMP-NANA-containing media (Fig. 1A, lower panel, lane marked lst-N). The 398 *lst* mutant lacked the ability to sialylate both LOS glycoforms. Collectively, these data examined together with the mass spectrometric analysis strongly suggest that this *lst* in gonoccci (i.e., one that is known to sialylate the LNT LOS [via an α(2,3) linkage] also mediates transfer of a sialic acid residue onto the terminal Gal in L1 LOS [via an α(2,6) linkage], thereby rendering it a bifunctional sialyltransferase.

Comparison of meningococcal and gonococcal *lst* gene products. Our data suggest that the gonococcal Lst is a bifunctional enzyme capable of adding an α(2,3)-linked sialic acid onto LNT LOS and an α(2,6)-linked sialic acid onto L1 LOS. Wakarchuk et al. have shown that Lst from meningococcal strain 126E is also a bifunctional enzyme with similar substrate specificities (51). An isoleucine (Ile) residue at position 168 was found to be critical for bifunctionality of the 126E Lst. Alignment of gonococcal strain FA1090 Lst (GenBank accession no. AE004969) with 126E Lst revealed an asparagine (Asn)

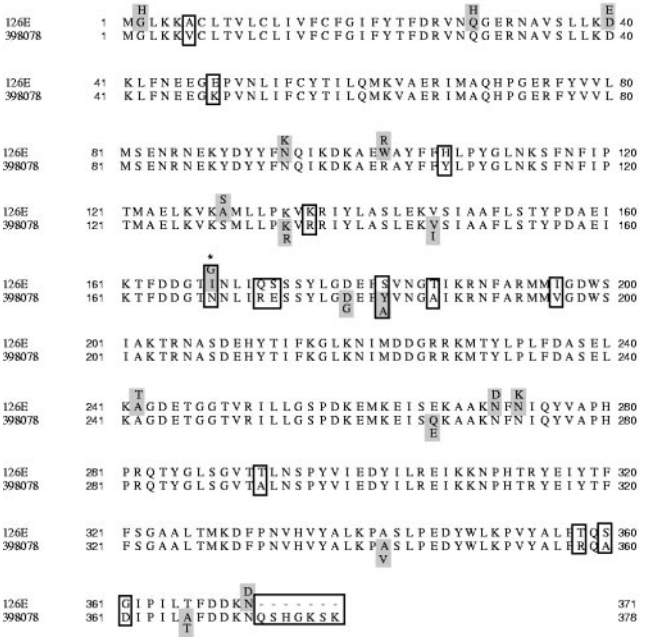

FIG. 2. Lst sequences from 6 meningococcal and 14 gonococcal strains were aligned using ClustalW. A representative alignment of the bifunctional Lsts from meningococcal strain 126E and gonococcal strain 398078 is shown. Species-specific variation occurred at 21 separate amino acid positions (boxed) distributed across the protein. Strain variation within a species (shaded) was noted at 11 meningococcal (above) and 7 gonococcal (below) amino acid positions. The isoleucine (Ile) residue at position 168, shown to be critical for bifunctionality of 126E Lst, is marked with an asterisk.

residue at amino acid position 168 in FA1090. Wakarchuk et al. demonstrated by site-directed mutagenesis that an Asn at position 168 of the MC58 Lst resulted in a protein with only about 25% of the α (2,6) sialytransferase activity of an Lst with an Ile at the same position (52). It is worth noting that the FA1090 Lst also contained 7 additional amino acids at the C-terminal end due to a T-to-C substitution at the site of the 126E TAA stop codon.

To further examine gonococcal Lst sequence variation, we amplified and sequenced *lst* from 14 gonococcal strains (including FA1090). The region sequenced encompassed the putative 7-amino-acid C-terminal extension found in FA1090. These sequences were aligned with six meningococcal Lst sequences available in GenBank (accession numbers U60662 through U60664 and NMB0922) using ClustalW. A representative alignment is shown in Fig. 2. We identified 21 amino acid positions that diverged between gonococci and meningococci. Within meningococci, Lst varied at 11 amino acid positions with a maximal diversity of 3%. The 7 C-terminal amino acids found in FA1090 were not observed in any meningococcal sequences. Among gonococcal strains, the Lst sequences diverged at 7 amino acids. The maximum difference noted between any two sequences was 4 amino acids. This implies at least a 98.97% homology in the Lst amino acid sequences among the strains we examined. In every gonococcal strain sequenced, including that from strain 398078, an Asn residue was noted at

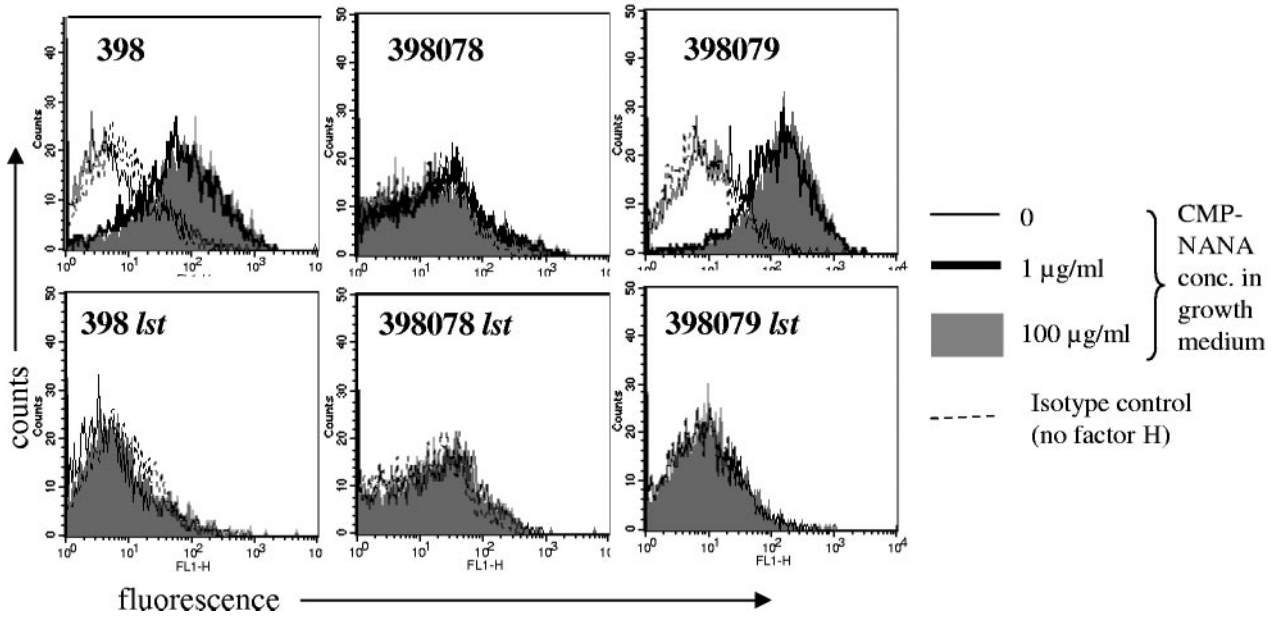

FIG. 3. Factor H binds only to gonococcal strains (398 and 398079) bearing sialylated lacto-*N*-neotetraose (3F11) LOS, but not to strain 398078 bearing sialylated L1 LOS. (Upper panel) Factor H binding to strains 398 (LNT and L1 LOS), 398078 (L1 LOS), and 398079 (LNT LOS) grown either without CMP-NANA (histogram represented by thin solid line) or in media containing 1 μg/ml (bold line) or 100 μg/ml (gray shaded histogram) CMP-NANA was quantified by flow cytometry. (Lower panel) Factor H binding to *lst* mutants of 398, 398078, and 398079 grown with (100 μg/ml; gray shaded histogram) or without CMP-NANA. The *x* axis represents fluorescence, and the *y* axis denotes the number of events. Isotype controls (antibodies alone, no factor H) are shown in each plot by thin broken lines. One representative experiment of duplicate experiments is shown.

position 168. All gonococcal *lst* genes contained the 7 additional amino acids first noted in FA1090.

Sialylation of the L1 LOS in *N. gonorrhoeae* does not enhance factor H binding. Sialylation of serum-sensitive gonococcal strains bearing the 3F11 LOS has been shown to enhance factor H binding and mediate serum resistance (37). We examined factor H binding to strains 398, 398079 (LNT LOS), and 398078 (L1 LOS) and their sialylated derivatives grown in media containing 0, 1, 10, or 100 μg/ml CMP-NANA) by flow cytometry (histogram for 10 μg/ml CMP-NANA has been omitted to simplify the illustration). We observed that factor H binding was enhanced only when 3F11 LOS-bearing strains 398 and 398079, but not strain 398078 (L1 LOS), was sialylated (Fig. 3, upper panel). Maximal factor H binding was seen even at 1 μg/ml CMP-NANA and was similar to binding observed with 100 μg/ml CMP-NANA. The *lst* mutant derivatives of these strains did not bind factor H when grown in CMP-NANA-containing media (Fig. 3, lower panel). These data suggest that enhancement of factor H binding to sialylated gonococci is restricted to strains that bear the LNT LOS species; enhancement was not seen in the strain that expressed only L1 LOS.

The above data show that levels of factor H binding to the LNT LOS-bearing strains were similar over CMP-NANA concentrations ranging from 1 to 100 μg/ml. We next quantified the proportion of LOS of strains 398078 (L1) and 398079 (mostly 3F11) that was sialylated at the different CMP-NANA concentrations to ensure that differences in the level of sialylation between the two LOS species did not account for the inability of sialylated L1 LOS to bind to factor H. Direct mass spectrometry showed that 5%, 25%, and 30% of glycoforms were sialylated when strain 398079 was grown in 1, 10, and 100 μg/ml CMP-NANA, respectively. The corresponding percentage of sialylated glycoforms in strain 398078 grown in the three concentrations of CMP-NANA (in order of increasing concentration) were 10%, 40%, and 55%, respectively. In a separate experiment, we repeated the sialic acid estimation in samples grown in 1 and 10 μg/ml of CMP-NANA using precursor ion scanning for the *m/z* 951 species, which represents the basic O-deacylated lipid A structure without additional phosphorylation. The percentages of 398079 LOS (predominantly 3F11) that was sialylated were 30% and 50%, and the percentages of sialylated L1 LOS in 398078 were 15 and 62.5 when organisms were grown in 1 and 10 μg/ml CMP-NANA, respectively. These data show that the two glycoforms were sialylated to similar extents and that only a small proportion of LOS sialylation was necessary for maximal factor H binding to the strain bearing the LNT LOS. Proportionally higher sialylation of L1 LOS did not result in factor H binding.

Differences in the efficiency of serum resistance conferred by sialylation of the 3F11 and L1 LOS. We examined the ability of strains 398079 (LNT LOS) and 398078 (L1 LOS) to resist the bactericidal action of 10% NHS when grown in media containing increasing concentrations of CMP-NANA. Figure 4A shows that LNT LOS-expressing strain 398079 demonstrated ~88% survival when grown in media containing 1 μg/ml CMP-NANA, while MAb L1-positive strain 398078 showed only ~17% survival under similar growth conditions. Survival of 398078 gradually increased with increasing CMP-NANA concentrations in the media. Strain 398, which expressed both LOS species, showed an intermediate level of serum resistance. The *lst* mutants of all three strains were completely killed in

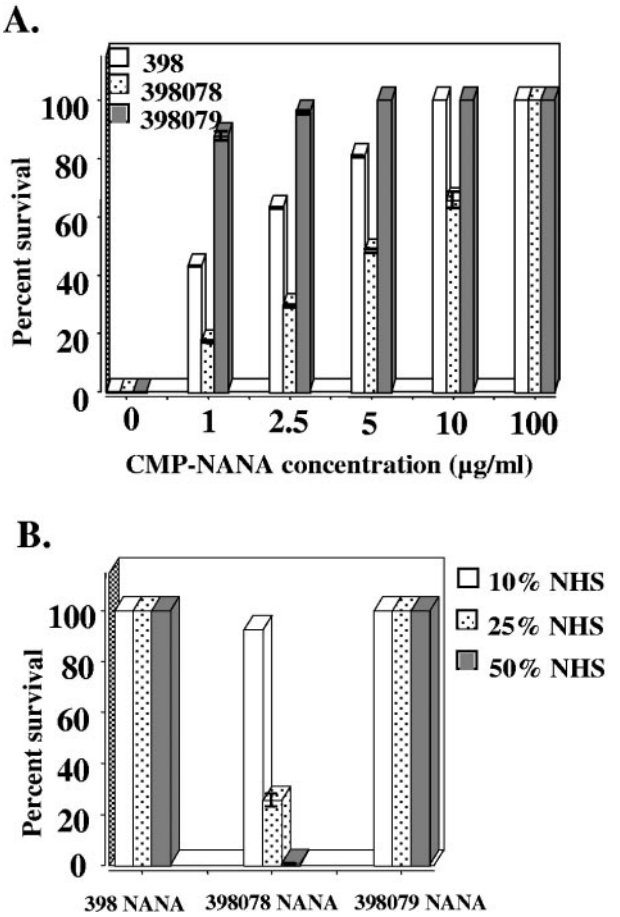

FIG. 4. (A) Serum resistance conferred by sialylation of LNT LOS occurs at lower CMP-NANA concentrations compared to L1 LOS sialylation. Bacteria were grown in the presence of increasing concentrations of CMP-NANA, and serum bactericidal assays were performed in the presence of 10% NHS. Strain 398079 showed 88% survival when grown in the presence of 1 μg/ml CMP-NANA, while 398078 showed only 17% survival under similar growth conditions. A gradual increase in serum resistance was seen with 398078 as CMP-NANA concentrations were increased. The mean (±standard deviation) of one representative experiment performed in duplicate is shown. (B) Sialylation of lacto-*N*-neotetraose LOS confers a higher level of serum resistance than sialylation of L1 LOS. Strains 398, 398078, and 398079 were grown in media containing 100 μg/ml CMP-NANA in an attempt to maximize LOS sialylation. Bacteria were then subjected to serum bactericidal testing in the presence of increasing serum concentrations. The mean (±standard deviation) of one representative experiment performed in duplicate is shown.

even 10% NHS when grown in media containing CMP-NANA at 100 μg/ml (data not shown).

In a separate experiment (Fig. 4B), we examined the degree of serum resistance that was conferred by sialylation of the two LOS species. LOS was fully sialylated by growth in media containing 100 μg/ml CMP-NANA, and bacteria were subjected to the effects of 10%, 25%, and 50% NHS in a serum bactericidal assay. We observed that sialylation of 398 and 398079 resulted in a higher degree of serum resistance (both strains fully resisted even 50% NHS), compared to sialylated 398078, which fully resisted only 10%, but was killed 75% and 99.5% in 25% and 50% NHS, respectively.

DISCUSSION

In order to survive the milieu of the genital tract and establish infection, *N. gonorrhoeae* must evade the innate immune system at the level of the genital mucosa. Naturally occurring antibody and complement are important effector arms of the immune response at the cervical mucosal level (33). Gonococcal LOS undergoes considerable phase variation in vivo (3, 12, 41), which could be advantageous in evading complement-mediated killing. For example, alteration in LOS structure may decrease binding of preexisting natural anti-LOS antibody to the bacterial surface. However, this could occur at the expense of the loss of a mechanism to evade complement.

Gonococci have developed a variety of mechanisms to evade complement-mediated killing. Sialylation of the lacto-*N*-neotetraose LOS of otherwise serum sensitive strains renders them resistant to bactericidal killing by NHS (31). This is termed "unstable" serum resistance, because it is a property that is lost when organisms are subcultured onto media that lack the donor molecule for sialic acid, CMP-NANA (30). Certain gonococcal strains are intrinsically resistant to killing by NHS, independent of LOS sialylation, a property termed "stable" serum resistance (38, 39). Stable serum resistance may in part be mediated by the ability of gonococcal porin to bind complement regulatory molecules such as factor H and/or C4b-binding protein (C4bp) (35, 36).

Prior work in our laboratory has shown that sialylation of gonococcal 3F11 (LNT) LOS enhances factor H binding, which results in regulation of the alternative pathway of complement (37). Factor H is a key soluble phase regulator of the alternative pathway of complement and acts as a cofactor in the factor I-mediated cleavage of C3b to the hemolytically inactive molecule, iC3b (29, 44, 56). Factor H also limits the amount of C3b deposited on the bacterial surface by virtue of its decay accelerating function, in which the factor Bb is irreversibly dissociated from C3-convertase, C3b,Bb (10, 29, 44, 53, 56).

NMR analysis of the sialylated LOS of 398078 showed an α(2,6)-linked sialic acid residue to the terminal Gal on HepI. This contrasted with the sialylation observed on the serologically identical and epidemiologically related strain, 398079 (LNT LOS), which as expected, possessed an α(2,3)-linked sialic acid. Inactivation of *lst* in 398078 and 398079 resulted in an inability of both strains to sialylate their LOS and suggests that the same Lst enzyme was capable of adding sialic acid in two distinct configurations on two different glycoforms. Bifunctionality of Lst has previously been described in meningococcal strain 126E. The presence of an Ile residue at position 168 of the 126E Lst was deemed critical for the ability to sialylate the L1 LOS at the 6-position. A comparison of the amino acid sequences of the Lst proteins of 398078 and 126E showed that the former had an Asn, but not an Ile residue at position 168. All gonococcal *lsts* that we sequenced encoded Asn at position 168. Another noteworthy feature of the gonococcal Lst was the presence of 7 additional C-terminal amino acids that were not seen in the meningococcal enzyme. Our data suggest that the molecular basis for bifunctionality of gonococcal Lst may differ from that reported for meningococci. The amino acid(s) that impart gonococcal Lst bifunctionality remains to be determined.

Meningococci that cause invasive disease are encapsulated, and LOS sialylation does not appear to impact virulence at least in animal models and may play only a minor role in meningococcal serum resistance (49, 50). In contrast, gonococci may depend more on LOS sialylation to survive the effects of complement, and this may be reflected in the maintenance of a relatively highly conserved and bifunctional enzyme among isolates. The Lst enzyme has recently been localized to the bacterial outer membrane (43), but the kinetics of the Lst enzymes of the two pathogenic neisserial species were not compared in situ; likewise, the differences in sialylation rates of the 3F11 and L1 LOS species are unknown.

We compared the abilities of strains bearing sialylated L1 LOS (NANAα2 → 6Galα1 → 3Galβ1 → 4Glcβ1 → 4HepI) and predominantly sialylated 3F11 LOS (NANAα2 → 3Galβ1→ 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4HepI) to bind factor H. As expected, the latter strain bound factor H well, but the sialylated L1 LOS did not enhance factor H binding to the gonococcal surface (Fig. 3). These data suggest that the binding of factor H to sialylated gonococci is highly specific and restricted to strains that possess sialylated lacto-*N*-neotetraose LOS. Specificity of factor H binding to polyanions is illustrated by the observation that factor H binds to heparin, sialic acid, dextran sulfate, chondroitin sulfate A, and type III and IV carrageenan, while little or no binding occurs to colominic acid, chondroitin sulfate C, keratan sulfate, hyaluronic acid, or polyaspartic acid (24). Ongoing work in our laboratory has shown that only sialylation of gonococcal, but not meningococcal, LNT LOS augments factor H binding. Preliminary evidence suggests that a cooperative mechanism between gonococcal porin and sialylated LNT LOS is required for factor H binding in this neisserial species (18).

Factor H binding to strain 398079 was not augmented as the proportion of sialylated LOS increased, possibly because the bacterial surface has already been "saturated" with factor H. Approximately 50,000 factor H molecules have been shown to bind to a single group A streptococcus (14). The factor H binding and serum bactericidal data obtained at lower CMP-NANA concentrations are likely to be biologically relevant because the amount of CMP-NANA obtained from cell lysates from whole blood is <1 μg/ml (26). Shifting of serum-sensitive *N. gonorrhoeae* to a serum-resistant phenotype was noticeable when bacteria were grown in media containing as little as 2×10^{-2} nM (~0.01 μg/ml) CMP-NANA (8).

Lipopolysaccharides of *Actinobacillus actinomycetemcomitans* (57), *Klebsiella pneumoniae* (1), and *Salmonella enterica* serovar Montevideo (16) have been shown to bind to C3b. Edwards et al. have identified gonococcal LOS as a target where C3b is converted to iC3b (7). Recently, we have identified *N. meningitidis* LOS as a target for complement C4b and have shown that alterations in HepI hexose substitutions and phosphoethanolamine residues on HepII can modulate the nature of the linkage (i.e., amide versus ester) of C4b with LOS (34). Sialylation of both 398078 and 398079 decreased C4 binding to a similar extent in a flow cytometry assay (data not shown), suggesting that LOS sialylation may also diminish or prevent C4b binding to LOS. The differences in the level (or degree) of serum resistance between 398078 and 398079, when grown in the presence of increasing concentrations of CMP-NANA (Fig. 4A), reflect the importance of factor H bound to sialylated LNT LOS on 398079 in down-regulating complement. Therefore any alternative pathway amplification that is initiated by the classical pathway is regulated by factor H bound directly to 398079. On the other hand, 398078 must rely on classical pathway regulation by obscuring targets for C4b. Such a mechanism would likely require a greater proportion of LOS to be sialylated and may be less efficient than a mechanism that involves binding of a complement regulator (in addition to obscuring targets for C3b and C4b).

In conclusion, we have demonstrated sialylation of the L1 (or P^K-like) LOS in gonococci and defined the nature of the sialic acid linkage. Although the chemistry of the sialylation of gonococcal LOS is similar to that seen in meningococci, we have noted differences in amino acid sequences of the bifunctional Lst enzymes in these two neisserial species. Our observations suggest that the bifunctionality of gonococcal Lst is not related to the Ile at position 168 as was reported in meningococci. Determining the requirements for the ability of gonococcal Lst to add sialic acid in two distinct configurations may shed light on the structure-function relationships of this enzyme. The ability of gonococci to sialylate two distinct LOS species would enable strains to undergo a phase variation of LOS from L1 to LNT (or vice versa), while retaining the ability to evade complement (albeit by different mechanisms). This may be important for strains such as 398078, which do not bind either factor H or other complement regulators such as C4bp in the native (unsialylated) state.

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ERRATUM

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

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Volume 73, no. 11, p. 7390–7397, 2005. Page 7390, abstract: "L1 (Galα1→3Galβ1→4Glcβ1→4HepI)" should read "L1 (Galα1→4Galβ1→4Glcβ1→4HepI)."

Column 2, lines 1 and 2: "L1 LOS (Galα1→3Galβ1→4Glcβ1→4HepI)" should read "L1 LOS (Galα1→4Galβ1→4Glcβ1→4HepI)."

Page 7392, column 2, line 1: "L1 LOS (Galα1→3Galβ1→4Glcβ1→4HepI)" should read "L1 LOS (Galα1→4Galβ1→4Glcβ1→4HepI)."

Column 2, line 7 from the bottom: "the Galα1→3Galβ1→4Glcβ1→ extension" should read "the Galα1→4Galβ1→4Glcβ1→ extension."

Page 7396, column 1, lines 13 and 14: "L1 LOS (NANAα2→6Galα1→3Galβ1→4Glcβ1→4HepI)" should read "L1 LOS (NANAα2→6Galα1→4Galβ1→4Glcβ1→4HepI)."