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Differential Expression and Transcriptional Analysis of the α-2,3-Sialyltransferase Gene in Pathogenic *Neisseria* spp.

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α-2,3-Sialyltransferase (Lst) is expressed on the outer membrane of *Neisseria gonorrhoeae* and *Neisseria meningitidis* and sialylates surface lipooligosaccharide (LOS), facilitating resistance to complement-mediated killing. The enzyme is constitutively expressed from a single gene (*lst*) and does not undergo antigenic or phase variation. We observed that Triton X-100 extracts of *N. gonorrhoeae* strain F62 contain about fivefold more sialyltransferase (Stase) activity than extracts of *N. meningitidis* strain MC58 ⊄3 a serogroup B acapsulate mutant. We confirmed and expanded upon this observation by showing that extracts of 16 random *N. gonorrhoeae* isolates contain various amounts of Stase activity, but, on average, 2.2-fold-more Stase activity than extracts of 16 *N. meningitidis* clinical isolates, representing several serogroups and nongroupable strains. Northern and real-time reverse transcription-PCR analysis of *lst* transcript levels in *N. gonorrhoeae* and *N. meningitidis* revealed that *N. gonorrhoeae* strains express more *lst* transcript than *N. meningitidis* strains. Although transcript levels correlate with average Stase activity observed in the two species, there was not a direct correlation between *lst* transcript levels and Stase activity among individual isolates of each species. Comparison of *lst* upstream (5′*lst*) regions of *N. gonorrhoeae* and *N. meningitidis* revealed striking sequence differences characteristic of the two pathogens. *N. gonorrhoeae* 5′*lst* regions possess 30-bp and 13-bp elements present as single elements or as tandem repeats that exist only as single elements in the 5′*lst* regions of *N. meningitidis* isolates. In addition, the 5′*lst* regions of *N. meningitidis* strains have 105-bp transposon-like Correia elements which are absent in *N. gonorrhoeae*. Chromosomal *N. gonorrhoeae* 5′*lst*::*lacZ* translational fusions expressed 4.75 ± 0.09-fold ($n = 4$) higher β-galactosidase (β-gal) activity than *N. meningitidis* 5′*lst*::*lacZ* fusions in a host-independent manner, indicating differential expression is governed at least in part by sequence variations in the 5′*lst* regions. Reporter fusion assays and promoter-mapping analysis revealed that *N. gonorrhoeae* and *N. meningitidis* use different promoters with different strengths to transcribe *lst*. In *N. gonorrhoeae*, a strong sigma 70 promoter 80 bp upstream of the translational start site is used to transcribe *lst*, whereas this promoter is inactive in *N. meningitidis*. In *N. meningitidis*, a weak sigma 70 promoter at the 3′ terminus of a 105-bp Correia repeat-enclosed element 99 bp upstream of the translational start site is used to transcribe *lst*. We conclude that differential Stase expression between *N. gonorrhoeae* and *N. meningitidis* is due at least in part to differential *lst* gene transcription.

The sialylation of lipooligosaccharide (LOS) in pathogenic *Neisseria* spp. is catalyzed by the outer membrane enzyme α-2,3-sialyltransferase (Lst) (15, 26). The importance of this enzyme for neisseria virulence is highlighted by the finding that Lst is found primarily in the pathogenic, as opposed to nonpathogenic, *Neisseria* spp.(14, 15). LOS sialylation is responsible for converting serum-sensitive strains of *Neisseria gonorrhoeae* to serum resistance by allowing gonococci to bind complement factor H (20). The role of LOS sialylation in mediating serum resistance of *N. meningitidis* is less well understood and thought to act in concert with capsule, which inhibits complement membrane attack complex insertion (19). In serum-sensitive meningococcal isolates, exogenous sialylation of LOS enhances serum resistance (8). In highly serum-resistant meningococcal disease strains, LOS sialylation appears dispensable for serum resistance (31). Thus, the need for LOS sialylation in the pathogenic *Neisseria* spp. varies among isolates and species.

Natural variations occur in the degree of LOS sialylation in different isolates of pathogenic *Neisseria* spp. (8, 15, 18, 28). The factors that could affect the degree of LOS sialylation include the availability of phase-variable terminal galactose sialylation targets (29, 30), the amount of available CMP–*N*-acetylneuraminic acid (CMP-NANA) (21, 34), and inherent specific activity or regulated expression of Lst. Regulation of sialyltransferase (Stase) expression has not been demonstrated within strains. In an effort to define the distribution of sialyltransferase activity among commensal and pathogenic strains of *Neisseria*, Mandrell et al. (15) observed that Triton X-100 extracts of *N. gonorrhoeae* F62 were more efficient at sialylating exogenous LOS than extracts of *N. meningitidis* L11 strain 7889, implying that *lst* may be expressed at different levels among pathogenic *Neisseria* spp.

In this paper, we describe differential Stase expression between *N. gonorrhoeae* and *N. meningitidis* and address the possibility that it is due to differential *lst* gene expression. To this end, we performed transcriptional analysis of six *N. gonor-*

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rhoeae and six *N. meningitidis* clinical isolates and found that *lst* transcript levels were more abundant in *N. gonorrhoeae* than in *N. meningitidis*. By reporter fusion assays and promoter-mapping analysis, we show that *N. gonorrhoeae* and *N. meningitidis* strains use different promoters with different strengths to transcribe *lst*. Overall, this study indicates that expression of different levels of *lst* by *N. meningitidis* and *N. gonorrhoeae* is controlled at least in part at the level of transcription.

(Observations on different sialyltransferase activities, distinctive *lst* upstream sequences, and differential *lst* reporter gene expression were presented by S. V. Liu, Y.-B. Liu, and R. F. Rest at the 11th International Pathogenic Neisseria Conference, 1998, Nice, France.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* F62, *N. meningitidis* MC58 3, and *Escherichia coli* XL1-Blue MRF′ were obtained from P. Frederick Sparling (University of North Carolina, Chapel Hill), E. Richard Moxon (University of Oxford, Oxford, United Kingdom), and Stratagene (La Jolla, Calif.), respectively. Random clinical isolates of *N. gonorrhoeae* were obtained from the City of Philadelphia Public Health Laboratories, and representative *N. meningitidis* strains (see Table 2 and Fig. 1C) were graciously donated by David Stephens, Emory University, Atlanta, GA. *N. gonorrhoeae* ST01 is an *lst* knockout mutant of *N. gonorrhoeae* F62 constructed by the insertion of a kanamycin cassette in the *lst* open reading frame (kind gift of Michael Jennings). *N. gonorrhoeae* ST01 does not express *lst* protein or sialyltransferase activity (26). Frozen stocks of *Neisseria* or *E. coli* cells were clonally passaged for up to 1 week by growing aerobically at 37°C in a humidified 5% CO_2 incubator (Forma Scientific, Marietta, Ohio) on GC agar (Difco, Detroit, Mich.) with supplement (12) or on Luria-Bertani (LB) agar, respectively.

Measurement of Stase activity in cell extracts. Stase activity was determined in freshly made *Neisseria* cell extracts using a method developed by Mandrell (15) with some modifications (17). Briefly, bacteria were harvested from overnight agar cultures, washed once, and suspended in sterile PBSGCM (phosphate-buffered saline containing 0.1% [wt/vol] gelatin, 0.1% [wt/vol] $CaCl_2$, and 0.1% [wt/vol] $MgCl_2$) to an optical density at 550 nm (OD_{550}) of 0.18 ($\sim 2 \times 10^8$ CFU/ml). Bacteria in 1.5 ml of PBSGCM were pelleted by centrifugation and suspended in 60 μl of 0.5% Triton X-100 in 0.5 mM phosphate buffer (pH 6.8). Cell suspensions were pipetted up and down at least 15 times, and cell extracts were obtained after centrifugation (10,000 × *g*, 4°C, 10 min). Stase activity in the cell extracts was determined by quantifying ^{14}C-labeled NANA transferred from CMP-[^{14}C]NANA onto purified LOS from *N. gonorrhoeae* F62, unless otherwise indicated after 15 min of incubation at 37°C (17).

PCR amplification of 5′*lst*. PCR primers were synthesized according to sequence information derived from the *lst* gene (accession no. U60660) and are listed in Table 1.

Construction of 5′*lst*::*lacZ* fusions. Various lengths of *Neisseria* 5′*lst* regions were amplified by PCR using primer pairs indicated in Table 1. PCR fragments contained a putative ribosome binding site (RBS) and 24 bp of *lst* coding region (including ATG). Translational fusions were created using the vector pLES94, a high-copy-number plasmid containing a *Neisseria* uptake sequence designed to effect allelic exchange into neisserial chromosomes (27). As templates for PCRs, we used supernatants prepared from a few colonies of *N. gonorrhoeae* or *N. meningitidis* boiled for 10 min in 50 μl H_2O followed by incubation at 37°C with RNase A (20 μg/ml) for 30 min. PCR-amplified 5′*lst* fragments were digested with BamHI and then ligated into BamHI- and shrimp alkaline phosphatase-treated pLES94. The resulting constructs were transformed by electroporation into *E. coli* XL1-Blue MRF′, prepared by washing log-phase *E. coli* in 10% glycerol at 4°C three times, and then stored at −70°C until use. Electroporation was performed at 2.5 keV, using 0.4-cm electroporation cuvettes in a Gene Pulser (Bio-Rad, Hercules, CA). The electroporated bacteria were incubated in 1 ml of super broth (25 g tryptone, 15 g yeast extract, and 5 g NaCl per liter of H_2O) at 37°C for 1 to 3 h in a shaking water bath, and then the bacteria were plated onto selective media. Transformants were selected on LB agar containing 100 μg/ml ampicillin and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) at 40 μg/ml. After overnight incubation, blue colonies were picked and plasmids were checked for inclusion of correct inserts by PCR analysis and DNA sequencing. Plasmids containing the correct insert in the proper orientation were transformed into *N. meningitidis* or *N. gonorrhoeae*. Transformants were selected on gonococcal agar containing supplements and chloramphenicol at 1 μg/ml for *N. gonorrhoeae* F62 or 5 μg/ml for *N. meningitidis* MC58 ⊄3.

Integration of fusions into neisserial chromosomes. Integration of fusions into the *N. gonorrhoeae* F62 chromosome was done by mixing donor DNA (~1 μg DNA in 20 μl H_2O) with 20 μl of *N. gonorrhoeae* cell suspension (made by suspending the pellet of 3 ml of a 0.18 A_{550} culture in 240 μl gonococcal broth (GCB) plus 20 mM $MgCl_2$) on GC agar plates and incubating the mixture at 37°C for 5 to 6 h. The *N. gonorrhoeae* cells were subsequently swabbed into 200 μl GCB and plated onto selective media. Integration of fusions into *N. meningitidis* MC58 ⊄3 was done by electroporation under the conditions described above for *E. coli*. Competent *N. meningitidis* cells were made by washing cells three times in ice-cold buffer containing 9% (wt/vol) sucrose and 15% (vol/vol) glycerol. After electroporation, cells were incubated in GCB with supplement at 37°C for 3 h and then plated on gonococcal agar containing chloramphenicol at 5 μg/ml. All neisserial integrants expressed levels of native Stase activity similar to those of wild-type strains (data not shown).

RNA isolation. Total RNA was extracted from exponential-phase broth-grown bacteria using QIAGEN mini RNAeasy isolation kits according to the manufacturer's instructions. If needed, RNA preparations were concentrated by addition of 0.5× volume of 1 M LiCl (Ambion) and incubation at −20°C for 30 min. Precipitated RNA was pelleted by centrifugation at 12,000 × *g* and washed with cold 70% (vol/vol) ethanol, before resuspension in diethylpyrocarbonate (Sigma)-treated double-distilled H_2O (ddH_2O) containing 1 μl of RNasin (40 U/μl; Promega). Concentrations of RNA were determined by optical density at 260 nm. All preparations were treated twice with DNase I (6 U, RNase free; Ambion) and stored at −70°C until use.

cDNA synthesis. cDNA was synthesized using the reverse transcriptase RNase H^- SuperScript III (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, 2 μg of DNase I-treated RNA in a 10-μl volume was mixed with 1 μl (250 ng) of random hexamers (Promega) and 1 μl (10 mM) of deoxynucleoside triphosphate (dNTP) mix (Promega). This mixture was incubated at 65°C for 5 min and quickly chilled on ice. A cocktail containing 4 μl of 5× First-Strand buffer, 2 μl of 0.1 M dithiothreitol and 1 μl of RNasin were then added to each tube. After a brief centrifugation, the tubes were incubated at room temperature for 10 min. The reaction mixtures were then preincubated at 50°C for 2 min, before adding 1 μl of SuperScript. cDNA synthesis was then allowed to proceed at 50°C for 50 min, followed by incubation at 70°C for 15 min to inactivate the reaction. Nuclease-free water (1 μl) was added to reaction mixtures in place of Superscript for controls.

Real-time PCR. The SYBR Green Master Mix kit (ABI) was used to perform real-time PCR assays. cDNA reaction mixtures (20 μl) were diluted to a final volume of 100 μl with nuclease-free ddH_2O. The diluted cDNA template (2 μl), in addition to 1:2 and 1:4 dilutions thereof, was then subjected to PCR amplification in a 7700 Sequence Detector (ABI) in a total volume of 25 μl containing 12.5 μl SYBR Green Master Mix, 1 μl of each primer (0.4 μM, final concentration), and 8.5 μl ddH_2O. The reactions were cycled according to the following parameters: 10 min at 95°C and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using the Sequence Detector v.1.7a software (ABI). The cycle threshold (C_T) was defined as the cycle number corresponding to the point where the amplification plot of all samples was linear. We used the comparative C_T method ($\Delta\Delta C_T$) for relative quantification of *lst* expression where 16S rRNA (*rrs*) expression served as the active reference control (normalizer). Quantification of relative *lst* expression included calculating the difference between the C_T values of the normalizer (16S rRNA) and the C_T values of individual samples: $\Delta C_T = C_{T(16S\ rRNA)} - C_{T(sample)}$. The difference between each sample's ΔC_T value and the ΔC_T value of *N. gonorrhoeae* F62 ($\Delta\Delta C_T$) was then used to obtain an absolute value for the difference (fold) in *lst* mRNA levels between samples ($2^{\Delta\Delta CT}$). Results are expressed as arbitrary units reflecting this difference. The sequences of primers used for this analysis are given in Table 1.

Primer extension. Primers were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). RNase H^- SuperScript III (Gibco BRL, Rockville, MD) was used for 5′ mapping. For most reactions, 10 μl of RNA (10 to 50 μg) was mixed with 1 μl of labeled primer (2 pmol) and incubated at 70°C for 10 min. The tubes were immediately cooled on ice for 2 to 3 min for primer annealing. A cocktail containing 4 μl of 5× first-strand buffer (Gibco BRL), 2 μl of 0.1 μM dithiothreitol, 1 μl 10 mM dNTP, and 1 μl of RNasin was then added to the annealing mixture to a final volume of 19 μl. The resulting mixture was incubated at 50°C for 2 min, before adding 1 μl of Superscript, and the primer extension was performed at 50°C for 50 min. The reaction was then ethanol precipitated, and the mixture was resuspended in 4 μl of Tris-EDTA and 4 μl of gel loading buffer (Promega). Extension products were analyzed on a 6% denaturing polyacrylamide gel containing 8 M urea. The migrations of the primer extension products were compared to sequencing ladders generated by PCR

TABLE 1. Primers used in this study

Primer	Sequence 5′→3′	Amplification target
Northern blot probe PCR		
Sta2 For	GCGTATGTTCAATTTGTCG	1,140-bp product from *lst* of *N. gonorrhoeae* and *N. meningitidis*
Sta3 Rev	CGTCAAATGTCAAAATCGG	
Real-time PCR		
LstRTF	AAACCCGCATACGAGGTATGA	100-bp product from *lst* of *N. gonorrhoeae* and *N. meningitidis*
LstRTR	AAGCCGGTTTCAATGCGTAA	
16S RT F	GCGTGGGTAGCAAACAGGAT	100-bp product from *rrs* gene of *N. gonorrhoeae* and *N. meningitidis*
16S RT R	CGCGTTAGCTACGCTACCAAG	
Cloning[a]		
FusBam F1	CGCTGGATCCGACATCAATATCGG	496 bp of *N. gonorrhoeae* 5′*lst* including RBS sequence and 24 bp of *lst* including ATG
FusBam R1	CAAAGGATCCTTTTTCAAGCCC	586 bp of *N. meningitidis* 5′*lst* including RBS sequence and 24 bp of *lst* including ATG
Primer extension		
PE1	CGCATTCCTTTCCCCCTGATTTAC	3′ region of primers anneals 78, 16, and 24 bp downstream of *lst* ATG, respectively
PE2	CACAACACGGTCAAACAAGC	
PE3	CAATCAGGCACAACACGGTC	
RPA primers		
PA1	GATCGAGCTCGTTCGATCTTGGCGTGTTTG	292 bp of *N. gonorrhoeae* 5′*lst* excluding the RBS sequence; SacI site of PA1 is denoted by double underline
PA2	GATCGGATCCCTCCATTCCGACAAATTGAAC	
PA3	AATTAACCCTCACTAAAGGG	397-bp product from pSKII including the cloned 292-bp *N. gonorrhoeae* 5′*lst* insert
PA4	GTAATACGACTCACTATAGGG	
RT-PCR: Fig. 7		
P1	CGGGATCCGGCTTTCCCGCGTTTGCCGG	5′ region of the primer anneals upstream of CREE; 282 bp upstream of *lst* ATG
P2	CGGGATCCCGCCTTGTGCCTGATGTGCG	5′ region of the primer anneals upstream of CREE; 252 bp upstream of *lst* ATG
P3	CGGGATCCTTTCGGTAAAATTGATTTTA	5′ region of the primer anneals upstream of CREE; 222 bp upstream of *lst* ATG
P4	CGGGATCCAACTGTCGGAATATCTGCTA	5′ region of the primer anneals downstream of CREE; 91 bp upstream of *lst* ATG
P5	CGGGATCCTTTTTCCGTCCCGGGACAC	5′ region of the primer anneals downstream of CREE; 61 bp upstream of *lst* ATG
P6	CGGGATCCACACTCGGGGCGTATGTTCA	5′ region of the primer anneals downstream of CREE; 41 bp upstream of *lst* ATG
P7	CGGGATCCAGGGATATGGGCTTGAAAAAG	5′ region of the primer anneals downstream of CREE; 6 bp upstream of *lst* ATG
C_{rev}	CTCCGCCATCGTCGGAAT	Common reverse primer used in conjunction with primers P1 to P7 and the 3′ region of the primer anneals 372 bp downstream of lst ATG
RT-PCR: Fig. 8		
IP1	TTATTCTCTCTTGTAGGTTGG	Generates a 212-bp product of 5′*icd* upstream region; P4 and Crev primers used in Fig. 7 were used in Fig. 8
IP2	TGCCGCCGCACATCAGGCACA	

[a] Primers FusBam F1 and FusBam R1 were used to include regions of the *N. gonorrhoeae* or *N. meningitidis* 5′*lst* region including the RBS and 24 bp of the *lst* gene including ATG to create translational *lacZ* fusions in pLES94. The BamHI restriction sites are underlined with single lines.

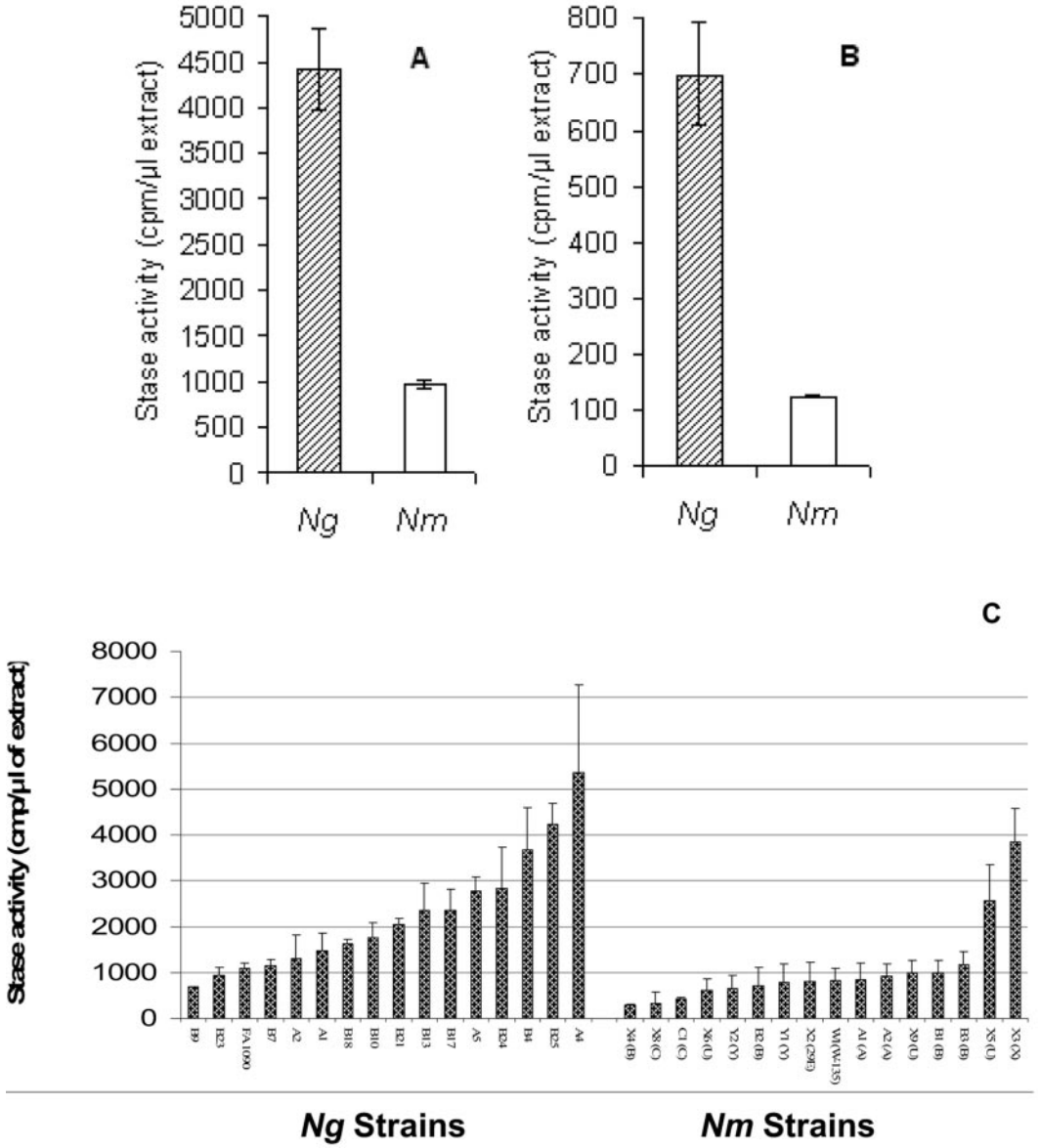


FIG. 1. Sialyltransferase activity of *N. gonorrhoeae* (*Ng*) F62 and *N. meningitidis* (*Nm*) MC58 measured as described in Materials and Methods with LOS prepared from *N. gonorrhoeae* F62 (A) or *N. meningitidis* MC58 (B). A graphical representation of data in Table 2 comparing the Stase activities of *N. gonorrhoeae* and *N. meningitidis* clinical isolates is shown in panel C.

(*f*mole DNA Cycle Sequencing Sample; Promega, Madison, Wis.) using the same primer. A constant current of 1,800 V was applied to the sequencing gel in 1× Tris-borate-EDTA buffer. After electrophoresis, the gel was dried for 1 h at 80°C in a vacuum gel dryer, and the gel was exposed to X-ray film overnight before development.

Northern blot analysis. Northern blot analysis was performed using a 1.2% MOPS (morpholinepropanesulfonic acid)–formaldehyde agarose gel essentially as described by Maniatis et al. Before loading the agarose gel, RNA samples were mixed with 3× volumes of loading buffer (Ambion) containing 10 μg/ml ethidium bromide and heated at 68°C for 15 min. RNA was transferred using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto Nytran N (Schleicher and Schuell, Keene, NH) and hybridized with horseradish peroxidase-labeled PCR fragments (≈1.1 kb) (North2South; Pierce) specific for *lst*.

DNA sequencing. DNA fragments to be sequenced were amplified by PCR from *Neisseria* chromosomes with appropriate primers and run on agarose gels. Bands of the expected size were cut from gels and purified using the Wizard PCR Prep DNA purification system (Promega, Madison, Wis.). Purified DNA fragments were mixed with the appropriate primer, and nucleotide sequences were determined by direct automated fluorescent DNA sequencing at facilities at either the University of Pennsylvania or Drexel University College of Medicine.

Assay for β-Gal activity in cell extracts. β-Galactosidase (β-Gal) activity was determined by the Miller method with minor modifications. Bacteria were grown on plates or in broth to the mid-log phase. *E. coli* transformants were grown in the presence of ampicillin (100 μg/ml) and tetracycline (15 μg/ml). Broth cultures or suspensions of cells harvested from plates were adjusted to an OD_{600} of 0.4. For *Neisseria*, 0.5 ml of the OD_{600} 0.4 suspension was mixed with 0.5 ml of Z buffer. For *E. coli*, 0.1 ml of the OD_{600} 0.4 suspension was mixed with 0.9 ml Z buffer. Bacterial cells were disrupted by adding 20 μl of chloroform and 10 μl of 0.1% sodium dodecyl sulfate. Cell extracts were incubated in a 28°C water bath for 5 min, and assays were started by adding 0.2 ml of *O*-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml). Reactions were stopped when an OD_{420} of about 0.6 to 0.9 developed. The absorbance of reaction mixtures was determined at 420 nm. Miller units were calculated according to the formula $1{,}000 \times OD_{420}/[\text{time (min)} \times \text{volume (ml)} \times OD_{600}]$.

RPAs. RNase protection assays (RPAs) were performed using Ambion's RPA III kit following the manufacturer's directions. Briefly, 2.5 μg to 15 μg of total

RNA from *N. gonorrhoeae* F62 or *N. gonorrhoeae* FA1090 was mixed with ^{32}P-labeled antisense RNA probe (generated using Ambion's in vitro transcription kit, MAXIscript) in 10 μl of Hybridization Buffer III and incubated overnight at 42°C. Samples were digested with 1:100 dilution of RNase A/T_1 mix for 30 min at 37°C. RNase was inactivated by Inactivation/Precipitation Solution III, and the protected fragments were resolved along with an end-labeled Promega ΦX174 DNA size marker by 6% polyacrylamide gel electrophoresis (PAGE) with 8 M urea.

Generation of ^{32}P-labeled antisense RNA probe. The *lst* upstream region of *N. gonorrhoeae* F62 was PCR amplified with the primer pair PA1 and PA2 and cloned into pSKII at the SacI and BamHI sites. The T7 promoter with the cloned *lst* fragment was PCR amplified from pSKII with the primer pair PA3 and PA4 and used as a template for in vitro transcription reactions following Ambion's MAXIscript directions. The 380-bp ^{32}P-labeled antisense riboprobe generated from the T7 promoter was resolved by 5% PAGE with 8 M urea, and gel-purified full-size probe was used in RPAs.

RESULTS

Stase activity in Triton X-100 extracts of *N. gonorrhoeae* is greater than that in extracts of *N. meningitidis*. Triton X-100 extracts of *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ⊄3 were prepared and tested for Stase activity as described in Materials and Methods. *N. gonorrhoeae* F62 extracts contained 4.5-fold-more Stase activity than *N. meningitidis* MC58 ⊄3 extracts (*N. gonorrhoeae*, 4,414 ± 444 cpm/μl; *N. meningitidis*, 974 ± 39 cpm/μl) (Fig. 1A). The difference in Stase activity was independent of LOS source, since *N. gonorrhoeae* F62 extracts also had greater Stase activity than *N. meningitidis* MC58 ⊄3 extracts when measured using LOS purified from *N. meningitidis* MC58 ⊄3 (*N. gonorrhoeae*, 699 ± 91 cpm/μl; *N. meningitidis*, 124 ± 2 cpm/μl) (Fig. 1B). To extend this finding to other strains of *Neisseria*, Triton X-100 extracts of 16 random clinical isolates each of *N. gonorrhoeae* and *N. meningitidis* were prepared and tested for Stase activity. Triton extracts of these isolates expressed a range of Stase activities (*N. gonorrhoeae*, 685 to 5,367 cpm/μl; *N. meningitidis*, 288 to 3,845 cpm/μl) (Table 2 and Fig. 1C). On average, extracts of *N. gonorrhoeae* strains contained 2.2-fold more Stase activity than extracts prepared from *N. meningitidis* strains (Mann-Whitney U test, $P < 0.001$). We were interested in determining if variation in Stase activities between *N. gonorrhoeae* and *N. meningitidis* extracts was regulated at the level of transcription.

***lst* transcript levels are more abundant in *N. gonorrhoeae* than in *N. meningitidis* by Northern blot analysis.** We initially analyzed *lst* transcript levels in *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ⊄3 by Northern blot analysis as described in Materials and Methods. Blots were probed with a 1.1-kb *lst* fragment; the *N. gonorrhoeae lst* gene is 1,116 bp (10). Single bands of ≈1.2 kb were detected in total RNA isolated from *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ⊄3 (Fig. 2A). These bands were not detected in total RNA isolated from the *lst*-negative mutant *N. gonorrhoeae* ST01 (data not shown). Furthermore, the *lst* band was less intense for *N. meningitidis* MC58 ⊄3 than for *N. gonorrhoeae* F62, indicating greater *lst* mRNA levels in *N. gonorrhoeae* F62 than in *N. meningitidis* MC58 ⊄3. *lst* transcript levels were also evaluated in three additional *N. gonorrhoeae* and *N. meningitidis* isolates. The *lst* mRNA bands were more intense for strains of *N. gonorrhoeae* than for strains of *N. meningitidis* (Fig. 2B). Surprisingly, *N. gonorrhoeae lst* transcript levels were similar among all four isolates and did not reflect differences in Stase activities of Triton extracts (Fig. 2B). For example, *N. gonorrhoeae* A4 extracts contained 7.8-fold-more Stase activity than extracts of *N. gonorrhoeae* B9 (A4 = 5,367 ± 1,915 cpm/μl; B9 = 685 ± 12 cpm/μl; Table 2), but there was less than a twofold difference in the intensities of *lst* bands for these strains. *N. meningitidis* strains X3 and X4 expressed more *lst* mRNA than strains X5 and MC58 ⊄3. Similar to *N. gonorrhoeae*, the transcript levels found in *N. meningitidis* strains did not reflect their differences in Stase activity. For example, *N. meningitidis* X3 extracts contained 13.3-fold-more Stase activity than extracts of *N. meningitidis* X4 (X3 = 3,845 ± 731 cpm/μl, X4 = 288 ± 23 cpm/μl; Table 2), but there was less than a 2-fold difference in the intensities of *lst* bands for these strains. Therefore, although differences in Stase activities in extracts were the original impetus for our studies, Stase activity in Triton extracts does not directly correlate with *lst* transcript levels when compared between isolates of the same species. Regardless, overall, *N. gonorrhoeae* strains express more *lst* transcript than do *N. meningitidis* strains.

TABLE 2. Stase activity in Triton X-100 extracts of *N. gonorrhoeae* and *N. meningitidis* clinical isolates[a]

Strain	Mean Stase activity ± SD (cpm/μl)
N. gonorrhoeae	
B-9	685 ± 12
B-23	945 ± 159
FA1090	1,092 ± 117
B-7	1,154 ± 129
A-2	1,310 ± 508
A-1	1,482 ± 371
B-18	1,631 ± 88
B-10	1,762 ± 331
B-21	2,044 ± 133
B-13	2,357 ± 581
B-17	2,357 ± 458
A-5	2,768 ± 304
B-24	2,836 ± 896
B-4	3,671 ± 924
B-25	4,223 ± 473
A-4	5,367 ± 1,915
N. meningitidis (serogroup)	
X4 (B)	288 ± 23
X8 (C)	330 ± 252
C1 (C)	427 ± 23
X6 (U)	611 ± 252
Y2 (Y)	642 ± 293
B2 (B)	709 ± 399
Y1 (Y)	784 ± 410
X2 (29E)	807 ± 412
W1 (W-135)	814 ± 272
A1 (A)	844 ± 357
A2 (A)	927 ± 259
X9 (U)	999 ± 267
B1 (B)	1,003 ± 259
B3 (B)	1,176 ± 280
X5 (U)	2,561 ± 783
X3 (X)	3,845 ± 731

[a] A graphical representation of the tabulated data comparing the Stase activities of *N. gonorrhoeae* and *N. meningitidis* clinical isolates is shown in Fig. 1C.

***lst* transcript levels are more abundant in *N. gonorrhoeae* than in *N. meningitidis* by real-time PCR.** To corroborate our Northern blot studies and for a more quantitative assessment of *lst* transcript levels in *N. gonorrhoeae* and *N. meningitidis*, we used semiquantitative real-time PCR. In addition to strain *N. gonorrhoeae* F62 (Stase activity = 4,414 ± 444 cpm/μl) and *N.*

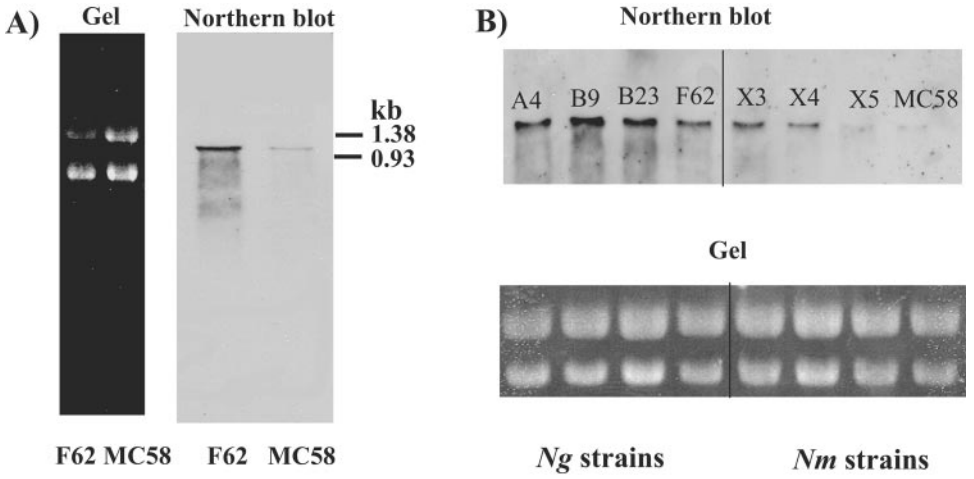


FIG. 2. Northern analysis of *lst* transcripts in *N. gonorrhoeae* and *N. meningitidis*. (A) Total RNA (20 μg) isolated from *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ⊄3 was fractionated on a 1.2% formaldehyde agarose gel, blotted onto Nytran N, and localized with an *lst*-specific horseradish peroxidase-labeled probe as described in Materials and Methods. (B) Total RNA (20 μg) prepared from *N. gonorrhoeae* (*Ng*) strains A4, B9, B23, and F62 and *N. meningitidis* (*Nm*) strains X3, X4, X5, and MC58 ⊄3 analyzed by Northern analysis. Molecular sizes are expressed in kb.

meningitidis MC58 (Stase activity = 974 ± 39), three additional isolates of each species exhibiting low Stase activities and two exhibiting high Stase activities were used for real-time PCR analysis: *N. gonorrhoeae* low, B9 = 685 ± 12, B23 = 945 ± 159, and FA1090 = 1,092 ± 117 cpm/μl, and *N. gonorrhoeae* high, B25 = 4,223 ± 473 and A4 = 5,367 ± 1,915 cpm/μl; and *N. meningitidis* low, X4 = 288 ± 23, X8 = 330 ± 252, and C1 = 427 ± 23 cpm/μl and *N. meningitidis* high, X5 = 2,561 ± 783 and X3 = 3,845 ± 731 cpm/μl. Expression of 16S rRNA was measured as an internal reference, and the primers used are given in Table 1. Differences in C_T values (ΔC_T) are given for each isolate used to calculate relative RNA levels (Fig. 3A). Consistent with results of our Northern analysis, relative differences in *lst* mRNA levels among strains of *N. gonorrhoeae* were less than 2-fold and *lst* transcript levels in *N. meningitidis* were 2.5- to 10-fold less than those in *N. gonorrhoeae* F62 (Fig. 3B). As with Northern analysis, variations in *lst* transcript levels detected in *N. meningitidis* strains were much more evident than in *N. gonorrhoeae* strains. In particular, *N. meningitidis* X3 produced 3.7 ± 0.3-fold more *lst* transcript than *N. meningitidis* MC58 ⊄3 and X8, and *N. meningitidis* C1 and X4 produced 2.2 ± 0.2-fold more *lst* transcript than MC58 ⊄3 and X8. Thus, real-time PCR results confirmed Northern blot analyses that Stase activity in Triton extracts does not correlate with *lst* transcript levels when compared within isolates of the same species (Fig. 3C).

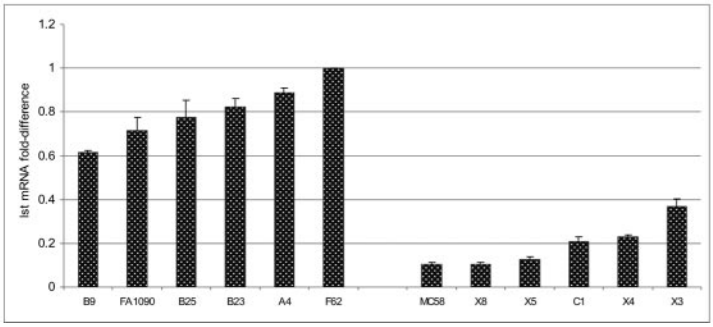
Analysis and comparison of sequences upstream of *N. gonorrhoeae* and *N. meningitidis lst*. Next, we wanted to identify *lst* upstream (5′*lst*) sequences that might influence *lst* transcription. We sequenced and compared the *lst* upstream regions of 28 *N. gonorrhoeae* and 17 *N. meningitidis* strains. Primers FusBam F1and FusBam R1 (Table 1) were used to amplify the upstream regions of *lst* genes. To our surprise, different size fragments were amplified from chromosomal DNA of *N. gonorrhoeae* strains, whereas an apparently constant size fragment was amplified from *N. meningitidis* strains (Fig. 4A). Analysis of DNA sequences revealed significant differences contained in a conserved background. Examination of the 5′*lst* regions revealed that *N. meningitidis* strains possess the 105-bp Correia repeat-enclosed element (CREE), while it is absent from the 5′*lst* regions of all *N. gonorrhoeae* strains (13). For simplicity of sequence comparison, we used *N. gonorrhoeae* F62 and *N. meningitidis* MC58 as representative strains for the respective *Neisseria* species. In the *lst* promoter region, *N. gonorrhoeae* F62 has two near perfect tandem repeats of 30 bp and 13 bp, whereas *N. meningitidis* MC58 has a single copy of each element; other single-base-pair variations also exist (Fig. 4B). In addition, 11 of the 28 *N. gonorrhoeae* strains had single 13- or 30-bp elements, in place of the tandem repeats observed in *N. gonorrhoeae* F62 (13). We also found single-base-pair differences. Overall, the 5′*lst* region of *N. gonorrhoeae* strains varied considerably, whereas the *N. meningitidis* 5′*lst* region remained relatively conserved.

β-Galactosidase activities of *lst* promoter fusions of *N. gonorrhoeae* are higher than those of *N. meningitidis*. To further examine whether differences in promoter regions of *N. gonorrhoeae* and *N. meningitidis* were associated with transcript levels or enzyme activity in Triton extracts, 5′*lst* fragments were amplified with primers FusBam F1 and FusBam R1; incorporated into the promoterless *lacZ* vector, pLES94; and subsequently integrated into *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ⊄3 chromosomes as described in Materials and Methods. The *N. gonorrhoeae* F62 5′*lst*::*lac*Z fusion expressed 4.75- or 5.5-fold-higher levels of β-Gal activity, respectively, than the *N. meningitidis* MC58 5′*lst*::*lac*Z fusion (Fig. 5A) when expressed in the chromosome of *N. gonorrhoeae* F62 (*N. gonorrhoeae* = 118 ± 12 Miller units, *N. meningitidis* = 25 ± 3 Miller units) or *N. meningitidis* MC58 (*N. gonorrhoeae* = 98 ± 6 Miller units, *N. meningitidis* = 18 ± 3 Miller units) (n = 4). Although to a lesser degree than when expressed in neisseria, the *N. gonorrhoeae* fusions also exhibited greater β-Gal activity than the *N. meningitidis* fusions when expressed as multicopy plasmids in *E. coli* (Fig. 5B) (*N. gonorrhoeae* = 3,498 ± 300 Miller units, *N. meningitidis* = 1,600 ± 100 Miller units) (n =

A)

Ng strain	ΔC_T	RNA fold-difference	*Nm* strain	ΔC_T	RNA fold-difference
F62	12.92 ± 0.08	1	MC58⊄3	16.20 ± 0.26	0.10 ± 0.02
A4	13.10 ± 0.28	0.89 ± 0.02	X8	16.19 ± 0.17	0.10 ± 0.01
B23	13.19 ± 0.14	0.82 ± 0.04	X5	15.89 ± 0.10	0.13 ± 0.01
B25	13.28 ± 0.07	0.77 ± 0.08	C1	15.19 ± 0.15	0.21 ± 0.03
FA1090	13.40 ± 0.05	0.72 ± 0.06	X4	15.03 ± 0.03	0.23 ± 0.01
B9	13.62 ± 0.09	0.61 ± 0.01	X3	14.35 ± 0.14	0.37 ± 0.03

B)



C)

	Ng strains	*Nm* strains
Stase Activity	A4>F62>B25> FA1090>B23> B9	X3>X5>MC58 >C1>X8>X4
lst transcript levels	F62>A4>B23> B25>FA1090> B9	X3>X4>C1> X5>MC58=X8

FIG. 3. Real-time PCR analysis of *lst* expression in *N. gonorrhoeae* (*Ng*) and *N. meningitidis* (Nm). (A) Real-time PCR was repeated on 3 days with similar results. The table contains ΔC_T values representing the amount of *lst* sample RNA normalized to the endogenous reference, 16S rRNA. As described in Materials and Methods, relative RNA levels were determined using the ΔC_T value for *N. gonorrhoeae* F62 as a baseline for comparison. (B) Graphical representation of RNA difference (fold) given in the table. (C) Comparison of Stase activity and *lst* transcript levels in *N. gonorrhoeae* and *N. meningitidis*.

4). The difference was magnified when 5′*lst* fusions were expressed in *E. coli* in a low-copy plasmid (data not shown). *N. gonorrhoeae* 5′*lst*::*lacZ* fusions created from strains that possessed single 30-bp and 13-bp elements exhibited β-Gal activity comparable to that in strains containing these elements as tandem repeats (data not shown). All 5′*lst*::*lacZ* fusions of *N. gonorrhoeae* strains (A4, B9, B23, B25, and FA1090), irrespective of their Stase activities, have high β-Gal fusion activity levels similar to 5′*lst*::*lacZ* fusions of *N. gonorrhoeae* F62 (*N. gonorrhoeae* = 118 ± 12 Miller units). Similarly, all 5′*lst*::*lacZ* fusions of *N. meningitidis* strains (C1, X3, X4, X5, and X8) have low β-Gal activity levels comparable to 5′*lst*::*lacZ* fusions of *N. meningitidis* MC58 (*N. meningitidis* = 25 ± 3 Miller units). GenBank accession numbers for the 5′*lst* of six *N. gonorrhoeae* and *N. meningitidis* strains run from DQ375987 to DQ375998. Although the results of this study did not rule out a role for host-specific *trans* factors in modulating 5′*lst* promoter activity, the host independence and the similar high and low expression of 5′*lst* β-Gal fusion levels of *N. gonorrhoeae* and *N. meningitidis* suggest that species-specific sequence differences in the 5′*lst* are important for the differential 5′*lst* promoter activity between *N. gonorrhoeae* and *N. meningitidis*.

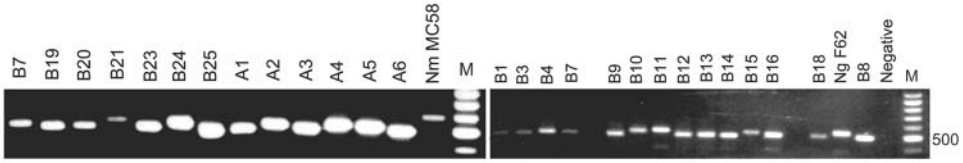
***N. gonorrhoeae lst* is transcribed from a σ^{70} promoter.** *lacZ* reporter studies showed that the 5′*lst* regions of *N. gonorrhoeae* and *N. meningitidis* have different promoter strengths as indicated above. The size of *N. gonorrhoeae* and *N. meningitidis lst* RNA in Northern blots indicates that *lst* transcription starts at identical or similar sites in both species. To examine this possibility, primer extension analysis, RPAs, reverse transcription-PCR (RT-PCR), and mutational analyses were performed with *N. gonorrhoeae* and *N. meningitidis* strains to identify the *lst* promoter(s).

Primer extension analysis of total RNA isolated from *N. gonorrhoeae* F62 with primer PE1 (Table 1) produced a major band starting at an adenine residue 74 bp upstream of ATG and 6 bp downstream of the −10 sequence of the putative sigma 70 promoter (Fig. 6A and C). An equivalent band was also evident using total RNA isolated from *E. coli* harboring 5′*lst*::*lacZ* fusions using a *lacZ*-specific primer (data not shown).

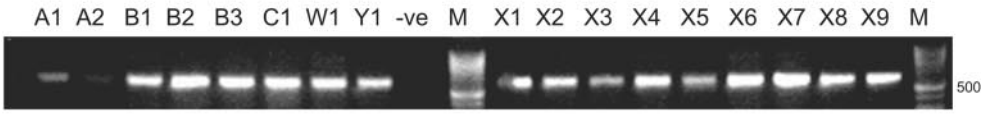
To confirm our primer extension results, we performed RPA analysis of different concentrations of total RNA isolated from *N. gonorrhoeae* strains F62 and FA1090. A protected probe fragment size of 65 bp was obtained from reaction mixtures containing total RNA from either *N. gonorrhoeae* F62 or *N. gonorrhoeae* FA1090 and was consistent with the transcriptional start site obtained from primer extension analysis (Fig. 6B): i.e., 74 bp upstream of *lst* ATG. Probe protection was

A

PCR amplification of 5' *lst* region of 28 *Ng* strains



PCR amplification of 5' *lst* region of 17 *Nm* strains



B

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                                  Nm MC58  GGTTTTGACATCAATATCGG 20
                                  Ng F62   GGTTTTGACATCAATATCGG

Nm MC58  CGTGGCGGGCAAAAGCCTGCACAATCGGCAGCAGCGATTGGGTCGCCAGC 70
Ng F62   CGTGGCGGGCAAAAGCCTGCACAATCGGCAGCAGCGATTGGGTCGCCAGC
                                                  <-- icd
Nm MC58  GCGGGTGCTTCGTCGGTATGGGTATAAACAATGGTGGATTTTTGAGTCAT 120
Ng F62   GCGGGTGCTTCGTCGGTATGGGTATAAACAATGGTGGATTTTTGAGTCAT
                                                     IC
Nm MC58  AGGATTATTCTCTTGTAGGTTGGTTTTTTCTTTTGGAACACATTGCGCGG 170
Ng F62   AGGATTATTCTCTTGTAGGTTGGTTTTTTCTTTTGAAACACATTGGGCGG
                                                 *       *

Nm MC58  GGAATGTGCGTGGCTATTATGGCATATTTTGGCGGCTTTGTTCGCGCTTT 220
Ng F62   GGAATGTGCGCGGCTATTATGGCATATTTTGGCGGCTTTGTTCGCGCTTT
               *

Nm MC58  GTTCGATCTTGGCGTGTTTG[------------30bp------------] 270
Ng F62   GTTCGATCTTGGCGTGTTTGAACGCGGCGGCGGGAAAGGAAAGGGGAAAT

         [------------30bp------------]
Nm MC58  AACGCGGCGGCGTGAAAGGAAGGGGGAAATGGTTTTCCCGCGTTTGGCGG 320
Ng F62   AACGCGGCGGCGGGAAAGGAAGGGGGAAATGGCTTTCCCGCGTTTGCCGG
                 *                   *             *

Nm MC58  CGG--TCGGAGGTGCTGTGCCTGATGTGCGGCGGCATATTTTCGGTGAAA 370
Ng F62   CGGCGTCGGCGGCGTTGTGCCTGATGTGCGGCGGCACATTTTCGGTAAAA
              *  * *                  *         *

Nm MC58  TTGATTTTATAGTGGTTTAAATTTAAACCAGTACAGCGTTGCCTCGCCTT 420
Ng F62   TTGATTTTA[----------------------------------------

Nm MC58  GTCGTACTGCTTGTACTGTCTGCGGCTTCGTCGCCTTGTCCTGATTTAAA 470
Ng F62   ----------------------105bp-------------------------
                                    Nm lst tsp
Nm MC58  TTTAAACCACTATAATATTCGGTAACTGTCGGAATATCTGCTAAAATTCC 520
Ng F62   -------------]ATATTCGGCAACTGTCGGAATATCTGCTAAAATTCC
                          *
                       [----13bp---]
Nm MC58  GCATT[---12bp---]TTTCCGTCCCGGGACACTCGGGGCGTATGTTCA 570
Ng F62   GCATTTTCCGCACCGGGTTTCCGCACCGGGACACTCGGGGCGTATGTTCA
          Ng lst tsp      **
                                  lst -->
Nm MC58  ATTTGTCGGAATGGAGTTTT-AGGGATATGGCTTGAAAAAGGCTTGTTTG--->
Ng F62   ATTTGTCGGAATGGAGTTTTTAGGGATATGGCTTGAAAAAGGCTTGTTTG--->
                                SD    IC
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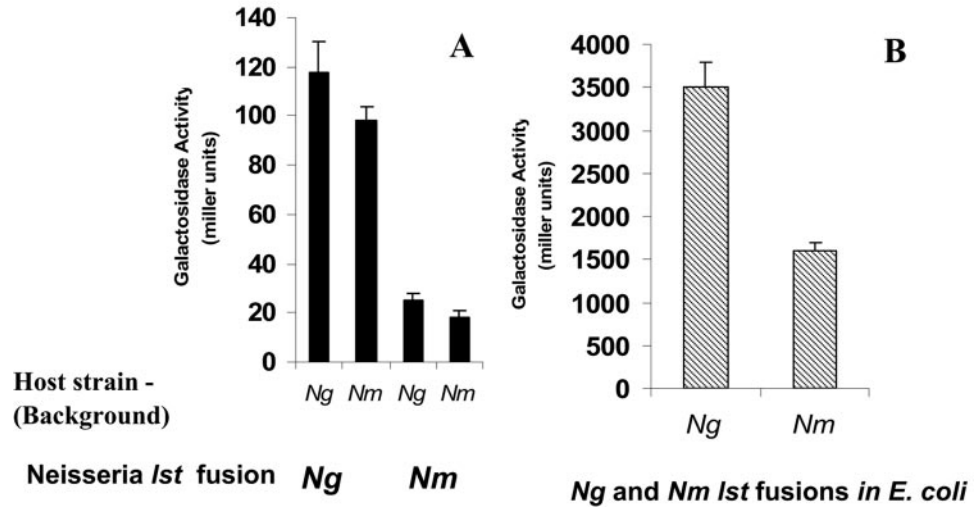

FIG. 5. β-Gal activity of *N. gonorrhoeae* (*Ng*) F62 and *N. meningitidis* (*Nm*) MC58 5′*lst* reporter fusions. *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ⊄3 5′*lst*::*lac*Z fusions were constructed with primers FusBam F1 and FusBam R1 (Table 1) as described in Materials and Methods. β-Gal activity of each fusion was determined in *N. gonorrhoeae* and *N. meningitidis* as chromosomal integrates (A) and in *E. coli* XL1-Blue as plasmid fusions (B).

linear with increasing concentrations of total RNA and specific for *lst*, since protection was absent when yeast total RNA was used in the reaction (Fig. 6B).

Finally, mutation of the sigma 70 promoter, from TAAAAT to ATTTTA in an *N. gonorrhoeae* 5′*lst*::*lacZ* construct abrogated β-Gal activity, confirming the function of this region as the only active promoter for *lst* in *N. gonorrhoeae* (*N. gonorrhoeae* = 118 ± 12 Miller units, *N. gonorrhoeae* TATA mutant = 5 ± 3 Miller units).

***N. meningitidis lst* is transcribed from a σ^{70} promoter present at the 3′ end of a CREE.** CREEs are repetitive elements scattered randomly in the pathogenic *Neisseria* spp. (2, 4, 13). CREEs act as promoters for neisserial genes like *uvrB* (1), *drg* (3) and IS*1106*Tip (24), or they serve as an RNA processing elements (5, 16, 23). All 17 *N. meningitidis* clinical isolates studied have the 105-bp CREE upstream proximal to the sigma 70 promoter that would drive *lst* transcription in *N. gonorrhoeae* (Fig. 4B). We investigated the possible modulatory role of the CREE element in *lst* transcription in *N. meningitidis*.

RT-PCR was used to map the *lst* promoter region in *N. meningitidis*. We asked whether amplification products could be obtained using a common reverse primer (C_{rev}) in conjunction with oligonucleotide primers P1 through P7, spaced 30 to 60 bp apart (Table 1), that anneal upstream and downstream of the CREE (Fig. 7B). Amplification products were obtained only with primers that annealed downstream of the CREE (P4 to P7), suggesting that the promoter for *lst* transcription does not lie upstream of the CREE. An amplification product was obtained with primer P4, which anneals downstream of the CREE but upstream of the position of the *N. gonorrhoeae lst* promoter, suggesting that the 3′ end of the CREE is part of a promoter for *lst* in meningococci (Fig. 7B). The identities of the amplified products were confirmed by sequencing (data not shown).

It was evident from RT-PCR results that the 3′ end of CREE is part of a promoter but the transcriptional status of a second *N. meningitidis* promoter (the one used in *N. gonorrhoeae*) was unclear. To investigate the possibility that *N. meningitidis* strains have two *lst* promoters, primer extension analysis were performed on total RNA isolated from three *N. meningitidis* strains (MC58, X3, and X4). The extension reactions using primer PE2 (Table 1) produced a single major product that mapped to a thymine residue 92 bp upstream of ATG and 7 bp downstream of the −10 sequence of the putative sigma 70 promoter. No extension product was obtained that corresponded to the *N. gonorrhoeae lst* promoter (Fig. 7A). In addition, the extension product was more intense for X3 than MC58, in agreement with the amount of *lst* transcript found in these strains, and was specific for *lst*, since no band was formed when yeast RNA was used in the primer extension reaction. A single extension product was generated using a second primer, PE3 (Table 1), which was consistent with these results (data not shown). Analogous to the sigma 70 promoter 5′ of *uvrB* (1), the *N. meningitidis lst* sigma 70 promoter was also created by the insertion of the CREE (Fig. 4B).

FIG. 4. Analysis and comparison of sequences upstream of *N. gonorrhoeae* (*Ng*) and *N. meningitidis* (*Nm*) *lst*. (A) The 5′*lst* upstream regions of 28 *N. gonorrhoeae* strains and 17 *N. meningitidis* strains were PCR amplified using primer pair FusBam F1 and FusBam R1, and the amplicons were resolved in a 1.5% agarose gel. (B) Nucleotide sequence comparison of the 5′*lst* regions of *N. meningitidis* MC58 and *N. gonorrhoeae* F62: DNA fragments absent or duplicated are indicated by dashes within or above the sequence, respectively. Individual base differences are indicated by asterisks. The −10 and −35 sequences are in boldface and underlined. The putative Shine-Dalgarno (SD) sequence for *lst*, and the initiation codons (IC) for *lst* and *icd* (isocitrate dehydrogenase) are italicized and in boldface. The transcriptional start sites (tsp) downstream of the −10 sites are in boldface, italic, and indicated with small arrows.

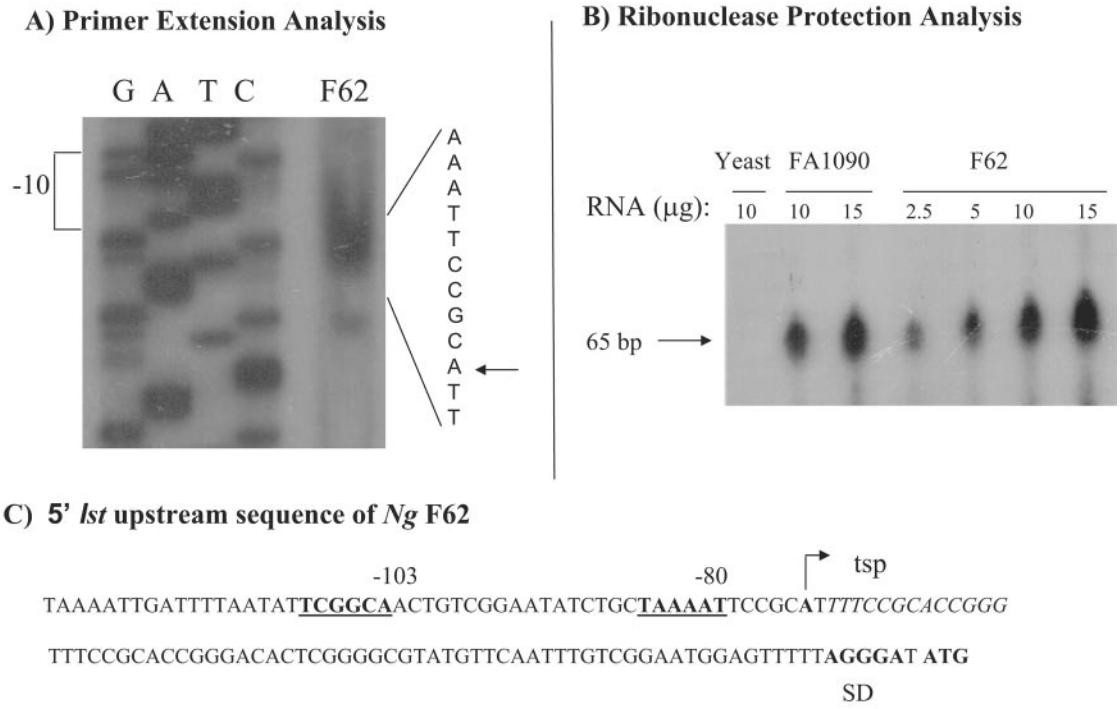

FIG. 6. Analysis of the *N. gonorrhoeae lst* promoter. (A) Primer extension analysis. Total RNA (20 μg) was isolated from *N. gonorrhoeae* strain F62, and primer extension analysis was performed using primer PE1. The extension product along with a sequencing reaction with the same primer was resolved by 6% PAGE with 8 M urea. The arrow points to the transcriptional start site. (B) RNase protection assay. ^{32}P-labeled RNA probe was hybridized with total RNA from *N. gonorrhoeae* strains F62 and FA1090 or yeast RNA (negative control) and digested with a 1:100 dilution of RNase A/T_1 mix. The protected fragments were resolved along with a DNA size marker by 6% PAGE with 8 M urea. (C) The 5′*lst* upstream sequence of *N. gonorrhoeae* F62. The −10 and −35 sequences of the promoter are boldface and underlined, and their positions relative to the translational start site are given. The transcriptional start site (tsp) is marked by an arrow; the putative Shine-Dalgarno (SD) sequence and translational start site are in boldface.

Taken together, these data show that *lst* transcription in *N. meningitidis* proceeds from a single sigma 70 promoter present at the 3′ end of the CREE and that the *N. gonorrhoeae lst* promoter is inactive in *N. meningitidis*.

Evidence of divergent transcription from CREE ends. The ends of CREE are complementary to each other, and DNA sequence analyses predict there may be divergent promoters directing transcription from both ends of CREEs (Fig. 8A). The 3′ terminus of the CREE found on the *lst* coding strand is highly similar in sequence to the 3′ terminus of CREE found on the isocitrate dehydrogenase (*icd*) coding strand (Fig. 8B). Since the 3′ terminus of the CREE on the *lst* coding strand possesses an active promoter, we speculated that the highly similar 3′ terminus of the CREE found on the *icd* coding strand could act as a promoter to transcribe *icd*. We used RT-PCR to investigate this possibility. Similar to mapping of the *lst* promoter, we used oligonucleotide primer pairs that anneal upstream and downstream from both the ends of CREE (i.e., with reference to the *icd* coding strand and the *lst* coding strand) (Fig. 8C). Amplified products were obtained only with primers that annealed downstream of the CREE with respect to each coding strand (Fig. 8C). The RT-PCR results suggest that the ends of the CREE possess promoters for the divergent transcription of *icd* and *lst*.

DISCUSSION

N. gonorrhoeae and *N. meningitidis* have similar and distinct systems for evading human immune factors during infection. Expression of the LOS-specific Lst is required in vitro for serum-sensitive strains of *N. gonorrhoeae* to resist complement-mediated killing (6, 7, 9, 34), while surface sialylation may enhance the resistance of *N. meningitidis* during periods of infection when capsule is not expressed (32).

Natural variations occur in the degree of LOS sialylation in different strains of pathogenic *Neisseria* spp. (8, 15, 18, 28). The factors that could affect the degree of LOS sialylation include the availability of phase-variable terminal galactose sialylation targets (29, 30), the amount of available CMP-NANA (21, 34), and inherent specific activity or regulated expression of Lst in these strains. In this study, we examined the regulation of *lst* gene transcription. We observed that extracts of *N. gonorrhoeae* F62 contain 5-fold more Stase activity than *N. meningitidis* MC58 ⊄3 and that among clinical neisseria strains that exhibit variable Stase activities, *N. gonorrhoeae* expresses about 2.2-fold more Stase activity than strains of *N. meningitidis*. Since the *lst* gene is 95 to 98% homologous between most strains (unpublished observation), we hypothesized that differences in transcriptional control may be the determinant for variations in Stase activities between *N. gonorrhoeae* and *N. meningitidis.* Analysis of transcript levels in *N. gonorrhoeae* and *N. meningitidis* by Northern blot analysis and real-time PCR showed that *N. gonorrhoeae* strains express more *lst* transcript than strains of *N. meningitidis* and correlated with Stase activity observed in the two species. However, there was not a direct correlation between levels of *lst* mRNA and Stase activity among strains of each species. The possible reasons for poor

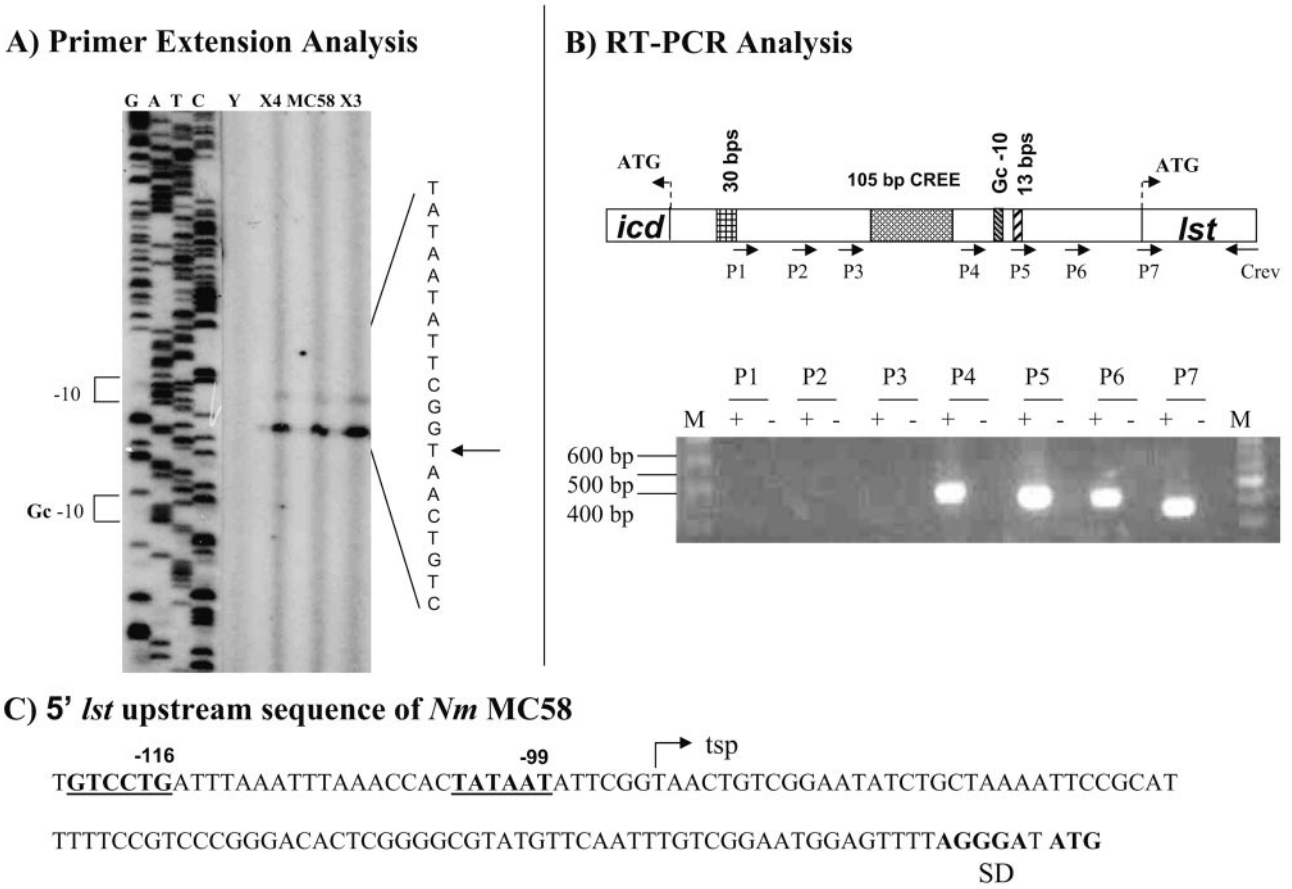

FIG. 7. Analysis of the *N. meningitidis lst* promoter. (A) Primer extension analysis. Total RNA (20 μg) was isolated from yeast (negative control) and *N. meningitidis* strains X4, MC58, and X3, and primer extension analyses were performed using primer PE2 (Table 1). The extension products along with a sequencing reaction with the same primer were resolved by 6% PAGE with 8 M urea. The arrow points to the transcriptional start site. The *N. meningitidis* −10 element is indicated as "−10," and the inactive gonococcal −10 element is indicated as "Gc −10." (B) RT-PCR analysis. *N. meningitidis* MC58 total RNA (20 μg) was reverse transcribed with and without the reverse transcriptase and was used in PCRs as template with seven forward primers (P1 to P7) and a common reverse primer (C_{rev}). The amplified products were resolved in a 1.5% agarose gel along with DNA size markers. A diagram of the intergenic region between *lst* and *icd* is shown (not drawn to scale). The 30-bp and 13-bp elements, the 105-bp CREE, and the gonococcal −10 element are indicated as shaded boxes. The opposing dotted arrows indicate the translational start sites of *lst* and *icd*. The small arrows pointing to the right denote the forward primers (P1 to P7) and their relative position where they anneal to the *lst* upstream region. (C) 5′*lst* upstream sequence of *N. meningitidis* MC58. The −10 and −35 sequences of the promoter are in boldface and underlined, and their positions relative to the translational start site are given. The transcriptional start site is marked by an arrow, and a putative Shine-Dalgarno (SD) sequence and translational start site are in boldface.

correlation observed between mRNA and Stase activities when comparing individual strains could be due to: (i) differences in inherent specific activity of Stases from different strains of *N. gonorrhoeae* or *N. meningitidis* due to point mutations, which we know exist from our sequencing efforts of the *lst* gene and recent data from others (11); (ii) Stase activity being affected by some unknown neisserial factor(s), perhaps, protein that binds either to Stase or LOS; or; (iii) the conformation of LOS and the interaction of enzyme (Stase) with its substrate (LOS) (33).

Real-time PCR analysis revealed that there was less than a 2-fold (1.6-fold) difference in *lst* transcript levels among the various *N. gonorrhoeae* strains examined, whereas transcript levels among *N. meningitidis* strains varied considerably; *lst* levels in *N. meningitidis* were as follows: strains MC58 ⊄3 and X8 < C1 and X4 (2.2 ± 0.2-fold more than MC58 ⊄3 and X8) < X3 (3.7 ± 0.3-fold more than MC58 ⊄3 and X8). It is possible that *N. meningitidis* X3 could have a mutation(s) or a host-specific factor(s) that enhances *lst* transcription. The observation that *lst* transcript levels in *N. meningitidis* strains vary while they are nearly unchanged in *N. gonorrhoeae* strains suggests that mechanisms for regulating this outer membrane protein are different between these two species. Another example of an outer membrane protein that is regulated differently between *N. gonorrhoeae* and *N. meningitidis* includes the *mtrCDE*-encoded efflux pump. Expression of the *mtrCDE* operon in *N. gonorrhoeae* is induced by the AraC-like protein, MtrA (22), and negatively regulated by MtrR (25). In *N. meningitidis*, the *mtr* system is subject to transcriptional regulation by integration host factor (IHF) and posttranscriptional regulation by cleavage in the inverted repeat of the Correia element (23) and not subjected to MtrR or MtrA regulatory schemes.

Sequence comparison of the 5′*lst* regions of *N. gonorrhoeae* and *N. meningitidis* strains reveals striking species-specific differences. The 5′*lst* region of *N. gonorrhoeae* varies, whereas the

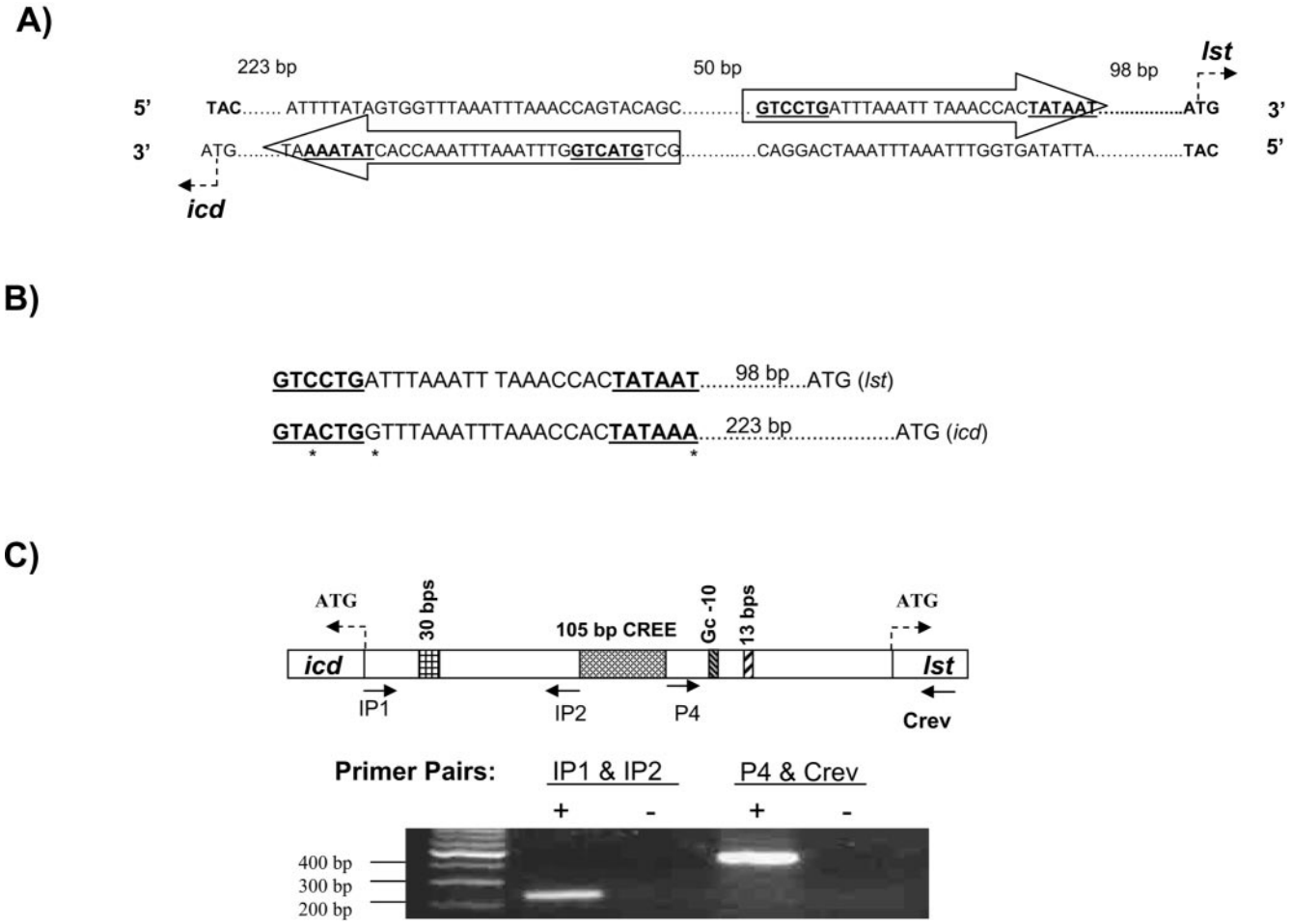

FIG. 8. Evidence of divergent transcription from CREE ends. (A) Diagram of the intergenic region between *lst* and *icd* (not drawn to scale). The small opposing dotted arrows indicate the translational start sites of *lst* and *icd*. The number of bases denoted by dots that link sequences are given above the dots. The large open arrows pointing in the opposite directions represent inverted repeats (IRs) that are highly similar. The sequence comparison of them is shown in panel B, and individual base differences in the putative promoter regions are indicated by asterisks. (C) *N. meningitidis* MC58 total RNA (20 μg) was reverse transcribed with (+) and without (−) the reverse transcriptase and was used in PCRs as a template with two sets of primer pairs (IP1 and IP2 and P4 and C_{rev}). The amplified products were resolved in 1.5% agarose gel along with DNA size markers. A diagram of the intergenic region between *lst* and *icd* is shown (not drawn to scale). The 30-bp, 13-bp, and 105-bp CREEs and the gonococcal −10 elements are indicated as hatched boxes. The opposing dotted arrows indicate the translational start sites of *lst* and *icd*. The two sets of primer pairs (IP1 and IP2 and P4 and Crev) are denoted as small arrows, and the relative positions where they anneal to the *lst* upstream and coding region are shown.

N. meningitidis 5′*lst* region remains relatively conserved. Some *N. gonorrhoeae* 5′*lst* regions contained a 13-bp and a 30-bp tandem repeat element that exists as single copies in other strains of *N. gonorrhoeae* and all strains of *N. meningitidis*. Most importantly, the 5′*lst* regions of all *N. meningitidis* strains have 105-bp CREEs that are absent in the 5′*lst* regions of all *N. gonorrhoeae* strains examined. To determine how these sequence differences might contribute to differential transcript levels in *Neisseria* spp., we created *N. gonorrhoeae* and *N. meningitidis* translational 5′*lst*::*lacZ* fusions and expressed them in the chromosomes of *N. gonorrhoeae* F62 and *N. meningitidis* MC58 ℄3. The 5′*lst* region of *N. gonorrhoeae* was fivefold more active than that of *N. meningitidis* MC58 whether expressed in *N. gonorrhoeae* or *N. meningitidis*. Furthermore, this pattern of expression was observed in *E. coli* indicating the difference in promoter activity was predominantly a consequence of some intrinsic property of the DNA and was not caused by regulatory factors present in the host per se.

It is evident from the *lacZ* reporter studies that the 5′*lst* regions of *N. gonorrhoeae* and *N. meningitidis* have different promoter strengths. Promoter-mapping studies showed that *N. gonorrhoeae* and *N. meningitidis* use different promoters to transcribe *lst* under the growth conditions used in this study. In *N. gonorrhoeae*, a sigma 70 promoter 80 bp upstream of the translational start site is used to transcribe *lst*. In *N. meningitidis*, this promoter is inactive and is replaced by a sigma 70 promoter present at the 3′ terminus of a 105-bp CREE 99 bp upstream of the translational start site. The CREEs act as promoters for neisserial genes like *uvrB* (1), *drg* (3), and IS*1106*Tip (24). The promoter found at the 3′ terminus of the CREE of *uvrB* (1) is highly homologous to the promoter we mapped upstream of *lst* in *N. meningitidis*.

drg is transcribed only in strains that have CREE inserted in their upstream region and is silent in strains that do not have the CREE in their upstream region. Thus, regulation of *drg* in *Neisseria* spp. occurs by promoter insertion (3). Regulation of *lst* between the pathogenic *Neisseria* spp. occurs by promoter replacement. In *N. gonorrhoeae*, *lst* is transcribed from a strong sigma 70 promoter. In *N. meningitidis*, the strong gonococcal promoter is silent and is replaced by a weaker sigma 70 promoter formed by CREE insertion. In addition, for the first time, we provide evidence for the CREEs to act as divergent promoters directing transcription from both ends of inverted repeats. Overall, promoter-mapping studies and LacZ reporter studies show that *lst* transcription in *N. gonorrhoeae* and *N. meningitidis* occurs from different promoters with different strengths. We conclude that the differential Stase expression between *N. gonorrhoeae* and *N. meningitidis* is due at least in part to differential *lst* gene transcription.

The biological significance of the observation that *N. gonorrhoeae* strains transcribe more *lst* than *N. meningitidis* strains remains to be seen. One possible explanation could be that dependence of *N. gonorrhoeae* strains on sialylation for protection against complement-mediated killing would be higher than in *N. meningitidis* strains which have capsule for protection against complement-mediated killing. Sialylation of the *N. gonorrhoeae* lactoneotetraose (Lnt) epitope results in uniform enhanced resistance to normal human serum in serum-sensitive strains; however, sialylation of *N. meningitidis* Lnt does not result in enhanced serum resistance in all strains (Madico et al., abstr., p. 230, 14th Int. Pathogenic Neisseria Conf., Milwaukee, Wis., September 2004). Different *lst* transcript levels observed in *N. gonorrhoeae* and *N. meningitidis* and variations observed in *lst* transcripts among *N. meningitidis* strains may explain the contrasting behavior of Lnt sialylation in *N. gonorrhoeae* and *N. meningitidis*. Future experiments will address the probable role of differential sialylation of strains in protection against complement-mediated killing and association with epithelial cells. Posttranscriptional regulatory mechanisms and reasons for loss of correlation between Stase activity and *lst* expression among strains in each species will be examined.

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REFERENCES

1. **Black, C. G., J. A. M. Fyfe, and J. K. Davies.** 1995. A promoter associated with the neisserial repeat can be used to transcribe the *uvrB* gene from *Neisseria gonorrhoeae*. J. Bacteriol. **177:**1952–1958.
2. **Buisine, N., C. M. Tang, and R. Chalmers.** 2002. Transposon-like Correia elements: structure, distribution and genetic exchange between pathogenic Neisseria sp. FEBS Lett. **522:**52–58.
3. **Cantalupo, G., C. Bucci, P. Salvatore, C. Pagliarulo, V. Roberti, A. Lavitola, C. B. Bruni, and P. Alifano.** 2001. Evolution and function of the neisserial dam-replacing gene. FEBS Lett. **495:**178–183.
4. **Correia, F. F., S. Inouye, and M. Inouye.** 1986. A 26-base-pair repetitive sequence specific for *Neisseria gonorrhoeae* and *Neisseria meningitidis* genomic DNA. J. Bacteriol. **167:**1009–1015.
5. **De Gregorio, E., C. Abrescia, M. S. Carlomagno, and P. P. Di Nocera.** 2002. The abundant class of nemis repeats provides RNA substrates for ribonuclease III in Neisseriae. Biochim. Biophys. Acta **1576:**39–44.
6. **de la Paz, H., S. J. Cooke, and J. E. Heckels.** 1995. Effect of sialylation of lipopolysaccharide of Neisseria gonorrhoeae on recognition and complement-mediated killing by monoclonal antibodies directed against different outer-membrane antigens. Microbiology **141:**913–920.
7. **Elkins, C., N. H. Carbonetti, V. A. Varela, D. Stirewalt, D. G. Klapper, and P. F. Sparling.** 1992. Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. Mol. Microbiol. **6:**2617–2628.
8. **Estabrook, M. M., J. M. Griffiss, and G. A. Jarvis.** 1997. Sialylation of *Neisseria meningitidis* lipooligosaccharide inhibits serum bactericidal activity by masking lacto-*N*-neotetraose. Infect. Immun. **65:**4436–4444.
9. **Frangipane, J. V., and R. F. Rest.** 1993. Anaerobic growth and cytidine 5′-monophospho-*N*-acetylneuraminic acid act synergistically to induce high-level serum resistance in *Neisseria gonorrhoeae*. Infect. Immun. **61:**1657–1666.
10. **Gilbert, M., D. C. Watson, A. M. Cunningham, M. P. Jennings, N. M. Young, and W. W. Wakarchuk.** 1996. Cloning of the lipooligosaccharide alpha-2,3-sialyltransferase from the bacterial pathogens Neisseria meningitidis and Neisseria gonorrhoeae. J. Biol. Chem. **271:**28271–28276.
11. **Gulati, S., A. Cox, L. A. Lewis, F. St. Michael, J. Li, R. Boden, S. Ram, and P. A. Rice.** 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.
12. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and D. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.
13. **Liu, S. V., N. J. Saunders, A. Jeffries, and R. F. Rest.** 2002. Genome analysis and strain comparison of Correia repeats and Correia repeat-enclosed elements in pathogenic *Neisseria*. J. Bacteriol. **184:**6163–6173.
14. **Mandrell, R. E., and M. A. Apicella.** 1993. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. Immunobiology **187:**382–402.
15. **Mandrell, R. E., H. Smith, G. A. Jarvis, J. M. Griffiss, and J. A. Cole.** 1993. Detection and some properties of the sialyltransferase implicated in the sialylation of lipopolysaccharide of Neisseria gonorrhoeae. Microb. Pathog. **14:**307–313.
16. **Mazzone, M., E. De Gregorio, A. Lavitola, C. Pagliarulo, P. Alifano, and P. P. Di Nocera.** 2001. Whole-genome organization and functional properties of miniature DNA insertion sequences conserved in pathogenic Neisseriae. Gene **278:**211–222.
17. **McGee, D. J., and R. F. Rest.** 1996. Regulation of gonococcal sialyltransferase, lipooligosaccharide, and serum resistance by glucose, pyruvate, and lactate. Infect. Immun. **64:**4630–4637.
18. **McQuillen, D. P., S. Gulati, S. Ram, A. K. Turner, D. B. Jani, T. C. Heeren, and P. A. Rice.** 1999. Complement processing and immunoglobulin binding to Neisseria gonorrhoeae determined in vitro simulates in vivo effects. J. Infect. Dis. **179:**124–135.
19. **Ram, S., F. G. Mackinnon, S. Gulati, D. P. McQuillen, U. Vogel, M. Frosch, C. Elkins, H. K. Guttormsen, L. M. Wetzler, M. Oppermann, M. K. Pangburn, and P. A. Rice.** 1999. The contrasting mechanisms of serum resistance of Neisseria gonorrhoeae and group B Neisseria meningitidis. Mol. Immunol. **36:**915–928.
20. **Ram, S., A. K. Sharma, S. D. Simpson, S. Gulati, D. P. McQuillen, M. K. Pangburn, and P. A. Rice.** 1998. A novel sialic acid binding site on factor H mediates serum resistance of sialylated Neisseria gonorrhoeae. J. Exp. Med. **187:**743–752.
21. **Rest, R. F., and R. E. Mandrell.** 1995. Neisseria sialytransferases and their role in pathogenesis. Microb. Pathog. **19:**379–390.
22. **Rouquette, C., J. B. Harmon, and W. M. Shafer.** 1999. Induction of the mtrCDE-encoded efflux pump system of Neisseria gonorrhoeae requires MtrA, an AraC-like protein. Mol. Microbiol. **33:**651–658.
23. **Rouquette-Loughlin, C. E., J. T. Balthazar, S. A. Hill, and W. M. Shafer.** 2004. Modulation of the mtrCDE-encoded efflux pump gene complex of Neisseria meningitidis due to a Correia element insertion sequence. Mol. Microbiol. **54:**731–741.
24. **Salvatore, P., C. Pagliarulo, R. Colicchio, P. Zecca, G. Cantalupo, M. Tredici, A. Lavitola, C. Bucci, C. B. Bruni, and P. Alifano.** 2001. Identification, characterization, and variable expression of a naturally occurring inhibitor protein of IS*1106* transposase in clinical isolates of *Neisseria meningitidis*. Infect. Immun. **69:**7425–7436.
25. **Shafer, W. M., J. T. Balthazar, K. E. Hagman, and S. A. Morse.** 1995. Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of Neisseria gonorrhoeae that are resistant to faecal lipids. Microbiology **141:**907–911.
26. **Shell, D. M., L. Chiles, R. C. Judd, S. Seal, and R. F. Rest.** 2002. The

Neisseria lipooligosaccharide-specific α-2,3-sialyltransferase is a surface-exposed outer membrane protein. Infect. Immun. **70:**3744–3751.
27. **Silver, L. E., and V. L. Clark.** 1995. Construction of a translational lacZ fusion system to study gene regulation in Neisseria gonorrhoeae. Gene **166:**101–104.
28. **Tsai, C.-M., and C. I. Civin.** 1991. Eight lipooligosaccharides of *Neisseria meningitidis* react with a monoclonal antibody which binds lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Infect. Immun. **59:**3604–3609.
29. **Tsai, C. M., G. Kao, and P. Zhu.** 2002. Influence of the length of the lipooligosaccharide α chain on its sialylation in *Neisseria meningitidis*. Infect. Immun. **70:**407–411.
30. **van Putten, J. P.** 1993. Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of Neisseria gonorrhoeae. EMBO J. **12:**4043–4051.
31. **Vogel, U., H. Claus, G. Heinze, and M. Frosch.** 1999. Role of lipopolysaccharide sialylation in serum resistance of serogroup B and C meningococcal disease isolates. Infect. Immun. **67:**954–957.
32. **Vogel, U., S. Hammerschmidt, and M. Frosch.** 1996. Sialic acids of both the capsule and the sialylated lipooligosaccharide of Neisseria meningitidis serogroup B are prerequisites for virulence of meningococci in the infant rat. Med. Microbiol. Immunol. **185:**81–87.
33. **Wakarchuk, W. W., D. Watson, F. St. Michael, J. Li, Y. Wu, J. R. Brisson, N. M. Young, and M. Gilbert.** 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.
34. **Wetzler, L. M., K. Barry, M. S. Blake, and E. C. Gotschlich.** 1992. Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. Infect. Immun. **60:**39–43.

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