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Phase-Variable Expression of *lptA* Modulates the Resistance of *Neisseria gonorrhoeae* to Cationic Antimicrobial Peptides

Justin L. Kandler,[a] Sandeep J. Joseph,[b] Jacqueline T. Balthazar,[a] Vijaya Dhulipala,[a] Timothy D. Read,[b] Ann E. Jerse,[c] William M. Shafer[a,d]

Departments of Microbiology and Immunology[a] and Medicine[b], Emory University School of Medicine, Atlanta, Georgia, USA; Department of Microbiology and Immunology, F. Edward Herbert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA[c]; Laboratories of Microbial Pathogenesis, Veterans Affairs Medical Center, Decatur, Georgia, USA[d]

Phosphoethanolamine (PEA) decoration of lipid A produced by *Neisseria gonorrhoeae* has been linked to bacterial resistance to cationic antimicrobial peptides/proteins (CAMPs) and *in vivo* fitness during experimental infection. We now report that the *lptA* gene, which encodes the PEA transferase responsible for this decoration, is in an operon and that high-frequency mutation in a polynucleotide repeat within *lptA* can influence gonococcal resistance to CAMPs.

Neisseria gonorrhoeae is a strict human pathogen that has caused the sexually transmitted infection termed gonorrhea for thousands of years. Over the millennia, *N. gonorrhoeae* has developed multiple mechanisms to resist innate host defenses, including cationic antimicrobial peptides/proteins (CAMPs) produced by phagocytes and epithelial cells (1). Phosphoethanolamine (PEA) decoration of the lipid A possessed by *N. gonorrhoeae* and *N. meningitidis* has been shown to contribute to their resistance to CAMPs by a mechanism that likely involves a reduction in ionic interactions of CAMPs with the bacterial surface (1–6), resistance of *N. gonorrhoeae* to complement-mediated killing by normal human serum (3, 4), *N. gonorrhoeae* fitness during experimental infection in mice and humans (5, 7), and the proinflammatory potential of *N. gonorrhoeae* (7, 8). Most commensal *Neisseria* do not encode *lptA* (8), but *N. gonorrhoeae* and *N. meningitidis* (2, 3, 8) typically contain *lptA* and produce multiple isoforms of lipid A that differ in PEA decoration at the 4′ and/or 1 position, though the basis of these isoforms has not been fully defined. We now provide evidence that gonococcal *lptA* is within an operon and that *N. gonorrhoeae* resistance to a model CAMP (polymyxin B; PMB) is modulated by high-frequency mutation due to a phase-variable (PV) polynucleotide stretch in the *lptA* coding sequence.

Organization and expression of the *lptA* locus in *N. gonorrhoeae*. Bioinformatic analysis of the DNA sequence of the *N. gonorrhoeae* FA 1090 chromosome (http://www.genome.ou.edu/gono.html) suggested that *lptA* is transcriptionally linked to two upstream genes (*serC* and a hypothetical gene annotated as NGO1282) and a downstream gene (*nfnB*) (Fig. 1). This hypothesis was confirmed by results from reverse transcription-PCR (RT-PCR) experiments (Fig. 2A) that demonstrated transcriptional linkage of *lptA* with the *serC*, hypothetical, and *nfnB* genes; details of the experimental procedures and a list of oligonucleotide primers are provided in the legends of Fig. 1 and 2 and in Table 1, respectively. However, primer extension analysis of total *N. gonorrhoeae* RNA performed as described previously (9) identified a transcriptional start point (TSP) positioned 61 nucleotides (nt) upstream of the *lptA* translational start codon and four nt downstream of near-consensus −10 and −35 elements (Fig. 1 and 2B). Thus, we tentatively conclude that *lptA* expression in *N. gonorrhoeae* can be initiated by two promoters upstream of *serC* and *lptA*, respectively. The mechanisms that control use of these promoters are now under investigation.

Analysis of the online FA 1090 genome sequence indicated that the *lptA* coding sequence contains a polynucleotide tract consist-

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Address correspondence to William M. Shafer, wshafer@emory.edu.

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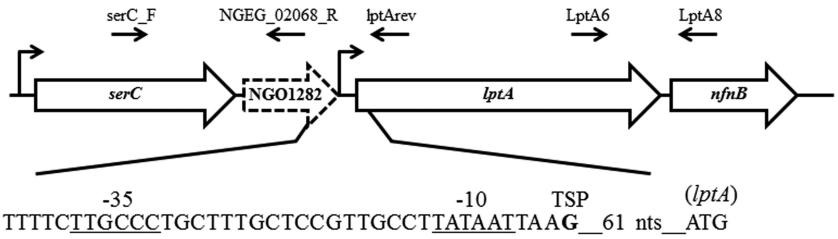

FIG 1 Genetic context of *lptA* in *N. gonorrhoeae* FA19. The 3.8-kb region of the FA19 genome shown corresponds to nucleotides 1236150 to 1232381 in *N. gonorrhoeae* FA 1090 (http://www.genome.ou.edu/gono.html and GenBank accession number AE004969.1). *serC* encodes a putative phosphoserine aminotransferase, NGO1282 encodes a hypothetical gene, *lptA* encodes a lipid A phosphoethanolamine transferase, and *nfnB* encodes a putative nitroreductase. The locations of the *serC* (undefined) and *lptA* (defined in the Fig. 2B legend) promoters are depicted with bent arrows. The *lptA* transcriptional start point, −10, and −35 promoter elements are shown below the illustration. The approximate sites of annealing for oligonucleotides used in RT-PCR experiments (Table 1) are shown with arrows.

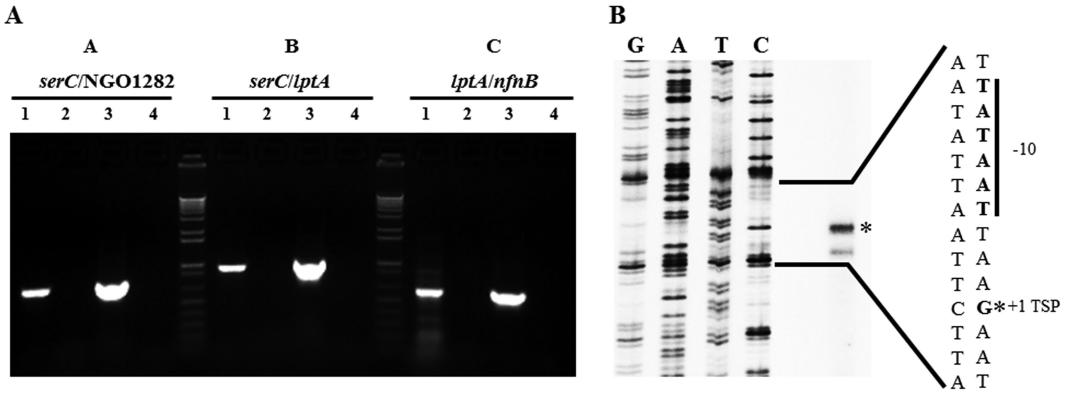

FIG 2 Transcription of the *lptA* coding sequence. (A) Transcriptional linkage between *serC*, NGO1282, *lptA*, and *nfnB*. All RT-PCRs were performed on purified RNA harvested (RNeasy minikit; Qiagen) from a log-phase culture of strain FA19 grown as described previously (9). First-strand cDNA was generated using SuperScript II reverse transcriptase (Invitrogen) and a gene-specific reverse primer (LptA8) that binds in the *nfnB* gene and primes elongation of a single-stranded cDNA toward *serC*. PCR was then performed to confirm transcriptional linkage between pairs of genes. Sections A, B, and C of the gel are grouped by forward- and reverse-primer locations and separated by 1-kb PLUS DNA ladders (Invitrogen). Lane 1, FA19 cDNA; lane 2, −RT negative control (RT omitted); lane 3, FA19 genomic DNA positive control; lane 4, no-template negative control. Section A, "*serC*" = serC_F; "NGO1282" = NGEG_02068_R. Section B, "*serC*" = serC_F; "*lptA*" = lptArev. Section C, "*lptA*" = LptA6; "*nfnB*" = LptA8. (B) Primer extension of the *lptA* transcript. Primer extension analysis was performed as described previously (9) using 20 μg of FA19 total RNA as the template and a radioactively ([γ-^{32}P]ATP) labeled reverse primer (LptA7_R) that anneals 67 bp downstream of the *lptA* start codon. RNA was purified and cDNA generated as described for panel A. Radioactive single-stranded cDNA products were separated on a polyacrylamide gel alongside sequencing reactions that used the same reverse primer (LptA11 was the forward primer used for generation of the sequenced *lptA* promoter template). The TSP corresponds to the band labeled with an asterisk and is 4 bp downstream of a consensus σ^{70}-type −10 element. A second band appeared running approximately 4 to 5 nucleotides shorter than the proposed +1 TSP band and could be due to a degraded mRNA transcript.

ing of seven Ts (T-7), which would result in production of a truncated LptA enzyme due to a new translational stop codon (Fig. 3). However, our independent sequencing of a PCR product containing the *lptA* gene from FA 1090 as well from strain FA19 showed the presence of a T-8 tract (data not presented and Fig. 3A), which would result in production of a full-length LptA enzyme (Fig. 3B). Moreover, analysis of the online (http://www.broadinstitute.org/annotation/genome/neisseria_gonorrhoeae/GenomesIndex.html) whole-genome sequences of 13 other gonococcal strains indicated that their *lptA* gene contains the T-8 tract (data not presented). In addition, the genome sequence for 73 *N. gonorrhoeae* clinical isolates from patients with symptomatic gonorrhea was determined using Illumina technology; the details of this genome shotgun sequencing effort will be published separately. The nucleotide sequence of the FA19 *lptA* gene was searched against a BLAST database of all the whole-genome *de novo*-assembled contigs of these clinical isolates using BLASTN in WUBLAST, in order to identify the genome location of the gene within each of the strains. We used the default blastn parameters and specified hspsepSmax (maximum separation allowed between HSPs [high-scoring segment pair] along subject) to be 100 bp. Sequences of *lptA* genes were then extracted and screened for the presence of a T-8 tract on both the forward and reverse strands of the gene using pattern matching. The results showed that all strains contained a T-8 tract and a full-length *lptA* sequence with 100% nucleotide identity to FA 1090 (data not presented). Accordingly, we propose that possession of an in-frame *lptA* gene is a common feature of *N. gonorrhoeae* isolates.

***lptA* behaves as a PV gene in *N. gonorrhoeae*, and phase-off variants are hypersusceptible to PMB.** The presence of the T-8 tract in the 5′ end of the *lptA* coding sequence suggested to us that it is a member of the PV gene family possessed by *N. gonorrhoeae* (10). If so, production of a full-length LptA, PEA decoration of lipid A, and CAMP resistance could differ within a population of gonococci. To test this possibility, we employed a PMB screen/selection procedure since loss of *lptA* expression renders *N. gonorrhoeae* hypersusceptible to this model CAMP (3, 5, 7). After replica plating approximately 3,000 colonies of strain FA 1090 (T-8 tract and PMB MIC of 100 μg/ml) onto gonococcal base (GCB) agar plates with or without PMB selection, we identified (approximate frequency of 3.3×10^{-4}) a colony (strain BB22) that was unable to grow on GCB agar plates containing 10 μg/ml

TABLE 1 Oligonucleotide primers used in this study

Primer name	Primer sequence	Purpose
LptA6	5′-CGGTTTTGTATGTGGATCAGTT-3′	Transcriptional linkage
LptA7	5′-GCCTTTCTTTCCCTGTATTCTT-3′	Sequencing of the poly-T tract
LptA7_R	5′-AAGAATACAGGGAAAGAAAGGC-3′	Primer extension
LptA8	5′-ACGTTGCAATCCTACCTCGC-3′	Transcriptional linkage
LptA11	5′-CCGGTTCGAATTTTGCTTACG-3′	Primer extension
LptAdelL	5′-TGCAGGTACATCATGAAATTAGAC-3′	Sequencing of the poly-T tract
lptAJK4	5′-TAAGAATCTTTTTCAATAATCCGGAT-3′	Sequencing of the poly-T tract
lptArev	5′-GCCTCAGGTTCGGTTTTATC-3′	Transcriptional linkage
LptAstart	5′-TCTAGAAAGCTTCATCGACTTGT-3′	Sequencing of the poly-T tract
NGEG_02068_R	5′-GCGGGCAAAGCATTTCATAT-3′	Transcriptional linkage
serC_F	5′-CGACTACGGACTGATTTACG-3′	Transcriptional linkage

A

Strain	Poly-T tract length	Sequence	Spontaneous mutation frequency	PMB MIC (μg/mL)
FA 1090	8T	…GTGCTGTTTTTTTAAGTAA…	N/A	100
BB22	9T	…GTGCTGTTTTTTTTAAGTAA	~3.3×10^{-4}	0.2
BB22R	8T	…GTGCTGTTTTTTTAAGTAA…	10^{-5}	100

B

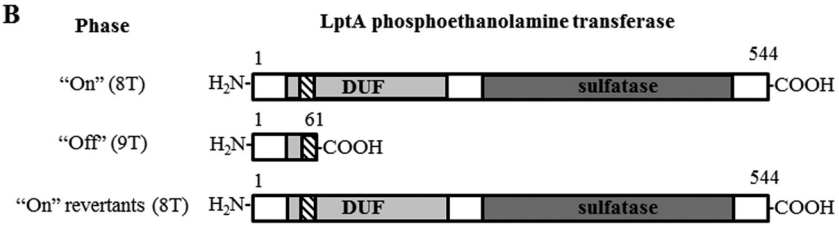

FIG 3 The *lptA* PV poly-T tract impacts LptA protein length and function. (A) Summary of the *lptA* PV poly-T tract. The PV poly-T tract comprises nucleotides 172 to 179 of the phase-on *lptA* open reading frame. Analysis of phase-on and phase-off *lptA* coding sequences using the ExPASy Translate tool (http://web.expasy.org/translate/) revealed that insertion of a ninth T nucleotide (bolded) in the poly-T tract of strain BB22 results in a frameshift mutation, which would generate a "UAA" stop codon shortly after this PV tract in the *lptA* mRNA transcript. (B) Impact of the PV poly-T tract on LptA protein length. When in the phase-on state, the *lptA* poly-T tract (hatched boxes) has 8 nucleotides and *lptA* encodes a protein 544 amino acids long. Frameshift of the *lptA* open reading frame (ORF) due to an insertion of a single T nucleotide within this poly-T tract would result in a premature stop codon and subsequent truncation of the LptA nascent polypeptide at just 61 amino acids. Phase-off variants are not predicted to translate the C-terminal sulfatase domain of the LptA protein (13). DUF, domain of unknown function.

of PMB. The PMB MIC against BB22 was 0.2 μg/ml (Fig. 3A), which is similar to the PMB MIC against an *lptA* deletion mutant of FA 1090 described previously (5). DNA sequence analysis of the *lptA* sequence of BB22 revealed that it possessed a T-9 tract that would result in premature truncation of LptA (Fig. 3). We then selected for spontaneous variants of BB22 that would grow on GCB agar containing 10 μg/ml of PMB. In four separate experiments, spontaneous PMB-resistant variants arose at a frequency of approximately 10^{-5}; in contrast, spontaneous erythromycin-resistant mutants (selected at 1 μg/ml) were recovered at a frequency of 10^{-8} (data not presented). The PMB MIC against PMB-resistant mutants of BB22 was, like that seen with parental strain FA 1090, 100 μg/ml (see strain BB22R data in Fig. 3A). DNA sequencing of the *lptA* PV tract from sixteen randomly picked PMB-resistant revertants of BB22 showed that all possessed a wild-type T-8 tract (see BB22R in Fig. 3A) and would produce a full-length LptA (Fig. 3B). Based on this reversion frequency, we estimate that the poly-T tract in *lptA* phase varies at an approximate frequency of 10^{-5}. This frequency is 2 to 3 orders of magnitude lower than that seen with other PV genes of *N. gonorrhoeae*, which may be due to its shorter tract (8 nt) and A/T characteristics that reduce slipped-strand mispairing events compared to the results seen with longer, G/C-rich repeats (10–12).

Conclusions. Production of PEA-decorated lipid A by *N. gonorrhoeae* has been linked with bacterial resistance to mediators of innate host defense, the capacity of *N. gonorrhoeae* to elicit a pro-inflammatory response, and *in vivo* fitness (3–5, 7, 8). The structurally variable lipooligosaccharide (LOS) chemotypes produced by gonococci have been linked to PV genes that encode enzymes responsible for adding carbohydrates within the branched-chain oligosaccharide region (11, 12). Our work now extends this PV expression property of LOS to the lipid A isoforms and emphasizes the complexity of LOS structures that can be presented by *N. gonorrhoeae*. Importantly, to our knowledge, this is the first direct evidence that gonococcal resistance to CAMPs can be modulated by a PV process.

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We declare that we have no conflicts of interest.

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