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Phase-Variable Expression of \textit{lptA} Modulates the Resistance of \textit{Neisseria gonorrhoeae} to Cationic Antimicrobial Peptides

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\textit{Neisseria gonorrhoeae} is a strict human pathogen that has caused the sexually transmitted infection termed gonorrhea for thousands of years. Over the millennia, \textit{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 \textit{N. gonorrhoeae} and \textit{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 \textit{N. gonorrhoeae} to complement-mediated killing by normal human serum (3, 4), \textit{N. gonorrhoeae} fitness during experimental infection in mice and humans (5, 7), and the proinflammatory potential of \textit{N. gonorrhoeae} (7, 8). Most communal \textit{Neisseria} do not encode \textit{lptA} (8), but \textit{N. gonorrhoeae} and \textit{N. meningitidis} (2, 3, 8) typically contain \textit{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 \textit{lptA} is within an operon and that \textit{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 \textit{lptA} coding sequence.

\textbf{Organization and expression of the \textit{lptA} locus in \textit{N. gonorrhoeae}.} Bioinformatic analysis of the DNA sequence of the \textit{N. gonorrhoeae} FA 1090 chromosome (\url{http://www.genome.ou.edu/gono.html}) suggested that \textit{lptA} is transcriptionally linked to two upstream genes (\textit{serC} and a hypothetical gene annotated as \textit{NGO1282}) and a downstream gene (\textit{nfnB}) (Fig. 1). This hypothesis was confirmed by results from reverse transcription-PCR (RT-PCR) experiments (Fig. 2A) that demonstrated transcriptional linkage of \textit{lptA} with the \textit{serC}, hypothetical, and \textit{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 \textit{N. gonorrhoeae} RNA performed as described previously (9) identified a transcriptional start point (TSP) positioned 61 nucleotides (nt) upstream of the \textit{lptA} translational start codon and four nt downstream of near-consensus −10 and −35 elements (Fig. 1 and 2B).

Thus, we tentatively conclude that \textit{lptA} expression in \textit{N. gonorrhoeae} can be initiated by two promoters upstream of \textit{serC} and \textit{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 \textit{lptA} coding sequence contains a polynucleotide tract consist-
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-32P]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 3'-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.

TABLE 1 Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LptA6</td>
<td>5'-CGGTTTTTGTATGTTGATCAGTT-3'</td>
<td>Transcriptional linkage</td>
</tr>
<tr>
<td>LptA7</td>
<td>5'-GGCCTTCTCTTTCCTGTATCTT-3'</td>
<td>Sequencing of the poly-T tract</td>
</tr>
<tr>
<td>LptA7_R</td>
<td>5'-AAGAATACAGGGAAGAAGAAGGC-3'</td>
<td>Primer extension</td>
</tr>
<tr>
<td>LptA8</td>
<td>5'-AGGTGGCAATCTCATCCTCAGC-3'</td>
<td>Transcriptional linkage</td>
</tr>
<tr>
<td>LptA11</td>
<td>5'-CGGGTCGAATTITGCTTTACG-3'</td>
<td>Primer extension</td>
</tr>
<tr>
<td>LptAdelL</td>
<td>5'-TGCGAGGTACATCATGAATTAGAC-3'</td>
<td>Sequencing of the poly-T tract</td>
</tr>
<tr>
<td>lptAIK4</td>
<td>5'-TAAGAATTTTTTTCAATATCCGGAT-3'</td>
<td>Sequencing of the poly-T tract</td>
</tr>
<tr>
<td>lptArev</td>
<td>5'-GGCTCGAGGTCTGCTTTATAC-3'</td>
<td>Transcriptional linkage</td>
</tr>
<tr>
<td>LptAstart</td>
<td>5'-TCCTAGAAGGCTTATGCAGTGT-3'</td>
<td>Sequencing of the poly-T tract</td>
</tr>
<tr>
<td>NGEG_02068_R</td>
<td>5'-GGCCGGCAAGACTTCTTTCATAT-3'</td>
<td>Transcriptional linkage</td>
</tr>
<tr>
<td>serC_F</td>
<td>5'-GGACTACGGAGCTTATCCAG-3'</td>
<td>Transcriptional linkage</td>
</tr>
</tbody>
</table>
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^-9; 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 thank Jo-Anne Dillon, Carlos del Rio, and Magnus Unemo for providing *N. gonorrhoeae* strains and DNA used in whole-genome sequencing (WGS).

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We declare that we have no conflicts of interest.

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