Transcriptional control of the gonococcal *ompA* gene by the MisR/MisS two-component regulatory system

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*Neisseria gonorrhoeae*, the causative agent of gonorrhea, is an exclusive human pathogen whose growing antibiotic resistance is causing worldwide concern. The increasing rise of antibiotic resistance expressed by gonococci highlights the need to find alternative approaches to current gonorrhea treatment such as vaccine development or novel therapeutics. The gonococcal OmpA protein was previously identified as a potential vaccine candidate due to its conservation and stable expression amongst strains of *Neisseria gonorrhoeae*. However, factors that might modulate levels of OmpA and therefore potential vaccine efficacy are unknown. Earlier work indicated that *ompA* is part of the MisR/MisS regulon and suggested that it was a MisR-activated gene. Herein, we confirmed MisR/MisS regulation of *ompA* and report that the MisR response regulator can bind upstream of the *ompA* translational start codon. Further, we describe the contribution of a DNA sequence upstream of the *ompA* promoter that is critical for MisR activation of *ompA* transcription. Our results provide a framework for understanding the transcription of gonococcal *ompA* through a regulatory system known to be important for survival of gonococci during experimental infection.

The strict human pathogen *Neisseria gonorrhoeae* (*Ng*) is the etiologic agent of gonorrhea, which is the second most common sexually transmitted infection in the United States and causes an estimated 87 million infections globally per year.¹² Historically, *Ng* has developed clinical resistance to every antibiotic introduced for therapy of gonorrhoea.¹³ Worryingly, extensively drug-resistant *Ng* strains have been reported globally that are resistant to azithromycin and/or ceftriaxone, which are currently used in dual antibiotic therapy in the United States and elsewhere.¹⁴ The current crisis of antibiotic resistance expressed by *Ng* strains and overall reduced industrial efforts to develop new antimicrobial drugs has renewed interest in developing a gonorrhea vaccine.⁹ In this respect, several surface-exposed, conserved and stably produced *Ng* antigens have been proposed as vaccine candidates; included in this list is a 23 kDa outer membrane protein termed OmpA that is similar to OmpA in other Gram-negative bacteria.⁸³⁰ OmpA-like proteins have been considered as vaccine targets.¹¹-¹³ For instance, mucosal immunization of mice with purified OmpA elicted protective immunity against multi-drug resistant *Acinetobacter baumanii*.¹⁶ *Ng* OmpA was initially discovered by *in silico* screening of the FA1090 genome database for potentially surface-exposed proteins that could be vaccine antigens for an *Ng* vaccine.¹⁹ As *Ng* OmpA is present and conserved by all examined *Ng* strains and not subject to phase or antigenic variation, it is an ideal target for recognition by the immune system. Relevantly, sera from *Ng*-infected patients recognized OmpA indicating its expression during natural human infection.¹⁶¹⁷ Consideration of OmpA as a vaccine candidate is further supported by findings that it facilitates *Ng* adhesion to and invasion of human cervical and endometrial cells, resistance to phagocytosis and survival during experimental infection of the lower genital tract of female mice.¹⁸ Taken together, these studies implicate OmpA as a virulence factor that could be exploited as part of a vaccine to protect at-risk individuals from gonorrhea.

Notably, *ompA* was shown to be amongst the approximately 17% of *Ng* genes differentially expressed during symptomatic, natural cervical infection in women compared to *Ng* (strain NCCP11945) grown in chemically

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defined broth\textsuperscript{18}. Thus, understanding mechanisms of \(N_g\) transcriptional control of \(ompA\) expression could help to advance knowledge regarding the role of OmpA in \(N_g\) pathogenesis during infection and advance vaccine development efforts. In this respect, there is evidence from studies with other bacteria that \(ompA\)-like genes are subject to transcriptional control systems. For instance, \(ompA\) has been reported to be regulated by Hfq and small RNAs such as MicA and SSr1 in \textit{Escherichia coli} and \textit{Shigella flexneri}\textsuperscript{19–21}. However, \(N_g\) \(ompA\) does not appear to be regulated by Hfq or any of the predicted \(N_g\) sRNAs\textsuperscript{18,22}. Nevertheless, evidence for transcriptional regulation of \(N_g\) \(ompA\) is suggested by results from two independent transcriptional profiling studies that included \(ompA\) as a gene that can be activated by the MisR/MisS sensory two-component regulatory system (TCS)\textsuperscript{23,24}. MisR/MisS is similar to CpxR/CpxA possessed by other bacteria\textsuperscript{25} and consists of the MisR response regulator and MisS sensory histidine kinase (MisS) responsible for phosphorylation of MisR. Although environmental signals that activate MisR/MisS remain unknown, this TCS was reported to be essential for \(N_g\) survival during experimental infection of the lower genital tract of female mice\textsuperscript{24}. Accordingly, we sought to define the mechanism of MisR regulation of \(ompA\) and herein provide a model for MisR activation of this \(N_g\) virulence gene.

**Results and Discussion**

**Confirmation of MisR/MisS regulation of \(ompA\).** Our previous work and that of others that defined the \(N_g\) MisR/MisS regulon identified \(ompA\) as being transcriptionally activated by MisR\textsuperscript{23,24}. To confirm these observations, we examined \(ompA\) transcript and protein levels in wild-type (WT) strain FA19, its isogenic misR-null (JK100) and complemented strain (JK101) at the mid-logarithmic phase of growth. Results from qRT-PCR analysis showed a significant reduction in the \(ompA\) transcript level due to the loss of MisS (Supplementary Fig. S1), which was reversed by complementation with the WT misS gene expressed in trans. Thus, both MisR and MisS participate in activation of \(ompA\) expression.

![Figure 1](https://example.com/figure1.png) **Figure 1.** MisR is necessary for expression of \(ompA\). (a) qRT-PCR analysis of \(ompA\) transcripts in FA19, misR-null (JK100), and complemented strain (JK101) at the mid-logarithmic phase of growth. (b) qRT-PCR analysis of \(ompA\) transcripts in strains FA1090, MS11, and HO41 and their respective misR-null mutants. Error bars represent standard deviations from the means of 3 independent experiments. Normalized Expression Ratios (NER) were calculated using 16S rRNA expression. The statistical significance of the results was determined by Student’s t-test, \(*P < 0.05, **P < 0.001.\)
MisR directly regulates \textit{ompA} expression. We next determined if MisR regulation of \textit{ompA} is direct. For this purpose, an electrophoretic mobility shift assay (EMSA) was performed using in vitro phosphorylated MisR (MisR--P) and 400 bp of the DNA sequence upstream of the \textit{ompA} translational start codon that contains putative promoter elements. The results showed that MisR--P could bind to the target DNA (Fig. 2 and Supplemental Fig. 3). As a control, we determined if another gonococcal transcriptional regulatory protein, MpeR, could bind to the examined \textit{ompA} promoter. The regulon of MpeR has some overlap with genes regulated by MisR/MisS\textsuperscript{28} but does not regulate \textit{ompA}. We confirmed that MpeR does not bind \textit{ompA} target DNA, suggesting that the interaction between \textit{ompA} and MisR is specific (Supplemental Fig. S4).

To ensure that MisR--P binding to the target DNA was specific, we performed a competitive EMSA using unlabeled specific (\textit{ompA}) and non-specific (\textit{rnpB}) probes; \textit{rnpB} is not regulated by MisR\textsuperscript{23}. Importantly, only the specific unlabeled probe could compete with the labeled \textit{ompA} probe for MisR-binding (Fig. 2) indicating that such binding was specific.

Identification of MisR target sites upstream of \textit{ompA}. After confirmation of MisR binding to the upstream \textit{ompA} DNA, we sought to identify the \textit{ompA} promoter and MisR-binding sites important for the regulation of \textit{ompA} expression. Accordingly, we first mapped the \textit{ompA} promoter by identifying the transcriptional start site (TSS) by primer extension (PE) analysis using total RNA isolated from strains FA19 and JK100 (FA19 \textit{misR}:\textit{kan}). The PE assay protocol generated a single peak from the FA19 RNA that was absent when such RNA was treated with RNaseA or when RNA from JK100 was used. The PE product obtained with FA19 RNA was positioned approximately 23 bp upstream of the ATG start codon and 11 bp downstream of a –10 sequence of the putative sigma 70 promoter element (Fig. 3a, top panel). Interestingly, the separation of the predicted TSS site was removed from the FL fusion (Fig. 4c). By comparison, disruption of the S1 site reduced expression of \textit{ompA} by 85% in the \textit{misR}--Δ\textit{S1} null strain (JK100) compared to WT strain FA19 indicating that MisR interaction with the \textit{ompA} promoter containing region is essential for WT levels of \textit{ompA} expression (Fig. 4b). Importantly, the presence of only the S1 site in the Trunc fusion resulted in significantly reduced expression compared to the FL fusion in both the WT and \textit{misR}:\textit{kan} backgrounds (Fig. 4b).

To further assess the contribution of S1 and S2 with respect to MisR control of \textit{ompA} expression, we constructed additional \textit{ompA}-\textit{lacZ} fusions in WT strain FA19 that had mutations in each site (Fig. 4a). Thus, we deleted the entire S2 site (14 bp) to create FLΔ\textit{S2}. Given that the S1 site overlaps the putative –10 promoter element, we removed 8 bp of the binding site (5′-GTACGGTT-3′) and inserted 8 bp of non-consensus sequence (5′-ACCTTAC-3′) to create FLΔ\textit{S1} and TruncΔ\textit{S1}; the region of the sequence changed in the S1 site is shown in italics in Fig. 3b. This fusion construct allowed for loss of the S1 binding site while maintaining the integrity of the –10 element and the TSS. With these fusion strains, we noted an 85% decrease in \textit{ompA} expression when the S2 site was removed from the FL fusion (Fig. 4c). By comparison, disruption of the S1 site reduced expression of the \textit{ompA} promoter to a lesser extent (ca. 50%) (Fig. 4c). Further, there was no significant difference in \textit{ompA}-\textit{lacZ} expression between the Trunc or TruncΔ\textit{S1} fusions, although there was still a significant reduction compared to
the FL fusion containing both S1 and S2. Thus, although both putative MisR-binding sites may contribute to regulating ompA expression, the results suggested that S2 plays a more predominant role in interactions with MisR. To test this hypothesis, we performed a competitive EMSA using the disrupted S1 or S2 site DNAs as unlabeled competitors to the radiolabeled FL probe. As expected, the unlabeled FL probe competed with the radiolabeled FL probe (Fig. 5 and Supplemental Fig. 5) as did the FLΔS1 probe, albeit to a lesser extent. Consistent with the lacZ fusion data, the FLΔS2 probe did not compete with the FL probe. Thus, we concluded that the S2 site is required, but not sufficient, for full MisR activation of ompA expression in Ng.

This work was stimulated by previous observations that collectively suggested important roles for both OmpA and MisR/MisS in the ability of Ng to survive during experimental lower genital tract infection of female mice\(^{23,24}\). Since ompA expression was found in two different studies to be part of the MisR regulon\(^{23,24}\), we sought to define the mechanistic basis for MisR/MisS regulation of ompA expression. The results presented herein indicate a direct role for MisR control of ompA. We propose that while both the S1 and S2 putative MisR-binding sites (Fig. 3b) participate in MisR activation of ompA expression the latter plays a more predominant role in this regulation. The location of the S2 site immediately upstream of the −35 hexamer suggests that bound MisR assists recruitment of RNAP to the promoter, which has a sub-optimal spacing between the −10 and −35 hexamers, for transcription of ompA. However, we cannot discount a role for the downstream S1 site as disruption of it in WT strain FA19 significantly reduced ompA expression even when S2 was present (Fig. 4c).

In conclusion, this is the first report that characterizes regulation of the Ng ompA gene, which encodes a candidate vaccine antigen. We propose that MisR/MisS directly enhances ompA expression. Taken together, the intrinsic linkage of MisR/MisS and OmpA could be exploited for vaccine or chemotherapeutic development purposes.
Methods

Bacterial strains, plasmids, and primers. Ng strain FA19 and its isogenic mutant strains, along with the plasmids used and their Escherichia coli hosts, are listed in Table 1. The oligonucleotide primers used in this study are listed in Supplementary Table S1. E. coli strains were routinely cultured on Luria-Bertani (LB) agar or in LB broth (Difco, Sparks, MD) containing 50 µg/mL kanamycin, 100 µg/mL ampicillin or 100 µg/mL chloramphenicol as necessary. Gonococci were grown on gonococcal base (GCB) agar (Difco, Sparks, MD) containing 100 µg/mL kanamycin and 100 µg/mL ampicillin.

Figure 4. Regulation of ompA by MisR. (a) The organization of the ompA-lacZ fusion constructs are depicted. The approximate locations of the predicted MisR-binding sites are indicated by boxes S1-S2. The –10 and –35 hexamers are notated. (b) Regulatory effect of the misR mutation on the expression of ompA. The specific β-galactosidase activity per mg of total protein in cell extracts of reporter strains containing the ompA-lacZ fusions (FL and Trunc) in the FA19 and misR null (JK100) backgrounds. (c) Effect of disruption of MisR binding sites on the expression of ompA. The specific β-galactosidase activity per mg of total protein in cell extracts of reporter strains containing the ompA-lacZ fusions (FL and Trunc) and the disrupted MisR binding site fusions (FLΔS2, FLΔS1, and TruncΔS1) in the FA19 background. Results are the average of three independent experiments. Statistical significance was determined by ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5. Mapping of primary MisR binding sites. Competitive EMSA demonstrating preferential MisR binding to specific sites in the promoter region. Lane 1 and 9, radiolabeled probe alone (5 ng); Lane 2, radiolabeled probe plus MisR~P (1.5 µg); Lanes 3–4, radiolabeled probe plus unlabeled FL competitor probes; Lanes 5–6 radiolabeled probe plus unlabeled FLΔS2 competitor probes; Lanes 7–8 radiolabeled probe plus unlabeled FLΔS1 competitor probes.
supplements I and II at 37 °C under 5.0% (v/v) CO₂. Liquid cultures of gonococci were begun by inoculating plate-grown cells in pre-warmed GCB broth containing Kellogg’s supplements I and II and 0.043% (w/v) sodium bicarbonate and grown at 37 °C with shaking. Liquid cultures of gonococci contained a final concentration of 10 mM MgCl₂.

**Generation of ompA and misR-null mutants.** Construction of the FA19ompA::ermC mutant (strain CH10) was performed as described below using an erythromycin resistance cassette. CH10 was constructed by transforming WT FA19 with a purified PCR product made from genomic DNA from the NgFA1090ompA::ermC mutant constructed previously and generously donated by Ann Jerse (Uniform Services University, Bethesda, MD). Plate transformations were performed as described previously and transformants selected on GCB agar containing erythromycin 1 μg/mL. Insertion of the ermC cassette was confirmed by PCR using primers HFLF2 and HFLR2 and verified by sequencing of PCR product made from genomic DNA. MisR::kan mutants in strains FA1090, HO41 and MS11 were constructed by inactivating the misR gene using the nonpolar aphA-3 kanamycin cassette as described previously. Loss of misR was confirmed by PCR and sequencing using primers misRkanup and misRkandown.

**Complementation of the ompA::ermC mutant.** Ng strain CH10 was complemented as follows. In FA1090ompA::ermC C’, the entire ompA gene and its native promoter are inserted into an intergenic region in the chromosome between NGO0077 and NGO0078. The complemented coding region was amplified using

| Strain or plasmid | Genotype or description | Reference or source |
|-------------------|------------------------|---------------------|
| N. gonorrhoeae    |                        |                     |
| FA19              | WT strain              | 38                  |
| CH10              | FA19ompA::ermC         | This Study          |
| CH11              | CH10 complementation   | This Study          |
| JK100             | FA19misR::kan          | 10                  |
| JK101             | JK100 complementation  | (FA19misR::kan/pGCC4-misR) |
| FA1090            | WT strain              | 30                  |
| FA1090 ΔompA      | FA1090ompA::ermC       | 10                  |
| FA1090 ΔompA C’   | FA1090ompA::ermC       | 10                  |
| FA1090 misR::kan  | FA1090misR::kan        | 23                  |
| MS11              | WT strain              | 40                  |
| MS11 misR::kan    | MS11misR::kan          | This Study          |
| HO41              | WT strain              | 41                  |
| HO41 misR::kan    | HO41misR::kan          | This Study          |
| FA19::PFL        | FA19 containing a translational fusion of 123 bp of the promoter region of ompA to the lacZ gene | This Study          |
| FA19::PFL127      | FA19 containing a translational fusion of 123 bp of the promoter region of ompA to the lacZ gene and S1 disruption | This Study          |
| FA19::PFL227      | FA19 containing a translational fusion of 123 bp of the promoter region of ompA to the lacZ gene and S2 deletion | This Study          |
| FA19::PTrunc      | FA19 containing a translational fusion of 81 bp of the promoter region of ompA to the lacZ gene | This Study          |
| FA19::PTrunc241   | FA19 containing a translational fusion of 81 bp of the promoter region of ompA to the lacZ gene and S1 disruption | This Study          |

**Escherichia coli**

| Strain or plasmid | Genotype or description | Reference or source |
|-------------------|------------------------|---------------------|
| One Shot TOP10    | F’ merA Δ(mrr-hsdRMS-merBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 (ara leu)7697 galU galK rpsL (Str) endA1 nupG | Invitrogen (Carlsbad, CA) |
| BL21(DE3)         | fhuA2 [lac] ompT gal (λ DE3) [ΔlacS λ] ΔhsdS λ | New England Biolabs (Ipswich, MA) |

**Plasmids**

| Strain or plasmid | Genotype or description | Reference or source |
|-------------------|------------------------|---------------------|
| pET-15b           | Bacterial expression vector with T7lac promoter, N-terminal His-tag | Merck Millipore (Burlington, MA) |
| pCH1              | pET-15b containing FA19ompA coding region | This Study          |
| pLES94            | pUC18 derivative containing a truncated lacZ gene for use in translational fusions; recombines at the proAB locus of the gonococcal chromosome | 46 |
| pCH22             | pLES94 containing 123 bp upstream of ompA (FL) | This Study |
| pCH23             | pLES94 containing 81 bp upstream of ompA (Trunc) | This Study |
| pCH24             | pCH22 with disrupted S1 site | This Study          |
| pCH25             | pCH22 with deleted S2 site | This Study          |
| pCH26             | pCH23 with disrupted S1 site | This Study          |

Table 1. Bacterial Strains and plasmids used in this study.
primers PNG0077 and PNG0078 to ensure recombination of the complement in the correct locus. Transformants of CH10 were selected on GCB agar using chloramphenicol 10 μg/ml and verified by PCR and sequencing.

qRT-PCR analysis of Ng transcripts. For measurement of target gene expression, gonococci were harvested at mid- or late-log phase and the pellets were stored at −70 °C. RNA was purified by Trizol extraction as per manufacturer instructions (Thermo Fisher Scientific, Waltham, MA) followed by Turbo DNA-free (Ambion, Austin, TX) treatment. cDNA was generated using a QuantiTect reverse transcriptase kit (Qiagen, Venlo, Netherlands). We validated our qRT-PCR methods by examining primer efficiency, primer specificity (melt temperature) and linear dynamic range for each primer pair utilized herein. For additional information about our validation results, see Supplemental Fig. S6. For qRT-PCR analysis, the normalized expression of each target gene was calculated using 16S rRNA as a housekeeping reference gene33. As an additional internal control, significance was confirmed using recA as the reference gene (data not shown). All qRT-PCRs were performed in technical and biological triplicates.

Purification of recombinant His-OmpA protein and preparation of polyclonal antiserum. The coding sequence of ompA was amplified with primers His-OmpAF and His-OmpAR. The PCR product was digested with BamHI and XhoI and then cloned into pET-15b which had been digested with the same enzymes to yield pCH1. The plasmid was purified and transformed into E. coli expression strain BL21(DE3). A His-OmpA fusion protein was produced using a hybrid purification method denaturing the protein to enable solubilization first and renaturing the protein on the column prior to elution as per manufacturer’s protocol (Millipore Sigma, Burlington, MA). The fusion protein was purified using a nickel-nitrilotriacetic acid (Ni2+-NTA) column. His-OmpA was eluted with buffer containing 100 and 200 mM imidazole. The fractions were dialyzed to remove imidazole using 10 mM PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) and concentrated. Dithiothreitol (DTT) and glycerol were added to a final concentration of 1 mM and 10% (v/v), respectively. The purity of recombinant OmpA was confirmed by SDS-PAGE electrophoresis staining with Coomassie blue. A rabbit polyclonal anti-OmpA antibody was generated (Pacific Immunology, Ramona, CA) using a small peptide corresponding to amino acids 145–159 of the OmpA protein (Cys-NHHTDNTGSDAVNPNP). The specificity of the antibody was tested against whole cell lysates purified from FA19 and ompA mutant strains as well as the purified His-OmpA protein.

Western blotting. Gonococci grown to late-log phase in broth were pelleted by centrifugation at 10,000 rpm for 2 min, and whole-cell lysates were prepared in 2X SDS loading dye (100 mM Tris-HCl, pH 6.8, 4% [wt/vol] SDS, 0.2% [wt/vol] bromophenol blue, 20% glycerol, 200 mM dithiothreitol [DTT]). Protein levels were normalized by use of a NanoDrop spectrophotometer and BCA Protein Assay. Equivalent loading was confirmed by Coomassie staining on a 12% SDS-polyacrylamide gel. Blots were blocked in 5% (wt/vol) nonfat dried milk in 1X TST buffer (0.01 M Trizma base, 0.150 M NaCl, 0.05% [vol/vol] Tween 20) and probed with primary antibody against OmpA O/N at 4 °C using a 1:1000 dilution. Blots were then washed with 1X TST before incubation with secondary antibody conjugated to AP and developed with NBT/BCIP.

EMSA for detection of MisR binding to target DNA. A DNA probe containing the putative ompA promoter region (Fig. 3b) was amplified by PCR from FA19 genomic DNA using the primers pOmpA2F and pOmpAR. For radiolabeled probes, the indicated PCR product was labeled with [γ-32P]-dATP using T4 polynucleotide kinase (New England Biolabs, Ipswitch, MA). The labeled DNA fragments (5 ng) were incubated with 1.5 μg of MpcR and MisR that had been phosphorylated with acetyl phosphate in a 30 μl reaction buffer at room temperature34. For MisR competition assays, the unlabeled ompA probe or an unlabeled PCR product (5 ng) using RnpB1F and RnpB1R primers (non-specific rnpB probe) were incubated with protein for 15 minutes prior to the addition of the radiolabeled probe. Samples were subjected to electrophoresis in a 6% native polyacrylamide gel at 4 °C, followed by autoradiography.

Primer extension analysis. The ompA TSS was identified by primer extension using a 5′-fluorescently labeled HEX primer and analysis on an automated capillary electrophoresis instrument as described previously35 with modifications. FA19 and JK100 were grown to an OD600 of 1.0, and 1 mL of the culture was resuspended in 200 μl of RNAlater solution (Ambion, Austin, TX). Total RNA was isolated by using the RNeasy Plus Minikit (Qiagen, Venlo, Netherlands), contamination with genomic DNA was removed using the Turbo DNA-free Kit (Ambion, Austin, TX) following the company protocol. Two μl of the primer extension reactions were combined with 7.5 μl Hi-Di formamide and 0.5 μl GeneScan 600 LIZ size standard (Applied Biosystems, Waltham, MA) and detected with a 3730 capillary DNA analyzer (Applied Biosystems, Waltham, MA) running a default temperature treatment. cDNA was generated using the SuperScript II Reverse Transcriptase system (Invitrogen, Carlsbad, CA) following the company protocol. Two μl of the primer extension reactions were combined with 7.5 μl Hi-Di formamide and 0.5 μl GeneScan 600 LIZ size standard (Applied Biosystems, Waltham, MA) and detected with a 3730 capillary DNA analyzer (Applied Biosystems, Waltham, MA) running a default genotyping module. The length and abundance (height and area below the peaks) of the HEX-labeled cDNA primer extension products were analyzed by using Applied Biosystems GeneMapper Software version 4.0 (https://www.thermofisher.com/order/catalog/product/44409154/4440915).

To accurately assign a nucleotide base to the peaks detected in the primer extension reaction, a sequence ladder was generated by using the Thermo Sequenase Cycle Sequencing Kit (Applied Biosystems, Waltham, MA). Briefly, a DNA template was generated by PCR using primers OmLacFL and Om-LR-R2. Sequencing reactions were conducted with 200 fmol of template DNA and 2 pmol of Hex-OmpA (template strand) according to
the manufacturer’s instructions. Each of the four deoxyribose reaction mixtures was diluted 5-fold in water, and 2μl was loaded onto the 3730 DNA analyzer (Applied Biosystems, Waltham, MA). The electropherograms of the sequencing reactions were horizontally aligned with those generated in the primer extension using GeneMapper 4.0 (Applied Biosystems, Waltham, MA).

Construction of the ompA-lacZ fusions.  ompA-lacZ translational fusions were generated using the pLES94 system36. Briefly, putative promoter regions of ompA were amplified using primers Omlac21, Omlac1 and OmlacRev and used to generate translational fusions of ompA to the truncated, promoter-less lacZ gene in pLES94. The constructs were transformed into One Shot TOP10 chemically competent E. coli cells (Invitrogen, Carlsbad, CA) by heat shock, and transformants were selected on LB agar containing 100μg/mL ampicillin and 40μg/mL X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The plasmids were confirmed, purified, and transformed into the wild type strain FA19 to generate strains FA19::Pfl, and FA19::Ptrans. Gonococcal transformants were selected on GCB agar containing 1μg/mL of chloramphenicol and further verified by PCR.

For the construction of the disrupted site fusions, the suspected binding sites were deleted (ΔS2) or altered (ΔS1) using primers S2dis/F and S1dis/F respectively. The full-length promoter was amplified from a PCR product containing the disruptions and cloned into pLES94. The resulting plasmids were transformed into FA19 to generate strains FA19::PflΔS2, FA19::PflΔS1, FA19::PtransΔS1. misR-null strains were constructed by deleting misR in the WT fusions strains. All strains were confirmed by sequencing.

Preparation of cell extracts and β-galactosidase assays. Ng strains containing lacZ translational fusions were grown overnight on GCB agar plates containing 1μg/mL of chloramphenicol. Cells were scraped, washed once with phosphate-buffered saline (pH 7.4), and resuspended in lysis buffer (0.25mM Tris [pH 8.0]). Cells were then broken by three freeze-thaw cycles. The cell debris was removed by centrifugation at 15,000 × g for 10 min at 4°C. β-Galactosidase assays were performed as previously described37.

Statistical methods. All the data were expressed as means with standard deviation (SD). Statistical significance between all quantitative data are analyzed by Student t-tests or one-way ANOVA followed by Tukey’s honestly significant difference post-hoc test. Significance was set at P < 0.05.

Data availability
The datasets that supported the findings of this study are available from the corresponding author upon request.

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Author contributions

C.L.H. and W.M.S. conceived and designed the study. Primer extension was performed by J.C.A. All other data and supplementary information was analyzed by C.L.H. All authors participated in writing the manuscript and approved its submission.

Competing interests

The authors declare no competing interests.

Additional information

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