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MECHANISMS OF RESISTANCE

cis- and *trans*-Acting Factors Influence Expression of the *norM*-Encoded Efflux Pump of *Neisseria gonorrhoeae* and Levels of Gonococcal Susceptibility to Substrate Antimicrobials

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ABSTRACT The gonococcal NorM efflux pump exports substrates with a cationic moiety, including quaternary ammonium compounds such as berberine (BE) and ethidium bromide (EB) as well as antibiotics such as ciprofloxacin and solithromycin. The *norM* gene is part of a four-gene operon that is transcribed from a promoter containing a polynucleotide tract of 6 or 7 thymidines (T's) between the −10 and −35 hexamers; the majority of gonococcal strains analyzed in this study contained a T-6 sequence. Primer extension analysis showed that regardless of the length of the poly(T) tract, the same transcriptional start site (TSS) was used for expression of *norM*. Interestingly, the T-6 tract correlated with a higher level of both *norM* expression and gonococcal resistance to NorM substrates BE and EB. Analysis of expression of genes downstream of *norM* showed that the product of the *tetR*-like gene has the capacity to activate expression of *norM* as well as *murB*, which encodes an acetylenolpyroylglucosamine reductase predicted to be involved in the early steps of peptidoglycan synthesis. Moreover, loss of the TetR-like transcriptional regulator modestly increased gonococcal susceptibility to NorM substrates EB and BE. We conclude that both *cis*- and *trans*-acting regulatory systems can regulate expression of the *norM* operon and influence levels of gonococcal susceptibility to antimicrobials exported by NorM.

KEYWORDS NorM, efflux pumps, gonorrhea, resistance

Neisseria gonorrhoeae is a strict human pathogen and is the etiologic agent of the sexually transmitted infection (STI) termed gonorrhea, which is the second most prevalent bacterial STI in the United States and had a worldwide incidence in 2012 of an estimated 78 million infections (1). The gonococcus has adapted numerous strategies to survive attack by antimicrobials, including classical antibiotics used in treatment of infection and those of host origin that participate in innate host defense. In this respect, gonococci use efflux pumps to resist the antimicrobial action of beta-lactam and macrolide antibiotics as well as cationic antimicrobial peptides and long-chain fatty acids (2–4). The capacity of gonococci to utilize efflux pumps to resist clinically useful antibiotics is of interest given the emergence of strains resistant to current and past front-line antibiotics (2, 5–7). The contribution of efflux pumps in aiding bacterial evasion of antimicrobials can be enhanced by mutations that derepress expression of efflux pump-encoding genes (8). With respect to gonococci, previous work revealed that overexpression of the *mtrCDE* efflux pump operon due to *cis*- or *trans*-acting mutations can contribute to clinically relevant levels of antibiotic resistance (9, 10) and

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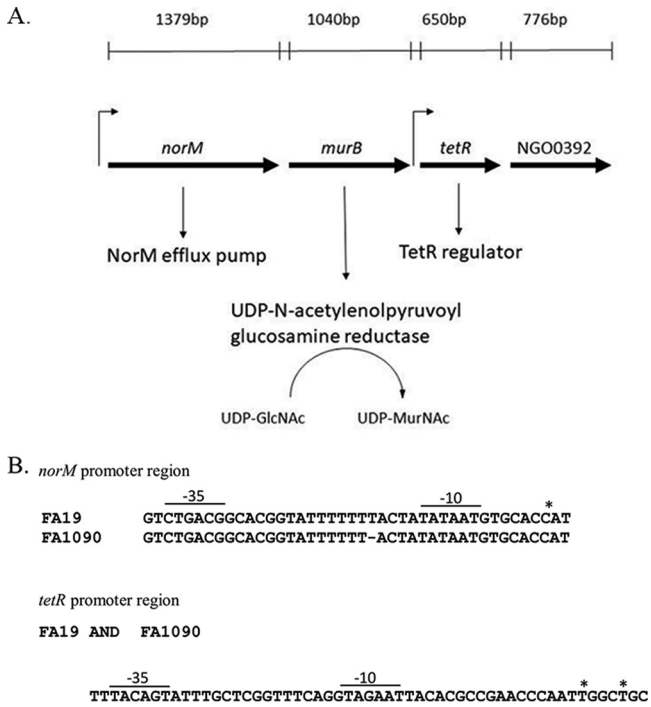

FIG 1 (A) The organization of the *norM* operon is depicted. The length and transcriptional direction (arrows) of the genes are shown. (B) Sequences of the *norM* and *tetR* promoter regions from strains FA19 and FA1090. The −10 and −35 hexamers are indicated. The asterisk represents the TSS.

increase bacterial fitness during experimental infection of the lower genital tract of female mice, presumably due to enhanced resistance to host antimicrobials (11, 12).

In this study, we investigated the regulation of the gonococcal *norM* gene. NorM belongs to the multidrug and toxic compound extrusion (MATE) family of efflux proteins, which are Na^+- or H^+-coupled transporters and are present in all living organisms (13). Gonococcal NorM is highly similar (56%) to NorM of *Vibrio parahaemolyticus* (14). We previously reported that NorM can export substrates with a cationic moiety, including berberine (BE), ciprofloxacin (CIP), and ethidium bromide (EB) (15). Additionally, the loss of the NorM efflux pump in multidrug-resistant strain H041 was found by Golparian et al. (6) to increase gonococcal susceptibility to solithromycin. In this study, we investigated *cis*- and *trans*-acting regulatory mechanisms that influence *norM* expression and the consequence of such on antimicrobial resistance. Importantly, we identified a heretofore undescribed TetR-like regulator that activated the *norM* gene as well as a single-base-pair deletion that resulted in a stronger *norM* promoter.

RESULTS AND DISCUSSION

***cis*-Acting transcriptional regulation of *norM* in *N. gonorrhoeae* and influence on antimicrobial resistance.** Bioinformatics analysis (http://www.ncbi.nlm.nih.gov/gene) indicated that *norM* (NGO0395) is the first gene of an operon that also contains three downstream genes annotated as *murB* (NGO0394), which encodes a putative UDP-*N*-acetylenolpyruvoylglucosamine reductase involved in the initial steps of the peptidoglycan synthesis (Fig. 1A) (24), NGO0393, which encodes a TetR-like family transcriptional regulator homolog, and NGO0392, which encodes a hypothetical protein. Using total RNA prepared from strain FA19Str^R in reverse transcription-PCR (RT-PCR) experiments, we confirmed transcriptional linkage of *norM* and *murB* as well as *murB* and *tetR* (see Fig. S1 in the supplemental material), which supports the hypothesis that the genes form an operon. Primer extension analysis of this RNA indicated the presence of 2 distinct transcriptional start sites (TSSs). One TSS was located upstream of *norM* that corresponded to that described previously by our group (15) as well as another TSS

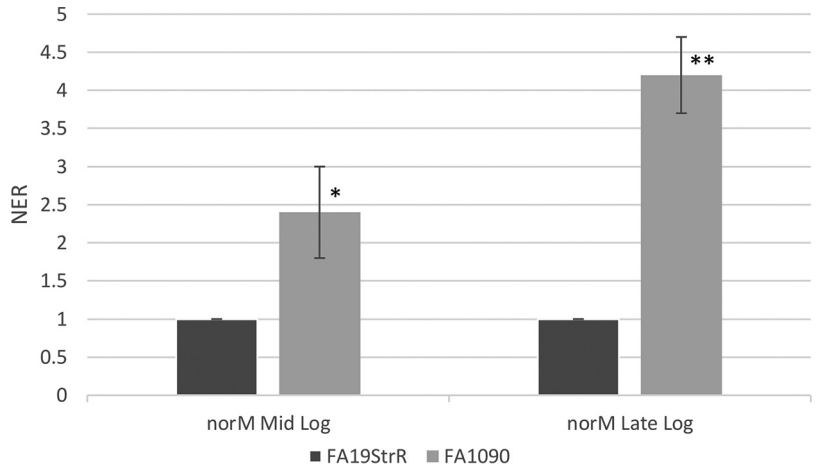

FIG 2 Quantitative RT-PCR results with *norM* in strains FA19Str^R and strain FA1090 at the mid-logarithmic and late logarithmic phases of growth. Error bars represent SDs from the means of 3 independent experiments. Normalized expression ratios (NER) were calculated using 16S rRNA expression. *, $P = 0.018$; **, $P = 0.004$ (for comparison of values of FA1090 versus FA19Str^R). The statistical significance was determined by Student's *t* test.

located upstream of *tetR*. This result suggests the presence of two distinct promoters that express genes within the operon, one capable of directing transcription of the entire operon and the second driving the transcription of *tetR* and possibly NGO0392 (Fig. 1B).

DNA sequencing of the *norM* promoter region of strain FA19Str^R revealed the presence of a stretch of 7 T's between the −10 and −35 hexamers (Fig. 1B). In order to learn if this poly(T) stretch is common among gonococci, we performed a bioinformatics analysis of a 200-bp region upstream of the *norM* translational start codon using 31 gonococcal whole-genome sequences that are available online (http://blast.ncbi.nlm.nih.gov). This analysis revealed that the majority (77.4%) of gonococcal strains had a stretch of 6 T's (including strains FA1090 and certain WHO reference strains), while the rest (22.6%) had 7 T's (including strains FA19 and MS11). Using a PCR-generated product, we also sequenced this *norM* upstream region from 10 clinical isolates and found that 9 strains had the T-6 sequence (Table S1). Thus, we conclude that the T-6 sequence predominates in gonococci. In contrast, our analysis of whole-genome sequences of 86 *Neisseria meningitidis* strains that are publicly available (http://blast.ncbi.nlm.nih.gov) revealed that 85 (99%) have a *norM* promoter with a T-7 repeat sequence (data not presented).

Despite the difference in T repeat length, primer extension analysis revealed that the TSS positioned upstream of the *norM* promoter was possessed by strains FA19 (T-7) and FA1090 (T-6), which was identified as a C residue located 6 bp downstream of the −10 hexamer (data not presented; summarized in Fig. 1B). The level of the *norM* transcript in strains FA19Str^R and FA1090 was determined by quantitative RT-PCR (qRT-PCR) analysis using total RNA prepared from mid-logarithmic and late logarithmic cultures, which showed that the *norM* transcript was 2.4- and 4.2-fold higher in strain FA1090 than in FA19Str^R at the mid-logarithmic and late logarithmic phases, respectively (Fig. 2). Previous studies on the regulation of the *mtrCDE* efflux pump-encoding operon revealed that the distance between the −10 and −35 promoter hexamers can significantly influence transcription and levels of gonococcal resistance to antimicrobials exported by MtrCDE (9, 16). Guided by this work, we constructed *norM* mutants of FA1090 and FA19Str^R by insertional inactivation with the nonpolar *aphA-3* cassette and found that while the loss of *norM* influenced gonococcal susceptibility to NorM substrates (BE and EB), the impact was greater in strain FA1090 (T-6 promoter) than in strain FA19Str^R (T-7 promoter) (Table 1). In order to determine if inactivation of *norM* would increase susceptibility of a more recent gonococcal clinical isolate displaying

TABLE 1 Susceptibility of gonococcal strains to NorM substrates

Strain	MIC (μg/ml)[a]	
	BE	EB
FA19Str^R	5	1.25
FA19Str^R *norM*::*kan*	1.25	0.625
FA19Str^R n*orM*::*kan*C3	5	1.25
FA19Str^R *tetR*::*kan*	2.5	0.625
FA19Str^R *tetR*::*kan*C4	5	1.25
FA19Str$^R_{PnorMFA1090}$	10	2.5
FA 1090	20	5
FA 1090 *norM*::*kan*	1.25	0.625
FA 1090 *tetR*::*kan*	20	5

[a]All results are representative of those from from 3 or more independent determinations. BE, berberine; EB, ethidium bromide.

resistance to multiple antibiotics (6), we constructed a *norM*::*kan* transformant of strain H041 (T-6 promoter) (Table S1). We found that the loss of the NorM efflux pump decreased resistance of H041 to both BE and EB as well as solithromycin (4-fold decrease in MIC [Table S2]).

In order to determine the influence of the *norM* promoter T repeat sequence on gonococcal expression of the *norM* operon and resistance to NorM substrates, we exchanged the *norM* promoter region of FA19Str^R with that of FA1090 by transformation. DNA sequencing of a PCR fragment from a representative transformant strain (FA19Str$^R_{PnorMFA1090}$) confirmed the presence of the T-6 instead of T-7 repeat element (data not presented). Importantly, FA19Str$^R_{PnorMFA1090}$ displayed a 5-fold increase in expression of *norM* as assessed by qRT-PCR (Fig. 3) and displayed a 2-fold increase in resistance to EB and BE compared to parental strain FA19Str^R (Table 1). These combined results indicated that the length of the T tract can influence levels of gonococcal expression of *norM* and resistance to NorM substrates.

***trans*-Acting transcriptional regulation of *norM* and influence on antimicrobial resistance.** Bioinformatics analysis revealed that the putative TetR-like protein (216 amino acids) encoded by a gene within the *norM* operon is highly conserved in gonococci. This finding is exemplified by 100% amino acid identity of the protein that would be produced by strains FA19 and FA1090; the protein is also highly similar (97% identical) to a counterpart encoded by meningococci (data not presented). That this TetR-like protein can act as a transcriptional regulator was suggested by the presence of a helix-turn-helix DNA-binding domain at the N terminus (amino acids 15 to 61).

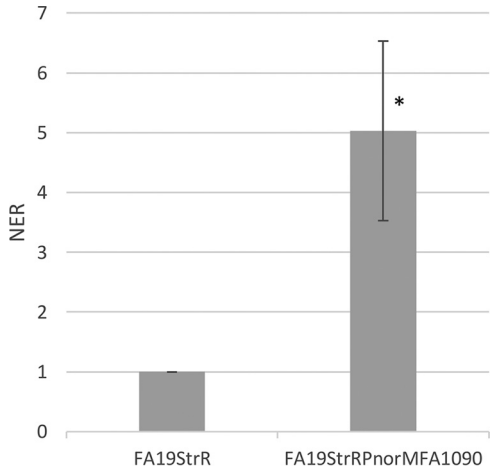

FIG 3 Quantitative RT-PCR results with *norM* in strains FA19Str^R and strain FA19Str$^R_{PnorMFA1090}$ at mid-logarithmic phase of growth. Error bars represent SDs from the means of 3 independent experiments. NER were calculated using 16S rRNA expression. *, $P = 0.01$. The statistical significance of the results was determined by Student's *t* test.

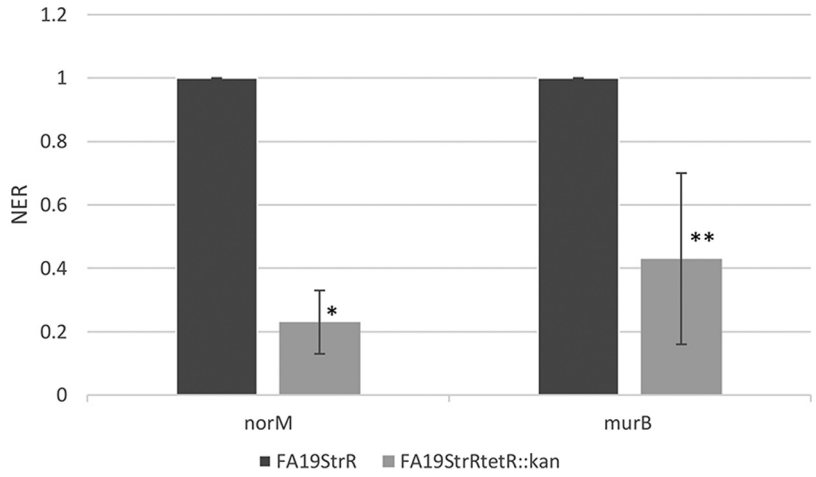

FIG 4 Quantitative RT-PCR results with *norM* and *murB* in strain FA19Str^R and strain FA19Str^R*tetR*::*kan* at the mid-logarithmic phase of growth. Error bars represent SDs from the means of 3 independent experiments. NER were calculated using 16S rRNA expression. *, $P = 0.0004$; **, $P = 0.022$ (for comparison of values of FA19Str^R *tetR*::*kan* versus FA19Str^R). The statistical significance of the results was determined by Student's *t* test.

Further, the position of *tetR* downstream of *norM* suggested that the TetR-like protein might exert transcriptional control of *norM* and other genes (e.g., *murB*) in the operon. In order to test this possibility, *tetR*::*kan* mutants of strains FA19Str^R and FA1090 were constructed and analyzed for changes in susceptibility to NorM substrates and levels of gene transcripts within the operon. We noted that with strain FA19Str^R, but not FA1090, insertional inactivation of *tetR* reproducibly resulted in 2-fold decrease in gonococcal resistance to BE and EB (Table 1). Although the impact of loss of the TetR-like protein was modest, complementation of the FA19Str^R *tetR*::*kan* strain with a pGCC4 construct bearing the wild-type *tetR* gene expressed at the *aspC-lctP* locus from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter restored wild-type levels of antimicrobial susceptibility (Table 1).

Based on the above-described results, we used strain FA19Str^R to ascertain if the TetR-like protein could regulate the *norM* operon. Results from qRT-PCR experiments indicated that loss of the TetR-like protein decreased expression of both *norM* and *murB* (Fig. 4), which is consistent with the transcriptional linkage of these genes by a promoter upstream of *norM*. To investigate if the TetR homolog could directly activate transcription of the *norM* operon, a recombinant His-tagged TetR protein was purified and employed in competitive electrophoretic mobility shift assays (EMSAs) that used a radiolabeled PCR probe containing 344 bp of DNA upstream of *norM*. The results from DNA binding experiments showed that TetR could bind to the probe in a specific manner, as binding could be inhibited by the unlabeled *norM* PCR product, but not by a nonspecific PCR probe (Fig. 5). Thus, this gene regulator serves as a transcriptional activator of the *norM* operon.

As a member of the MATE family of efflux pumps, the gonococcal NorM efflux pump has the capacity to export antimicrobial quaternary ammonium compounds (reference 15 and Table 1). The conservation of *norM* among gonococci suggests a role for NorM in the survival of gonococci. Thus, we hypothesized that NorM might also export host-derived antimicrobials and promote survival of gonococci during infection. However, using the established female mouse model of lower genital tract infection previously employed to determine the biological significance of the gonococcal MtrCDE efflux pump and cognate regulatory systems (11, 17), we did not detect a fitness or survival defect of gonococci (FA19Str^R and FA1090) bearing a null mutation in *norM* when competed with the wild-type parent strains (Fig. S2). It is important to note that this model may not fully recapitulate the repertoire of antimicrobials present at human female or male mucosal surfaces. Moreover, the infection model employed is

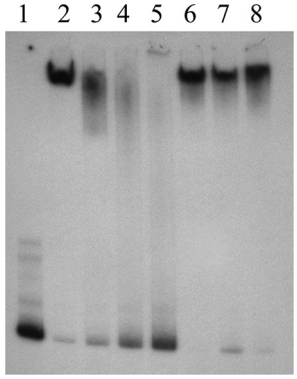

FIG 5 Competitive EMSA. The purified TetR-His protein binds to the *norM* promoter from strain FA19 in a specific manner. Lane 1, hot probe N11/N14* alone; lane 2, hot probe N11/N14* plus 8 μg of TetR-His; lanes 3 to 5, hot probe N11/N14* plus 8 μg of TetR-His plus 100×, 200×, and 400×, respectively, unlabeled N11/N14; lanes 6 to 8, hot probe N11/N14* plus 8 μg of TetR-His plus 100×, 200×, and 400×, respectively, unlabeled *rnpB*.

limited to the lower genital tract of female mice, and distinct antimicrobials in the upper tract that might serve as NorM substrates could exist. For instance, differences in the presence and level of antimicrobial peptides have been reported at mucosal surfaces of the human ectocervix and endocervix (18). Hence, the possibility that NorM promotes survival of gonococci during human infection by promoting resistance to a host antimicrobial cannot be discounted.

As with other bacterial efflux pump-encoding genes (8, 9, 16), we conclude that the gonococcal *norM* gene is subject to transcriptional control that influences its expression and levels of bacterial resistance to antimicrobials that can be exported by NorM. It is of interest that both *cis*- and *trans*-acting regulatory processes identified in this study can modulate expression of *norM* and that these regulatory schemes seem dependent on the length a poly(T) tract in the *norM* promoter. The majority of gonococci contain a T-6 tract in the promoter that seems to enhance transcription of *norM*. In contrast, strain FA19, which we have used extensively in our work on gonococcal efflux pumps (3, 4, 15–19), is representative of the minority of gonococcal strains harboring a T-7 sequence. Since mutations that increase or decrease spacing between the −10 and −35 hexamers can influence the fidelity of gene expression due to impacting interactions of RNA polymerase, as has been observed with nucleotide deletions or insertions within the *mtrR* promoter (16, 19), it is likely that the single T difference can impact *norM* expression in gonococcal strains with a T-6 or T-7 sequence by influencing promoter recognition by RNA polymerase.

In addition to this *cis*-acting regulatory mechanism, the TetR-like protein encoded by a gene within the *norM* operon can influence expression of *norM* in strain FA19. Importantly, the TetR DNA-binding protein also activates expression of *murB*, which is consistent with its transcriptional linkage with *norM*. It is of interest that a gene (*murB*) encoding an enzyme involved in the earliest steps of peptidoglycan biosynthesis (19) is coregulated with *norM* by both *cis*- and *trans*-acting regulatory schemes. Thus, the fidelity of early stages of peptidoglycan biosynthesis may be modulated by transcriptional control systems that also influence expression of *norM* and levels of gonococcal resistance to antimicrobials exported by NorM.

The chemical characteristics of known substrates of the gonococcal NorM efflux pump suggest that the clinical efficacy of future antimicrobials having similar properties may be influenced by constitutive or inducible changes in *norM* expression. We hypothesize that increased expression of *norM* coupled with other mutations could result in clinical resistance to antibiotics used in the future for treatment of gonorrhea. Based on earlier work with multidrug-resistant strain H041 by Golparian et al. (6) and our findings with this clinical isolate, this possibility should be considered for solithro-

mycin and its future use in treatment of gonorrhea. In a broader sense, derepression of bacterial efflux pump genes due to constitutive mutations as well as inducible activation systems should be a contributing factor by which gonococci (or other bacteria) might develop clinical resistance to antibacterials under development.

MATERIALS AND METHODS

Gonococcal strains, growth conditions, and determination of susceptibility to antimicrobial agents. Strains FA19, FA19Str^R and FA1090 were the primary gonococcal strains used in this study. These strains and their genetic derivatives as well as their susceptibilities to antimicrobials are presented in Table 1. We also sequenced the *norM* promoter regions from 10 clinical isolates (Table S1; see below). Gonococcal strains were grown overnight at 37°C under 5% (vol/vol) CO_2 on GC agar containing defined supplements I and II (9). The susceptibilities of test strains to antibiotics were determined by the agar dilution method and reported as the MIC (20). IPTG was added at a final concentration of 1 mM to MIC plates to allow complementation by the pGCC4 vector (21). Antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO). Solithromycin was obtained from Med Chem Express (Monmouth, NJ). E. coli strains were grown overnight at 37°C on LB agar.

Sequencing of the *norM* promoter. The *norM* promoter region was PCR amplified from gonococci using primers norMPac1 (5′-GATCTTAATTAACAATGCCGTCAAGTCGTTAAA-3′) and N10 (5′-CATCACGGT ATCGACGAAACGATGCCC-3′). The resulting PCR product was sequenced using norMPac1.

Construction of the *norM* and *tetR*-negative mutants and their complemented strains. The pBAD*norM*::*kan* construct (15) was transformed into FA19Str^R, and transformants were selected on GC agar supplemented with 50 μg/ml of kanamycin (Kan). FA19Str^R *norM*::*kan* transformants were verified by PCR and sequencing. The pGCC3 vector (21) was used to complement FA19Str^R *norM*::*kan*. This complementation system allows the integration of a wild-type copy of *norM* under its own promoter at the transcriptionally silent intergenic region between *lctP* and *aspC*. norMPac1 and norMpme1 (5′-GATCGTTTAAACTATCGGATG GGTTGCATGGT-3′) were used to amplify the *norM* gene and its own promoter. The resulting PCR product was cloned into the pGCC3 vector. The pGCC3*norM* construct was verified by sequencing and then transformed into FA19Str^R *norM*::*kan*. FA19Str^R *norM*::*kan*C3 transformants were selected on GC agar plates supplemented with 1 μg/ml of erythromycin (Ery) and verified by colony PCR and sequencing. The *norM* gene from FA1090 was amplified using primers N6 (5′-TCGGTATCGGATGGGTTGC-3′) and N4 (5′-ATGCTGCTCGACCTCGACC-3′), and the resulting PCR product was cloned into pBAD. pBAD*norM* was then digested by NaeI, and a nonpolar Kan resistance cassette from pUC18K (22) was inserted. The resulting construct was transformed into FA1090, and transformants were selected on GC agar plates supplemented with 50 μg/ml of Kan. FA1090 *norM*::*kan* transformants were verified by colony PCR and sequencing. To construct the *tetR*-negative mutant, pUC19 vector was digested by BamHI and EcoRI and PCR was performed on FA19 genomic DNA with primers E1tetR (5′-GGAATTCCTGTATGGGCAGGTTGATGTC-3′) and Sma1R (5′-TCCCCCGGGGGATCGCCCAACAATTCGGCAC-3′) and B1tetR (5′-CGCGGATCCGCGCTGAAGGGCTTCCAAATCGG-3′) and Sma1F (5′-TCCCCCGGGGGAACACA ATACCTTTACCCAAGC-3′). The resulting PCR products were ligated into pUC9 digested with BamHI and EcoRI to create a Sma1 site 356 bp downstream the ATG of the *tetR* gene. The resulting construct was verified by PCR and then digested by SmaI. The Kan resistance cassette was PCR amplified with *Pfu* using primers AphF (5′-GTGACTAACTAGGAGGAATAAAT-3′) and AphR (5′-GGTCATTATTCCCTCCAGGTA-3′) and pUC18K (21) as a template. The Kan resistance cassette was then cloned into the SmaI-digested pUC19*tetR*. The ligation was transformed into E. coli DH5α, and transformants were selected on LB agar plates supplemented with 50 μg/ml of Kan. The resulting construct was then verified by sequencing and used to transform strains FA19Str^R and FA1090 for resistance to Kan (50 μg/ml). The pGCC4 vector was used to complement FA19Str^R *tetR*::*kan*. This complementation system allows the integration of a wild-type copy of *tetR* under the control of an IPTG-inducible promoter at the transcriptionally silent intergenic region between *lctP* and *aspC*. tetRpac1 (5′-GATCTTAATTAAAGCCTGTAAATCCAAGGAGTA-3′) and tetRpme1 (5′-GATCCGTTTAAACCGTCTGAAGGCTG ATTCGG-3′) were used to amplify the *tetR* gene. The resulting PCR product was cloned into the pGCC4 vector. The pGCC4*tetR* construct was verified by sequencing and then transformed into FA19Str^R *tetR*::*kan*. Transformant FA19Str^R *tetR*::*kan*C4 was obtained by selection with 1 μg/ml of chloramphenicol (CMP), and the genotype was verified by colony PCR.

Construction of FA19Str$^R_{P norM FA1090}$. Primers N5 (5′-GGATGAACATCGGCACCTTG-3′) and norMPac1 (5′-GATCTTAATTAACAATGCCGTCAAGTCGTTAAA-3′) were used to amplify the *norM* promoter region of strain FA1090. The resulting 1,385-bp PCR product was then transformed into strain FA19Str^R, and transformants were selected on GC agar containing defined supplements I and II supplemented with EB (1 μg/ml). Transformants were then verified by DNA sequencing of a PCR product generated using primers N5 and norMPacI.

Mapping transcriptional start sites by primer extension analysis. Total RNA from strains FA19 and FA1090 was prepared at the late logarithmic phase of growth in GC broth as described above by the TRIzol method as directed by the manufacturer (Thermo Fisher Scientific, Waltham, MA). Primer extension experiments were performed as described previously (9, 16) on 6 μg of total RNA with primer N11 (5′-CGGTCAGCAGGCGGATTTCTTTCAGG-3′) for *norM* and primer tetRPE (5′-TGGCGTCGATGATGCGGG-3′) for *tetR*. Primer extension transcription start sites (TSSs) were determined by electrophoresis of the extension products on a 6% (wt/vol) DNA sequencing acrylamide gel adjacent to reference sequencing reactions.

Qualitative and quantitative RT-PCR. For RT-PCR and qRT-PCR analyses of transcript levels, RNA was extracted from strains FA19Str^R, FA1090, their respective *norM*-negative and *tetR*-negative mutants, and FA19Str$^R_{P norM FA1090}$ grown in GC broth plus supplements to mid-logarithmic and late logarithmic

phases by the TRIzol method as directed by the manufacturer (Thermo Fisher Scientific, Waltham, MA). Genomic DNA (gDNA) was removed by RNase-free DNase treatment and gDNA Wipeout (Qiagen, Germantown, MD). The resulting RNA was then reverse transcribed to cDNA using the QuantiTect reverse transcriptase kit (Qiagen). Quantitative real-time RT-PCR was performed using the generated cDNA, and results were normalized to 16S rRNA expression for each strain. Primers 16Smai_qRTF (5′-CCATCGGTA TTCCTCCACATCTCT-3′) and 16Smai_qRTR (5′-CGTAGGGTGCGAGCGTTAATC-3′) were used for the 16S rRNA, while primers tetR_qRTR (5′-TTCCACATCAGAGGGCAACA-3′) and tetR_qRTF (5′-GCAACATCAGCA CCAACCAT-3′) were used for the *tetR* gene. Primers N4 and N10 (5′-CATCACGGTATCGACGAAACCGATG CCC-3′) were used for the *norM* gene. Primers murB_qRTF (5′-TAAACACGCCGACGAATTGC-3′) and murB_qRTR (5′-TCTCGCGTATGCCCTTGTTT-3′) were used for the *murB* gene. All qRT-PCRs were performed in experimental duplicates and biological triplicates. For RT-PCR, random hexamers were used for the reverse transcription, while murB_qRTF and tetRSma1R (5′-TCCCCCGGGGGATCGCCCAACAATTCGG CAC-3′), N8 (5′-CCGTTCGGACTGACAGCG-3′), and murB_qRTR were used for PCRs on the cDNA, for the controls without reverse transcriptase, and for the genomic DNA.

Purification of the TetR protein. Construction of pET15b*tetR* was done by amplifying the *tetR* open reading frame using the primers pETtetR_F (5′-TCGATCCATATGCCCGTGACCCGCATTG-3′) and pETtetR_R (5′-GATCGGATCCTTACGGGTTGCCGTTGCCG-3′). The resulting PCR product along with the pET15b vector was digested with NdeI and BamHI, ligated overnight, and transformed into *E. coli* DH5α. The pET15b*tetR* construct was confirmed by sequencing with vector-specific primers T7F (5′-TTAATACGACTCACTATAGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′). For protein expression, pET15b*tetR* was transformed into *E. coli* BL21(DE3) cells. Cultures (5 ml) of BL21(DE3)-pET15b*tetR* were grown overnight at 30°C and added to 500 ml of LB broth the next morning. The culture was grown at 30°C until mid-log phase, then induced with 0.3 mM IPTG, and grown overnight at 30°C. Cells were harvested and resuspended in 20 ml of 10 mM Tris (pH 7.5)–200 mM NaCl, and EDTA-free protease inhibitor was added to the bacterial suspension. The cells were lysed by use of a French press cell as described previously (23), membranes and unbroken cells were removed by centrifugation at 100,000 $\times$ *g*, and the supernatant was collected and filtered. TetR-His was purified over a 2-ml nickel-nitrilotriacetic acid (Ni^{2+}-NTA) column. After the supernatant was allowed to flow over the Ni^{2+}-NTA column, the resin was washed successively with buffer containing 20 mM and 50 mM imidazole to remove contaminants and weakly bound proteins, and TetR-His was eluted successively with buffer containing 100 and 200 mM imidazole. The fractions containing TetR-His were concentrated, and the imidazole-containing buffer was removed by dialysis into storage buffer (10 mM Tris-HCl [pH 7.5], 200 mM NaCl, and 1 mM EDTA). Dithiothreitol and glycerol were added to final concentrations of 1 mM and 10% (wt/vol), respectively.

EMSA. A DNA probe encompassing the *norM* promoter region was amplified by PCR from FA19 genomic DNA using either of the upstream primers N11 (5′-CGGTCAGCAGGCGGATTTCTTTCAGG-3′) and N14 (5′-TCTGCCTTCTGTTTTATCCTG-3′). When making radioactive probes, the desired PCR products were labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (New England BioLabs). The labeled DNA fragments were incubated with 8 μg of TetR-His in 30 μl of reaction buffer at room temperature. For the competition assays, the same nonlabeled probe or a nonlabeled PCR product using primers rnpBF1 (5′-CGGGACGGGCAGACAGTCGC-3′) and rnpBR1 (5′-GGACAGGCGGTAAGCCGGGTTC-3′) were added in the reaction. Samples were subjected to electrophoresis in a 6% native polyacrylamide gel at 4°C, followed by autoradiography as described previously (23).

Competitive infection of female mice to measure gonococcal fitness. The female mouse model of lower genital tract infection was used to assess whether loss of NorM imposed an *in vivo* fitness cost or benefit. Mice were inoculated vaginally with equal numbers of CFU of parent strains FA19Str^R and FA1090 with their respective n*orM*::*kan* transformants, and the relative numbers of mutant and wild-type bacteria recovered were compared. The details of the experimental procedures have been described previously (11, 12, 23). Animal experiments were conducted in the laboratory animal facility at USUHS, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the USUHS Institutional Animal Care and Use Committee.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00821-18.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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We have no competing interest to declare.

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