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Control of *gdhR* Expression in *Neisseria gonorrhoeae* via Autoregulation and a Master Repressor (MtrR) of a Drug Efflux Pump Operon

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**ABSTRACT** The MtrCDE efflux pump of *Neisseria gonorrhoeae* contributes to gonococcal resistance to a number of antibiotics used previously or currently in treatment of gonorrhea, as well as to host-derived antimicrobials that participate in innate defense. Overexpression of the MtrCDE efflux pump increases gonococcal survival and fitness during experimental lower genital tract infection of female mice. Transcription of *mtrCDE* can be repressed by the DNA-binding protein MtrR, which also acts as a global regulator of genes involved in important metabolic, physiologic, or regulatory processes. Here, we investigated whether a gene downstream of *mtrCDE*, previously annotated *gdhR* in *Neisseria meningitidis*, is a target for regulation by MtrR. In meningococci, GdhR serves as a regulator of genes involved in glucose catabolism, amino acid transport, and biosynthesis, including *gdhA*, which encodes an L-glutamate dehydrogenase and is located next to *gdhR* but is transcriptionally divergent. We report here that in *N. gonorrhoeae*, expression of *gdhR* is subject to autoregulation by GdhR and direct repression by MtrR. Importantly, loss of GdhR significantly increased gonococcal fitness compared to a complemented mutant strain during experimental murine infection. Interestingly, loss of GdhR did not influence expression of *gdhA*, as reported for meningococci. This variance is most likely due to differences in promoter localization and utilization between gonococci and meningococci. We propose that transcriptional control of gonococcal genes through the action of MtrR and GdhR contributes to fitness of *N. gonorrhoeae* during infection.

**IMPORTANCE** The pathogenic *Neisseria* species are strict human pathogens that can cause a sexually transmitted infection (*N. gonorrhoeae*) or meningitis or fulminating septicemia (*N. meningitidis*). Although they share considerable genetic information, little attention has been directed to comparing transcriptional regulatory systems that modulate expression of their conserved genes. We hypothesized that transcriptional regulatory differences exist between these two pathogens, and we used the *gdh* locus as a model to test this idea. For this purpose, we studied two conserved genes (*gdhR* and *gdhA*) within the locus. Despite general conservation of the *gdh* locus in gonococci and meningococci, differences exist in noncoding sequences that correspond to promoter elements or potential sites for interacting with DNA-binding proteins, such as GdhR and MtrR. Our results indicate that implications drawn from studying regulation of conserved genes in one pathogen are not necessarily translatable to a genetically related pathogen. 

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**Neisseria gonorrhoeae** 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 of an estimated 78 million infections in 2012 (1). The capacity of gonococci to develop resistance to antibiotics is now of great concern with the recent emergence of strains resistant to current and past frontline antibiotics (2–5). With respect to the clinical efficacy of antibiotic treatment regimens, evidence has been presented that overproduction of the gonococcal MtrCDE efflux pump due to cis- or trans-acting mutations that elevate transcription of mtrCDE can contribute to clinically relevant levels of antibiotic resistance (6–10). The mtrR gene, which encodes the master repressor (MtrR) of the mtrCDE efflux pump operon (8–10), is located immediately upstream of the mtrCDE operon (Fig. 1). The mtrR and mtrCDE genes are oriented away from each other and have overlapping promoters. Transcription of mtrCDE is repressed when MtrR is bound to the mtrCDE promoter, which overlaps the mtrR promoter (7, 8). Point mutations in the MtrR-binding site (8, 10), a single base pair deletion within a 13-bp inverted repeat sequence in the mtrR promoter (7), a point mutation that creates a new promoter (9), or missense mutations that cause radical amino acid replacements within the helix-turn-helix DNA-binding motif of MtrR can increase mtrCDE expression and antimicrobial resistance (8, 10). Such elevated expression of mtrCDE also increased gonococcal fitness in vivo when assessed by use of an experimental female murine lower genital tract infection model (11). In addition to regulating mtrCDE, MtrR serves as a global regulator of gonococcal genes (12) and directly or indirectly activates or represses at least 65 genes outside the mtrCDE locus. Included in these so-called "off-target" genes are those that are involved in the stress response (ppgH), amino acid synthesis (glnA and glnE), peptidoglycan biosynthesis (ponA), and regulation of gene expression (farR); the regulatory properties of MtrR have been summarized elsewhere (2, 13). Our analysis of the whole-genome sequence of strain FA1090 (http://www.genome.ou.edu) revealed an open reading frame (termed NGO 1360) positioned 943 bp downstream of the mtrCDE operon that encodes a transcriptional regulator previously annotated GdhR in Neisseria meningitidis (Fig. 1). GdhR belongs to the bacterial GntR family of proteins, which serve as gene regulators and contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain involved in effector binding and oligomerization (14). In N. meningitidis, which causes often deadly meningitis or fulminant septicemia (15), GdhR regulates the expression of a number of genes, some of which are involved in metabolism (16, 17). Meningococcal GdhR has been reported to activate gdhA, which encodes an NADP-specific L-glutamate dehydrogenase (16). Given the prominent role of MtrR in modulating gonococcal resistance to antimicrobials, its control of genes involved in metabolism, its influence on the fitness of gonococci in an experimental infection model, and its proximity to gdhR, we tested the capacity of MtrR to regulate expression of gdhR in N. gonorrhoeae, as well as the ability of GdhR to regulate genes in the gdh locus (gdhR and gdhA). Our results suggest that gonococcal and meningococcal GdhRs have distinct regulatory properties that are driven by differences in promoter utilization of regulated genes and emphasize
the importance of bacterial species-specific studies for examining regulatory properties of a common DNA-binding protein.

RESULTS

The gdh locus in N. gonorrhoeae. Similar to N. meningitidis, the gdh locus in N. gonorrhoeae FA19 is positioned 954 bp downstream from the mtr locus (Fig. 1) and contains two open reading frames, gdhR, which encodes a GntR-like DNA-binding protein, and gdhA, which encodes L-glutamate dehydrogenase. The gonococcal GdhR and GdhA proteins are 97% and 98% identical, respectively (data not shown) to the equivalent proteins described for meningococci (16). The end of gdhA is positioned 238 bp from the end of gdhR and is transcribed in the opposite direction; transcription of gdhA has been reported to be activated by GdhR in meningococci (16).

Bioinformatic analysis (http://www.ncbi.nlm.nih.gov) revealed that the 200 bp up-stream of gdhR in five gonococcal strains (FA19, FA1090, MS11, FA6140, and F89) were identical, except for a C-to-T change in FA1090 29 bp upstream of the translation start codon but after the transcription start site (TSS) (see below). In these same gonococcal strains, 100% identity was noted for the 500-bp sequence upstream of gdhA (data not shown). When the same regions from N. meningitidis strain MC58 in the corresponding upstream regions of gdhR and gdhA were used in a BLAST search against whole-genome sequences, two other meningococcal isolates (LNP21362 and H44/76) were found to have identical sequences, while 10 others showed 99% identity (data not shown). Thus, our use of gonococcal strain FA19 for comparison to meningococcal strain MC58 is suitable for determining differences in regulation of the gdh locus in these pathogens. Although the DNA sequences of the gdh loci in gonococci and meningococci are very similar, important differences exist, especially in the location of promoters and potential cis-acting regulatory sequences (see Fig. 3 for the CE insertion in the meningococcal gdhR and see Fig. 6 for the gdhA promoters, respectively). We hypothesized that the differences in these sequences between gonococci and meningococci could impact GdhR-mediated regulation of gene expression in gonococci and influence gonococcal biology. Accordingly, we sought to identify a phenotype that is linked to GdhR production in gonococci and then to examine regulation of gdhR expression and the capacity of GdhR to control model genes.

Loss of GdhR impacts in vivo fitness of gonococci independently of the mtr locus. Given the close location of gdhR to the mtr locus (Fig. 1), we determined whether expression of GdhR influences transcription of mtrCDE and resistance of gonococci to antimicrobials recognized by the MtrCDE efflux pump (2, 6–12). For this purpose, we constructed a gdhR null mutant as well as a complemented strain. We found that the wild-type parent (FA19), the gdhR::kan mutant, and the complemented strain displayed identical levels of susceptibility to antimicrobials (erythromycin [Erm] MIC, 0.25 μg/ml; penicillin MIC, 0.015 μg/ml; Triton X-100 MIC, 100 μg/ml), and these MICs varied according to the levels of the MtrCDE efflux pump (6, 7, 11, 18). Moreover, results from quantitative reverse transcription-PCR (qRT-PCR) experiments indicated that expression of mtrC (the first gene in the mtrCDE operon [Fig. 1]) was not impacted by loss of GdhR (data not shown).

We also assessed the fitness of the gdhR mutant relative to wild-type and complemented mutant bacteria during competitive infection of the lower genital tract of female BALB/c mice. Mice were inoculated vaginally with mixed bacterial suspensions containing similar numbers of the strains to be subjected to competition (total CFU per mouse, 10^9), and the number of each strain of bacteria recovered from vaginal swab suspensions on days 1, 3, and 5 postinoculation was expressed as a competitive index (CI), as described in Materials and Methods. Ninety percent of the mice were colonized throughout the 5-day study period, with 10^4 to 10^5 CFU/vaginal swab suspension recovered from the majority of mice on day 1 postinoculation and >10^4 CFU/ml on day 5 postinoculation (see Fig. S1 in the supplemental material). There was no difference in the relative fitness of the wild-type parental strain bacteria and the complemented gdhR::kan mutant strain (Fig. 2C). Interestingly, however, the gdhR::kan mutant strain
was significantly more fit than the complemented gdhR mutant strain on days 1, 3, and 5 postinoculation (geometric mean CIs, 18, 49, and 81, respectively) (Fig. 2B) compared to the CIs for mice infected with the complemented mutant versus the wild-type strain (Fig. 2C). Competitive infections between the gdhR::kan mutant strain and the parental strain also showed elevated CIs (geometric mean CIs, 3.4, 30, and 21 on days 1, 3, and 5 postinoculation) (Fig. 2A), although the differences were not statistically significantly different from those for the complemented mutant strain versus the wild-type strain. Consistent with the gdhR mutant strain outcompeting the GdhR-expressing bacteria in vivo, only mutant CFU were recovered from some mice inoculated with wild-type or complemented mutant bacteria mixed with the gdhR::kan mutant bacteria on days 3 and 5 (Fig. 2A and B, open circles). These results indicated that GdhR production negatively influences the in vivo fitness of gonococci; importantly, loss of GdhR did not impact the growth rate of gonococci in GC broth (data not shown). This result
suggested that GdhR may be a negative regulator of in vivo fitness; therefore, we sought to determine how gdhR is regulated in gonococci and if GdhR controls expression of model genes (gdhR and gdhA) previously studied in meningococci (16). MtrR is a direct repressor of gdhR expression. MtrR exerts transcriptional repression on the mtrCDE operon by binding to a promoter located upstream of mtrC (8, 19). Given the close proximity of gdhR to the mtr locus in both gonococci and meningococci, we asked if MtrR regulates gdhR expression in gonococci. Although gdhR was not previously assigned to be an MtrR-regulated gene in an earlier transcriptional profiling study that employed gonococcal RNA prepared from mid-logarithmic-phase cultures (12), we reexamined MtrR control of gdhR for two reasons. First, the presence of a putative MtrR-binding site upstream of the gdhR gene (Fig. 3A) suggested such control is possible. This MtrR-binding site (boxed in Fig. 3A) was 60% homologous to the MtrR-binding site on the mtrC promoter region (Fig. 3B). Second, the work of Mercante et al. (20) showed that a different transcriptional factor (MpeR) expressed in gonococci displays growth phase-dependent regulons.

Results from qRT-PCR experiments indicated that deletion of mtrR increases gdhR transcription, supporting the hypothesis that MtrR controls gdhR expression in gonococci by functioning as a repressor of this gene in the late-logarithmic phase of growth (Fig. 3C). Using primer extension (PE) analysis (see Fig. S2), we identified three gdhR TSSs, positioned 73, 72, and 70 nucleotides upstream of the start of translation of gdhR. These TSSs allowed us to identify a promoter element (5′-TAGAAT-3′ for the 10 hexamer and 5′-TTGACG-3′ for the -35 hexamer) 81 bp upstream of the ATG translational start (Fig. 3A). Importantly, the putative MtrR-binding site overlapped the -10 hexamer sequence of the predicted gdhR promoter. Based on this promoter mapping and the identification of a predicted MtrR-binding site within the putative gdhR promoter, we tested if MtrR bound in a specific manner upstream of the gdhR coding sequence, and we used an electrophoretic mobility shift assay (EMSA) for this purpose. We found that 2 μg of MBP-MtrR was sufficient to completely shift a 32P-labeled probe, termed R3/R2, that consisted of 393 bp of sequence upstream of the gdhR translational start (Fig. 4, lane 2). In order to better localize the MtrR-binding site(s) within this region, we performed a competitive EMSA with nonradioactive fragments of the R3/R2 probe used in the aforementioned EMSA. Binding competition assays showed that a smaller probe encompassing the gdhR promoter and its downstream region (probe R4/R2 [Fig. 3A]) competed with MtrR binding to the labeled R3/R2 probe.
(Fig. 4, lanes 3 and 4), while a fragment located upstream of the promoter (R3/R5 [Fig. 3A]) did not (Fig. 4, lanes 5 and 6). Accordingly, we propose that MtrR represses gdhR expression by binding within the promoter sequence that contains the predicted MtrR-binding site.

GdhR regulation of model genes in gonococci. We selected two GdhR genes for study: gdhR and gdhA, which constitute the gdh locus (Fig. 1). We chose these 2 genes to test if gdhR is subject to autoregulation by its gene product and because gdhA has been reported to be a GdhR-activated gene in meningococci (16) and is positioned near gdhR in both pathogens.

Pagliarulo et al. (16) suggested that the meningococcal gdhR transcript originates in a Correia element (CE) (21) located upstream of the gdhR gene. An examination of 23 publicly available gonococcal genome sequences revealed that this CE is absent in the gdhR promoter region (data not shown). However, a putative GntR-like binding site (5′-TGTCATTA-3′) was identified between the −10 and the −35 sites of the gonococcal gdhR promoter overlapping the predicted MtrR-binding site (Fig. 3A, underlined in green). In order to investigate autoregulation of gdhR, qRT-PCR analysis of total RNA prepared from mid- and late-log-phase cultures of strains FA19 and FA19 gdhR::kan was performed. The expression of gdhR was increased by 4-fold at mid-log phase and by a little more than 2-fold at late-log phase in the GdhR-negative mutant compared to the parental strain (Fig. 5A). We hypothesize that insertion of a CE upstream of gdhR in meningococci, but not in gonococci, results in the utilization of distinct gdhR promoters.

**FIG 4** Competitive EMSAs. The MtrR-binding site located on fragment R4/R2 has the highest affinity for the MtrR protein. Lanes: 1, probe R3/R2* alone; 2, probe R3/R2* plus 2 μg of MtrR; 3, probe R3/R2* plus 2 μg of MtrR plus 50× unlabeled R3/R2; 4, probe R3/R2* plus 2 μg of MtrR plus 100× unlabeled R3/R2; 5, probe R3/R2* plus 2 μg of MtrR plus 50× unlabeled R3/R5; 6, probe R3/R2* plus 2 μg of MtrR plus 100× unlabeled R3/R5; 7, probe R3/R2* plus 2 μg of MtrR plus 50× unlabeled R4/R2; 8, probe R3/R2* plus 2 μg of MtrR plus 100× unlabeled R4/R2; 9, probe R3/R2* plus 2 μg of MtrR plus 50× rnpB; 10, probe R3/R2* plus 2 μg of MtrR plus 100× rnpB. An asterisk indicates a radioactive probe. The location of the different gdhR probes are shown in Fig. 3A.

**FIG 5** Quantitative RT-PCR results with gdhR (A) or gdhA (B) in wild-type (WT) and gdhR-negative strains at the mid- and late-logarithmic phases of growth. Error bars represent standard deviations from the means of three independent experiments. Normalized expression ratios (NER) were calculated using 16S rRNA expression. *, P = 0.011; **, P = 0.008; NS, not significant.
by these two related pathogens. Therefore, competing mechanisms of gdhR regulation by MtrR and GdhR itself may not occur in meningococci. In this respect, it is important to note that most meningococci encode an MtrR protein that contains loss-of-function mutations in mtrR (22, 23), while nearly 80% of gonococci encode a wild-type MtrR (reviewed in reference 2). Previous work indicated that expression of gdhA in meningococci is directed by two promoters, only one of which is regulated by GdhR (16). The GdhR-activated promoter of gdhA in meningococci has a putative GdhR-binding site (5′-TGTCAACA-3′) upstream of the −35 hexamer, based on similarity to the known GntR-binding site (5′-TGTcaACA-3′; the lowercase letters refer to nucleotides that differ from consensus GntR-binding site) in other bacteria (14); this site is also located in the corresponding gonococcal sequence (underlined in Fig. 6). In order to investigate whether GdhR binds to this site in gonococci, as was shown previously in meningococci, we performed EMSA competition analysis using purified gonococcal His-tagged GdhR protein. The results showed that gonococcal GdhR binds specifically to a DNA fragment encompassing the GntR-binding site present upstream of gdhA in gonococci (Fig. 7).

FIG 6  Alignment of gdhA promoters from gonococcal strain FA19 (top) and meningococcal strain MC58 (bottom). The TSSs determined by primer extension experiments for strain FA19 identified in this study and that of MC58 as reported by Pagliarulo et al. (16) are represented in blue, green, and red, with their respective putative promoter elements. The consensus binding sequence for the GntR protein is underlined. The gdhA translation start site is represented in purple.
In meningococci, the presence of another gdhA TSS was detected 207 bp upstream of the ATG translational start codon (represented in blue in Fig. 6). However, a GdhR-binding site was not identified within or near this distal promoter in meningococci. Interestingly, we did not detect a TSS 207 bp upstream of the ATG in gonococci. This could be due to the presence of a mutation which changes the −10 hexamer from 5′-TAATTA-3′ in meningococcal strain MC58 to 5′-TAACCTA-3′ in gonococcal strain FA19. To determine if gonococci have an additional promoter(s) for gdhA transcription, we used PE analysis to identify transcription start sites. The results suggested the presence of two promoters (Fig. 6). We identified a TSS located 8 bp downstream from a −10 hexamer that constitutes the homolog of the above-mentioned meningococcal promoter (shown in red). We also identified three TSSs located upstream of a non-consensus −10 hexamer (5′-ATTATTT-G-3′) that is spaced 17 nucleotides from a weak −35 hexamer (5′-ATATGGG-3′) (represented in green in Fig. 6). Importantly, this putative promoter has the previously identified GdhR-binding site (underlined sequence in Fig. 6) between its −10 and −35 hexamer sequences. This second gonococcal promoter was not identified in meningococci. Based on the location of the two putative gdhA promoters in gonococci, GdhR could impact expression of gdhA from both promoters through interaction with the identified GdhR-binding site.

Taken together, our promoter mapping studies suggest that differences exist regarding gdhA transcription in gonococci and meningococci and that a GdhR-binding site influences gdhA transcription in gonococci. In order to assess promoter utilization in gonococci and any influence of GdhR on gdhA transcription, we performed qRT-PCR analysis on RNA prepared from strain FA19 and its isogenic gdhR::kan mutant at mid- and late-logarithmic phases of growth. Unlike its influence on gdhR expression, loss of GdhR did not impact gdhA expression in either mid-log- or late-log-phase cultures (Fig. 5B). One explanation for why we did not observe changes in gdhA expression is that GdhR binds upstream of the most proximal promoter (represented in red in Fig. 6) and inhibits the binding of the RNA polymerase to the second gdhR promoter (represented in green in Fig. 6), but it does not interfere with transcription from the proximal promoter, just as in meningococci. Consequently, when GdhR is present, the most proximal promoter (represented in red in Fig. 6) is the primary promoter used for transcription of gdhA. When GdhR is absent, the most distal promoter (green in Fig. 6) becomes the primary promoter for gdhR transcription.

**DISCUSSION**

Our interest in gdhR was spurred by its close location to the mtr locus, which encodes the tripartite RND-type efflux pump MtrC-MtrD-MtrE and a transcriptional repressor (Fig. 1). We hypothesized that GdhR and MtrR might have cross-regulatory activities on the mtr and gdh loci, respectively. While we did not find evidence that GdhR regulates mtrCDE or antimicrobial resistance, we did find that its loss significantly increased fitness of gonococci during an experimental infection of the lower genital tract of female mice. This experimental model of infection has been used by us to show the importance of the MtrCDE pump for gonococcal survival in vivo and that gradients of fitness can be observed (11, 24), depending on the presence of distinct cis- or trans-acting mutations that influence mtrCDE expression (2).

Although the observed increase in fitness in the gdhR-negative mutant of strain FA19 was not as dramatic as previous findings (11) with an mtrR-negative mutant (5- to 10-fold versus ca. 100-fold), the impact on fitness was reproducible and significant. Therefore, we sought to define regulatory systems that influence gdhR expression in gonococci, and we compared our results with those obtained by others working on regulation of meningococcal genes controlled by GdhR. We discovered two trans-acting regulatory systems that were not previously observed in meningococci: (i) MtrR, which can directly repress expression of gdhR (Fig. 3), and (ii) GdhR, which can repress itself (Fig. 4). In the first instance, MtrR regulation of gdhR emphasizes the global regulatory properties of this DNA-binding protein in gonococci. It is noteworthy that this mechanism does not extend to most meningococcal isolates, since they harbor loss-of-function mutations in mtrR (22, 23). In the second
instance, GdhR autoregulation of gdhR may be unique to gonococci, because the presence of a CE in this region in meningococci, but not gonococci, likely influences promoter utilization and GdhR binding.

GdhR has been previously studied in meningococci for its capacity to regulate genes involved in metabolism, but heretofore it has not been investigated for its regulatory properties in gonococci. Although GdhR has been reported to activate expression of gdhA and gltT, which encodes an L-glutamate transporter that appeared to be essential for full virulence in a rodent model of invasive meningococcal disease (25), its capacity to autoregulate its own gene or be controlled by trans-acting factors has not been elucidated in either pathogen. The work presented here illustrates that two genetically related pathogens can encode the same transcription factor (e.g., GdhR) and have conserved target genes (e.g., gdhA), gene regulatory principles that have evolved for one pathogen may not necessarily apply to the related pathogen. Thus, although the GdhRs in meningococci and gonococci are identical, regulation of one target, gdhA, is distinct. While gdhA is a GdhR-activated gene in meningococci, based on quantitative analysis of levels of mRNA transcripts, our work failed to reveal differences in gdhA transcript levels in isogenic GdhR-positive and -negative gonococci. This does not mean that GdhR cannot activate gdhA in gonococci. We draw this conclusion because PE analysis suggested the presence of two promoters in gonococci that could direct transcription and be differentially impacted (activated or repressed) by GdhR, thereby giving the impression of lack of gdhA regulation. We propose that differences in the DNA sequence in the gdh locus in gonococci versus meningococci result in distinct promoter utilization and regulation.

Additional studies are needed to define the GdhR regulon in gonococci in order to understand the role of this DNA-binding protein in controlling genes important for metabolism and in vivo fitness of N. gonorrhoeae. In this respect, the increased fitness of the gdhR mutant observed on days 3 and 5 (Fig. 2) corresponds to the time inflammation is detected in the mouse model (26). With the protocol we use, proinflammatory cytokines and chemokines begin to increase on day 3 and peak on day 5, along with a peak polymorphonuclear leukocyte influx on day 5; expression of antimicrobial peptides also peaks on day 5 (A. E. Jerse et al., unpublished data). Thus, it is possible that gdhR may downregulate genes important in the invasion of innate defenses. Depression or induction of genes that are important in growth and metabolism could also contribute to the increased fitness observed with the gdhR mutant. With these possibilities in mind, which form the basis for future studies, our results emphasize that pathogen-specific regulatory actions of a common DNA-binding protein likely exist even between closely related bacteria (e.g., gonococci versus meningococci) and that differences in gene control, which could be influenced by cis-regulatory elements, may have consequences for the overall biology of members in same genus.

MATERIALS AND METHODS

Gonococcal strains, growth conditions, and determination of susceptibility to antimicrobial agents. Strains used in this study are presented in Table 1. Gonococcal strains were grown overnight at 37°C under 5% (vol/vol) CO₂ on GC agar containing defined supplements I and II (27). Determination of susceptibility of test strains to antibiotics was performed by the agar dilution method, and results were reported as the MIC (18). Antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO). Escherichia coli strains were grown overnight at 37°C on LB agar.
Constriction of the gdhR-negative mutant and its complemented strain. The plasmid construct used to insertionally inactivate the gdhR gene was created in pUC18s, which is pUC18 containing the 10-bp uptake sequence preceding the HindIII site in the polylinker. Overlap extension PCR was used to amplify the gdhR gene containing an internal Xbal site by using the upstream primer S′-GepR-new-Bam (S′-AGAGATCTCAGAAAGCTGTAGAACCCGCTACGA-3′) and midstream reverse primer 3′-GepR-mid-Xbal (3′-CTTCCTCAAACTTTTCTAGACAAAACCGAATCCGC-3′) to amplify the first half of the gene and the midstream forward primer S′-GepR-mid-Xbal (S′-GCGGATTCCGGTTTTGTCTAGAATAAGAAAATTGGAAGAAG-3′) and the downstream reverse primer 3′-gepR-EcorI (S′-AGAAGATTCAATACCTCCAAATCCTGGCAC-3′) to amplify the second half of the gene. The two midstream primers are complementary to one another, and so in the second round of PCR, aliquots of each amplification product were used as the template with the forward upstream and reverse downstream primers to generate the gdhR gene containing an Xbal site in the middle of the gene. This modified gdhR construct was digested with EcoRI and BamHI and cloned into similarly digested pUC18s in which the existing Xbal site was destroyed by cutting with Xbal, filling in the 5′-overhangs with Klenow fragment, and religation. To create the gdhR inactivation construct, a blunt-ended kanamycin (Kan) resistance cassette derived from pLG338 (28) was ligated into the pUC18s-gdhR plasmid at the filled-in Xbal site in the middle of the gdhR gene. This construct was linearized by digestion with EcoRI and used to transform FA19Strr (FA19 containing the rpsL allele from FA1090 that confers resistance to streptomycin [Str]), with transformants selected on GC agar plates containing 50 μg/ml Kan and verified by colony PCR and sequencing.

The pGCC3 vector (29) was used to complement FA19Strr gdhR::kan because it allows the integration of a wild-type copy of gdhR under its own promoter at the transcriptionally silent intergenic region between ictP and aspc. (pGCC3 is a derivative of pUCE258 that encodes pIctP) (5′-GATCTTTAAGACCGGATCCGATGTTTTT-3′) and pm1gepIPM (5′-GATCTTTAAGACCGGATCCGATGTTTTT-3′) were used to amplify the gdhR gene. The resulting PCR product was cloned into the pGCC3 vector. The pGCC3-gdhR construct was verified by sequencing and then transformed into FA19Strr gdhR::kan. FA19Strr gdhR::kanC3 transformants were selected on GC agar plates supplemented with 1 μg/ml of Erm and verified by colony PCR.

Competitive murine infection. Mixed bacterial inocula containing similar numbers of the two strains being tested were prepared by harvesting test strains from GC agar plates grown for 18 to 21 h and suspending the bacteria in 4 to 5 ml of 1 × phosphate-buffered saline (PBS). The suspensions were passed through a 1.2-μm filter to remove bacterial aggregates and, using previously filtered determined standard values for each strain relating readings of the optical density at 600 nm (OD600) to CFU counts, the bacterial suspensions were diluted to ~5 × 10⁹ CFU/ml before being mixed in a 1:1 ratio (actual ratios were determined by plating as described below). Female NCI BALB/c mice (6 to 8 weeks old; Charles River) were injected intraperitoneally with 0.5 mg of Premarin (Pfizer) on days −2, 0, and +2. On day 0, the mice were inoculated vaginally with 20 μl of the mixed suspension (~1 × 10⁹ to 2 × 10⁹ CFU/mouse). Mice were also treated with Str, vancomycin, and trimethoprim as described elsewhere (30) to suppress the overgrowth of commensal flora that occurs under the influence of estrogen. The vaginas were gently swabbed with a PBS-moistened sterile swab on days +1, +3, and +5 postinfection, and the swab material was suspended in 1 ml of PBS. Serial dilutions were performed in GC broth with 0.05% saponin, and dilutions were plated on GC agar with 100 μg/ml of Str for determination of total CFU. GC agar with 100 μg/ml of Str and 50 μg/ml Kan for determination of FA19Strr gdhR::kan CFU, or 1 μg/ml Erm for FA19Strr gdhR::kanC3 CFU. Plates were incubated overnight at 37°C under 7% (vol/vol) CO₂, and colonies were counted after 24 to 48 h. The number of CFU recovered on GC-Str plus Kan or GC-Str plus Em agar plates was subtracted from the number of CFU recovered on GC-Str agar to determine the number of wild-type/parent CFU recovered. The CI was calculated according to the following equation: [(CFU mutant/CFU parent)output/(CFU mutant/CFU parent)input]. A value of 20 CFU (limit of detection) was used to calculate the CI for cultures from which CFU from one of the two strains were not recovered. Competitive infections were repeated, and the data were combined to test reproducibility and increase the statistical power. The Kruskall-Wallis test with Dunn’s multiple-comparisons test (GraphPad Prism) was used to compare the differences in the CIs for mice inoculated with each mixture.

Animal experiments were conducted in the laboratory animal facility at USUHS, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the USUHS Institutional Animal Care and Use Committee.

Quantitative RT-PCR. For qRT-PCR analysis of transcript levels, RNA was extracted from strains FA19, JF1 (FA19 ΔmtrR) (12), and FA19 gdhR::kan grown in GC broth with supplements to late-logarithmic phase 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 use of 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 TCTTCCACATCTCT-3′) and 16Smai_qRTR (5′-CTTAGGGTGGCAGGTATTA-3′) were used for the 16S rRNA, while primers gdhR-qRTF-R2 (5′-AAACGCAATCGCTCTTAAAACGGC-3′) and gepR_qRTFI (5′-ATCCAG GTATTGCCGTATTGGAAGG-3′) were used for the gdhR gene. Genes gdhA_qRTF (5′-TCCATCGCAGCTTGAGAA-3′) and gdhA_qRTI (5′-TTGCTGCTCTGCACGGTTA-3′) were used for the gdhA gene. Both gdhR-qRT-F-R2 and gepR_qRTF1 anneal upstream of the kanamycin cassette insertion, allowing their use for qRT-PCR experiments that employ RNA extracted from the gdhR::kan mutant. Primers mtrC_qRT_F (5′-CGCATTGCGCCGTATACAA-3′) and mtrC_qRT_R (5′-TAATGGCCACAGGTCGACA-3′) were used for the mtrC gene. All qRT-PCR reactions were performed in experimental duplicates and biological duplicates or triplicates.
Mapping transcriptional start sites by primer extension analysis. Total RNA from strain FA19 was prepared at the late-logarithmic phase of growth in GC broth as described above, using the method of Baker and Yanofsky (31). Primer extension experiments were performed as described previously (7) with 6 μg of total RNA with primers PEgntR (5’-CCGCGTGATTCATCTCTC-3’) and PEgdhA (5’-TTTGGGTCGCAAAACGGG-3’). The AMV reverse transcriptase primer extension system from Promega (Madison, WI) was used as described by the manufacturer. The TSSs were determined via electrophoresis of the extension products on a 6% (wt/vol) DNA sequencing acrylamide gel adjacent to reference sequencing reaction mixtures.

Purification of the GdhR protein. Construction of pET15bgdhR was done by amplifying the gdhR open reading frame using the primers gdhR_F (5’-GATCGCCCATATGAACTGGTAGGACTCAG-3’) and gdhR_R (5’-GCCGCGTACCATACTCCTCCTCTC-3’). The resulting PCR product along with the pET15b vector were digested with NdeI and BamHI, ligated overnight, and transformed into E. coli DH5α. The pET15bgdhR construct was confirmed by sequencing with vector-specific primers T7F (5’-TTTATATAGAGATCTAGGACACTTGGT-3’) and T7R (5’-GCTAGTATTGTCAGCCGGA-3’).

For protein expression, pET15bgdhR was transformed into E. coli BL21(DE3) cells. Cultures (5 ml) of BL21(DE3)-pET15bgdhR cells 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 and then induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside 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 then EDTA-free protease inhibitor was added to the bacterial suspension. The cells were lysed by use of a French press cell as described elsewhere (32), membranes and unbroken cells were removed by centrifugation at 100,000 × g, and the supernatant was collected and stored. GdhR-His was purified over a 2-ml nickel-nitrilotriacetic acid (Ni2+-NTA) column. After flowing the supernatant over the Ni2+-NTA column, the resin was washed successively with buffer containing 20 mM and 50 mM imidazole to remove contaminants and weakly bound proteins, and GdhR-His was eluted successively with buffer containing 100 and 200 mM imidazole. The fractions containing GdhR-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%, respectively. To verify the stability and purity of the GdhR and MtrR fusion proteins, we subjected 1 µl of reaction buffer at room temperature. For the competition assays, the same nonlabeled probe or a 100-fold excess of cold competitor was included in the reaction mixture. EMSAs were amplified by PCR from FA19 genomic DNA using the upstream primer R3 (5’-CGGGACGGGCAGACAGTCGC-3’) and the downstream primer R2 (5’-CCGTGTAGTTATTGCTCAGCGG-3’) for gdhR (relative to the direction of transcription) and P1958F (5’-GATCGCCATATGAACTGGTAGGACTCAG-3’) and P1538R (5’-GTGATCCCTGAGAGATCCCTTCTT3’) for gdhA (5’-TTTGGGTCGCAAAACGGG-3’). For protein expression, pET15bgdhR was transformed into E. coli BL21(DE3) cells. Cultures (5 ml) of BL21(DE3)-pET15bgdhR cells 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 and then induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside 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 then EDTA-free protease inhibitor was added to the bacterial suspension. The cells were lysed by use of a French press cell as described elsewhere (32), membranes and unbroken cells were removed by centrifugation at 100,000 × g, and the supernatant was collected and stored. GdhR-His was purified over a 2-ml nickel-nitrilotriacetic acid (Ni2+-NTA) column. After flowing the supernatant over the Ni2+-NTA column, the resin was washed successively with buffer containing 20 mM and 50 mM imidazole to remove contaminants and weakly bound proteins, and GdhR-His was eluted successively with buffer containing 100 and 200 mM imidazole. The fractions containing GdhR-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%, respectively. To verify the stability and purity of the GdhR and MtrR fusion proteins, we subjected 1 µg of purified proteins to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% (wt/vol) polyacrylamide gel (33), and then stained the resolved proteins with Coomassie brilliant blue (CBB). Each protein preparation contained a single CBB-staining band; the respective proteins migrated in the SDS-PAGE gel with a molecular mass consistent with their fusion protein status (32.0 kDa for GdhR-His and 65 kDa for MtrR-MBP [data not shown]).

EMSAs. DNA probes encompassing the gdhR or the gdhA promoter regions that were used in the EMSAs were amplified by PCR from FA19 genomic DNA using the upstream primer R3 (5’-CGGCGTAGTGCCGTAGTTTTT-3’) or R4 (5’-CTGCATCTCCTCTCCTTCTT-3’) and the downstream primer R2 (5’-GGTTTCACTCCTCCTTCCTTTA-3’) or R5 (5’-CGGTCCGGCGCTCAACGGG-3’) for gdhR (relative to the direction of transcription) and P1958F (5’-GATCGCCATATGAACTGGTAGGACTCAG-3’) and P1538R (5’-CGCATCCTGAGAGATCCCTT3’) for gdhA (5’-TTTGGGTCGCAAAACGGG-3’). For gdhR, when making radioactive probes, the indicated PCR products were labeled with [32P]dATP using T4 polynucleotide kinase (New England Biolabs). The labeled DNA fragments were incubated with 2 µg of MtrR-MBP, purified as described previously (8, 22), or with 1 µg of GdhR-His, in 30 µl of reaction buffer at room temperature. For the competition assays, the same nonlabeled probe or a nonlabeled PCR product along with mpBR1 (5’-CCGGACGGGCAGACATGTCG-3’) and mpBR1 (5’-GGA CAGGGCGTGATTCATCTC-3’) primers were added to the reaction mixture. Samples were subjected to electrophoresis in a 6% native polyacrylamide gel at 4°C, followed by autoradiography.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00449-17.

FIG S1, TIF file, 10.6 MB
FIG S2, TIF file, 2.8 MB.

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We have no competing interests to declare.

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