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RESEARCH ARTICLE

Gonococcal Clinical Strains Bearing a Common *gdhR* Single Nucleotide Polymorphism That Results in Enhanced Expression of the Virulence Gene *lctP* Frequently Possess a *mtrR* Promoter Mutation That Decreases Antibiotic Susceptibility

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ABSTRACT GdhR is a transcriptional repressor of the virulence factor gene *lctP*, which encodes a unique L-lactate permease that has been linked to pathogenesis of *Neisseria gonorrhoeae*, and loss of *gdhR* can confer increased fitness of gonococci in a female mouse model of lower genital tract infection. In this work, we identified a single nucleotide polymorphism (SNP) in *gdhR*, which is often present in both recent and historical gonococcal clinical strains and results in a proline (P)-to-serine (S) change at amino acid position 6 (P6S) of GdhR. This mutation (*gdhR6*) was found to reduce GdhR transcriptional repression at *lctP* in gonococcal strains containing the mutant protein compared to wild-type GdhR. By using purified recombinant proteins and *in vitro* DNA-binding and cross-linking experiments, we found that *gdhR6* impairs the DNA-binding activity of GdhR at *lctP* without an apparent effect on protein oligomerization. By analyzing a panel of U.S. (from 2017 to 2018) and Danish (1928 to 2013) clinical isolates, we observed a statistical association between *gdhR6* and the previously described adenine deletion in the promoter of *mtrR* (*mtrR*-P A-del), encoding the repressor (MtrR) of the *mtrCDE* operon that encodes the MtrCDE multidrug efflux pump that can export antibiotics, host antimicrobials, and biocides. The frequent association of *gdhR6* with the *mtrR* promoter mutation in these clinical isolates suggests that it has persisted in this genetic background to enhance *lctP* expression, thereby promoting virulence.

IMPORTANCE We report the frequent appearance of a novel SNP in the *gdhR* gene (*gdhR6*) possessed by *Neisseria gonorrhoeae*. The resulting amino acid change in the GdhR protein resulted in enhanced expression of a virulence gene (*lctP*) that has been suggested to promote gonococcal survival during infection. The mutant GdhR protein expressed by *gdhR6* had a reduced ability to bind to its target DNA sequence upstream of *lctP*. Interestingly, *gdhR6* was found in clinical gonococcal strains isolated in the United States and Denmark at a high frequency and was frequently associated with a mutation in the promoter of the gene encoding a repressor (MtrR) of both the *mtrCDE* antimicrobial efflux pump operon and *gdhR*. Given this frequent association and the known impact of these regulatory mutations, we propose that virulence and antibiotic resistance properties are often phenotypically linked in contemporary gonococcal strains.

KEYWORDS *Neisseria gonorrhoeae*, *gdhR*, *lctP*, *mtrR*, antibiotic resistance

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Gonorrhea is the second most common of the reported bacterial sexually transmitted infections (STI) worldwide, with ca. 82 million cases estimated among adults in 2020 and rising incidences in many countries during the past decade (1). Since the 1930s, antibiotic therapy has been the mainstay for both curing infection and reducing the spread of *Neisseria gonorrhoeae* in the community. Complicating the high global burden of gonorrhea is the emergence of gonococci expressing resistance to all new antibiotics brought into clinical practice (reviewed in references 2 and 3). Recently, in the United States and the United Kingdom, a dual therapy regimen of ceftriaxone (Cro) and azithromycin (Azm) was replaced by Cro monotherapy due to the increasing prevalence of *N. gonorrhoeae* strains being nonsusceptible to Azm, which is defined by a MIC of >1 μg/mL (4, 5).

N. gonorrhoeae often accumulates spontaneous or acquired mutations under the selective pressure of antibiotics at key genes required for antimicrobial resistance (AMR) (reviewed in references 6 and 7). Among these genes are those located within the *mtr* locus that include *mtrCDE* (NGO1365-1363), encoding the antimicrobial efflux pump MtrCDE (8–10), and *mtrR* (NGO1366), which encodes the repressor (MtrR) of *mtrCDE* (9, 11) (Fig. 1A). In addition to serving as a repressor of *mtrCDE*, MtrR also negatively or positively controls expression of multiple *N. gonorrhoeae* genes, including *gdhR*, which is positioned just downstream of the *mtrCDE* efflux pump operon (12, 13). Previous work showed that MtrR is a direct repressor of *gdhR* and that loss of GdhR can enhance gonococcal fitness during experimental infection of the lower genital tract of female mice (12), suggesting that it is important for mouse virulence. GdhR is a GntR-type transcriptional regulator that can regulate multiple *N. gonorrhoeae* genes, including direct repression of *lctP* (14). This regulatory pathway consisting of MtrR and GdhR is of interest, as the regulation of *lctP* likely impacts pathogenesis, given that loss of LctP, which encodes an L-lactate permease, was shown to reduce gonococcal survival in the female mouse model of infection (15).

We hypothesized that since many current *N. gonorrhoeae* strains have mutations that greatly diminish MtrR production or its activity (9, 16), which would lead to increased expression of *gdhR* and repression of *lctP*, *N. gonorrhoeae* may develop mechanisms to bypass GdhR repression of *lctP*. In this regard, we now report a *gdhR* single nucleotide polymorphism (SNP) in gonococcal clinical strains that interferes with the DNA-binding activity of GdhR, resulting in enhanced *lctP* expression. Further, we present results that show that strains bearing this mutation frequently have a well-described single-base-pair deletion mutation in the *mtrR* promoter that is known to abrogate *mtrR* expression and decrease gonococcal susceptibility to beta-lactam and macrolide antibiotics (9, 17) (Fig. 1B).

RESULTS AND DISCUSSION

Identification of a *gdhR* SNP that reduces GdhR repression of *lctP*. To determine if *gdhR* SNPs occur in commonly used *N. gonorrhoeae* laboratory strains, we aligned the predicted amino acid sequence of GdhR from the international reference strains FA19, FA1090, F62, H041, and MS11. The predicted GdhR amino acid sequences from these five strains were identical, with the exception of a proline (P)-to-serine (S) change at amino acid position 6 in strain MS11 (Fig. 2A). To determine whether this GdhR P6S or other *gdhR* SNPs resulting in amino acid changes in GdhR are present in recent clinical isolates, we analyzed whole-genome sequences of a panel of 300 gonococcal isolates collected in 2017 to 2018 by the U.S. Gonococcal Isolation Surveillance Project (GISP) (18–20). From the alignment of the *gdhR* alleles, in addition to the GdhR P6S-encoding allele *gdhR6*, we found only 3 new SNPs resulting in GdhR amino acid changes (G28K, V44I, and E214K) (Table 1; see also Table S1 in the supplemental material). The *gdhR6* allele was found at the highest frequency (19.7% compared to 0.3 to 1.0% for the other SNPs resulting in amino acid changes in GdhR).

Given that the *gdhR6* allele was the most prevalent *gdhR* mutant allele of recent U.S. clinical isolates, we asked if the mutant GdhR retained repressive activity on *lctP* gene expression. First, we compared the level of *lctP* expression in strains producing the GdhR

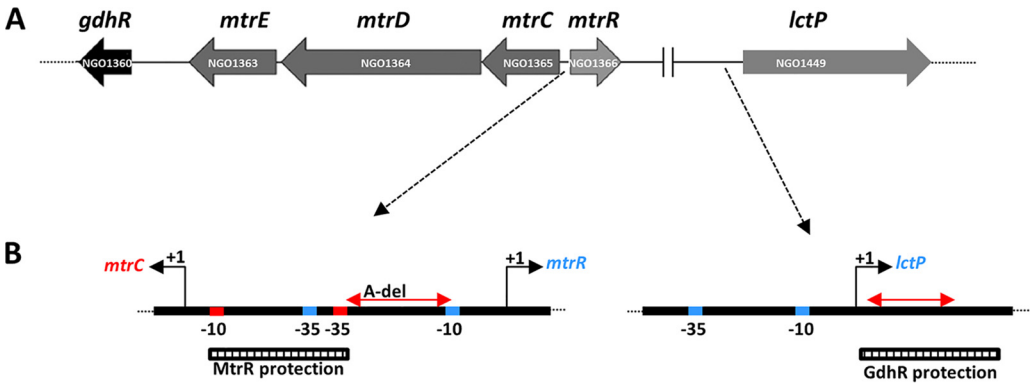


FIG 1 Genomic organization of the *Neisseria gonorrhoeae gdhR*, *mtr*, and *lctP* loci. (A) Relative position of the genes within the FA1090 strain genome (GenBank assembly accession number GCA_000006845.1). (B) Depiction of the transcriptional regulatory elements present within the intergenic region between *mtrC-mtrR* (left) and upstream of *lctP* (right). Promoter elements (−10 and −35) are depicted in blue for *mtrR* and *lctP* and in red for *mtrC*. +1 represents previously determined transcriptional start sites for *mtrC* (11), *mtrR* (17), and *lctP* (14). Striped boxes represent DNase I protected regions on both strands previously determined for MtrR (36) and GdhR (14). Double-headed arrows represent the inverted repeat sequences present at the spacer of the *mtrR* promoter elements, where the adenine deletion mutation (A-del) is located (37), and at the *lctP* 5′ untranslated region, which is the DNA sequence requirement for GdhR binding (14). Not drawn to scale.

wild type (WT) (FA19, F62, and H041) versus the GdhR P6S (MS11) by quantitative reverse transcription-PCR (qRT-PCR) and found that the *gdhR6*-bearing strain MS11 had significantly higher levels of expression (Fig. 2B). To determine if *gdhR6* is responsible for increased *lctP* expression in MS11, we complemented FA19 and MS11*gdhR* null mutants (see Materials and Methods for strain construction) with chromosomally inserted plasmid constructs expressing *gdhR* WT or *gdhR6* from its own promoter in *trans* (Fig. 3). To monitor *lctP* expression, we used previously constructed *lctP-lacZ* fusion reporter strains (14). In the FA19 background, complementation of the null *gdhR* mutant with *gdhR* WT restored WT levels of *lctP* expression, while expression of *gdhR6* failed to repress *lctP* to WT levels (Fig. 3). Similarly, in the MS11 background, complementation of the null *gdhR* mutant with *gdhR* WT overrepressed *lctP* compared to WT levels, while expression of *gdhR6* restored *lctP* expression to the parental level.

The GdhR P6S protein has reduced binding to the *lctP* promoter region. To analyze the molecular mechanism by which the P6S mutation diminishes GdhR repression of *lctP*, we purified GdhR WT and P6S proteins from recombinant *Escherichia coli* cultures and determined their binding activity at the *lctP* promoter by electrophoresis mobility shift assay (EMSA). This analysis showed that the GdhR P6S had very limited DNA-binding activity compared to the GdhR WT *in vitro* (Fig. 4A). Although the P6S change is just upstream of the predicted DNA-binding domain, we hypothesized that the presence of the serine residue in GdhR P6S could influence flexibility of the adjacent helix-turn-helix region and thereby reduce GdhR binding to the target DNA sequence. To evaluate this possibility, we constructed a GdhR structure model by sequence homology using the resolved structure of *Streptococcus agalactiae* GntR (21) (Fig. 4D). Based on this model, we predict that the P6S change would alter the secondary structure of GdhR, likely impacting the flexibility of the adjacent DNA-binding domain.

An important feature of GntR-type regulators is the dimerization activity mediated by the C-terminal metabolite-binding and oligomerization domain (22, 23). Accordingly, to determine whether the observed impact of the P6S amino acid change on the GdhR DNA-binding activity is due to a distal effect on the protein dimerization activity, we carried out glutaraldehyde cross-linking experiments to capture the oligomerization state of GdhR WT and P6S (Fig. 4B). The result showed that both proteins were capable of forming dimers. To determine whether GdhR P6S has a dominant-negative phenotype over the GdhR WT protein, we conducted an EMSA in which GdhR WT and mutant proteins were mixed and incubated with the *lctP* promoter (Fig. 4C). This result showed that addition of increasing amounts of GdhR P6S to the WT did not displace bound GdhR WT protein

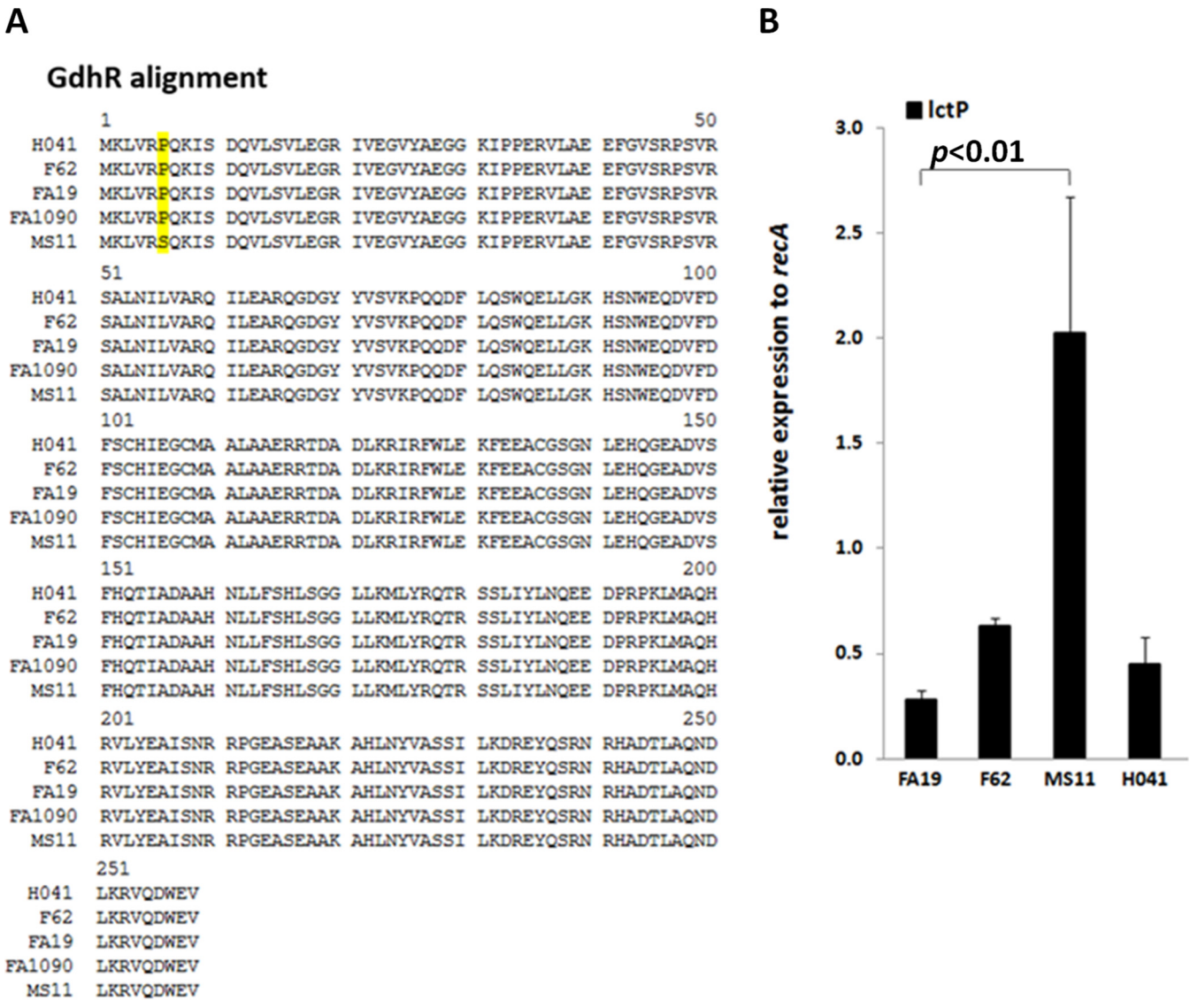


FIG 2 Expression of *lctP* in laboratory strains of *Neisseria gonorrhoeae*. (A) Alignment of the GdhR sequence from different *N. gonorrhoeae* laboratory strains using the ClustalW algorithm. (B) Relative levels of *lctP* were determined by qRT-PCR using *recA* as an internal reference gene. Total RNA samples were collected from cells grown in GC broth to late exponential phase. Data are presented as the means (bar) plus the standard deviations (error bar) from 3 biological samples. Significant statistical differences were determined by a nonparametric Kruskal-Wallis test and Dunn's posttest.

from the *lctP* promoter probe and did not change the remaining amount of unbound free DNA. However, we noticed that it did interconvert the amount of the lower nucleoprotein complex to the higher complex, which suggests that GdhR P6S can oligomerize with the GdhR WT protein without affecting its DNA-binding activity. This is most likely achieved through a second oligomerization interface that allows the protein to form tetramers. This hypothesis is consistent with our earlier observation that only a single GdhR-binding site exists upstream of *lctP* (14), and other lactate utilization operon repressors appear to form dimers and tetramers when bound to target DNA (24).

Clinical isolates bearing *gdhR6* often have increased AMR in the context of a coresident *mtrR* promoter mutation that decreases antimicrobial susceptibility. Analysis of the antimicrobial susceptibility of the 300 U.S. 2017–2018 GISP isolates showed that those bearing *gdhR6* were, on average, less susceptible to Azm and Cro (Table 2), both of which are substrates for the MtrCDE efflux pump (9, 25). Because loss of GdhR does not impact levels of gonococcal susceptibility to these antibiotic classes (12) and the presence of *gdhR6* does not alter antibiotic susceptibility in transformant

TABLE 1 Frequency of *gdhR* SNPs in GISP 2017–2018 clinical strains

SNP[a]	Amino acid change	Frequency[b] [% (no. positive/total no.)]	Localization in protein (amino acids)
C(16)T	Pro-6-Ser	19.7 (59/300)	Outside wHTH DNA-binding domain (8–73)
G(82)A	Glu-28-Lys	1.0[c] (3/300)	wHTH DNA-binding domain (8–73)
G(130)A	Val-44-Ile	0.3 (1/300)	wHTH DNA-binding domain (8–73)
G(640)A	Glu-214-Lys	0.3 (1/300)	Dimerization domain (97–225)

[a]Relative to the *gdhR* start codon.
[b]Out of 300 GISP clinical isolates from 2017 and 2018.
[c]Only present in isolates containing *gdhR6*.

derivatives of strain F62 (Table S2), we hypothesized that clinical isolates bearing *gdhR6* might, more frequently than those with a *gdhR* WT allele, have additional mutations that impact antibiotic susceptibility. To assess this, we analyzed the *mtrR* allele of the 300 GISP strain collection for mutations known to increase Azm resistance (Fig. 5A and Table S3). Indeed, this analysis revealed that isolates with *gdhR6* more frequently contained the well-described single-base-pair deletion in the 13-bp inverted repeat (IR) sequence within the *mtrR* promoter region (termed *mtrR*-P A-del) (Fig. 5B). This promoter mutation is known to result in elevated levels of *mtrCDE* expression and affords a higher level of antimicrobial resistance than mutations in the *mtrR* coding region (17).

The analysis of the distribution of frequencies of the *mtrR* mutations stratified by the *gdhR* genotype showed that this *mtrR* promoter mutation is highly represented in the

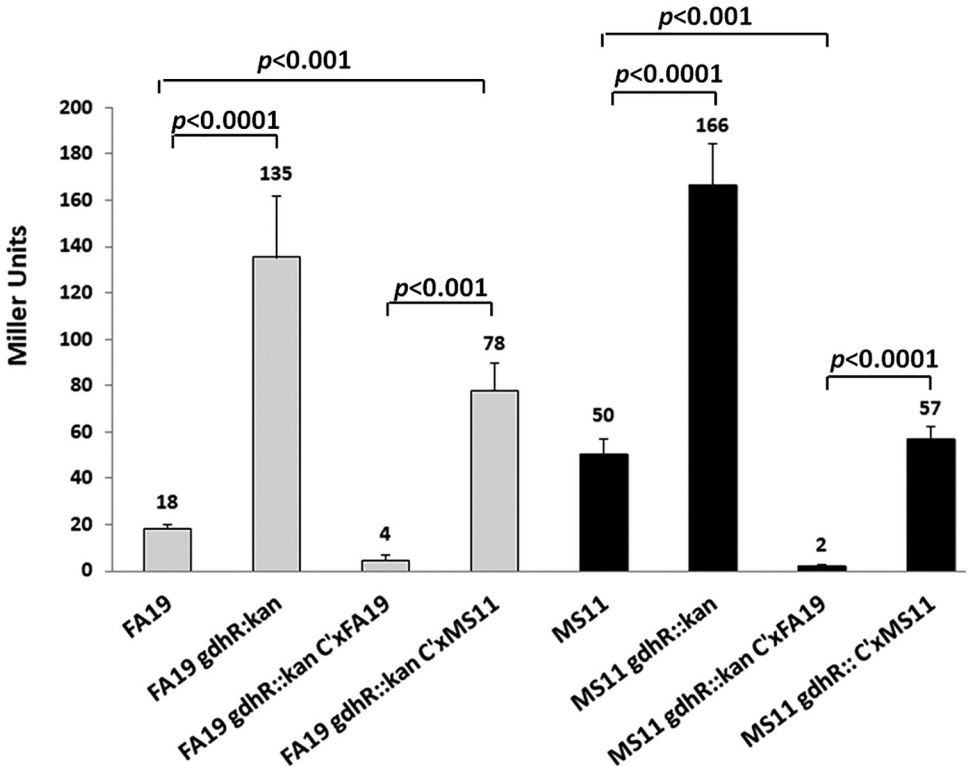


FIG 3 Effect of a *gdhR* missense mutation on *lctP* expression. Genetic complementation experiment in which the *gdhR* mutants (*gdhR::kan*) of gonococcal strains FA19 and MS11 were complemented in *trans* with either the *gdhR* allele of FA19 (C′xFA19) or the MS11 allele encoding a P6S change in GdhR (C′xMS11). All wild-type, mutant, and complemented strains contain an *lctP-lacZ* fusion in reporter vector pLES94-*lctP* and were grown to mid-exponential phase on GC broth. β-Galactosidase was expressed from the *lctP* transcriptional and translational signals, and its activity was determined in Miller units. Data are presented as the means (bar) plus the standard deviations (error bar) from 3 biological samples and two technical replicates each. Significant statistical differences were determined by an ANOVA test and Tukey's multiple-comparison posttest.

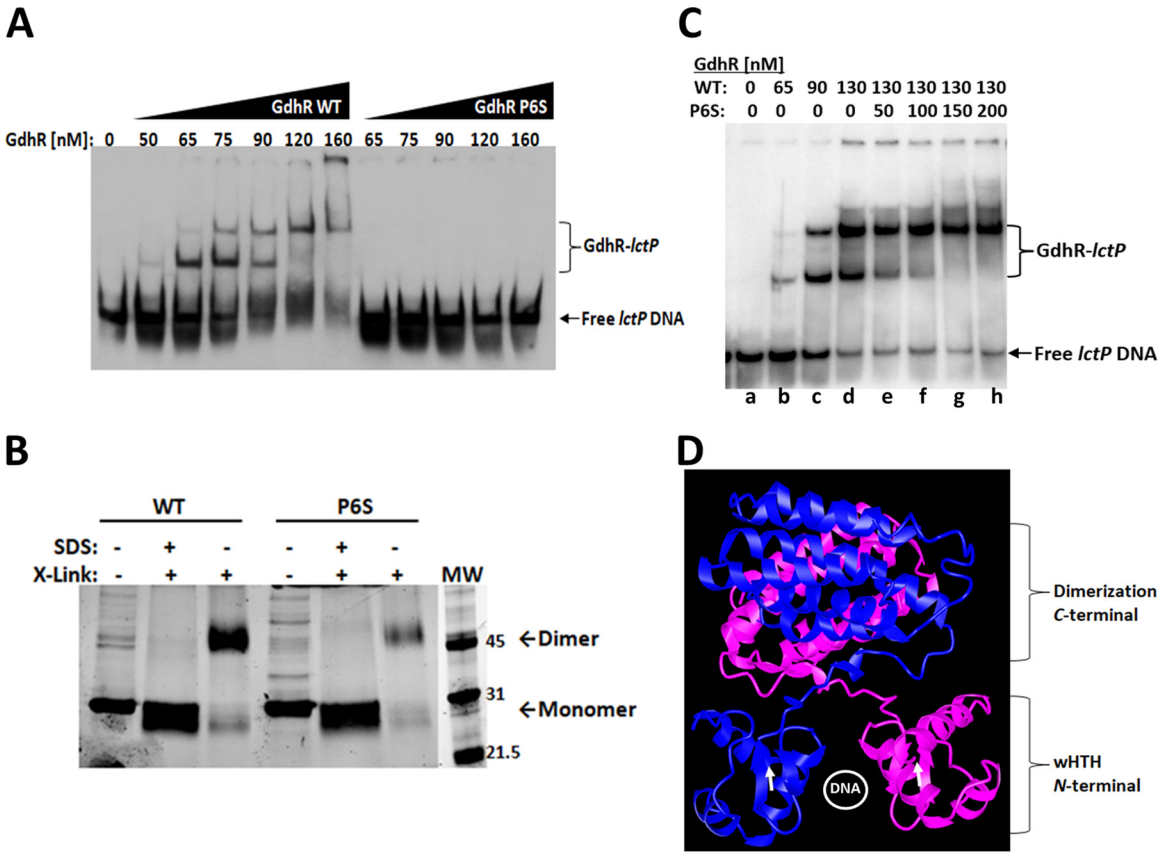


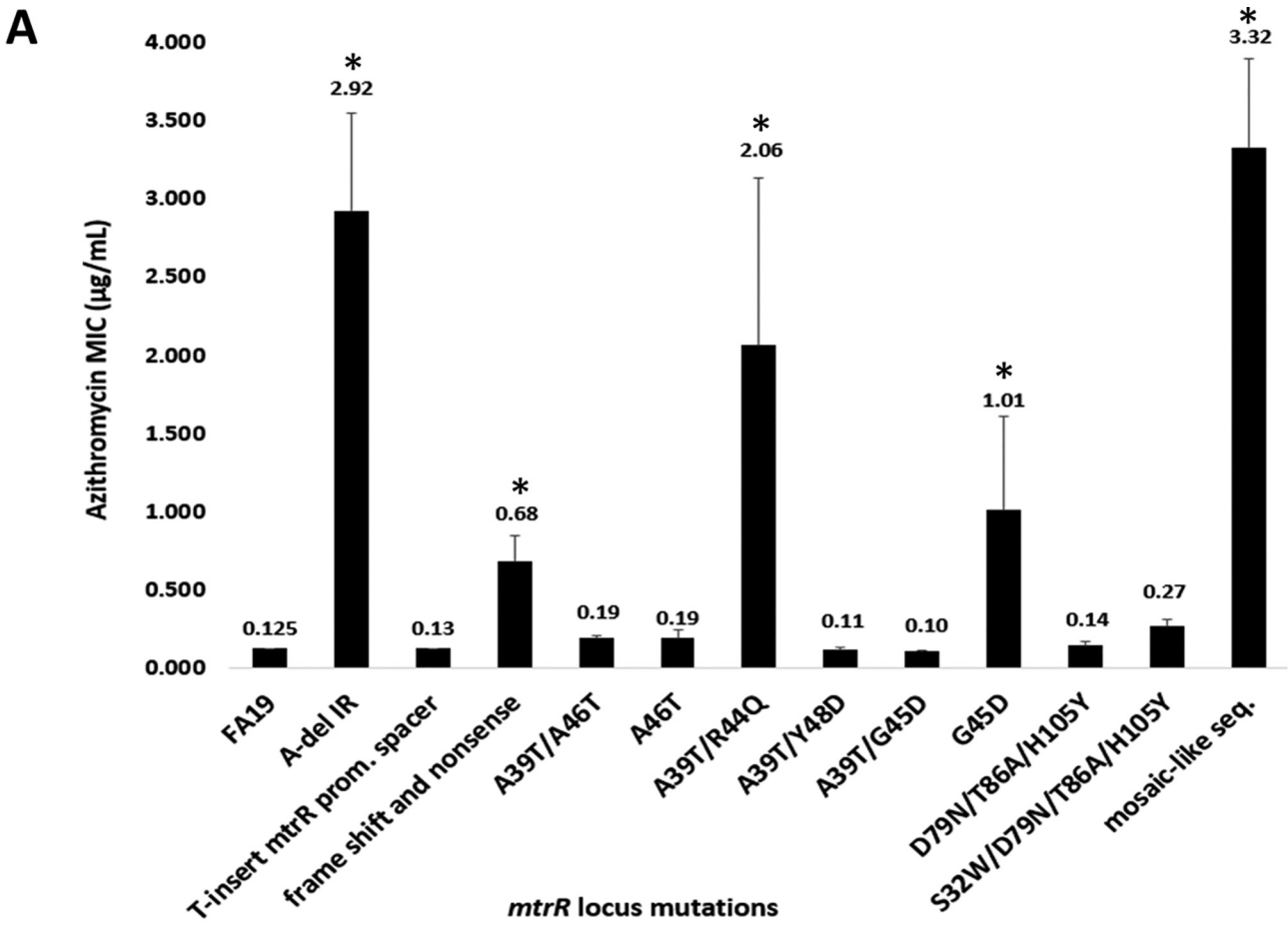
FIG 4 GdhR P6S mutation impairs the DNA-binding activity of the protein *in vitro* but not dimerization activity. (A) Binding of GdhR wild type (WT) and P6S mutant to the *lctP* promoter. A digoxigenin-labeled DNA fragment encoding the *lctP* promoter was reacted with increasing amounts of purified GdhR WT and mutant variants. The mobility of free DNA and of the nucleoprotein complexes was resolved in polyacrylamide gels as described for the EMSAs and indicated at the right of the gel. (B) Dimerization activity of GdhR protein variants. Purified GdhR WT and P6S were subjected to glutaraldehyde cross-linking in the presence or absence of SDS as a specificity control. The electrophoretic mobility of the resulting dimers/monomers was analyzed in UV-developed TGX stain-free gels with a Bio-Rad low-range molecular weight marker (MW). (C) The simultaneous binding of GdhR WT and P6S mutant to the *lctP* promoter was analyzed by EMSA. The DIG-labeled *lctP* promoter was incubated with increasing amounts of purified GdhR WT (lanes a to d) or with a fix concentration of GdhR WT and increasing amounts of GdhR P6S (lanes e to h). (D) GdhR protein structure was modeled by sequence homology using PDB entry 6AZ6 (21) and the SWISS-MODEL server (38). A GdhR dimer is represented in blue and cyan with the corresponding N-terminal winged helix-turn-helix (wHTH) and the C-terminal dimerization/substrate binding domains. The positions of proline-6 in each monomer are indicated by white arrows. The position of the recognizing DNA is shown transversally with a white circle.

GISP isolates containing the *gdhR6* allele (54%) compared to isolates containing WT *gdhR* (16%). To determine if the association between the *mtrR* promoter mutation and *gdhR*6 is a marker for the distribution of Azm MICs exhibited by isolates containing either WT or *gdhR6* mutant *gdhR* genotypes (as shown in Table 2), we analyzed the stratified Azm MIC of isolates bearing mutant or WT *gdhR* with either a WT *mtrR*-P IR or a *mtrR*-P A-del IR (Fig. 5C). We observed that the Azm MIC of *gdhR*6 isolates containing WT *mtrR*-P IR was not statistically different from that of the group containing WT *gdhR*. Therefore, the statistical association of the *gdhR6* allele with the *mtrR*-P A-del mutation in these clinical isolates explained the

TABLE 2 Mean azithromycin and ceftriaxone MIC in GISP isolates stratified by the *ghdR* genotype

	MIC (mean ± SEM, μg/mL) for:	
Genotype	**Azithromycin**	**Ceftriaxone**
WT	1.068 ± 0.164	0.012 ± 0.001
gdhR6	3.588[a] ± 0.733	0.020[a] ± 0.003

[a]Statistically different by either a parametric *t* test ($P < 0.001$) or by a nonparametric U test (Azi $P = 0.005$, Ceft $P = 0.048$).



B

mtrR mutation	*gdhR* WT	*gdhR* P6S	Significant?
A-del IR	16% (38/241)	54% (32/59)	Yes p<0.0001
A39T/R44Q	7% (18/241)	3% (2/59)	No p = 0.3850
G45D	3% (8/241)	10% (6/59)	Yes p = 0.0369
Mosaic-like seq.	19% (45/241)	0% (0/59)	Yes p < 0.0001
Frame shift and nonsense	23% (55/241)	2% (1/59)	Yes p < 0.0001

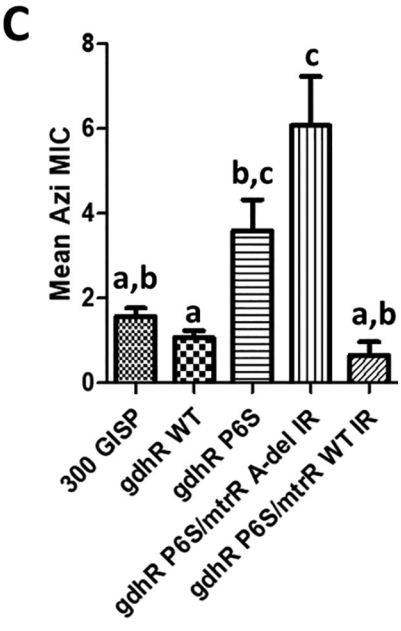


FIG 5 Relationship between the *gdhR6* mutation and *mtrR* mutations that impact gonococcal antibiotic resistance in the U.S. Gonococcal Isolate Surveillance Project (GISP) isolates. (A) Effect of different known *mtrR* locus mutations on the mean azithromycin MIC of the gonococcal clinical isolates. Data are represented as the means (bar) plus the standard errors of the means (error bar). An asterisk represents statistical differences ($P < 0.05$) from FA19 strain using the nonparametric Wilcoxon signed a rank test. (B) Statistical linkage between the *gdhR6* (P6S) mutation and *mtrR* mutations conferring the highest increase in azithromycin resistance among the 300 samples of GISP isolates. Gonococcal isolates bearing the *mtrR* mutations (adenine deletion in the *mtrR* promoter inverted repeat [A-del IR], missense mutations A39T/R44Q and G45D, mosaic-like *mtrR* sequence, and frameshift plus nonsense mutations grouped together)

(Continued on next page)

higher mean Azm MIC level shown by isolates containing *gdhR6*. Importantly, the mean MIC of strains with both mutations was above the CDC alert level (in 2018, alert MIC $\geq$ 0.125 [CRO], MIC $\geq$ 2.0 [AZM]), and their presence and spread in the community likely contributed to the decision to remove Azm from the treatment regimen in the United States and elsewhere (4).

Comparative evolution of *gdhR6* and *mtrR* promoter mutations. To extend the findings with the 2017–2018 GISP *N. gonorrhoeae* strains to an independent panel of clinical isolates from a different region, we analyzed the whole-genome sequences of a recently published collection of *N. gonorrhoeae* isolates that were collected in Copenhagen, Denmark, from 1928 to 2013 (26). Almost identical to U.S. GISP isolates, the *mtrR*-P A-del mutation was significantly more associated with *gdhR6* (53%) than with *gdhR* WT (7%) in the Danish isolates (Fig. 6A), and this association was reflected by significantly higher Azm MICs of *gdhR6 N. gonorrhoeae* (Fig. 6B). With the panel of Danish isolates, we could observe the evolution of these *mtrR* and *gdhR* mutations through the decades (Fig. 6C). This analysis showed that while *gdhR6* was present in clinical strains before the introduction of antibiotics, the *mtrR*-P A-del mutation, as previously recognized (26), started to accumulate in the 1960s, and from this point onward *gdhR6* followed the trend of accumulation of the *mtrR*-P A-del mutation. Given that the appearance of *gdhR6* preceded *mtrR* mutations, we hypothesized that *gdhR6* in these strains facilitated the selection for *N. gonorrhoeae* strains with *mtrR* mutations. However, we found that possession of *gdhR6* in a transformant of antibiotic-susceptible strain FA19 did not influence the spontaneous mutation frequency of *mtrR* mutations, including the single-base-pair promoter mutation studied here, in a statistically significant manner (mean frequency of *mtrR*-P A-del spontaneous mutations, WT at 2×10^{-9} versus *gdhR6* at 5×10^{-10}, U-test *P* value of 0.1). Hence, it is likely that independent selective pressures during infection and antibiotic chemotherapy accounted for their dual emergence in the 20th century. In this regard, we propose that the *mtrR*-P A-del mutation became more frequent in the 1960s due to selective pressure of antibiotic treatment and that strains with a coresident *gdhR6* allele expanded within this cohort. Thus, *gdhR6* likely provided a mechanism by which *N. gonorrhoeae* with a coresident *mtrR*-P A-del could enhance expression of *lctP*, thereby promoting survival during infection.

We have previously proposed that virulence and antibiotic resistance in *N. gonorrhoeae* are linked properties through the Mtr system (27). Our earlier observations that MtrR negatively regulates both the *mtrCDE* operon, which encodes a multidrug efflux pump that recognizes antibiotics and host antimicrobials (9, 17, 28) and *gdhR*, and that both the efflux pump and GdhR can influence *N. gonorrhoeae* fitness in the lower genital tract of female mice formed the basis for this hypothesis. The capacity of GdhR to directly and negatively regulate *lctP*, whose gene product (LctP) is required for L-lactate uptake and virulence during experimental murine infection (15), prompted us to examine the conservation of *gdhR* in more recent *N. gonorrhoeae* as well as in historical strains. Through this analysis, we identified a novel *gdhR* allele (*gdhR6*) that is frequently present in *N. gonorrhoeae* clinical isolates. The mutant GdhR protein encoded by *gdhR6* has a substantially reduced capacity to bind to the DNA sequence upstream of *lctP*, which likely explains why *lctP* expression is enhanced in strains bearing *gdhR6* compared to the WT gene. While GntR-like regulators form oligomers important for gene regulation, our data suggest that GdhR P6S retains the ability to oligomerize. Accordingly, we propose that the P6S change in GdhR has an influence on the secondary structure of the adjacent helix-turn-helix motif that negatively influences GdhR binding to the *lctP* promoter region.

FIG 5 Legend (Continued)
were divided by the wild-type and *gdhR6* genotypes. Statistical linkages of the *mtrR* and *gdhR6* mutations were determined by a Fisher exact test (*P* value shown) and a chi-square test. (C) The higher mean azithromycin MIC identified in isolates bearing *gdhR6* can be explained by their genetic association with the *mtrR*-P A-del IR mutation. The GISP isolate sample was stratified between the *gdhR* and the *mtrR* promoter inverted repeat genotypes, and the mean azithromycin MIC (bars) was computed along with the standard errors of the means (error bar). Letters represent different statistical population determined a Kruskal-Wallis test and a Dunn's multiple-comparison posttest.

A

mtrR mutation	*gdhR* WT	*gdhR* P6S	Significant?
A-del IR	6.8% (12/176)	53% (8/15)	Yes p<0.0001

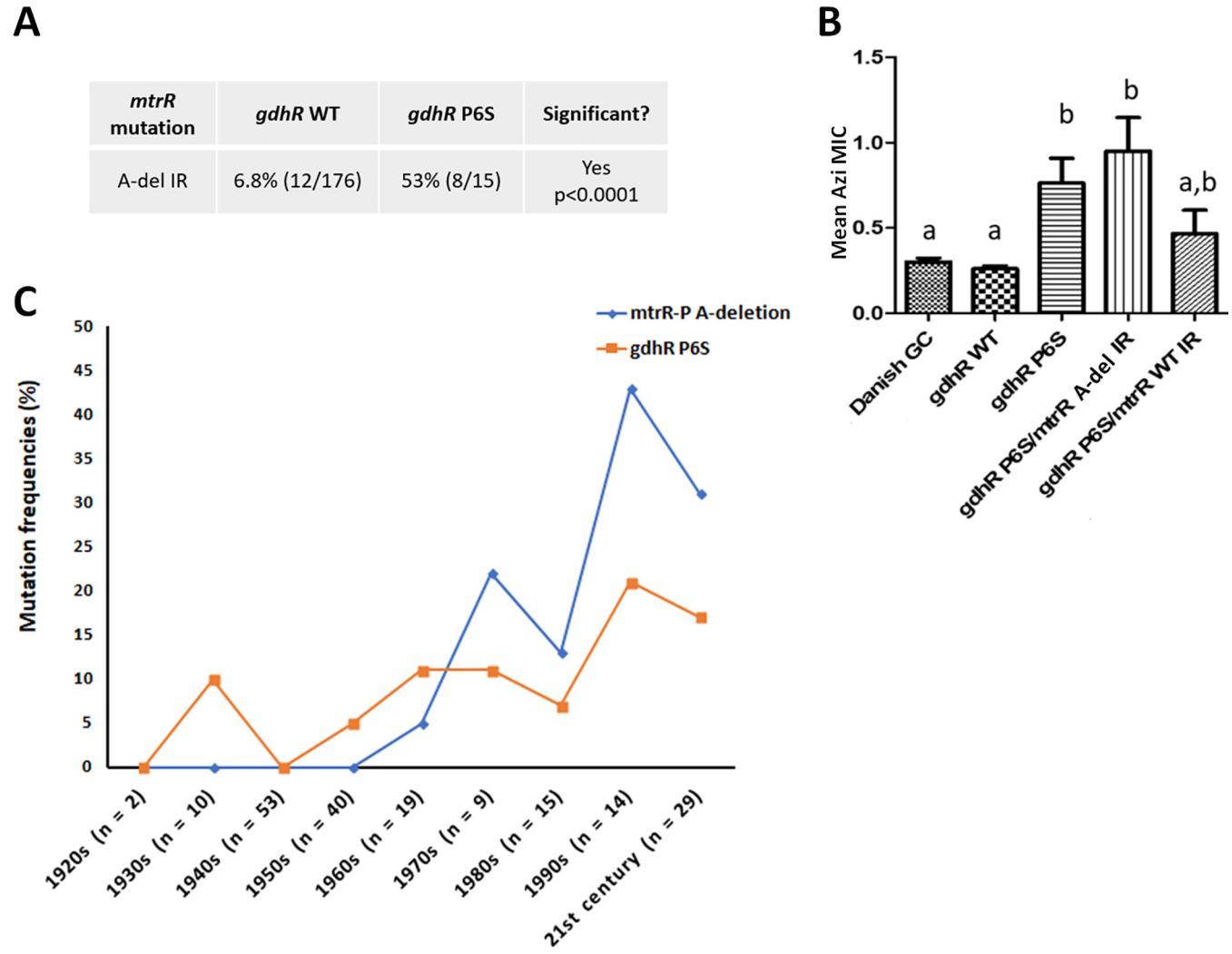


FIG 6 Prevalence of the *gdhR6* and *mtrR*-P A-del IR mutations in a cohort of gonococcal isolates from Denmark (1928 to 2013) (26). (A) Statistical association between the *gdhR6* (P6S) and the *mtrR*-P A-del IR mutations was analyzed in all 191 reported Danish gonococcal isolates using the PubMLST server (39) and the public data set with PubMed identifier 32013864. Statistical association was determined by a Fisher exact test and a chi-square test (*P* value shown for both tests). (B) Distribution of the mean azithromycin MIC in Danish gonococcal isolates based on the *gdhR* and *mtrR* promoter inverted repeat genotypes. Data are represented as the means (bar) plus the standard error of the means (error bar). Letters represent different statistical population determined by a Kruskal-Wallis test and a Dunn's multiple-comparison posttest. (C) Evolution of the frequency of the $gdhR^{P6S}$ and *mtrR*-P A-del IR mutations among the panel of Danish gonococcal isolates from different decades.

Our previous work on the *mtr* and *gdhR* loci as well as in gene regulation focused primarily on strain FA19, which was isolated in 1962 (29) during the Golden Age of antibiotic therapy when *N. gonorrhoeae* antibiotic-resistant strains were infrequent compared to the present time. The evolution of *mtrR* coding sequence and promoter mutations that are known to reduce MtrR repression of *mtrCDE* seems to have appeared in the 1960s, likely the result of antibiotic pressure (e.g., penicillin) (26). It is of interest that the *gdhR6* allele increased in frequency in the Danish strains in a manner resembling the presence of the single-base-pair deletion in the *mtrR* promoter (Fig. 6C). We suggest that the frequent coincidence of *gdhR6*, which would result in enhanced expression of *lctP*, with the *mtrR* promoter mutation is a molecular marker for linkage of virulence capability and AMR. GdhR represses at more than 2-fold, besides *lctP*, only three genes encoding a highly variable (usually frameshifted) fimbrial protein precursor and the type III restriction/modification system RmsR/ModA13 (14), in which the *modA13* allele is phase variable and usually inactive in most *N. gonorrhoeae* strains (30). Due to the narrow GdhR regulon and because the GdhR-*lctP* regulatory circuit is most likely a conserved trait in all gonococcal strains (14), we believe the main purpose for which the *gdhR6* allele accumulates in clinical isolates is to achieve *lctP* derepression.

At present, we cannot explain why clinical isolates in the United States from 2017 to 2018 and Denmark from 1928 to 2013 bearing both *gdhR6* and the *mtrR* promoter mutation have higher levels of Azm resistance than those strains containing only the *mtrR* promoter mutation; critically, they uniformly lack 23S rRNA mutations that are known to provide high-level (≥256 μg/mL) Azm resistance (data not presented). Indeed, transformants of antibiotic-susceptible strain F62 (Azm MIC of 0.0625 μg/mL) bearing both *gdhR6* and the *mtrR* promoter mutation display a level of Azm resistance equal to that of transformants with only the *mtrR* promoter mutation (0.25 μg/mL) (Table S2). Nevertheless, we conclude that contemporary *N. gonorrhoeae* strains may contain unique mutations that influence levels of susceptibility to antibiotics such as Azm not seen in historical strains (e.g., FA19) that are often used in *N. gonorrhoeae* antibiotic resistance basic research.

MATERIALS AND METHODS

Strains and media. *N. gonorrhoeae* strains used in this study are described in Table S4 in the supplemental material. Gonococcal strains were grown overnight at 37°C under 5% (vol/vol) CO_2-enriched atmosphere on GC-agar (Difco GC Medium Base) plates containing Kellogg's supplements I and II (31). Growth in liquid medium was at 37°C with orbital shaking (225 rpm) in 5 or 25 mL GC broth containing Kellogg's supplements I and II and 0.042% (wt/vol) sodium bicarbonate in 50- or 250-mL flasks, respectively. Liquid cultures were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 (or otherwise indicated) with biomass from overnight-grown GC-agar plates. When necessary, culture media were supplemented with ampicillin (Amp; 100 μg/mL), chloramphenicol (Cm; 0.5 to 1.0 μg/mL), kanamycin (Km; 50 μg/mL), erythromycin (Erm; 0.5 to 1 μg/mL), streptomycin (Str 100 μg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG; 1.0 mM), or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 20 μg/mL). *E. coli* TOP10 (Life Technologies, Carlsbad, CA) and ER2566 (New England BioLabs, MA, USA) were used for cloning and protein expression purposes, respectively, and grown on LB medium (32). Collection methods of GISP isolates in sentinel surveillance sites have been described (18–20). A CDC computer script randomly selected 150 isolates from 2017 and 2018, using the GISP database of isolates with available sequence and MIC data.

Construction of mutant and complementation strains. Plasmids and oligonucleotide primers used in this work are described in Tables S4 and S5, respectively. Genetic transformation of *N. gonorrhoeae* strains was carried out using spot transformation on agar plates or electroporation, as indicated and as described previously (33), with slight changes. Changes for electroporation were (i) all steps were carried out at 4°C; (ii) the resuspension and wash buffer consisted of 1 mM HEPES, pH 7.4, 137 mM sucrose, 10% glycerol; and (iii) centrifugations were performed at 9,000 rpm and 4°C for 3 min.

To construct an F62 WT strain with streptomycin resistance, the mutant *rpsL*^{K43R} allele from FA19 Str^R was recombined into native F62 *rpsL* (NGO1845). Briefly, a 553-bp PCR fragment encoding *rpsL* from FA19 Str^R was amplified with primers rpsL-F and rpsL-R and cleaned up from the agarose gel. The DNA fragment was used to electroporate F62, and the resulting strain F62 Str^R was selected on 100 μg/mL Str. To obtain LctP (NGO1449) expression reporter strains, the previously constructed reporter vector pLES94-*lctP* (14) was used to transform MS11 to obtain strain JC43. Transformants were selected on GC agar containing Cm at 6.0 μg/mL. To check the correct integration of the vector at the *proA* (NGO0850) locus, a PCR was carried out using primers proABFw and lacZRv, which anneal at *proA* and within the *lacZ* gene of the vector, respectively.

To make *gdhR* (NGO1360) insertional mutants, vector pUC18us-*gdhR*::*kan* (12) linearized with EcoRI was used to transform JC43 and to electroporate F62 Str^R to obtain strains JC47 and JC73, respectively. Transformants were selected on GC agar plates containing 60 μg/mL Km. To check the correct integration at the *gdhR* allele, two PCRs were performed with primer pairs pgntR3pac1/KmRv and KmRv/pme1gepR4, which amplify fragments at both flanking regions of the *kan* cassette. Both fragments were checked by Sanger sequencing using primers gdhR-pTX-F and pme1gepR4 for the 5′ and 3′ flanking fragments, respectively.

To complement *gdhR* insertional mutants, WT and P6S-encoding *gdhR* alleles were expressed from their own promoter in *trans* using vector pGCC3. Briefly, the *gdhR* promoter and coding sequence (CDS) were amplified by PCR with primers pgntR3pac1 and pme1gepR4 from FA19 and MS11 genomic DNA (gDNA). Both PCR fragments were digested with PacI-PmeI and ligated into similarly digested pGCC3 to create pGCC3-*gdhR* and pGCC3-*gdhR*^{P6S}. The ligated *gdhR* alleles were confirmed by sequencing with primers pgntR3pac1 and gepR_qRT_F. Vectors pGCC3-*gdhR* and pGCC3-*gdhR*^{P6S} were used to transform strain JC29, generating JC50 and JC51, respectively, and to transform JC47, generating strains JC52 and JC53, respectively. Transformants were selected on GC agar plates containing 1.0 μg/mL Erm for the FA19 background strains and 5.0 μg/mL for the MS11 background. The sequence of the integrated *gdhR* complement alleles was confirmed by PCR and Sanger sequencing using primers pgntR3pac1 and pme1gepR4.

To replace the *gdhR* WT allele with an unmarked mutant allele encoding GdhR P6S, we used the selectable and counterselectable *rpsL-cat* cassette method (34). Briefly, a PCR fragment encoding *gdhR* with SalI-XbaI restriction sites inserted after nucleotide 16 (from the start codon) was created by overlap extension PCR with primer pairs Eco-gdhR-Fw/MidgdhR-Rv and MidgdhR-Fw/Hin-gdhR-Rv using MS11

gDNA. The two obtained PCR fragments were mixed and used as a template to generate the final PCR fragment with primers Eco-gdhR-Fw and Hin-gdhR-Rv. Next, the PCR fragment was digested with EcoRI-HindIII and ligated into similarly digested pUC19 to generate pUC-*gdhR6*::SalI-XbaI. A SalI-XbaI-digested *rpsL-cat* cassette from pUNCH937 was ligated into similarly digested pUC-*gdhR6*::SalI-XbaI to obtain pUC-*gdhR6*::*rpsL-cat*. Additionally, a PCR fragment encoding *gdhR6* from MS11 was amplified with primers Eco-gdhR-Fw and Hin-gdhR-Rv and ligated into pUC19 as an EcoRI-HindIII fragment to generate pUC19-*gdhR6*. In a first step, vector pUC-*gdhR6*::*rpsL-cat* was linearized with NdeI-AatII and used to transform FA19 Str^R and to electroporate F62 Str^R, and transformants were selected on 0.5 μg/mL Cm. Integration of the *rpsL-cat* cassette into *gdhR* was confirmed by PCR with primers Eco-gdhR-Fw and Hin-gdhR-Rv. In a second step, the *rpsL-cat* cassette was replaced with a *gdhR6* allele using vector pUC19-*gdhR*^{P6S} linearized with NdeI-AatII and selection on GC-agar containing 100 μg/mL streptomycin to obtain strains JC69 and JC70. Replacement of the *gdhR* allele was confirmed by PCR with primers pgntR3pac1 and pme1gepR4 and by DNA sequencing with pgntR3pac1 and gepR_qRT_F.

To prepare transformants containing a single adenine deletion in the spacer of the *mtrR*-promoter elements (*mtrR*-P A-del), a PCR fragment was amplified with primers KH9#3 and CEL1 from gDNA of strain KH15 (11) and cleaned from the gel. The PCR fragment was used to electroporate strains F62 Str^R and JC70, and selection was done in GC-agar containing 0.5 μg/mL Erm to obtain JC72 and JC75, respectively. Transformants were confirmed by PCR and DNA sequencing using primers KH9#3 and CEL1.

Determination of the frequency of *mtrR*-P A-del spontaneous mutations. To determine the frequency of spontaneous *mtrR*-P A-del mutants arising from strain FA19 Str^R and its isogenic mutant containing *gdhR6* (JC69), approximately 5×10^8 CFU of each strain was plated on GC-agar containing Erm (0.5 μg/mL) in sextuplicate and octuplicate for FA19 and JC69, respectively. Arising colonies were quantified and isolated for further sequencing of the *mtrR* intergenic and CDS regions with primers KH9#3 and CEL1. The mutation frequency was calculated by dividing the number of resulting *mtrR*-P A-del mutants in each plate by 5×10^8 cells. The *gdhR* allele of *mtrR*-P A-del mutants obtained from FA19 was amplified with primers pgntR3pac1 and pme1gntR4 and sequenced with primers pgntR3pac1 and gepR_qRT_F, resulting in all keeping WT *gdhR*.

GdhR P6S expression and purification. GdhR WT protein was recombinantly expressed and purified from lysates of ER2566 *E. coli* cells using chitin bead affinity chromatography, as previously described (14). Similarly, to produce mutant GdhR P6S protein, the *gdhR6* allele from MS11 was cloned, expressed, and purified using the NEB IMPACT cloning and protein purification system by following the company protocol (New England Biolabs, MA, USA).

EMSA. EMSAs were conducted using the second-generation digoxigenin (DIG) gel shift kit (Roche Applied Sciences, Madison, WI) as previously described (14). In summary, a DNA fragment encoding the *lctP* promoter from −313 to −23 (relative to the start codon) was amplified with primers GdhR-EMSA-F/GdhR-EMSA-R from gDNA of strain FA19, and it was digoxigenin (DIG)-labeled using terminal transferase (Tdt) and DIG-11-ddUTP by following the manufacturer's protocol. DIG-labeled DNA was incubated with increasing concentrations of either GdhR WT or mutant purified proteins, and the resulting nucleoprotein complexes were resolved by polyacrylamide gel electrophoresis. The gel content was transferred to nylon membranes, UV-cross-linked, and developed using an anti-DIG Fab fragment-AP conjugate and chemiluminescence detection.

GdhR cross-linking studies. To study the dimerization activity of GdhR WT and mutant proteins, glutaraldehyde cross-linking assays were performed. Briefly, 2 μg of purified GdhR WT or mutant proteins was incubated in 18 μL of buffer containing 20 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 6.7, 0.1 M NaCl, 2% (vol/vol) glycerol with and without 2.6% (wt/vol) SDS (as a specificity control) for 5 min at room temperature. Next, 2.3 μL of 1.5% glutaraldehyde was added to the mix for a 17 mM final concentration, and the final reaction mixture was incubated for 30 min at 30°C. Finally, 6 μL of 6× Laemmli sample buffer containing β-mercaptoethanol (Bio-Rad) was added to the samples before being heated for 10 min at 90°C. Protein dimers and monomers were resolved using the TGX Stain-free fast cast 12% acrylamide kit (Bio-Rad). Gel images were developed with UV light and acquired using the Gel Doc XR molecular imager and the Image Lab software (Bio-Rad).

***β*-Galactosidase activity.** β-Galactosidase enzymatic activity was determined using the substrate *o*-nitro-phenyl-β-D-galactopyranoside (ONPG) as described by Miller (35). β-Galactosidase activities are given in Miller units using the formula $1{,}000 \times OD_{420nm}/(t \times v \times OD_{600nm})$, where t is the reaction time in min and v is the volume of cell lysates in milliliter per reaction mixture.

Extraction of total RNA and qRT-PCR. RNA extraction and qRT-PCR were performed as described before (14). Briefly, samples for RNA extraction were collected from liquid cultures (2 mL late exponential phase). Total RNA extraction was conducted using the RNeasy minikit (Qiagen) by following the manufacturer's protocol. Genomic DNA was removed using the Turbo DNA-free kit (Invitrogen). DNase I-digested total RNA samples were reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). qRT-PCR was conducted using the IQ SYBR green supermix and a CFX Connect real time-system (Bio-Rad Laboratories). Relative expression values for each sample were calculated as $2^{(CT\ internal\ reference\ -\ CT\ gene)}$, where CT is the fractional cycle threshold for the gene of interest and for the internal reference gene. The levels of *recA* mRNA were used as internal reference. The following primer pairs were used to quantify relative expression ratios: recAqFw/recAqRv for *recA* and lctPqFw/lctPqRv for *lctP*.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TABLE S1, XLSX file, 0.1 MB.

TABLE S2, DOCX file, 0.01 MB.

TABLE S3, XLSX file, 0.1 MB.
TABLE S4, DOCX file, 0.04 MB.
TABLE S5, DOCX file, 0.01 MB.

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We have no competing interest to declare.

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