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The MisR Response Regulator Is Necessary for Intrinsic Cationic Antimicrobial Peptide and Aminoglycoside Resistance in *Neisseria gonorrhoeae*

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During infection, the sexually transmitted pathogen *Neisseria gonorrhoeae* (the gonococcus) encounters numerous host-derived antimicrobials, including cationic antimicrobial peptides (CAMPs) produced by epithelial and phagocytic cells. CAMPs have both direct and indirect killing mechanisms and help link the innate and adaptive immune responses during infection. Gonococcal CAMP resistance is likely important for avoidance of host nonoxidative killing systems expressed by polymorphonuclear granulocytes (e.g., neutrophils) and intracellular survival. Previously studied gonococcal CAMP resistance mechanisms include modification of lipid A with phosphoethanolamine by LptA and export of CAMPs by the MtrCDE efflux pump. In the related pathogen *Neisseria meningitidis*, a two-component regulatory system (2CRS) termed MisR-MisS has been shown to contribute to the capacity of the meningococcus to resist CAMP killing. We report that the gonococcal MisR response regulator but not the MisS sensor kinase is involved in constitutive and inducible CAMP resistance and is also required for intrinsic low-level resistance to aminoglycosides. The 4- to 8-fold increased susceptibility of misR-deficient gonococci to CAMPs and aminoglycosides was independent of phosphoethanolamine decoration of lipid A and the levels of the MtrCDE efflux pump and seemed to correlate with a general increase in membrane permeability. Transcriptional profiling and biochemical studies confirmed that expression of *lptA* and *mtrCDE* was not impacted by the loss of MisR. However, several genes encoding proteins involved in membrane integrity and redox control gave evidence of being MisR regulated. We propose that MisR modulates the levels of gonococcal susceptibility to antimicrobials by influencing the expression of genes involved in determining membrane integrity.

*Neisseria gonorrhoeae* is a Gram-negative diplococcus and the causative agent of the sexually transmitted infection termed gonorrhea, which is currently the second most reported infection in the United States (1); an estimated 78 million new cases of gonorrhea occurred worldwide in 2012 (2). In addition to the high worldwide prevalence of gonorrhea, strains with resistance to currently or formerly used antibiotics have emerged, and concern has been voiced that without new effective antimicrobials, some cases of gonorrhea may be difficult to treat in future years (3). In addition to its ability to resist classical antibiotics used in treatment, gonococci have evolved mechanisms to evade the antimicrobial action of host compounds that participate in the innate host defense during infection. For instance, the ability of gonococci to resist the antibiotic-like action of host cationic antimicrobial peptides (CAMPs), such as defensins (4) or larger antimicrobial proteins (e.g., bactericidal permeability-increasing protein [5], cathepsin G [6], and CAP37 [7]), has been implicated in its survival within human polymorphonuclear granulocytes (PMNs) (8, 9).

Broadly, there are five known ways in which gonococci resist killing by CAMPs: (i) downregulation of host CAMP expression, (ii) delayed lysosomal fusion with gonococcal phagosomes, (iii) hindrance of CAMP access to the gonococcal surface, (iv) CAMP efflux, and (v) gonococcal surface modifications. These resistance mechanisms have been reviewed previously (10). Furthermore, recent evidence has shown that some gonococci can escape neutrophil extracellular traps (NETs) through the DNA-degrading action of a gonococcal thermonuclease, which is likely to diminish the bactericidal capacity of NET-associated antimicrobials, such as LL-37 and cathepsin G (11). Well-studied CAMP resistance mechanisms expressed by gonococci include efflux by the MtrCDE antimicrobial efflux pump (12) and surface modification at the lipid A moiety of lipooligosaccharide (LOS) with the small, positively charged molecule phosphoethanolamine (13). Both the efflux action of MtrCDE and phosphoethanolamine decoration of lipid A are important for gonococci to survive in the lower genital tract of experimentally infected female mice (14, 15), suggesting that these CAMP resistance systems are important for the *in vivo* survival of gonococci during genital tract infection in humans. In support of this hypothesis, Hobbs et al. showed that an *lptA*-null
Mutant was substantially less fit than the wild-type (WT) parental strain in the human male urethral infection model (16).

In addition to the presence and defined action of LptA and MtrCDE, gonococci have cis- and trans-acting control systems that modulate their expression at the transcriptional (mtrCDE) or translational (lptA) level; these regulatory systems have been reviewed elsewhere (10). In other bacteria, two-component regulatory systems (2CRS) consisting of a response regulator and a sensor kinase play prominent roles as regulators of CAMP resistance mechanisms. Well-studied examples include various outer membrane modifications of lipid A (e.g., decoration with small, positively charged molecules, such as 4-amino-4-deoxy-α-D-arabinopyranosylphosphate) through the action of the PhoP-PhoQ and PmrA-PmrB 2CRS in Gram-negative rods (17–19). Their sensing of environmental stimuli or stresses followed by transcriptional changes in target gene expression (e.g., ArnT, eptA) has received considerable attention, especially with respect to the control of bacterial virulence. The MisR-MisS 2CRS in N. meningitidis (20–24) shares some, but not all, properties of PhoP-PhoQ and also bears some similarity to another 2CRS involved in antimicrobial resistance termed CpxR-CpxA (25, 26). Accordingly, we hypothesized that gonococci might use MisR-MisS to sense and adapt to stresses imposed by CAMPs as an additional mechanism for resisting nonoxidative killing systems of the host. To our surprise, we found that MisR, but not MisS, contributes to gonococcal resistance to CAMPs as well as aminoglycosides by a mechanism independent of mtrCDE regulation or phosphoethanolamine decoration of lipid A. Furthermore, the loss of MisR decreased the potency of MtrCDE overexpression as a mechanism of resistance to some antimicrobials. We propose that MisR-dependent gonococcal antimicrobial resistance involves the regulation of many genes whose products collectively influence membrane permeability.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and primers.** N. gonorrhoeae strain FA19 and isogenic mutant strains, along with the plasmids used and their *Escherichia coli* hosts, are listed in Table 1. The primers used in this study are listed in Table S1 in the supplemental material. *E. coli* strains were routinely cultured on Luria-Bertani (LB) agar or in LB broth (Difco) containing 50 μg/ml kanamycin or 100 μg/ml ampicillin as necessary (liquid cultures were shaken at 200 rpm). Gonococci were grown on gonococcal base (GCB) agar (Difco) containing Kellogg’s supplements I and II (27) at 37°C under 5.0% (vol/vol) CO2. Liquid cultures of gonococci were begun by resuspending plate-grown cells in prewarmed 1× GCB broth; cells were then inoculated to a normalized optical density at 600 nm (OD_{600}) of 0.08 in prewarmed 1× GCB broth containing Kellogg’s supplements I and II and 0.043% (wt/vol) sodium bicarbonate and grown at 37°C with shaking at 200 rpm. Liquid cultures of gonococci contained a final concentration of 10 mM MgCl2 unless otherwise noted and were inoculated with plate-grown gonococci that were no more than 12 to 14 h old (we found

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>FA19</td>
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<tr>
<td>JK100</td>
<td>FA19 misR:kan</td>
<td>This study</td>
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<tr>
<td>JK101</td>
<td>JK100 complementation (FA19 misR:kan/pGCC4-misR)</td>
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<td>JK102</td>
<td>FA19 misS:kan</td>
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<tr>
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<td>JK102 complementation (FA19 misS:kan/pGCC4-misS)</td>
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<td>JK300</td>
<td>KH15 misR:kan</td>
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<td>FA19::P_{ptA-lacZ}</td>
<td>FA19 containing a translational fusion of the promoter region of lptA to the lacZ gene integrated at the proAB locus of the chromosome</td>
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<td>JK100::P_{ptA-lacZ}</td>
<td>FA19::P_{ptA-lacZ} misR:kan</td>
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<td>One Shot TOP10</td>
<td>F’ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔ15 ΔlacX74 recA1 araD139 ara leu7697 galU galK rpsL (Strr) endA1 nupG (catalog no. C4040-03; Invitrogen)</td>
<td>Invitrogen</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC19K</td>
<td>Plasmid containing the nonpolar aphA-3 kanamycin resistance cassette</td>
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<tr>
<td>pLES94</td>
<td>pUC18 derivative containing a truncated lacZ gene for use in translational fusions; recombines at the proAB locus of the gonococcal chromosome</td>
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<tr>
<td>pGCC4</td>
<td>Complementation vector for cloning of WT gene alleles downstream of an IPTG-inducible P_{lac} promoter; recombines between the lacT and aspC genes in the chromosome</td>
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<td>pLES94 containing the FA19 lptA promoter</td>
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<tr>
<td>pGCC4-misS</td>
<td>pGCC4 containing the WT FA19 misS gene under the control of P_{lac}</td>
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</table>
that misR::kan gonococci grew poorly in broth unless these conditions were met; in contrast, misS::kan gonococci did not have a growth defect [unpublished observations]). In experiments using strains containing WT copies of genes expressed ectopically from integrated pGCC4-based vectors (28), all cultures were supplemented with isopropyl β-D-1-thio-galactopyranosidase (IPTG) to a final concentration of 1 mM unless otherwise noted. When necessary, the presence of a single-base-pair deletion in the mtrR promoter (strain KH15 background) was confirmed by DNA sequencing of a PCR product representing the mtrR-mtrCDE intergenic region, generated using primers mtrC_promR and mtrJK1.

**Generation of misR and misS mutants and complemented strains.** Construction of the FA19 misR::kan mutant (here termed JK100) and FA19 misS::kan/pGCC4-misR (here termed JK101) complemented strains was performed as described below. The misR gene was inactivated using the nonpolar aphA-3 kanamycin resistance cassette (29). JK100 was constructed by transforming FA19 with meningooccal genomic DNA from the N. meningitidis misR::kan mutant S77T001 constructed previously (30). Plate transformations were performed as described previously (31). In general, misR-deficient transformants could be obtained with 3 to 4 days of incubation on GC agar containing kanamycin at 50 μg/ml. Loss of misR was confirmed by PCR using primers misR1, MisSR, and MisRev. Mutants were further verified by Western blotting (anti-MisR antisera were kindly provided by Yih-Ling Tzeng, Emory University). JK100 was complemented with a WT copy of misR cloned into pGCC4 using primers misRS3Pac and misR4Pmel and methods described previously (13) to generate strain JK101. MisR complementation in JK101 was confirmed by PCR and Western blotting.

Like misR, the misS gene was also inactivated using the nonpolar aphA-3 kanamycin resistance cassette. Strain JK102 (FA19 misS::kan) was constructed by transforming FA19 with genomic DNA from the N. meningitidis misS::kan mutant YT0310 constructed previously (24). The loss of misS in JK102 was confirmed by growth of the mutant on GCB agar containing kanamycin (50 μg/ml), followed by PCR and DNA sequencing across the misS5 operon using primers misR1 and MisSR, as well as KanB and KanD aphA-3 cassette primers. JK102 was complemented with a WT copy of misS cloned into pGCC4 using primers misSPlac and misSPmel and methods described previously (13) to generate strain JK103 (FA19 misS::kan/pGCC4-misS). Complementation in JK103 was confirmed by PCR.

**MIC assays.** For MIC assays, overnight (O/N) plate cultures of gonococci were resuspended in unsupplemented 1× GC broth, vortexed briefly, and normalized to an OD<sub>600</sub> of 0.1 before 5 μl was spot plated onto GCB agar containing 2-fold serial dilutions of the test antimicrobial. Incubated plates were grown for ~48 h before growth was analyzed. The MIC for each strain was considered to be the lowest concentration of antimicrobial required to significantly impact growth compared to that on a medium-only control plate.

**MBC assays and pretreatment of gonococci with PMB.** Polymyxin B (PMB) and LL-37 minimum bactericidal concentrations (MBCs) were determined as described previously (32) using gonococci grown in supplemented GCB broth containing 10 mM MgCl₂ and 1 mM IPTG. For PMB sublethal pretreatments, broth cultures were inoculated, grown as described above, and normalized to an OD<sub>600</sub> of 0.1 before 5 μl was added to GCB agar containing 2-fold serial dilutions of the test antimicrobial. Incubated plates were grown for ~48 h before growth was analyzed. The MIC for each strain was considered to be the concentration of antimicrobial at which ≥90% of the gonococci were killed. Purified synthetic LL-37 peptide was a kind gift from Jan Pohl (Biotechnology Core Facility Branch, Centers for Disease Control and Prevention, Atlanta, GA.)

**RNA-Seq analysis and qRT-PCR validation.** The transcriptome sequencing (RNA-Seq) experiment comparing the enriched mRNA transcriptomes of WT strain FA19 and JK100 (33) and the analyzed data set containing fold changes in gene expression (10; J. L. Kandler, R. Vélez Acvedo, M. K. Dickinson, D. R. Cash, W. M. Shafer, and C. N. Cornelis-sen, submitted for publication) have been reported previously. Gene expression fold changes (between JK100 and WT strain FA19) were considered significant if they met a ≥2-fold cutoff and had a Bonferroni-corrected P value of <0.05. For quantitative reverse transcription-PCR (qRT-PCR) measurement of MisR target gene expression, 1 ml of broth-grown gonococci was harvested at mid- or late log phase by centrifugation at 10,000 rpm for 2 min, and the pellets were stored at −70°C. RNA was purified by RNasey (Qiagen) and Turbo DNasefree (Ambion) treatment, and cDNA was generated using a Quantitect reverse transcriptase kit (Qiagen). The levels of MisR target gene transcription were determined by quantitative PCR in a 25-μl SYBR green (Bio-Rad) reaction mixture using 2 μl of 1:1,000 cDNA as the template. The normalized expression of each target gene was calculated by the 2<sup>−ΔΔC<sub>T</sub></sup> threshold cycle (C<sub>T</sub>) method (34) using 16S rRNA as a housekeeping reference gene. Mean fold change values were equivalent to the normalized expression ratio (MisR-repressed genes) or calculated as 1/normalized expression ratio (MisR-activated genes).

**Structure of the lptA-lacZ fusion and qualitative assessment of CPRG uptake and cleavage.** An lptA-lacZ translational fusion was generated using the pLES94 system (35). Briefly, the proximal promoter (36) of lptA was amplified using primers S’lptA-Z and S’lptA-Z and used to generate a translational fusion of lptA to the truncated, promoterless lacZ gene in pLES94. The pLES94 construct was transformed into One Shot TOP10 chemically competent E. coli cells (Invitrogen) by heat shock, and transformants were selected on LB agar containing 100 μg/ml ampicillin and 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and screened by PCR and sequencing. A confirmed pLES94-lptA plasmid was purified by use of a miniprep kit (Qiagen) and transformed into strain FA19 to generate strain FA19::P<sub>lptA-lacZ</sub>. Gonococcal transformants were selected on GCB agar containing 1 μg/ml chloramphenicol and further verified by PCR. Strain JK100::P<sub>lptA-lacZ</sub> was generated by transforming FA19::P<sub>lptA-lacZ</sub> with a PCR product spanning the misR::kan-misS operon from strain JK100, which was amplified using primers misR1 and MisSR. Transfected strains were selected on GCB agar containing 50 μg/ml kanamycin and then replica plated onto separate GCB agar plates containing 1 μg/ml chloramphenicol, 50 μg/ml kanamycin, or 25 μg/ml PMB (JK100 cannot grow in the presence of this PMB concentration). Transfectants with the correct antibiotic resistance phenotype were further confirmed for the absence of WT misR by PCR using primers misR1 and MisSR. Strains FA19 and JK100 with and without the lptA-lacZ fusion were spot plated onto supplemented GCB agar containing 2-fold serial dilutions of chlorophenol red-β-D-galactopyranoside (CPRG; catalog number 884308; Boehringer Mannheim) as described above for the MIC experiments. After 24 h of incubation, areas of gonococcal growth on plates containing 100 μg/ml CPRG were photographed to assess CPRG cleavage by LacZ and the release of chlorophenol red.

**Western blotting.** Mid-log-phase gonococci from broth cultures were pelleted by centrifugation at 10,000 rpm for 2 min, and whole-cell lysates were prepared in 1× Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄·6H₂O) by freeze-thawing 3 times in a dry ice-ethanol bath, followed by thorough vortexing. A 12% SDS-polyacrylamide gel (with a 5% stacking gel) was run in duplicate, with the levels of protein being normalized by use of a NanoDrop spectrophotometer prior to dilution and boiling for 10 min in 2× SDS loading dye (100 mM Tris-HCl, pH 6.8, 4% [wt/vol] SDS, 0.2% [wt/vol] bromophenol blue, 20% glycerol, 200 mM dithiothreitol [DTT]). One gel was Coomassie stained to show that the wells were loaded with an equivalent amount of protein. The other gel was transferred to a nitrocellulose membrane in Bjerrum-Schafer-Nielsen buffer (48 mM Tris, 39 mM glycine, 20% methanol [vol/vol]) on a Trans-Blot SD semidry transfer cell (Bio-Rad) and then was blocked O/N at 4°C using 3% [wt/vol] bovine serum albumin (MtrE blot) or 4% [wt/vol] nonfat dried milk (MisR blot) in 1× TST buffer (0.01 M Trizma base, 0.150 M NaCl, 0.05% [vol/vol] Tween 20). Blocked membranes were washed 3 times (10 min each) in 1× TST buffer.
and probed with primary antibody O/N at 4°C against MtrE or MisR using 1:10,000 rabbit polyclonal antiserum (antiserum were generously provided by Ann E. Jerse and Yih-Ling Tseng, respectively) diluted in 1× TST buffer. Anti-MtrE antiserum was generated using amino acids 110 to 120 of MtrE (RQGSLSGGNVS [37]). Anti-MisR antiserum was generated using purified MisR-His6X protein (24). The blots were washed as described above in 1× TST buffer and incubated with 1:2,500 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (product number 32460; Thermo Scientific) diluted in 1× TST buffer for 1 h at room temperature. The blots were given final washes as described above in 1× TST buffer before 1 min of development with a 1:1 luminol-peroxide solution (product number 32209; Thermo Scientific). Bands were visualized by exposure of the membranes to autoradiography film.

MALDI-TOF MS analysis of gonococcal lipid A. Overnight cultures of WT strain FA19, JK100, JK101, and FA19 lptA::spc (~12 liters per strain) were grown in shaking GCB broth containing 1 mM IPTG and 10 mM MgCl₂, pelleted by centrifugation at 7,500 rpm for 15 min at 4°C, washed 3 times in 1× phosphate-buffered saline (PBS; pH 7.2), and fixed in 10% formalin diluted in 1× PBS. Processed pellets were frozen at ~70°C, and LOS was extracted from the wet cell paste by the hot phenol-water method (38), followed by dialysis (3,500-Da-molecular-mass cutoff) against several exchanges of deionized H₂O. Phospholipids were removed by three washes and precipitation of LOS with chilled ethanol-H₂O (9:1, vol/vol). Nucleic acids and proteins were removed by treatment with DNase I, RNase A, and proteinase K (all reagents from Sigma-Aldrich) following the manufacturers’ instructions, the LOS material was dialyzed (3,500-Da-molecular-mass cutoff), and the dialysate was ultracentrifuged at 100,000 × g for 6 h at 4°C. Lipid A was extracted from LOS by mild hydrolysis with 1% acetic acid for 1 h at 100°C and recovered from the reaction mixture by extraction with chloroform. Purified lipid A was analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) on an AB Sciex TOF/TOF 5800 system in the negative reflector mode ([M-H]⁻).

RESULTS

MisR, but not MisS, is required for constitutive and inducible levels of gonococcal resistance to CAMPs. Earlier reports demonstrated that the loss of misR increases the susceptibility of N. meningitidis to a cationic lipopeptide antimicrobial, polymyxin B (PMB), which has served as a model CAMP (21, 22), and to the human defensin human neutrophil peptide-1 (HNP-1) (20). Since meningococcal MisR (GenBank accession number AAF41023.1; locus tag NMB0595) and gonococcal MisR (GenBank accession number EEZ45023.1; locus tag NGEG_00293) are 100% identical at the amino acid level (http://blast.ncbi.nlm.nih.gov/Blast.cgi), we hypothesized that gonococci would also display MisR-dependent CAMP resistance. Accordingly, we determined the MBC₉₀ of the human cationic LL-37 and the MIC of PMB against strain FA19 (WT) and the FA19 misR::kan mutant (JK100). We found that FA19 was 4- to 8-fold more resistant to LL-37 and PMB than JK100 and that the parental levels of LL-37 and PMB resistance could be restored if a WT copy of misR was expressed ectopically in complemented strain JK101 (FA19 misR::kan/pGCC4-misR) (Fig. 1A and B). Interestingly, the loss of misS in gonococcal strain JK102 (FA19 misS::kan) did not increase gonococcal susceptibility to PMB (Fig. 1B). Complementation of misS in strain JK103 (FA19 misS::kan/pGCC4-misS) also had no impact on PMB susceptibility.

The result presented above suggested that the response regulator action of MisR did not require input from its cognate sensor kinase, MisS, to provide constitutive levels of gonococcal resistance to CAMPs under the conditions tested. In order to determine if inducible CAMP resistance in gonococci is possible and if such resistance requires both MisR and MisS, we grew the test strains in the absence or presence of a sublethal level (0.1× plate MIC) of PMB in broth culture and then quantified the resulting PMB susceptibility by determining the MBC₉₀ for each strain. Briefly, we found that gonococci could indeed be induced to higher levels of PMB resistance (4-fold greater than that of carrier-treated WT strain FA19 gonococci) but that this induction required only MisR and not MisS (Fig. 2).

Gonococci exhibit MisR-dependent resistance to aminglycosides and antimicrobials recognized by the MtrCDE efflux pump. In order to determine if MisR-dependent antimicrobial resistance of gonococci was restricted to CAMPs, we assessed whether strain JK100 was more susceptible to other kinds of antimicrobials (i.e., antibiotic drugs, dyes, and detergents) than WT parent strain FA19. For this purpose, we also examined other genetic derivatives of strain FA19 that displayed increased CAMP susceptibility due to the loss of the MtrCDE efflux pump (strain
FIG 2 MisR, but not MisS, is required for constitutive and inducible resistance to PMB. MBC 90s for gonococci were determined after growth in broth in the presence or absence of sublethal levels of PMB. MBC 90s values compared to those for carrier-treated FA19 (WT) gonococci are represented. White columns, strains treated with carrier (ddH 2O); black columns, strains treated with a sublethal level of PMB (0.1%) on the plate MIC. Shown are modal results from three or more independent experiments (the JK103 [FA19 misS::kan/pGCC4::misS] complemented strain was tested in two independent experiments) performed in technical triplicate. MisR and MisS genotypes are noted in parentheses.

KH14 [FA19 mtrD::kan] or that lacked the ability to decorate lipid A with phosphoethanolamine (strain FA19 lptA::spc); the roles of the MtrCDE efflux system and phosphoethanolamine decoration of lipid A in gonococcal CAMP resistance have been described previously (12, 13, 16, 39–41). With respect to differences in CAMP susceptibility, the misR::kan and mtrD::kan mutations (strains JK100 and KH14, respectively) significantly increased PMB susceptibility 8- and 4-fold, respectively, though the strains with these mutations were not as exquisitely PMB sensitive as the FA19 lptA::spc mutant (256-fold increased susceptibility; Table 2). However, the loss of the MtrCDE efflux pump in KH14 did result in gonococcal hypersusceptibility to a number of antimicrobials known from previous work to be substrates for this pump (41). In contrast, strain JK100 displayed only a modest (2-fold) increase in susceptibility to most tested MtrCDE efflux pump substrates and was not hypersusceptible to Triton X-100 (TX-100), which has been shown to be exported by this pump (42). A notable exception to this trend was ceftiraxone, the MIC of which was decreased 4-fold in strain JK100 compared with that in strain FA19.

Since the repression of mtrCDE transcription by MtrR in the WT strain FA19 background (43, 44) might mask MisR’s impact on antimicrobial efflux, we also examined the effect of the misR::kan mutation in two genetic derivatives of strain FA19 (strain JF1 [FA19 ΔmtrR], which has a large deletion in the mtrR gene, and strain KH15 [FA19 mtrR::kan], which has a single-base-pair deletion in the mtrR-mtrCDE intergenic region [Table 2]). JF1 and KH15 have medium and high levels of expression of mtrCDE, respectively, and as a result display enhanced resistance to antimicrobials that are exported by MtrCDE. Strain JK200 (JF1 misS::kan) generally had 2-fold increased susceptibility to MtrCDE substrates compared to that of JF1. However, the MIC profile of strain JK300 (KH15 misR::kan) revealed the importance of MisR for the high-level resistance mediated by this efflux pump. Notable changes occurred in the MICs for crystal violet, erythromycin, penicillin G, and TX-100 (4-fold or more increased susceptibility in JK300 compared with that in KH15).

Having observed the impact of MisR on CAMP resistance and the efficient high-level efflux of antimicrobials, we wondered if gonococcal susceptibility to drugs that are not pump substrates of MtrCDE would also be affected by the loss of MisR. To test this, we determined the MICs of three aminoglycosides (gentamicin, tobramycin, and streptomycin) and an aminocyclitol (spectinomycin) against our test strains. As shown in Table 2, the loss of MisR in any mtrCDE background consistently increased gonococcal sensitivity to all aminoglycosides by 4- to 8-fold (in contrast to results for the aminocyclitol spectinomycin and the fluoroquinolone ciprofloxacin). We noted that this fold difference in aminoglycoside susceptibility was similar to that seen for CAMPs. Importantly, the loss of phosphoethanolamine decoration of lipid A in strain FA19 lptA::spc and the loss of MtrCDE efflux in strain KH14 resulted in only 2-fold increased susceptibility.

### TABLE 2 Susceptibility of misR-deficient gonococci to substrates of the MtrCDE efflux pump and aminoglycosides

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)a</th>
<th>CRO</th>
<th>Pen G</th>
<th>CV</th>
<th>TX-100</th>
<th>Cipb</th>
<th>Gent</th>
<th>Tob</th>
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<tbody>
<tr>
<td>FA19 (WT)</td>
<td>100</td>
<td>0.25</td>
<td>0.0006</td>
<td>0.016</td>
<td>0.625</td>
<td>125</td>
<td>0.0025</td>
<td>10</td>
<td>10</td>
<td>12.5</td>
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<tr>
<td>JK100 (FA19 misR::kan)</td>
<td>12.5</td>
<td>0.125</td>
<td>0.00015</td>
<td>0.008</td>
<td>0.313</td>
<td>125</td>
<td>0.0025</td>
<td>1.25</td>
<td>1.25</td>
<td>3.13</td>
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<td>0.0006</td>
<td>0.016</td>
<td>0.625</td>
<td>125</td>
<td>0.0025</td>
<td>10</td>
<td>10</td>
<td>25</td>
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<tr>
<td>JF1 (FA19 ΔmtrR)</td>
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<td>0.0006</td>
<td>0.016</td>
<td>1.25</td>
<td>250</td>
<td>0.0025</td>
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<td>10</td>
<td>12.5</td>
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<tr>
<td>JK200 (JF1 misR::kan)</td>
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<td>0.0003</td>
<td>0.008</td>
<td>0.625</td>
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<td>0.0025</td>
<td>1.25</td>
<td>1.25</td>
<td>3.13</td>
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<tr>
<td>KH15 (FA19 mtrD::kan)</td>
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<td>0.0006</td>
<td>0.032</td>
<td>2.5</td>
<td>800</td>
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<td>10</td>
<td>10</td>
<td>25</td>
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<tr>
<td>JF1 (FA19 mtrR::kan)</td>
<td>50</td>
<td>0.5</td>
<td>0.0003</td>
<td>0.008</td>
<td>0.625</td>
<td>125</td>
<td>0.0025</td>
<td>1.25</td>
<td>1.25</td>
<td>3.13</td>
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<tr>
<td>KH14 (FA19 mtrD::kan)</td>
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<td>0.0012</td>
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<td>FA19 lptA::spc</td>
<td>0.39</td>
<td>0.25</td>
<td>0.0006</td>
<td>0.032</td>
<td>0.625</td>
<td>125</td>
<td>0.0025</td>
<td>5</td>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

a Modal MIC values were determined from three or more independent experiments. Bold values indicate a 4-fold or greater increased susceptibility in a given misR-deficient mutant compared with the susceptibility of the parent strain. Antimicrobial abbreviations: PMB, polymyxin B; Ery, erythromycin; CRO, ceftriaxone; Pen G, penicillin G; CV, crystal violet; TX-100, Triton X-100; Cip, ciprofloxacin; Gent, gentamicin; Tob, tobramycin; Str, streptomycin; Spc, spectinomycin.

b The fluoroquinolone ciprofloxacin was included as a control drug that is not subject to MtrCDE efflux.

c The aminocyclitol spectinomycin was included as a control.

d Strain JK101 is resistant to erythromycin due to the ermC gene expressed by the pGCC4 construct.

e FA19 lptA::spc is resistant to both spectinomycin and streptomycin due to the adaA4 gene encoded in the Ω cassette that interrupts lptA (86).
ity to aminoglycosides, which is not considered significant in 2-fold agar dilution MIC assays.

To ensure that the increased CAMP and aminoglycoside susceptibility of gonococci due to the loss of MisR was not restricted to the FA19 genetic background, we constructed a misR::kan transformant of strain MS11, which has a higher level of PMB resistance (MIC, 400 µg/ml) due to mutations at the mtrR-mtrCDE locus (mtrR126 and mtrR3197) that result in overexpression of the mtrCDE efflux pump (45, 46). In agreement with the results obtained with MisR-negative variants of strains FA19, JF1, and KH15, we found that the loss of MisR in strain MS11 misR::kan resulted in an 8-fold increase in gonococcal susceptibility to both PMB and gentamicin compared to the susceptibility of the MS11 parent strain.

MisR control of CAMP and aminoglycoside resistance is independent of LptA and MtrCDE. The results from the MIC studies described above suggested that MisR modulation of gonococcal CAMP and aminoglycoside resistance is through a pathway independent of phosphoethanolamine decoration of lipid A or simple upregulation of MtrCDE efflux. To test this hypothesis, we asked if the loss of MisR might influence the lipid A structure (specifically, phosphoethanolamine decoration) or the levels of the MtrCDE efflux pump. With respect to lipid A, MALDI-TOF MS analysis of purified lipid A from gonococcal strains showed that phosphoethanolamine-decorated lipid A was readily detected in strains FA19, JK100, and JK101 (see Fig. S1 in the supplemental material). As expected, phosphoethanolamine-lipid A was undetectable in the FA19 lptA::spc control. No differences in lipid A attributable to altered MisR expression were found. To determine any impact of MisR on the levels of the MtrCDE efflux pump, we used anti-Mtre antisera to probe for the levels of the outer membrane channel protein, Mtre (which is encoded by the last gene in the mtrcde operon), in gonococcal whole-cell lysates. Results from Western immunoblotting experiments demonstrated that the levels of Mtre were identical in strain pairs FA19 and JK100, JF1 and JK200, and KH15 and JK300 (see Fig. S2 in the supplemental material). Finally, analysis of published RNA-Seq data comparing the mRNA-enriched transcriptomes of FA19 and JK100 (33; Kandler et al., submitted) confirmed that the loss of MisR did not alter the transcription of lpta or mtrcde, nor did it change the expression of accessory efflux genes mtrR, mtrA, and mtrF. This observation is consistent with the biochemical data described above showing that MisR does not control the expression of these CAMP resistance systems. Taken together, we conclude that MisR does not influence the expression of two established CAMP resistance systems (phosphoethanolamine decoration of lipid A and MtrCDE efflux of antimicrobials) and likely modulates the levels of gonococcal antimicrobial susceptibility through a different mechanism.

MisR influences global transcription patterns and membrane permeability. Additional analysis of a previously reported RNA-Seq data set (33; Kandler et al., submitted) revealed that 59% (55/94) of significantly MisR-regulated target genes (≥2-fold cut off; Bonferroni-corrected P value, <0.05) encoded proteins that are predicted or known to localize to the cell envelope (e.g., laz, dco, nadC, act) in the protein-folding/chaperone machinery (e.g., htx, ddbD), participate in protein secretion (e.g., tatB, tatC), or contribute to redox reactions within the cell (e.g., bfrA, bfrB, grx3, trx1) (see Tables S2A and B in the supplemental material). As is summarized in Table 3, we validated the RNA-Seq data using qRT-PCR of strains FA19 and JK100 to confirm the MisR activation or MisR repression of several genes in this regulon. For most genes tested, the MisR-dependent activation (tatC, htx, bfrA and bfrB, ddbD) or repression (nadC, clpB) observed in the RNA-Seq experiment was repeatable by qRT-PCR of total RNA prepared from both mid- and late-log-phase cultures. Additionally, complementation of MisR in strain JK101 returned the level of expression of these genes to nearly WT levels (see footnote d in Table 3). In contrast, grpE and dnaK expression was similar in FA19 and JK100 gonococci, as assessed by qRT-PCR. However, late-log-phase dnaK levels were ≥2-fold decreased in strain JK101 compared to those in FA19, indicating slight MisR repression of dnaK, which is consistent with the regulation seen in the RNA-Seq experiments. These observations highlight the importance of confirming transcriptomic data using alternative methods. Finally, a bioinformatics search for MisR consensus binding sites using the PRODORIC online tool (47) and a search for published DNA-binding experiments revealed the presence of a consensus or a nearly consensus MisR-binding site upstream of most of these genes, suggesting that they may be directly regulated by MisR. No such site was found upstream of grpE, which may explain the lack of MisR regulation as assessed by qRT-PCR.

Although the RNA-Seq data did not pinpoint a specific molecular mechanism of MisR-dependent antimicrobial resistance, it did suggest that the overall physiology and integrity of the cell envelope in MisR-negative gonococci differed from those in MisR-positive gonococci. Based on this hypothesis, we asked if the loss of MisR would increase the general permeability of gonococci. For a qualitative assessment of this possibility, we used strains FA19 and JK100 expressing an lpta-lacZ translational fusion to test the cleavage of the beta-galactosidase substrate chlorphenol red-β-d-galactopyranoside (CPRG), which is analogous to the more commonly used beta-galactosidase substrate o-nitrophenyl-β-d-galactopyranoside (ONPG). When hydrolyzed by the cytosolic enzyme beta-galactosidase, the yellow-orange CPRG substrate releases the red chromophore chlorphenol red. CPRG is more sensitive to beta-galactosidase activity than ONPG (48). Like ONPG, CPRG is membrane impermeant and has previously been used to assess membrane integrity (49–51); importantly, previous work with these strains confirmed their expression of beta-galactosidase, and MIC experiments demonstrated that CPRG lacks antigonococcal action against the test strains employed (data not shown). As is shown in Fig. 3, after 24 h of growth on plates containing 100 µg/ml CPRG, the JK100::lpta-lacZ strain had cleaved more CPRG than the FA19::lpta-lacZ strain, as demonstrated by the purple-red coloring of the cells and surrounding medium. In contrast, parent strains lacking beta-galactosidase were unable to cleave CPRG and generated no red coloring. This result is consistent with the notion that the loss of MisR increases the permeability of the gonococci.

DISCUSSION

We propose that the gonococcal MisR protein component of the MisR-MisS 2CRS functions as a transcriptional regulator of genes involved in determining cell envelope integrity. Herein, we provide phenotypic evidence that its loss increases gonococcal cell permeability and sensitizes gonococci to various antimicrobial agents, especially CAMPs and aminoglycosides. Additionally, we found that decoration of lipid A with phosphoethanolamine (a consequence of PhoP-PhoQ or PmrA-PmrB regulation in other
TABLE 3 Validation of the MisR RNA-Seq data set by qRT-PCR and bioinformatic prediction of MisR binding sites

<table>
<thead>
<tr>
<th>Fold change in expression between misR-deficient and WT strains by:</th>
<th>qRT-PCR$^d$</th>
<th>Position in FA 1090</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA-Seq$^c$</td>
<td>Mid-log phase</td>
</tr>
<tr>
<td>FA 1090 ORF$^a$ (gene)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGO0181 (tatC)</td>
<td>$-24.34$</td>
<td>$-3.61 \pm 1.34$</td>
</tr>
<tr>
<td>NGO0377 (nasC)</td>
<td>10.42</td>
<td>7.01 $\pm$ 2.73</td>
</tr>
<tr>
<td>NGO0399 (htpX)</td>
<td>$-3.84$</td>
<td>$-20.37 \pm 13.89$</td>
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<tr>
<td>NGO0794 (NGO0795)</td>
<td>$-3.26/-3.79$</td>
<td>$-6.36 \pm 3.48$/</td>
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<tr>
<td>NGO0978 (dsbD)</td>
<td>$-9.21$</td>
<td>$-51.30 \pm 27.47$</td>
</tr>
<tr>
<td>NGO1046 (clpB)</td>
<td>5.01</td>
<td>3.09 $\pm$ 1.50</td>
</tr>
<tr>
<td>NGO1422 (grpE)</td>
<td>2.62</td>
<td>1.44 $\pm$ 0.54</td>
</tr>
<tr>
<td>NGO1429 (dnaK)</td>
<td>2.20</td>
<td>1.53 $\pm$ 0.46</td>
</tr>
</tbody>
</table>

$^a$ ORF, open reading frame.
$^b$ Potential MisR binding sites were determined bioinformatically using the PRODORIC online tool (http://prodoric.tu-bs.de/). Input parameters were as follows: strain ATCC 708025, N. gonorrhoeae FA 1090 (NCBI reference sequence, GenBank accession number NC_002946); one-pattern IUPAC code, KWWWTGTAARGNNWH; mismatch tolerance, 2; maximum distance to gene, 3,000 bp; ignored match orientation and removed palindromic matches. MisR binding site nucleotides that match the consensus sequence reported previously (23) are in bold, and mismatches are underlined. The consensus sequence is KWWVTGAARGNNWH, where K is G or T, W is A or T, R is A or G, N is any nucleotide, and H is A, T, or C.
$^c$ Shown are the mean fold changes in the number of reads per kilobase per million reads (RPKM) comparing gene expression in JK100 and WT strain FA19 gonococci from three independent experiments. All RNA-Seq fold changes (between the misR-deficient strain and the WT) were statistically significant (Bonferroni-corrected $P$ value, $<0.05$). Negative fold changes indicate MisR activation, and positive fold changes indicate MisR repression.
$^d$ Shown are the mean ± standard deviation fold changes in expression between JK100 and WT strain FA19 gonococci at mid- or late log phase from three independent experiments. The mean normalized expression ratios comparing complemented strain JK101 and WT strain FA19 gonococci from three independent experiments are as follows: for mid-log-phase cells, 1.23 for tatC; 0.78 for nasC; 1.04 for hpcX; 0.79 for bfrA; 0.85 for bfrB; 0.83 for dsbD; 0.74 for clpB; 1.13 for grpE; and 0.59 for dnaK; for late-log-phase cells, 1.08 for tatC; 0.58 for nasC; 0.78 for hpcX; 0.75 for bfrA; 0.79 for bfrB; 1.04 for dsbD; 0.40 for clpB; 1.81 for grpE; and 0.46 for dnaK.
$^e$ Shown are the predicted MisR binding site upstream of dsbD in N. meningitidis (78). Note that the NCBI-annotated dsbD start codon in FA 1090 is upstream of the transcriptional start site experimentally determined previously (78) and is therefore likely to be incorrect. Thus, the numerical position of the MisR binding site shown here for dsbD is relative to that of the N. meningitidis dsbD start codon in the previous report (78).

FIG 3 Loss of MisR increases gonococcal envelope permeability. Gonococci expressing β-galactosidase were grown in the presence of the membrane-impermeant β-galactosidase substrate CPRG. Shown are buttons of gonococcal growth after 24 h of standard incubation on supplemented GCB agar containing 100 μg/ml CPRG. Note the increased cleavage of CPRG to release chlorophenol red for misR-deficient strain JK100::F$_{lpaC-lacZ}$. MisR genotypes are noted in parentheses.
MtrCDE substrates was apparent only when MtrCDE was overexpressed (compare the MICs for strains KH15 and JK300 in Table 2). Since we demonstrated that the levels of this efflux pump were likely the same regardless of MisR expression (see the Results and Fig. S2 in the supplemental material), we concluded that MisR is required for efficient MtrCDE function but not expression. Whether MtrCDE impairment by the loss of MisR is due to improperly localized/misfolded MtrCDE components, decreased pumping power due to possible effects on the proton motive force, or an expedited passage of MtrCDE substrates through a flawed phospholipid bilayer is not yet clear. We also note the curious finding that ceftriaxone is most potent against misR:kan gonococci in an FA19 background (Table 2). Ceftriaxone is currently the front-line therapy for gonococcal infections in the United States, but several instances of ceftriaxone resistance around the world (see Fig. 13 in reference 57) hasten the approach of more treatment failures (3, 58). To our knowledge, this is the first report of a 2CRS being involved in modulating the levels of gonococcal susceptibility to a 3rd-generation cephalosporin (59, 60).

While our data suggest that gonococcal MisR is not regulating CAMP and aminoglycoside resistance through known mechanisms, our RNA-Seq data show that it does regulate the genes of numerous envelope-localized proteins, proteases/chaperones, and redox factors. That MisR targets these particular categories of genes is reminiscent of 2CRS regulators in other Gram-negative organisms, such as CpxR in Vibrio cholerae (61) and E. coli (reviewed in reference 26), AmgR in Pseudomonas aeruginosa (62), and, indeed, MisR in N. meningitidis (23). Gonococcal MisR (GenBank accession number EEZ45023.1; locus tag NGEG_00293) is 100% identical to meningococcal MisR (GenBank accession number AAF41023.1; locus tag NMB0595) at the amino acid level, and a simple BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) reveals that CpxR in E. coli strain K-12 and V. cholerae O1 biovar El Tor (50% and 48% amino acid sequence identity, respectively) and, likewise, AmgR in P. aeruginosa strain PAO1 (41% amino acid sequence identity) are the closest orthologues to MisR in these species. Similar to MisR in gonococci, CpxR and AmgR are required for intrinsic aminoglycoside (26, 62–64) and CAMP (65, 66) resistance. In contrast, expression of the well-characterized PhoP-PhoQ and PmrA-PmrB systems appears to be important primarily for CAMP but not aminoglycoside resistance in enteric pathogens (reviewed in reference 67).

A shared characteristic of CAMPs and aminoglycosides is that both classes of antimicrobials have been proposed to enter bacteria via self-promoted uptake in a membrane integrity–dependent manner (68–70). As the concentration of aminoglycoside increases, misreading of mRNA results in an accumulation of improperly folded proteins, which, if integrated into the membrane, are hypothesized to fit poorly between phospholipids; inappropriate membrane integration may generate nonspecific hydrophilic channels through which more aminoglycoside molecules (normally unable to efficiently permeate the phospholipid bilayer) may pass with relative ease (69, 71). Similarly, CAMPs, which initially bind parallel to the plane of bacterial membranes at the interface of the hydrophilic head groups and hydrophobic fatty acid tails, may associate at higher concentrations into membrane-disruptive aggregates (72). Thus, one possible explanation for the defective membrane integrity seen in the gonococcal misR:kan mutants is that aberrant protein folding or faulty translocation through the membranes, perhaps resulting from dysregulation of protein quality control genes, may predispose gonococci to CAMP and aminoglycoside entry by disrupting the normally stable packing of membrane phospholipids. Notably, the gene encoding the HtpX membrane protease, which is important for degrading misfolded cell envelope proteins (73), is a shared target for strong activation by MisR (Table 3), CpxR (74, 75), and AmgR (62–64, 76) across three different bacterial genera.

Additionally, the loss of MisR may result in an atypical redox environment due to MisR regulation of genes encoding redox factors, iron controllers, electron transport components, etc. (see Table S2 in the supplemental material), whose products are associated with reduction/oxidation reactions or oxidative stress responses. For example, we demonstrated (Table 3; see also Table S2A in the supplemental material) that the bacterioferritin genes bfrA and bfrB are strongly activated by MisR and recently reported with our collaborators that bidirectional MisR regulation of the transferrin binding protein genes tlpB and tlpA is important for iron uptake control (Kandler et al., submitted). Furthermore, a hallmark target for MisR activation is the gene dsbD (Table 3), which encodes the thio-disulfide interchange protein DbSD important for proper disulfide bond formation in periplasmic proteins (23, 77). Published experiments performed with meningococci (78) have shown that dsbS expression is increased in response to the presence of a reducing agent (DTT) in a MisS-dependent manner, which could indicate that MisS can directly sense changes to the periplasmic redox balance. Further work is needed to address this possibility in gonococci.

CAMPs are increasingly recognized as important components of the innate host defense during infection. Over the millennia, these antimicrobials have exerted selective pressure on bacteria to develop mechanisms to resist their action (79). As was recently reviewed (80), CAMP resistance mechanisms expressed by bacteria can have a significant influence on bacterial survival or fitness during infection. One of the early observations validating this concept in gonococci was that the loss of the MtrD efflux pump protein increased gonococcal susceptibility to LL-37 by approximately 8-fold in vitro (12) and led to a >100-fold reduction in the amount of viable gonococci at 3 days postinfection in vivo (14). While information regarding the importance of MisR during gonococcal infection is lacking, previous studies on meningococci showed that the loss of MisS significantly decreased the virulence of N. meningitidis in an experimental murine infection model (21), and we suspect that the CAMP-susceptible phenotype of misR-deficient gonococci would produce similar results. It is also important to emphasize that because some CAMP resistance systems (e.g., efflux by MtrCDE) can also influence bacterial susceptibility to antibiotics used in therapy, the capacity of bacteria to regulate the expression or function of mechanisms involved in such resistance could impact the clinical efficacy of antibiotics. The latter point is of importance for gonorrhea because the absence of a vaccine requires the continued availability of effective antibiotics, which is now threatened by the emergence of strains resistant to previously used or current front-line antibiotics (3, 81). The characterization herein of a novel CAMP/aminoglycoside/MtrCDE-substrate resistance mechanism in gonococci is of special interest in light of the dwindling number of curative antibiotics for gonorrhea, clinical isolates that overexpress mtrCDE (46), and the potential implementation of an
aminoglycoside (gentamicin) as a first-line therapy in the United States (82).

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We have no competing financial interest to declare.

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