AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

COMMENTARY
Therapeutics and Prevention

Mosaic Drug Efflux Gene Sequences from Commensal *Neisseria* Can Lead to Low-Level Azithromycin Resistance Expressed by *Neisseria gonorrhoeae* Clinical Isolates

William M. Shafer[a,b,c]

[a]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia, USA
[b]The Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, Georgia, USA
[c]The Laboratories of Bacterial Pathogenesis, Medical Research Service, Veterans Affairs Medical Center, Decatur, Georgia, USA

ABSTRACT In a previous *mBio* article, Wadsworth and colleagues (mBio 9:e01419-18, 2018, https://doi.org/10.1128/mBio.01419-18) described *Neisseria gonorrhoeae* isolates that express low levels of azithromycin (Azi) resistance. Whole-genome sequencing and bioinformatic analysis suggested that the isolates had acquired DNA from commensal *Neisseria* spp. that caused numerous nucleotide changes in the *mtr* locus, which contains genes for a transcriptional repressor (MtrR) and three proteins (MtrC-MtrD-MtrE) that form a multidrug efflux pump known to export macrolides. Strong regions of linkage disequilibrium mapped to the overlapping *mtrR* and *mtrCDE* promoters and *mtrD*. Genetic analyses revealed that these mosaic-like sequences increased transcription of *mtrCDE* and MtrD function, respectively. These changes also had strong epistatic effects that collectively were responsible for decreased susceptibility to MtrCDE substrates, including Azi. The report emphasizes the importance of gene exchange among neisserial species and development of antibiotic resistance in gonococci, both of which have ramifications for detection of resistance markers and efficacy of antibiotic treatment regimens for gonorrhea.

KEYWORDS *Neisseria gonorrhoeae*, antibiotic resistance, gonorrhea, transformation

The efficacy of antibiotics for treatment of gonorrhea is now threatened by the global emergence of *Neisseria gonorrhoeae* strains with resistance to currently used antibiotics, including azithromycin (Azi) and ceftriaxone (Cro) (reviewed in reference 1). The threat of "untreatable infections" in the absence of new antibiotics is compounded by the over 78 million cases of gonorrhea estimated to occur worldwide each year (2). While spontaneous mutations in *N. gonorrhoeae* can contribute to antibiotic resistance in gonococci, earlier studies on acquisition of foreign resistance determinants (e.g., the beta-lactamase plasmid and *tetM* determinant) highlighted the ability of gonococci to acquire resistance from other microbes that can exist in the same ecological niche (reviewed in reference 3). Further, acquisition and recombination of similar, but not identical, DNA sequences from commensal *Neisseria* spp. by transformation can give rise to the formation of so-called mosaic sequences that can result in remodeling of an antibiotic target, making gonococci less susceptible to the drug. The best example of this is how gonococci developed decreased susceptibility (or even clinical resistance) to third-generation cephalosporins (e.g., Cro and cefixime) by acquiring *pbp2* sequences from commensal *Neisseria* spp. that result in numerous amino acid changes in penicillin-binding protein 2, decreasing the binding of beta-lactams (reviewed in reference 3).

In a recent article in *mBio*, Wadsworth et al. (4) extended this concept of gene mosaicism and gonococcal antibiotic resistance. They described a subset of strains

Published 11 September 2018

Citation Shafer WM. 2018. Mosaic drug efflux gene sequences from commensal *Neisseria* can lead to low-level azithromycin resistance expressed by *Neisseria gonorrhoeae* clinical isolates. mBio 9:e01747-18. https://doi.org/10.1128/mBio.01747-18.

Address correspondence to wshafer@emory.edu.

For the article discussed, see https://doi.org/10.1128/mBio.01419-18.

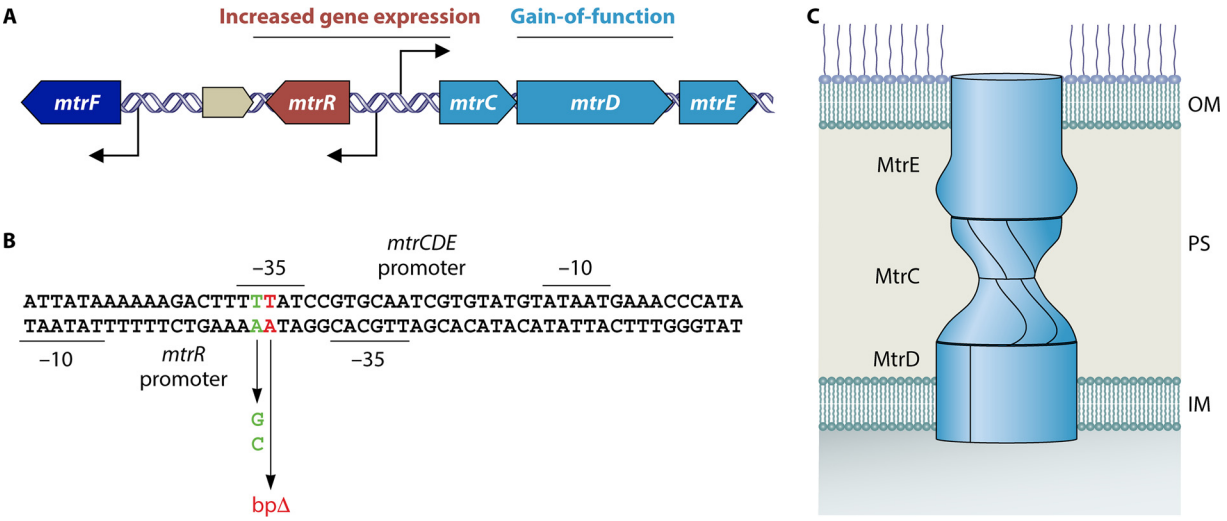

FIG 1 (A) Shown is the *mtr* locus of *N. gonorrhoeae* with horizontal lines above regions of strong disequilibrium that resulted from gonococcal recombination of DNA from *N. lactamica* or *N. meningitidis* and bent arrows showing sites of initiation of transcription. Examples of the likely impact of mosaic-like sequences within the *mtrR* and *mtrD* regions are noted above the horizontal lines. (B) The overlapping *mtrR* and *mtrCDE* promoters in *N. gonorrhoeae* nonmosaic strain FA19 are shown. The red-highlighted T:A base pair is representative of a site for the 1-bp deletion in many gonococcal strains displaying high levels of expression of *mtrCDE* and loss of *mtrR* expression (3). The adjacent green T:A base pair represents a nucleotide change within the *mtrR* or *mtrCDE* promoter that may impact *mtrR* or *mtrCDE* transcription as part of the mosaic-like sequence that can be generated by recombination. (C) The MtrC-MtrD-MtrE pump, showing the position of MtrD in the inner membrane (IM), MtrC in the periplasmic space (PS), and MtrE in the outer membrane (OM).

within a collection of 1,102 U.S. isolates that express decreased susceptibility to Azi (5). Although the MIC breakpoint for Azi has not been established, an MIC of 2 μg/ml (but probably lower for nongenital infections) has been suggested to result in clinical resistance. The clinical isolates examined in the report lacked known ribosomal mutations responsible for high-level Azi resistance. However, they contained DNA sequences most likely donated by transformation from *N. lactamica* or *N. meningitidis* that caused formation of mosaic-like sequences in the *mtr* (multiple transferrable resistance) efflux pump locus that resulted in Azi MICs of 1 to 4 μg/ml. Mosaic-like sequences in the *mtr* locus were present in nearly 10% of isolates in the larger collection, suggesting that such strains are relatively common (at least in the United States). Hence, these sequences and their contribution to antibiotic resistance could have significant implications for genomic approaches to detect resistance markers in the clinic (discussed below). Moreover, from analyses of the whole-genome sequence of the strains investigated, the authors noted considerable diversity in the mosaic *mtr* regions, suggesting unique donation events from commensals.

The *mtr* locus contains three genes within an operon encoding the MtrCDE efflux pump and an upstream, transcriptionally divergent gene encoding a transcriptional repressor (MtrR) of *mtrCDE* (Fig. 1). Mosaic-like sequences within the *mtrR* gene as well as overlapping promoter regions for *mtrR* and *mtrCDE* were identified and represented evidence of increasing expression of the *mtrCDE* operon. The *mtrR* and *mtrCDE* promoter regions and the *mtrD* gene showed the greatest degree of linkage disequilibrium and were targeted for detailed genetic and transcriptional studies. Increased transcription of the efflux genes could not alone account for the full level of Azi resistance expressed by the clinical isolates. In this respect, mosaic-like sequences within the *mtrD* gene, which encodes the inner membrane transporter component of the pump (Fig. 1), exerted strong epistatic effects that were needed for the donor level of Azi resistance. As emphasized below, the work has several levels of significant implications for advancing our knowledge regarding the mechanisms and emergence of antibiotic resistance expressed by gonococci. This is especially true for drug efflux pumps, such as MtrCDE, as they play a pivotal role in bacterial resistance to not only antibiotics but also host-derived antimicrobials (3).

Originally described by Maness and Sparling (6) in the early 1970s, the Mtr phenotype endows gonococci with increased resistance to multiple antimicrobials, typically hydrophobic drugs (including beta-lactams and macrolides) but also cationic antimicrobial peptides, progesterone, detergents (including the over-the-counter spermicide nonoxynol-9), and dyes. They noticed that increased gonococcal resistance to hydrophobic antimicrobials could be transferred *en bloc* to a susceptible recipient by transformation. From that study and other studies by the Sparling group, the reasonable assumption (at that time) was that resistance to structurally diverse antimicrobials was due to decreased cell envelope permeability. Work performed over 20 years ago (reviewed in reference 3) showed that the Mtr phenotype was due to increased expression of the *mtrCDE* efflux pump gene operon (Fig. 1). Overexpression of *mtrCDE* could be due to *cis*-acting promoter mutations that influenced expression levels of *mtrR* or *mtrCDE* or, but to a lesser extent, to *trans*-acting mutations that abrogated the repressor activity of MtrR (reviewed in reference 3). Notably, a 1-bp deletion in a 13-bp inverted repeat element within the *mtrR* promoter (Fig. 1B) has been observed in >80% of gonococcal isolates with the high-level Mtr phenotype. Mechanistically, this mutation abrogates transcription of *mtrR* while also promoting transcription of *mtrCDE* to a level above that seen due to loss of MtrR; presumably, because the promoters overlap, RNA polymerase can better interact with the *mtrCDE* promoter.

Until now, there had been no published reports on mutations in the genes within the *mtrCDE* efflux operon that could result in increased antimicrobial resistance or on how they might influence such resistance in the context of regulatory mutations. However, such mutations could, in theory, increase recognition or energy-dependent export of antimicrobials. While the report by Wadsworth et al. is not the first report of commensal neisserial DNA recombined into the gonococcal *mtr* locus (5, 7), the current findings suggest important mechanistic reasons for the manner in which mosaic-like sequences within *mtr* genes can decrease gonococcal susceptibility to Azi and other MtrCDE antimicrobial substrates. In brief, the imported sequences increased expression of *mtrCDE* due to promoter mutations not previously seen and to changes in the amino acid sequence of the MtrD transporter. The changes across MtrD likely impact its recognition and/or energy-dependent efflux of antimicrobials. Importantly for any future rapid point-of-care diagnostic test to inform physicians of a potential antibiotic resistance determinant in a clinical isolate, the nucleotide changes due to these mosaic-like sequences would be missed if such tests included only the known *mtrR* or *mtrCDE* promoter mutations (3).

With respect to the promoter regions for *mtrR* and *mtrCDE*, the recombined DNA resulted in increased expression of *mtrR* and *mtrCDE* in the absence of mutations shown previously to regulate these genes; this elevated gene expression did not require sublethal levels of Azi that could serve as an inducer. In the mosaic-like strains, the promoter region had a single nucleotide change within the spacer region of the *mtrR* promoter that contains the 13-bp inverted repeat element located within the overlapping −35 *mtrCDE* promoter region. It is noteworthy that transcription of *mtrR* was found to be enhanced in transformants bearing the mosaic *mtr* promoter sequence, but this may have been due to amino acid changes in MtrR that are also present in the donor and in transformants that diminish MtrR autorepressive activity. Nevertheless, from a gene expression view, the "take-home message" is that increased transcription of *mtrCDE* contributes to, but does not fully account for, the elevated Azi MIC of the clinical isolates or transformants generated by the clinical isolate's donor DNA that contains the mosaic *mtr* locus.

Perhaps the most significant aspect of the report lies in the context of the *mtrD* mosaic-like sequence that contained multiple nucleotide changes across the gene. Indeed, within the *mtr* locus, the *mtrD* gene showed the greatest degree of linkage disequilibrium compared to reference Azi-sensitive gonococci. In the absence of *mtrR* promoter mutations, a mosaic *mtrD* could elevate Azi resistance, but the highest level of such resistance (and cross-resistance to other antimicrobials) was seen in transformants with coresident mosaic sequences in both regions of the locus. Interestingly,

within *mtrD*, strong epistatic effects were detected due to mutations impacting the N- or C-terminal region of MtrD. Given that the MtrD structure has been solved (8) and has been shown to be similar but not identical to the AcrB protein possessed by Gram-negative enteric pathogens, it should be possible to identify important amino acids and sites that are involved in drug recognition and or efflux. In brief, amino acid changes at the N- and C-termini that could impact the MtrD structure in the central pore (PN1), which is important for stabilizing the trimeric MtrD complex, and in the outer periplasmic region (PC2), which could influence drug capture, seemed critical for raising the Azi MIC. The authors emphasize that the identified amino acid changes in MtrD have not been reported as contributing to efflux by orthologous protein AcrB or MexB possessed by enteric Gram-negatives or *Pseudomonas aeruginosa*, respectively. Thus, unique gain-of-function mutations in *mtrD* should be considered a mechanism by which gonococci could escape Azi action during treatment, especially at sites where the poor pharmacokinetic properties of this macrolide would promote clinical resistance.

In conclusion, Wadsworth et al. inform us on the power of combining tools of bioinformatics and genomics with molecular-method-based laboratory technologies and classic genetic approaches to understand how antibiotic resistance expressed by *N. gonorrhoeae* can emerge in the community. On a practical level, the article emphasizes the importance of sampling the oral cavity of patients suspected of having gonorrhea, as the local commensal *Neisseria* microbiota is a source of antibiotic resistance determinants. With the information provided in the report, new avenues of applied and basic research studies should follow that will help in countering the growing threat of antibiotic resistance expressed by gonococci.

ACKNOWLEDGMENTS

Special thanks to J. Reimche for providing critical reading of the manuscript prior to submission and Patrick Lane and C. Holley for help in preparing the figure.

I am the recipient of a Senior Research Career Scientist Award from the Biomedical Laboratory Research and Development Service of the U.S. Department of Veterans Affairs, and work in my laboratory on gonococcal drug efflux pumps is supported by NIH grant R37 AI21150-33.

The contents of this article are solely the responsibility of the author and do not necessarily reflect the official views of the National Institutes of Health, the U.S. Department of Veterans Affairs, or the United States government.

I have no competing interest to declare.

REFERENCES

1. Unemo M, del Rio C, Shafer WM. 2016. Antimicrobial resistance expressed by *Neisseria gonorrhoeae*: a major global public health problem in the 21st century, p 213–237. *In* Emerging Infections, vol 10, Chapter 12. ASM Press, Washington, DC.
2. Newman L, Rowley J, Vander Hoorn S, Saman Wijesooriya N, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M. 2015. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS One 10: e0143304. https://doi.org/10.1371/journal.pone.0143304.
3. Unemo M, Nicholas RA, Jerse AE, Davies C, Shafer WM. 2014. Molecular mechanisms of antibiotic resistance expressed by the pathogenic Neisseria, p 161–192. *In* Davies J, Kahler C, (ed), Pathogenic Neisseria: genomics, molecular biology and disease intervention. Horizon Scientific Press, Poole, United Kingdom.
4. Wadsworth CB, Arnold BJ, Sater MRA, Grad YH. 2018. Azithromycin resistance through interspecific acquisition of an epistasis-dependent efflux pump component and transcriptional regulator in *Neisseria* gonorrhoeae. mBio 9:e01419-18. https://doi.org/10.1128/mBio.01419-18.
5. Grad YH, Harris SR, Kirkcaldy RD, Green AG, Marks DS, Bentley SD. 2016. Genomic epidemiology of gonococcal resistance to extended-spectrum cephalosporins, macrolides, and fluoroquinolones in the United States, 2000–2013. J Infect Dis 214:1579–1587. 10.1093/infdis/jiw420
6. Maness MJ, Sparling PF. 1973. Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. J Infect Dis 128:321–330.
7. Trembizki E, Doyle C, Jennison A, Smith H, Bates J, Lahra M, Whiley D. 2014. A *Neisseria gonorrhoeae* strain with a meningococcal *mtrR* sequence. J Med Microbiol 63:1113–1115. https://doi.org/10.1099/jmm.0.074286-0.
8. Bolla JR, Su C-C, Do SV, Radhakrishnan A, Kumar N, Long F, Chou T-H, Delmar JA, Lei H-T, Rajashankar KR, Shafer WM, Yu EW. 2014. Crystal structure of the *Neisseria gonorrhoeae* MtrD inner membrane multidrug efflux pump. PLoS One 9:e97903. https://doi.org/10.1371/journal.pone.0097903.