Azithromycin resistance through interspecific acquisition of an epistasis dependent efflux pump component and transcriptional regulator in *Neisseria gonorrhoeae*

Crista B. Wadsworth[a]*, Brian J. Arnold[a,b], Mohamad R. Abdul Sater[a], and Yonatan H. Grad[a,c,]*

[a]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA 02115

[b]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA 02115

[c]Division of Infectious Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

*Corresponding Authors:

Crista Wadsworth (cwadsworth@hsph.harvard.edu), Yonatan Grad (ygrad@hsph.harvard.edu)

Keywords: *Neisseria gonorrhoeae*, gonorrhea, antibiotic resistance, macrolide, efflux pump, epistasis

ABSTRACT

Mosaic interspecifically acquired alleles of the multiple transferable resistance (*mtr*) efflux pump operon correlate with reduced susceptibility to azithromycin in *Neisseria gonorrhoeae* in epidemiological studies. However, whether and how these alleles cause resistance is unclear. Here, we use population genomics, transformations, and transcriptional analyses to dissect the relationship between variant *mtr* alleles and azithromycin resistance. We find that the locus encompassing the *mtrR* transcriptional repressor and the *mtrCDE* pump is a hotspot of interspecific recombination introducing alleles from *N. meningitidis* and *N. lactamica* into *N. gonorrhoeae*, with multiple rare haplotypes in linkage disequilibrium at *mtrD* and the *mtr* promoter region. Transformations demonstrated that resistance is mediated through epistasis between these two loci and that the full length of the mosaic *mtrD* allele is required. Gene expression profiling revealed the mechanism of resistance in mosaics couples the novel *mtrD* alleles with promoter mutations enhancing expression of the pump. Overall, our results demonstrate that epistatic interactions at *mtr* gained from multiple *Neisseria* has contributed to azithromycin resistance in the gonococcal population.

AUTHOR SUMMARY

Neisseria gonorrhoeae is the sexually transmitted bacterial pathogen responsible for over 100 million cases of gonorrhea worldwide each year. The incidence of reduced susceptibility to the macrolide class antibiotic azithromycin has increased in the past decade; however, a large proportion of the genetic basis of resistance to this drug remains unexplained. Recently, resistance has been shown to be highly associated with mosaic alleles of the multiple transferable resistance (*mtr*) efflux pump, which have been gained via horizontal gene exchange with other *Neisseria*. However, if and how these alleles caused resistance was unknown. Here, we demonstrate that resistance has been gained through epistasis between *mtrD* and the *mtr* promoter region using evidence from both population genomics and experimental genetic manipulation.

Epistasis also acts within the *mtrD* locus alone, requiring the full length of the gene for phenotypic resistance. Transcriptomic profiling indicates that the mechanism of resistance in mosaics is likely derived from both structural changes to *mtrD*, coupled with promoter mutations that result in regulatory changes to *mtrCDE*.

INTRODUCTION

The causal agent of gonorrhea, *Neisseria gonorrhoeae*, is a gram-negative diplococcus and an exclusively human pathogen. The prevalence of *N. gonorrhoeae* with reduced susceptibility to azithromycin has dramatically increased in recent years from just 0.6% in 2013 to 3.6% in 2016 in the United States [1], 0.8% in 2013 to 4.7% in 2016 in England and Wales [2], and 5.4% in 2013 to 7.1% in 2015 across Europe [3]. Additionally, reports in 2015 from both China and Japan have documented resistance in as high as 30% of the gonococcal population in some regions [4]. This spike in resistance is alarming, as azithromycin is one of the two first line drugs recommended as dual antimicrobial therapy for uncomplicated cases of gonococcal urethritis by the Centers for Disease Control (CDC). Azithromycin is a macrolide antibiotic that inhibits protein synthesis by binding to the 23s rRNA component of the 50S ribosome, and while the majority of resistance can be explained by mutations in the 23s rRNA azithromycin binding sites (i.e., C2611T and A2059G) [5-7] the genetic basis of ~36% of resistance is still unexplained within the U.S. population [7], thus limiting the potential for development of molecular-based resistance diagnostics and restricting our understanding of the evolutionary paths to reduced drug susceptibility.

Gonococci are adept at acquiring antimicrobial resistance as a result of their natural competence for transformation, allowing for the spread of resistance and other adaptively advantageous alleles, between lineages and even across species boundaries [8-11]. Extensive intragenus gene exchange has led to the concept of *Neisseria* as a consortium of species interconnected by allele sharing, with 'fuzzy' borders permitting rapid access to new adaptive

70 solutions [11,12]. In gonococci, intragenus recombination is an important source of novel genetic
71 variation with many observations of 'mosaic' loci gained from other neisserial species [13-16].
72 However, aside from horizontal gene transfer facilitating the evolution of resistance to third-
73 generation cephalosporins through acquisition of mosaic *penA* [7,14], allelic exchange has not yet
74 been proven to be the basis for resistance to any other antibiotic class in this species.
75 Recent epidemiological studies from the Unites States, Canada, and Australia have
76 reported an association between mosaic *multiple transferable resistance* (*mtr*) efflux pump alleles
77 and reduced susceptibility to azithromycin [7,17-19]. *Mtr* mosaics appear to have originated
78 through horizontal gene exchange from other *Neisseria*, and have been identified by high
79 sequence homology of the repressor of the pump (*mtrR*) to *N. meningitidis* and *N. lactamica*.
80 Mosaics have previously been associated with an outbreak of azithromycin resistance in Kansas
81 City, MO from 1999-2000 [20,21], and also the majority of azithromycin resistance reported in
82 New South Wales, Australia [19]. While correlation between mosaic *mtr* and azithromycin
83 resistance suggests causality, there is little experimental evidence to confirm the association.
84 The Mtr efflux pump is comprised of the MtrC-MtrD-MtrE cell envelope proteins, which
85 together export diverse hydrophobic antimicrobial agents such as such as antibiotics, nonionic
86 detergents, antibacterial peptides, bile salts, and gonadal steroidal hormones from the cell [22-26].
87 Mtr-mediated resistance to diverse antimicrobial agents in gonococcus is thought to act via
88 enhanced drug export as a result of overexpression of *mtrCDE*. Mutations that alter expression of
89 the pump include the $mtrC_{120}$ substitution, an adenine to guanine transition located 120 bp
90 upstream of the *mtrC* start codon which acts as an alternative promoter for *mtrCDE* [27,28]; an
91 A-deletion in the *mtrCDE* promoter that has been shown to repress the transcription of *mtrR*
92 while simultaneously enhancing transcription of *mtrCDE* [29]; and mutations that abrogate the
93 function of MtrR by inducing premature stop codons or radical amino acid substitutions in the
94 DNA-binding motif [20,30,31]. However, it is unclear if resistance in *mtr* mosaics is derived
95 from any of these mechanisms.

Here, we used a combination of population genomic and experimental approaches to dissect the mechanism of resistance in mosaics. We first assessed patterns of allelic diversity within the gonococcal population to define the boundaries of horizontal gene transfer at *mtrRCDE,* and found that the entire *mtr* region is a hotspot of interspecies recombination which has introduced multiple rare and divergent mosaic alleles from *N. meningitidis* and *N. lactamica* into the gonococcal population. Strong linkage disequilibrium at *mtrD* and the *mtr* promoter region suggested the maintenance of epistatic allelic combinations, thus we tested for interaction effects within and between *mtr* loci via transformation. We discovered epistatic interactions across almost the entirety of *mtrD,* and also between mosaic *mtrD* and mosaic *mtr* promoter regions, that synergistically enhanced azithromycin resistance. Furthermore, patterns of diversity in this region coupled with experimental evidence suggest antibiotic-mediated selection may be acting on these epistatic interactions. Finally, we tested for regulatory evolution of pump components, as previous mechanisms of azithromycin resistance through the Mtr efflux pump have been demonstrated to be expression-driven. Our results support that inheritance of mosaic promoter regions increases the expression of *mtrCDE* while gaining mosaic *mtrD* alone does not. Thus, the likely mechanism of resistance in mosaics is a structural change to *mtrD,* which enhances the capacity of the protein to recognize or transport azithromycin, coupled with increased efflux through the amplified production of pump components.

RESULTS

Allelic diversity suggests increased interspecies admixture at *mtrRCDE*

To gain insight into the evolutionary history of the *mtrR* transcriptional repressor and the *mtrCDE* pump, we analyzed patterns of diversity using the 1102 Gonococcal Isolate Surveillance Project (GISP) isolates described in Grad et al. [7,32]. A significant increase in allelic diversity was observed across *mtrRCDE* compared to the entire genome, with the highest diversity at *mtrD* (Figure 1a,b; Supplementary Table 1). We also detected a significant enrichment of rare alleles in

the population across *mtrRCDE* (Figure 1a,c; Supplementary Table 1). Linkage disequilibrium was strongest at *mtrD* and the *mtr* promoter region in a comparison of all pairs of single nucleotide polymorphisms (SNPs) within *mtrRCDE,* with higher linkage observed at pairs of variant sites within each of these loci (Figure 1d,f; Supplementary Table 1).

To define interspecific admixture events within *Neisseria*, we characterized the genealogical sorting index (*gsi*; [33]) to explore gene tree topology measures of species-specific phylogenetic exclusivity. *gsi* ranges from 0 (no exclusivity) to 1 (monophyletic), and serves as a metric to assess allele sharing that may arise through interspecific recombination or incomplete divergence from a recent split between species. We calculated *gsi* for genes in a 50 kb window encompassing *mtrRCDE* with conserved microsynteny between *N. gonorrohoeae* and other *Neisseria* (Supplementary Figure 1). This region included 29 core genes excluding *mtrRCDE*. To define the region-specific *gsi* background, we calculated *gsi* values across 100 bootstrap replicates for each gene in the *mtrRCDE* flanking region by species. Mean gonococcal *gsi* was 0.95 with *N. meningiditis*, 0.99 with *N. lactamica*, and 0.92 with *N. polysaccharea* for flanking genes (Figure 1e; Supplementary Table 2). Significant reductions in *gsi* were detected across *mtrRCDE* compared to the 29 loci within the surrounding 50 kb region (Figure 1e). Significant allele sharing between *N. gonorrhoeae* and *N. meningiditis* was exclusively observed at *mtrR* (Figure 1e; Supplementary Table 2), while significant allele sharing between *N. gonorrhoeae* and both *N. lactamica* and *N. polysaccharea* occurred across all *mtr* loci (Figure 1e; Supplementary Table 2).

There were multiple recombined mosaic haplotypes present spanning the full-length of *mtrD* (n=80), *mtrRCD* (n=9), *mtrRCDE* (n=20), and some isolates with partial mosaic *mtrD* with the majority of the gene homologous to native gonococcal sequence (n=13) (Figure 2). Of the 109 isolates with full-length mosaic *mtrD*, 4 were 99% identical to *N. meningitidis*, 5 had alleles with 94-96% identity to *N. lactamica,* and the remainder had alleles that aligned equally well to *N. meningitidis* and *N. lactamica* with identities ranging from 91-92%. Of the 29 isolates with

148 mosaic promoter regions identified by Grad et al. [7], 24 were 96-98% identical to *N. lactamica*,
149 4 were 99% identical to *N. meningitidis* with the presence of a 153-bp Correia element insertion
150 [20], and 1 was 92% similar to *N. meningitidis* but lacked the Correia element that was present in
151 the other four *N. meningitidis*-like isolates. All isolates with full-length mosaic *mtrD* had
152 azithromycin MICs ≥ 0.25 μg/ml, while all isolates with full-length mosaic *mtrD* and a mosaic
153 *mtr* promoter had MICs ≥ 1 μg/ml (Figure 2).

154 Of the twenty nine *mtr* mosaics described in Grad et al. [7], none had the $mtrC_{120}$
155 substitution, A-deletion, 23s ribosomal rRNA mutation A2059G, mutations in *rplD*, *rplV* tandem
156 duplications, or variants of the rRNA methylases *ermC* and *ermB* that have been associated with
157 or experimentally confirmed to be involved in azithromycin resistance [6,7,18,23,28,29,34].
158 However, four isolates had the premature stop codon mutations in *mtrR,* and five had the C2611T
159 23s rRNA mutation [5].

160

161 **Epistasis between multiple *mtr* loci and within *mtrD***

162 We exploited the natural competence of *Neisseria* to explore the potential for mosaic *mtr*
163 alleles to produce reduced susceptibility to azithromycin by transforming susceptible strains with
164 either genomic DNA (gDNA) or PCR-amplified products from mosaic donors. Susceptible
165 recipient strains for transformations included: 28Bl [20,35,36], GCGS0353, and GCGS0465
166 (MIC ≤ 0.125 μg/ml; Table 1). Three strains with reported mosaic *mtr* alleles and azithromycin
167 minimum inhibitory concentrations (MICs) ≥ 1 μg/ml were selected as donors for DNA transfer
168 (Table 1). These isolates included GCGS0276, GCGS0834, and GCGS0402. Of these mosaics,
169 GCGS0276 had a *N. meningitidis*-like *mtrR* sequence, while GCGS0834 and GCGS0402 had *N.*
170 *lactamica*-like *mtrR*s. None of the donor strains had premature stop codons in *mtrR* or the
171 C2611T mutation.

172 Genomic DNA from GCGS0276, GCGS0402, and GCGS0834 transformed multiple
173 susceptible isolates to resistance (Table 2a). To identify the locus responsible, we sequenced the

174 genomes of 28Bl cell lines transformed with gDNA from mosaic donors and characterized SNPs
175 that had been inherited from donor strains that were not present in the 28Bl recipient. The only
176 common region that had been inherited across all transformants was *mtrRCDE* (Supplementary
177 Figure 2).

178 Genomics results indicated the presence of linkage disequilibrium at *mtrD* and the *mtr*
179 promoter region (Figure 1 d,f). Thus, to test for possible interaction effects that contribute to
180 antibiotic-dependent fitness, and to further characterize the mechanism underlying reduced
181 susceptibility in *mtr* mosaics, we designed targeted amplicons for transformation from *N.*
182 *meningitidis*-like mosaic (GCGS0276) and a *N. lactamica*-like mosaic (GCGS0402). For
183 GCGS0276, the only locus within the *mtrRCDE* operon that was found to increase resistance to
184 azithromycin alone was *mtrD* (Figure 3a; Table 2b). GCGS0276 *mtrD* in the 28Bl background
185 raised the MIC to azithromycin by 3 fold, from 0.125 to 0.5 μg/ml, yet no single region of *mtrD*
186 was able to produce the 0.5 μg/ml phenotype (Figure 3b). However, inheriting amplicons that
187 contained both the 5' (18-356 bp) and 3' (2356-2724 bp) ends of GCGS0276 *mtrD* were together
188 sufficient to increase the 28Bl MIC to 0.5 μg/ml (Figure 3c-e). There were four changes at the
189 amino acid level between GCGS0276 and 28Bl in these regions, two in the PN1 domain of MtrD
190 (I48T and G59D) and two in the PC2 domain (K823D and F854L) (Supplemental Figure 3); and
191 in total twenty amino acid changes between the GCGS0276 and 28Bl proteins (Supplementary
192 Figure 3). GCGS0402 *mtrD* alone was not able to produce resistance in 28Bl.

193 Transformants that inherited the entire *mtrRCDE* operons of GCGS0276 and GCGS0402
194 had MICs of 1 and 2 μg/ml respectively, mirroring the donor strain phenotypes. Thus, amplicons
195 were designed for each of these strains to amplify the *mtrD* locus in combination with other
196 regions of the operon to determine the combination of loci that would reproduce the donor
197 resistance phenotypes. For both GCGS0276 and GCGS0402, we found that donor resistance
198 phenotypes of 1 and 2 μg/ml could be produced in 28Bl by transforming both *mtrD* and the *mtr*
199 promoter region together (Figure 4; Table 2b; Supplementary Figure 4).

Regulatory and structural mutations epistatically contribute to resistance

We tested for the contribution of transcript regulatory variation to the mechanism of resistance by profiling gene expression via RNA-seq of 28Bl, 28BlΔGCGS0276-mtrD, and 28BlΔGCGS0276-mtrRCDE. As expression of the *mtr* efflux pump is inducible by exposure to antimicrobial agents [37,38], we evaluated expression pre-azithromycin exposure and 120 minutes after the addition of a sub-MIC dose of azithromycin (0.125 μg/ml) to the culture media. Across 24 libraries, a total of 106 million 50 bp paired-end reads mapped to the FA1090 reference genome. Each library had on average 4.44±3.49 million mappable reads.

We assessed the impact of mosaic *mtrD* on *mtrRCDE* mRNA expression by comparing 28BlΔGCGS0276-mtrD transformants to 28Bl, and found no significant differential regulation of transcripts encoding *mtr* efflux pump components (Supplementary Figure 5; Supplementary Table 3). To determine the effect of the mosaic *mtr* promoter on pump expression, we compared 28BlΔGCGS0276-mtrD and 28BlΔGCGS0276-mtrRCDE transformants (Figure 5; Supplementary Table 3). Here, presence of a mosaic *mtr* promoter resulted in the significant upregulation of *mtrC*, *mtrD*, and *mtrE* across conditions (FDR < 0.0001) and upregulation of *mtrR* in the absence of azithromycin (FDR=0.003).

DISCUSSION

Genomic and surveillance efforts from across the globe have demonstrated an association between mosaic *mtr* alleles and low-level azithromycin resistance [7,17-19], but the causal role of these alleles in generating resistance has been unclear. Mtr-mediated decreased susceptibility to macrolides has been attributed exclusively to increased *mtrCDE* expression [27-30]. However, a majority of *mtr* mosaic isolates in the population genomic dataset have none of the variants that alter regulation of *mtrCDE,* such as the $mtrC_{120}$ substitution, A-deletion, or premature stop codon mutations in *mtrR* [20,27-31].

Using a combination of experimental and population genomic approaches, we demonstrated that the mosaic *mtr* alleles are responsible for resistance and showed that the mechanism of resistance involves multiple loci, including an epistatic interaction between the *mtrD* component of the pump and the *mtr* promoter. Population genomics and phylogenetic reconstruction demonstrated at least twelve independent acquisitions of mosaic *mtr* alleles, which have introduced multiple rare *mtr* haplotypes from *N. meningitidis* and *N. lactamica* into the gonococcal population (Figure 1 and 2).

Despite the sequence divergence (8%) between *N. meningitidis* and *N. lactamica*-like mosaics, *mtr* sequences from both generated azithromycin resistance through the same mechanism. For the *N. meningitidis* mosaic, *mtrD* alone was able to raise the MIC of 28Bl to 0.5 μg/ml independent of transcriptional changes to the pump's regulation; with the donor phenotype of 1 μg/ml only reproduced by adding the *N. meningitidis mtr* promoter region, which increased expression of *mtrCDE*. Similarly, although acquisition of the *N. lactamica mtrD* was not sufficient on its own to enhance resistance, transformation of both the *mtrD* and the *mtr* promoter region yielded the donor's azithromycin MIC of 2 μg/ml. Thus, the mechanism of resistance in mosaics is likely derived from both structural changes to *mtrD* coupled with promoter mutations that result in regulatory changes to *mtrCDE*.

The full-length *mtrD* was required for resistance in GCGS0276 transformants, indicating the role of within-gene epistasis, rather than a single point mutation, in generating azithromycin resistance. Two regions at the 5' and 3' ends of GCGS0276 *mtrD* together increased the MIC of 28Bl to 0.5 μg/ml (Figure 3). These two regions are part of the central pore of MtrD that stabilizes the trimeric organization of the protein (PN1) and the outer periplasmic region of the protein that may interact with MtrC to form a functional pump complex (PC2) [39]. Of note, none of the mutations observed between 28Bl and GCGS0276 have been shown to contribute to macrolide resistance in the orthologous proteins AcrB and MexB in other species, nor are they located in the direct contact site (residue 616) for macrolide recognition (e.g., [40-43]).

252 Within the *mtrCDE* operon, we observed local increases in linkage disequilibrium
253 coupled with increases of rare mutations (Figure 1). These signatures could be explained by the
254 recent acquisition of neutral diversity from closely related species, with too little evolutionary
255 time for the combined effects of recombination and mutation to break down linkage of sites
256 across imported DNA tracts, or the spread of these mutations to higher frequencies [44,45].
257 However, our experimental results confirm strong purifying selection on azithromycin plates after
258 inheritance of partial mosaic haplotypes, suggesting that some of the linkage within *mtrCDE*
259 observed in natural gonococcal populations may be driven by selection maintaining allelic
260 combinations that increase resistance to azithromycin.
261 Overall, our results defining the role of mosaic *mtr* in azithromycin resistance affirm the
262 importance of other *Neisseria* species as an antibiotic resistance reservoir for *N. gonorrhoeae*.
263 Moreover, whereas mosaic *penA* genes arise from interspecies recombinations within a single
264 gene and confer cephalosporin resistance through novel structural forms [14,46], our findings of
265 horizontally acquired epistasitically interacting structural and regulatory variants in *mtr* point to
266 the potential complexity by which antibiotic resistance can arise through the interactions of
267 multiple loci. Interspecies mosaicism will be an important consideration for future development
268 of sequence-based molecular resistance diagnostics, as markers designed to amplify gonococcal-
269 specific sequence will overlook or incorrectly diagnose resistance phenotype. Thus, as the
270 number of commensal neisserial genome sequences increase, analyses that map the patterns and
271 extent of interspecies recombination may be a valuable guide in understanding pathways to
272 resistance and in designing the appropriate diagnostic tools.
273

274 **MATERIALS AND METHODS**

275 **Genome sequencing and population genomics**

276 Sequencing libraries were prepared using a modification of Illumina's Nextera XP
277 protocol [47]. Samples were dual-indexed and pooled (n=15 per pool). Paired-end 150 bp

278 sequencing was conducted on an Illumina MiSeq (Illumina Corp., San Diego, C.A.) platform
279 located at the Harvard T.H. Chan School of Public Health to an average depth of 40x. Previously
280 sequenced read libraries were obtained from the NCBI's Short Read Archive (Project #
281 PRJEB2090) and the European Nucleotide Archive (Project #PRJEB2999 and PRJEB7904)
282 [7,32].

283 To determine the impact of interspecific recombination at *mtrRCDE*, we assessed
284 patterns of allelic diversity across the *mtrR* transcriptional repressor and the *mtrCDE* pump
285 compared to the rest of the genome for the 1102 GISP gonococcal isolate [7,32]. Reads were
286 aligned to the FA1090 reference using Bowtie2 v.2.2.4 [48], and variants were called using pilon
287 v.1.16 [49]. Vcftools v.0.1.12 [50] was used to merge resultant vcf files and calculate genome-
288 wide values of π and Tajima's D over 100-bp sliding windows, and r^2 linkage by site. Gubbins
289 v.2.2.0 [51] was used to predict regions of elevated SNP densities. For each isolate, BLASTn was
290 used to identify the top hit and highest percent sequence identity for *mtrD* and the *mtr* promoter
291 to all *Neisseria* within the NCBI database (*e*-value < 10^{-40}).

292 The extent of exclusive ancestry between *N. gonorrhoeae*, *N. meningitidis, N. lactamica,*
293 and *N. polysaccharea* was assessed using *gsi* [33] for each gene across a 25 kb window
294 surrounding *mtrRCDE*. In brief, we downloaded *de novo* assemblies and raw sequencing reads
295 from NCBI for *N. meningitidis* (n=431), *N. lactamica* (n=326), *N. polysaccharea* (n=37), and *N.*
296 *gonorrhoeae* (n=1102; [7,32]). Raw reads were assembled with SPAdes v.3.7.0 [52], and
297 assemblies were aligned to the *N. gonorrhoeae* FA1090 reference genome (AE004969.1) using
298 progressiveMauve ([53]; snapshot 2015-02-13 for linux-x64), since a multi-genome alignment for
299 all genomes was not computationally tractable. The sequences that were aligned to each gene
300 within 25 kb of *mtrRCDE* in FA1090 were then extracted with custom Perl scripts and realigned
301 with MAFFT v.7.309 [54]. This method of pairwise alignments of *de novo* assemblies to the
302 FA1090 reference identifies orthologs between *N. gonorrhoeae* and the other *Neisseria* using
303 both sequence identity and microsynteny, which is conserved in the genomic region surrounding

304 *mtrRCDE* (Supplementary Figure 1). We then used RAxML v.8.1.4 [55] to reconstruct the
305 phylogeny for each gene, using 50 bootstrap replicates and the GTRCAT substitution model.
306 With these multi-species phylogenies, we calculated *gsi* with the *genealogicalSorting* R
307 package[33,56].
308
309 **Bacterial culture conditions**
310 *N. gonorrhoeae* isolates were provided by the CDC (Table 1). Isolates were cultured on
311 GCB agar medium supplemented with 1% IsoVitaleX (Becton Dickinson Co., Franklin Lakes,
312 N.J.). After inoculation, plates were incubated at 37°C in a 5% CO_2 atmosphere incubator for 16-
313 18 hours. Antimicrobial susceptibility testing was conducted using the agar-dilution method at a
314 range of azithromycin concentrations from 0 to 16 μg/ml [1]. MICs were recorded after 24 hours
315 of growth. All isolate stocks were stored at -80°C in trypticase soy broth containing 20%
316 glycerol.
317
318 **Transformation of mosaic *mtr* alleles**
319 Genomic DNA was extracted from isolates by lysing growth from overnight plates in TE
320 buffer (10 mM Tris pH 8.0, 10 mM EDTA) with 0.5 mg/μl lysozyme and 3 mg/μl proteinase K
321 (Sigma-Aldrich Corp., St. Louis, M.O.). DNA was purified using the PureLink Genomic DNA
322 Mini Kit, treated with RNase A (Thermo Fisher Corp., Waltham, M.A.), and stored in water.
323 Primers were designed to amplify regions of *mtrRCDE*. For primer pairs that did not amplify over
324 a region containing a DNA uptake sequence, to enhance transformation efficiency the 12-bp AT-
325 DUS was added to the forward primer (5'-ATGCCGTCTGAA-3') [57]. PCR reactions were
326 conducted in 50 μl volumes using Phusion High-Fidelity DNA Polymerase (New England
327 Biolabs Inc., Ipswitch, M.A.) using the conditions listed in Supplementary Table 4. Amplified
328 products were run on a 0.8% agarose gel, excised, and purified with the QIAEX II Gel Extraction
329 Kit (Qiagen Inc., Valencia, C.A.) to remove gDNA contamination.

Transformations were conducted in GCP liquid broth (7.5 g Protease peptone #3, 0.5 g soluble starch, 2 g dibasic K_2HPO_4, 0.5 g monobasic KH_2PO_4, 2.5 g NaCl, ddH_2O to 500 ml; Becton Dickinson Co., Franklin Lakes, N.J.) supplemented with 1% IsoVitaleX and 10 μM $MgSO_4$ (Sigma-Aldrich Corp., St. Louis, M.O.). Naturally competent cells were incubated for 10 minutes with gDNA or purified PCR products to allow for DNA uptake and homologous recombination. Cells were plated on GCB with 1% IsoVitaleX and incubated for 4 hours at 37°C in a 5% CO_2 atmosphere to allow for expression of novel alleles. Cells on expression plates were resuspended in tryptic soy broth (Becton Dickinson Co., Franklin Lakes, N.J.) and selected on 0.38-1 μg/ml AZI GCB plates containing 1% IsoVitaleX. After 18 hours, single colonies were picked. Sanger sequencing performed using the GeneWiz sequencing service (GeneWiz Inc., Cambridge, M.A.) confirmed successful transformation.

Transcriptome construction

Cells harvested from overnight plates were suspended in GCP supplemented with 1% IsoVitaleX and 0.042% sodium bicarbonate. Cultures were incubated at 37°C for 2 hours to mid-log phase and then exposed to a sub-lethal dose of AZI (0.125 μg/ml). RNA was extracted at 0 minutes (pre-AZI) and 120 minutes (post-AZI) exposure using the Direct-Zol kit (Zymo Research, Irvine, C.A.). Transciptome libraries were prepared at the Broad Institute at the Microbial 'Omics Core using a modified version of the RNAtag-seq protocol [58]. 500 ng of total RNA was fragmented, depleted of genomic DNA, dephosphorylated, and ligated to DNA adapters carrying 5'-AN_8-3' barcodes of known sequence with a 5' phosphate and a 3' blocking group. Barcoded RNAs were pooled and depleted of rRNA using the RiboZero rRNA depletion kit (Epicentre, Madision, W.I.). Pools of barcoded RNAs were converted to Illumina cDNA libraries in 2 main steps: 1) reverse transcription of the RNA using a primer designed to the constant region of the barcoded adaptor with addition of an adapter to the 3' end of the cDNA by template switching using SMARTScribe (Clontech, Mountain View, C.A.) as described [59]; 2)

356 PCR amplification using primers whose 5' ends target the constant regions of the 3' or 5'
357 adaptors and whose 3' ends contain the full Illumina P5 or P7 sequences. cDNA libraries were
358 sequenced on the Illumina Nextseq 500 platform to generate 50-bp paired end reads.
359 Barcode sequences were removed, and reads were aligned to the FA1090 reference
360 genome. Reads counts were assigned to genes and other genomic features using custom scripts.
361 For the FA1090 we mapped reads to either the sense or anti-sense strand for coding domain
362 sequences (CDSs, n=1894), tRNAs (n=55), and rRNAs (n=12). For intergenic regions (IGRs,
363 n=1722), we mapped to each antiparallel strand. Differential expression analysis was conducted
364 in DESeq2 v.1.10.1 [60].
365
366 **ACKNOWLEDGEMENTS**
367 We thank David Trees and Steve Johnson for training on gonococcal transformation design and
368 Jonathan Livny for his technical assistance with transcriptome library preparation.
369
370 **FINANCIAL SUPPORT**
371 This work was supported by the Smith Family Foundation and NIH R01 AI132606.
372
373 **POTENTIAL CONFLICTS OF INTEREST**
374 All authors: No reported conflicts.
375
376 **REFERENCES**
377 1. Centers for Disease Control. Gonococcal Isolate Surveillance Project (GISP). Accessed 24
378 April 2018. Available: http://www.cdc.gov/std/gisp/

379 2. Public Health England. Surveillance of antimicrobial resistance in *Neisseria gonorrhoeae*
380 in England and Wales: Key findings from the Gonococcal Resistance to Antimicrobials

Surveillance Programme (GRASP). Antimicrobial resistance in *Neisseria gonorrhoeae*: Data to June 2017. 2017 pp. 1-24. Available: http://www.gov.uk/government/publications/gonococcal-resistance-to-antimicrobials-surveillance-programme-grasp-report

3. European Centre for Disease Prevention and Control. Gonococcal antimicrobial susceptibility surveillance in Europe. ECDC Surveillance Report. 2015 pp. 1–44. Available: http://www.ecdc.europa.eu

4. The World Health Organization. Gonococcal antimicrobial resistance in the Western Pacific Region. WHO Report. 2017 Sept pp. 1-4. Available: http://www.wpro.who.int/hiv/topics/gasp/

5. Ng LK, Martin I, Liu G, Bryden L. Mutation in 23S rRNA associated with macrolide resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Ch. 2002;46: 3020–3025.

6. Chisholm SA, Dave J, Ison CA. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. Antimicrob Agents Ch. 2010;54: 3812–3816.

7. Grad YH, Harris SR, Kirkcaldy RD, Green AG, Marks DS, Bentley SD, et al. Genomic epidemiology of gonococcal resistance to extended-epectrum cephalosporins, macrolides, and fluoroquinolones in the United States, 2000–2013. J Infect Dis. 2016;214: 1579–1587.

8. Smith JM, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria? P Natl A Sci; 1993;90: 4384–4388.

9. O'Rourke M, Stevens E. Genetic structure of *Neisseria gonorrhoeae* populations: A non-clonal pathogen. J Gen Microbiol. 1993;139: 2603–2611.

10. O'Rourke M, Spratt BG. Further evidence for the non-clonal population structure of *Neisseria gonorrhoeae*: Extensive genetic diversity within isolates of the same electrophoretic type. Microbiol. 1994;140: 1285–1290.

11. Corander J, Connor TR, O'Dwyer CA, Kroll JS, Hanage WP. Population structure in the *Neisseria*, and the biological significance of fuzzy species. J Roy Soc Interface. 2011;9: 1208–1215.

12. Fussenegger M, Rudel T, Barten R, Ryll R, Meyer TF. Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae* – A review. Gene. 1997;192: 125–134.

13. Halter R, Pohlner J, Meyer TF. Mosaic-like organization of *IgA* protease genes in *Neisseria gonorrhoeae* generated by horizontal genetic exchange in vivo. EMBO J. 1989;8: 2737–2744.

14. Spratt BG, Bowler LD, Zhang QY, Zhou J, Smith JM. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. J Mol Evol. 1992;34: 115–125.

15. Feavers IM, Heath AB, Bygraves JA, Maiden MC. Role of horizontal genetic exchange in the antigenic variation of the class 1 outer membrane protein of *Neisseria meningitidis*. Mol Microbiol. 1992;6: 489–495.

16. Zhou J, Spratt BG. Sequence diversity within the *argF*, *fbp* and *recA* genes of natural isolates of *Neisseria meningitidis*: Interspecies recombination within the *argF* gene. Mol Microbiol. 1992;6: 2135–2146.

17. Trembizki E, Doyle C, Jennison A, Smith H, Bates J, Lahra M, et al. A *Neisseria gonorrhoeae* strain with a meningococcal *mtrR* sequence. J Med Microbiol. 2014;63:

1113–1115.

18. Demczuk W, Martin I, Peterson S, Bharat A, Van Domselaar G, Graham M, et al. Genomic epidemiology and molecular resistance mechanisms of azithromycin-resistant *Neisseria gonorrhoeae* in Canada from 1997 to 2014. J Clin Microbiol. 2016;54: 1304–1313.

19. Whiley DM, Kundu RL, Jennison AV, Buckley C, Limnios A, Hogan T, et al. Azithromycin-resistant *Neisseria gonorrhoeae* spreading amongst men who have sex with men (MSM) and heterosexuals in New South Wales, Australia, 2017. J Antimicrob Chemoth. 2018;17: 148–5.

20. Johnson S. Mutations causing in vitro resistance to azithromycin in *Neisseria gonorrhoeae*. Int J Antimirob Ag. 2003;21: 414–419.

21. McLean CA, Wang SA, Hoff GL, Dennis LY, Trees DL, Knapp JS, et al. The emergence of *Neisseria gonorrhoeae* with decreased susceptibility to azithromycin in Kansas City, Missouri, 1999 to 2000. Sex Transm Dis. 2004;31: 73–78.

22. Pan W, Spratt BG. Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. Mol Microbiol. 1994;11: 769–775.

23. Hagman KE, Shafer WM. Transcriptional control of the *mtr* efflux system of *Neisseria gonorrhoeae*. J Bacteriol. 1995;177: 4162–4165.

24. Delahay RM, Robertson BD, Balthazar JT, Shafer WM, Ison CA. Involvement of the gonococcal MtrE protein in the resistance of *Neisseria gonorrhoeae* to toxic hydrophobic agents. Microbiol. 1997;143: 2127–2133.

25. Lucas CE, Balthazar JT, Hagman KE, Shafer WM. The MtrR repressor binds the DNA

448 sequence between the *mtrR* and *mtrC* genes of *Neisseria gonorrhoeae*. J Bacteriol.
449 1997;179: 4123–4128.

450 26. Hagman KE, Lucas CE, Balthazar JT, Snyder L, Nilles M, Judd RC, et al. The MtrD
451 protein of *Neisseria gonorrhoeae* is a member of the resistance/nodulation/division
452 protein family constituting part of an efflux system. Microbiol. 1997;143: 2117–2125.

453 27. Warner DM, Shafer WM, Jerse AE. Clinically relevant mutations that cause derepression
454 of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE Efflux pump system confer different
455 levels of antimicrobial resistance and *in vivo* fitness. Mol Microbiol. 2008;70: 462–478.

456 28. Ohneck EA, Zalucki YM, Johnson PJT, Dhulipala V, Golparian D, Unemo M, et al. A
457 novel mechanism of high-level, broad-spectrum antibiotic resistance caused by a single
458 base pair change in *Neisseria gonorrhoeae*. mBio. 2011;2: e00187–11.

459 29. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of
460 *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE*
461 efflux system. Microbiol. 1995;141: 611–622.

462 30. Shafer WM, Balthazar JT, Hagman KE, Morse SA. Missense mutations that alter the
463 DNA-binding domain of the MtrR protein occur frequently in rectal isolates of *Neisseria*
464 *gonorrhoeae* that are resistant to faecal lipids. Microbiol. 1995;141: 907–911.

465 31. Zarantonelli L, Borthagaray G, Lee EH, Veal W, Shafer WM. Decreased susceptibility to
466 azithromycin and erythromycin mediated by a novel *mtr(R)* promoter mutation in
467 *Neisseria gonorrhoeae*. J Antimicrob Chemoth. 2001;47: 651–654.

468 32. Grad YH, Kirkcaldy RD, Trees D, Dordel JD, Harris SR, Goldstein E, et al. Genomic
469 epidemiology of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime in the

USA: A retrospective observational study. Lancet Infect Dis. 2014;14: 220–226.

33. Cummings MP, Neel MC, Shaw KL. A genealogical approach to quantifying lineage divergence. Evol. 2008;62: 2411–2422.

34. Roberts MC, Chung WO, Roe D, Xia M, Marquez C, Borthagaray G, et al. Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal *Neisseria spp*. carry known rRNA methylase genes. Antimicrob Agents Ch. 1999;43: 1367–1372.

35. Morse SA, Johnson SR, Biddle JW, Roberts MC. High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal *tetM* determinant. Antimicrob Agents Ch. 1986;30: 664–670.

36. Johnson SR, Grad Y, Ganakammal SR, Burroughs M, Frace M, Lipsitch M, et al. *In vitro* selection of *Neisseria gonorrhoeae* mutants with elevated MIC values and increased resistance to cephalosporins. Antimicrob Agents Ch. 2014;58: 6986–6989.

37. Rouquette C, Harmon JB, Shafer WM. Induction of the *mtrCDE*-encoded efflux pump system of *Neisseria gonorrhoeae* requires MtrA, an AraC-like protein. Mol Microbiol. 1999;33: 651–658.

38. Zalucki YM, Dhulipala V, Shafer WM. Dueling regulatory properties of a transcriptional activator (MtrA) and repressor (MtrR) that control efflux pump gene expression in *Neisseria gonorrhoeae*. mBio. 2012;3: e00446–12.

39. Bolla JR, Su C-C, Do SV, Radhakrishnan A, Kumar N, Long F, et al. Crystal structure of the *Neisseria gonorrhoeae* MtrD inner membrane multidrug efflux pump. PLoS One. 2014;9: e97903–8.

40. Wehmeier C, Schuster S, Fahnrich E, Kern WV, Bohnert JA. Site-directed mutagenesis reveals amino acid residues in the *Escherichia coli* RND efflux pump AcrB that confer macrolide resistance. Antimicrob Agents Ch. 2008;53: 329–330.

41. Eicher T, Cha H-J, Seeger MA, Brandstatter L, El-Delik J, Bohnert JA, et al. Transport of drugs by the multidrug transporter AcrB involves an access and a deep binding pocket that are separated by a switch-loop. P Natl Acad Sci. 2012;109: 5687–5692.

42. Cha HJ, Muller RT, Pos KM. Switch-loop flexibility affects transport of large drugs by the promiscuous AcrB multidrug efflux transporter. Antimicrob Agents Ch. 2014;58: 4767–4772.

43. Ababou A, Koronakis V. Structures of gate loop variants of the AcrB drug efflux pump bound by erythromycin substrate. PLoS One. 2016;11: e0159154–8.

44. Gogarten JP, Townsend JP. Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol. 2005;3: 679–687.

45. Theunert C, Slatkin M. Distinguishing recent admixture from ancestral population structure. Genome Biol Evol. 2017;9: 427–437.

46. Tomberg J, Unemo M, Davies C, Nicholas RA. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae*: Role of epistatic mutations. Biochemistry. 2010;49: 8062–8070.

47. Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony R. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One. 2015;10: e0128036–15.

513 48. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Meth. 2012;9:
514 357–359.

515 49. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: An
516 integrated tool for comprehensive microbial variant detection and genome assembly
517 improvement. PLOS One. 2014;9: e112963–14.

518 50. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al. The variant
519 call format and VCFtools. Bioinformatics. 2011;27: 2156–2158.

520 51. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, et al. Rapid
521 phylogenetic analysis of large samples of recombinant bacterial whole genome sequences
522 using Gubbins. Nucleic Acids Res. 2015;43: e15–e15.

523 52. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes:
524 A new genome assembly algorithm and its applications to single-cell sequencing. J
525 Comput Biol. 2012;19: 455–477.

526 53. Darling AE, Mau B, Perna NT. progressiveMauve: Multiple genome alignment with gene
527 gain, loss and rearrangement. PLoS One. 2010;5: e11147–17.

528 54. Katoh K, Misawa K, Kuma K-I, Miyata T. MAFFT: A novel method for rapid multiple
529 sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30: 3059–
530 3066.

531 55. Stamatakis A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of
532 large phylogenies. Bioinformatics. 2014;30: 1312–1313.

533 56. Team RC. R: A language and environment for statistical computing. R Foundation for
534 Statistical Computing, Vienna, Austria.

535 57. Ambur OH, Frye SA, Tønjum T. New functional identity for the DNA uptake sequence in
536 transformation and its presence in transcriptional terminators. J Bacteriol. 2007;189:
537 2077–2085.

538 58. Shishkin AA, Giannoukos G, Kucukural A, Ciulla D, Busby M, Surka C, et al.
539 Simultaneous generation of many RNA-seq libraries in a single reaction. Nat Meth.
540 2015;12: 323–325.

541 59. Zhu YY, Machleder EM, Chenchik A, Li R, Siebert PD. Reverse transcriptase template
542 switching: A SMART approach for full-length cDNA library construction.
543 BioTechniques. 2001;30: 892–897.

544 60. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
545 RNA-seq data with DESeq2. Genome Biol. 2014;15: 21–31.

546 61. Seemann, T. Prokka: Rapid prokaryotic genome annotation. Bioinformatics 2014;30:
547 2068-2069.

548 **TABLES AND FIGURES**

Table 1. Properties of strains used in the study

Strain	Source	AZI[a] MIC (µg/ml)*
GCGS0276	GISP[b] isolate, Kansas City	1
GCGS0402	GISP[b] isolate, Miami	2
GCGS0834	GISP[b] isolate, Los Angeles	2
GCGS0353	GISP[b] isolate, Dallas	0.03
GCGS0465	GISP[b] isolate, Phoenix	0.06
28Bl	Disseminated gonococcal infection isolate (CDC, 1974)	0.125

a AZI, azithromycin

b GISP, Gonococcal Isolate Surveillance Project

*MIC scores are the average of three independent replicate tests.

549

550

Table 2. MIC values of transformant strains

Strain	Recipient	Transformation Substrate	AZI MIC (μg/ml)*	mosaic *mtrD*	mosaic *mtrp*
2a. Genomic DNA (gDNA) Transformant Strains					
N. meningitidis*-like *mtrRCDE					
28BlΔGCGS0276-gDNA	28Bl	GCGS0276 gDNA	2	+	+
GCGS0353ΔGCGS0276-gDNA	GCGS0353	GCGS0276 gDNA	1	+	+
GCGS0465ΔGCGS0276-gDNA	GCGS0465	GCGS0276 gDNA	2	+	+
N. lactamica*-like *mtrRCDE					
28BlΔGCGS0402-gDNA	28Bl	GCGS0402 gDNA	2	+	+
GCGS0353ΔGCGS0402-gDNA	GCGS0353	GCGS0402 gDNA	2	+	+
GCGS0465ΔGCGS0402-gDNA	GCGS0465	GCGS0402 gDNA	2	+	+
28BlΔGCGS0834-gDNA	28Bl	GCGS0834 gDNA	2	+	+
GCGS0353ΔGCGS0834-gDNA	GCGS0353	GCGS0834 gDNA	2	+	+
GCGS0465ΔGCGS0834-gDNA	GCGS0465	GCGS0834 gDNA	2	+	+
2b. PCR Product Transformant Strains					
28BlΔGCGS0276-mtrRCDE	28Bl	GCGS0276 *mtrRCDE*	1	+	+
28BlΔGCGS0276-mtrD	28Bl	GCGS0276 partial *mtrC* (1185-1241 bp) and *mtrD* (1-3174 bp)	0.5	+	-
28BlΔGCGS0276-mtrD/18-3174	28Bl	GCGS0276 partial *mtrD* (18-3174 bp)	0.5	+	-
28BlΔGCGS0276-mtrD/+262-2724	28Bl	GCGS0276 partial *mtrC* (1185-1241 bp) and *mtrD* (1-2724 bp)	0.5	+	-
28BlΔGCGS0276-mtrDp	28Bl	GCGS0276 mtrD + promoter	1	+	+
28BlΔGCGS0402-mtrRCDE	28Bl	GCGS0402 *mtrRCDE*	2	+	+
28BlΔGCGS0402-mtrDp	28Bl	GCGS0402 mtrD + promoter	2	+	+

*MIC scores are the average of three independent replicate tests.

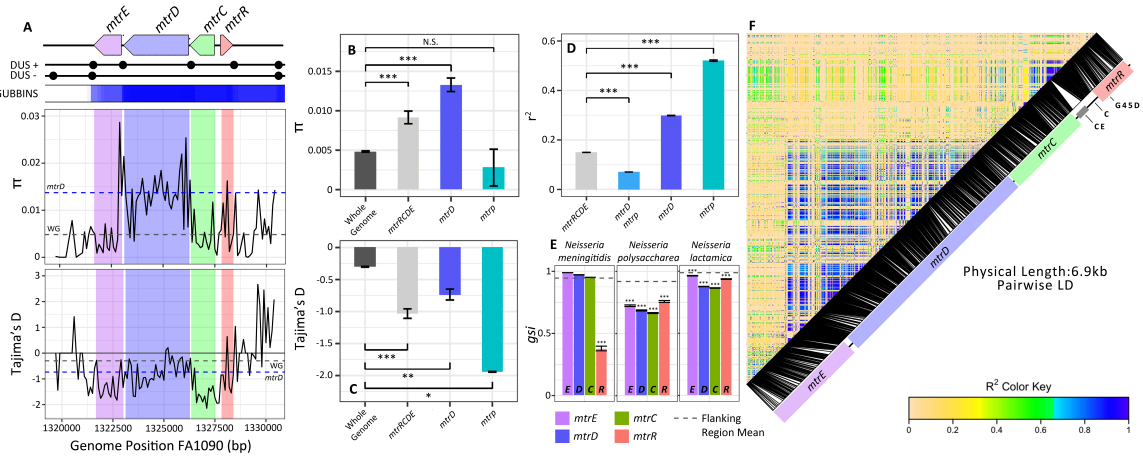


Figure 1. Horizontal gene transfer (HGT) of *mtr* introduces novel adaptive genetic variation into *Neisseria gonorrhoeae*. (A-C) The U.S. gonococcal population (n=1102 isolates) shows patterns of elevated allelic diversity across *mtrRCDE*, with the highest diversity at *mtrD*, compared to the rest of the genome. An excess of rare alleles across *mtrRCDE* (Tajima's D<0) suggests the introduction of new genetic variation in this region has occurred recently, possibly after a genome-wide selective sweep or population contraction. (E) Depressed *gsi* values indicate importation of divergent alleles from multiple neisserial species into gonococcus across all *mtr* loci. (D,F) The strongest linkage was observed within *mtrD* and the *mtr* promoter regions.

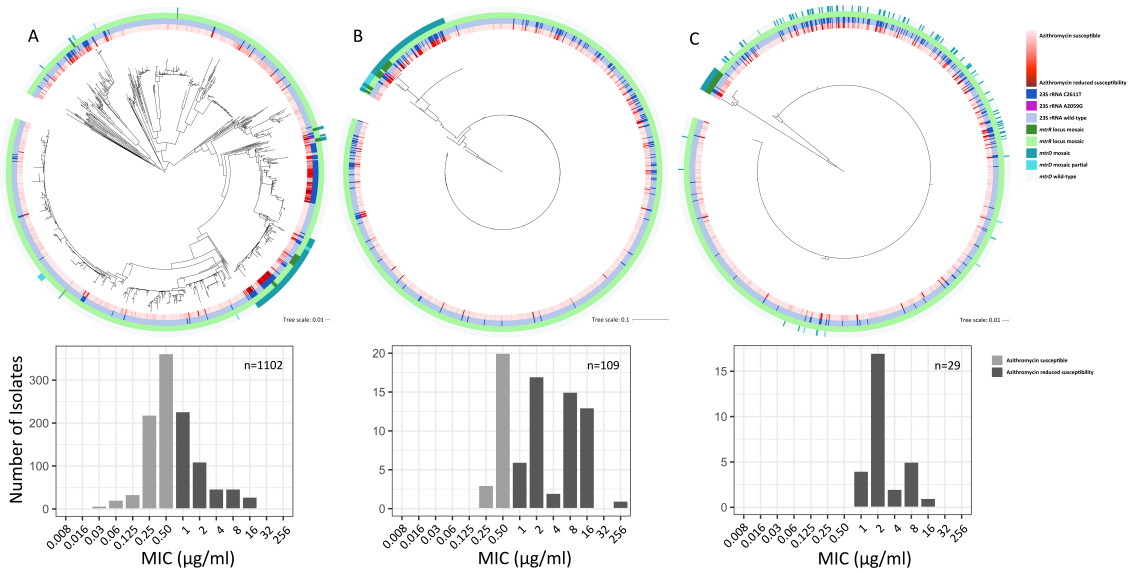
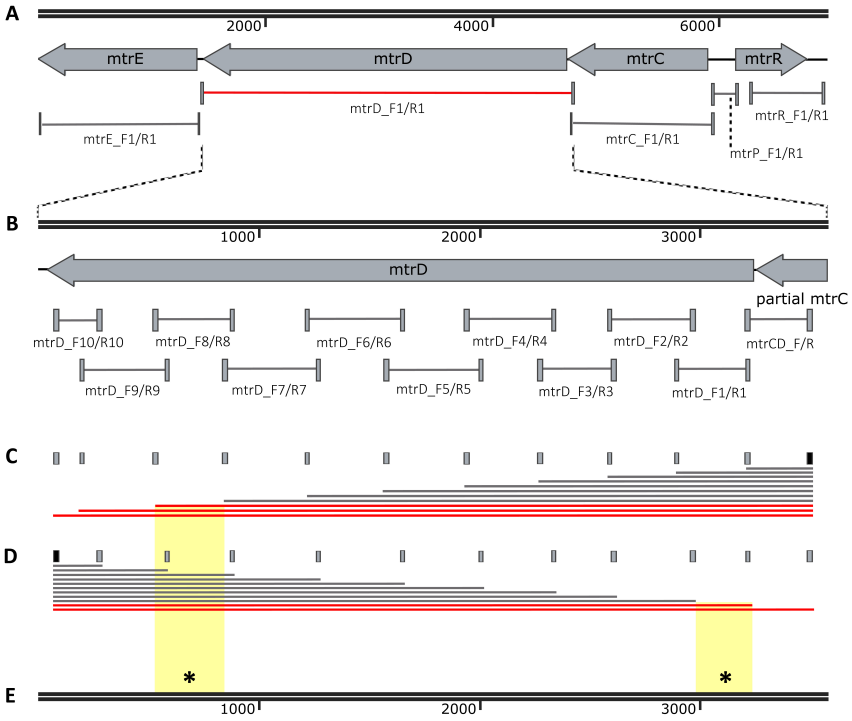


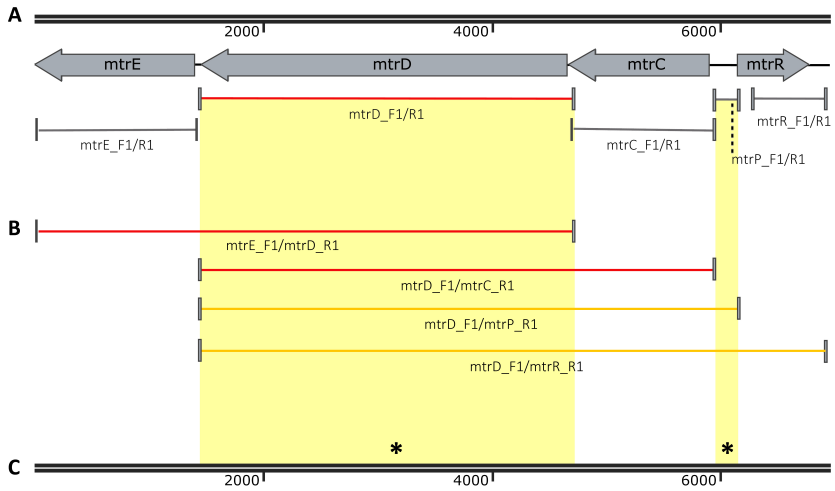
Figure 2. Divergent *mtrD* and *mtr* promoter haplotypes are associated with step-wise increases in MIC to azithromycin. (A) A maximum likelihood whole-genome-sequence phylogeny of 1102 *Neisseria gonorrhoeae* isolates, based on single-nucleotide polymorphisms generated from mapping to the FA1090 reference genome (Grad et al. 2016), is associated with a distribution of MIC values which fall both above and below the defined resistance threshold (MIC ≥ 1 µg/ml). The inner annotation ring shows MICs to azithromycin on a continuous scale, the following annotation ring indicates isolates with at least 2 copies of the C2611T 23S ribosomal RNA (rRNA) mutation or isolates with 4 copies of the A2059G 23S rRNA mutation, the next annotation ring shows isolates that were identified as interspecies mosaics based on their sequence at *mtrR* by Grad et al. (2016), and the outermost annotation ring shows isolates identified as *mtrD* mosaics in this study. (B) A maximum likelihood phylogeny built on *mtrD* alignments show 109 isolates with full-length mosaic alleles at this locus associated with elevated MICs to azithromycin. (C) A maximum likelihood phylogeny built on the *mtr* promoter region identifies all 29 mosaics with reduced susceptibility to azithromycin identified by Grad et al. [7] also have inherited mosaic *mtrD*.



577

578 **Figure 3. Epistatic interactions between multiple domains of *mtrD* contribute to elevated**
579 **MICs.** (A) GCGS0276 *mtrD* in the 28Bl background elevated MIC from 0.125 μg/ml to 0.5
580 μg/ml (red). (B) Primer pairs designed to amplify ~300 bp fragments over the length of *mtrD*
581 resulted in no observed transformants on 0.38 μg/ml selection plates, suggesting multiple
582 mutations across *mtrD* are needed for resistance. To determine the regions that contributed to
583 resistance, multiple fragment sizes were constructed by (C) holding the rightmost forward primer
584 (black) constant while adding different reverse primers (grey), and (D) holding the leftmost
585 reverse primer (black) constant while adding forward primers (grey) to separate reactions. (E) At
586 minimum SNPs at base pair positions 18 to 356 coupled with SNPs at positions 2356 to 2724 (*)
587 were needed to raise the MIC from the 0.125 μg/ml of the recipient 28Bl strain to 0.5 μg/ml.

588



589

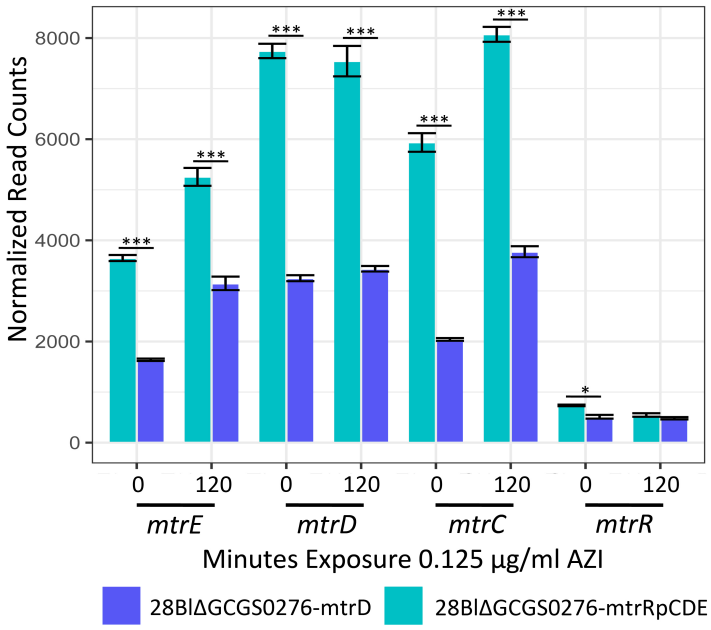
590 **Figure 4. Epistasis between *mtrD* and the *mtr* promoter region is causal to reduced**

591 **susceptibility.** (A) GCGS0276 *mtrD* was the only region that could independently raise MIC

592 from 0.125 to 0.5 μg/ml in the 28Bl background (red). (B) Only the addition of the *mtr* promoter

593 raised MIC to the donor strain phenotype of 1 μg/ml (yellow lines).

594



595

596 **Figure 5. The *N. meningitidis*-like GCGS0276 mosaic *mtr* promoter sequence upregulates**
597 **expression of *mtr* efflux pump component mRNAs.** 28Bl transformants with mosaic
598 GCGS0276 *mtrD* (blue) or mosaic GCGS0276 *mtrRCDE* (teal) were exposed to sub-MIC (0.125
599 µg/ml) concentrations of azithromycin for 120 minutes. In both the presence and absence of drug,
600 the presence of the *mtr* promoter region results in significantly upregulated pump component
601 mRNAs (FDR < 0.0001).

602