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Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*

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ABSTRACT

Strains of *Neisseria gonorrhoeae* with reduced susceptibility to ceftriaxone and cefixime (ceph^{I} strains) all contain a mosaic *penA* allele encoding penicillin-binding protein 2 (PBP 2) with nearly 60 amino acid differences compared to wild type PBP 2, together with a set of resistance determinants (i.e. *mtrR*, *penB*, and/or *ponA1*) that are required for high-level penicillin resistance. To define the individual contributions of these determinants to reduced susceptibility to ceftriaxone and cefixime, we created isogenic strains containing the mosaic *penA* allele from the ceph^{I} strain 35/02 (*penA35*) together with one or more of the other resistance determinants, and determined the MICs of penicillin G, ceftriaxone, and cefixime. The majority of cefixime resistance is conferred by the *penA35* allele, with only a small contribution from *mtrR* and *penB*, whereas ceftriaxone resistance is nearly equally dependent upon both. Unlike high-level penicillin resistance, the *ponA1* allele does not appear to be important for ceph^{I} resistance. A strain containing all four determinants has increased resistance to ceftriaxone and cefixime, but not to the levels of the donor ceph^{I} strain, suggesting that ceph^{I} strains, similar to high-level penicillin-resistant strains, contain an additional unknown determinant that is required to reach donor levels of resistance. Our data also suggest that the original ceph^{I} strains arose from transformation of *penA* genes from commensal Neisseria species into a penicillin-resistant strain already harboring *mtrR*, *penB*, *ponA1*, and the unknown gene(s) involved in high-level penicillin resistance.

Key words: *Neisseria gonorrhoeae*; reduced susceptibility to cephalosporins; *penA*; mosaic *penA*; *mtrR*; *penB*; *ponA1*

INTRODUCTION

Neisseria gonorrhoeae is an obligate human pathogen that is the etiologic agent of the sexually transmitted infection, gonorrhea. In 2007, over 350,000 gonorrhea infections were reported in the U.S., but it is estimated that the actual number of infections may be nearly twice that number (5). *N. gonorrhoeae* primarily infects mucosal surfaces in the lower genital tract, with females often being asymptomatic. If left untreated, gonorrhea can cause more serious infections such as pelvic inflammatory disease and disseminated gonococcal infections, leading to sterility and even death. Since there currently is no vaccine available for *N. gonorrhoeae*, antibiotic therapy is the mainstay for treating infections.

For nearly 40 years, penicillin was the antibiotic of choice for treating gonorrhea, until resistance reached levels that necessitated a switch to other antibiotics. Gonococci utilize two main mechanisms of resistance: plasmid-mediated expression of a TEM-1-like β-lactamase and acquisition of chromosomally mediated resistance determinants that collectively render the organism resistant to clinically achievable levels of penicillin (3). During the same time, tetracycline was also removed as a treatment option, as strains of *N. gonorrhoeae* had emerged with either plasmid-mediated expression of the protective TetM determinant (21) or chromosomally mediated mutations that conferred resistance to the antibiotic (17). One of the antibiotics used in lieu of penicillin and tetracycline, ciprofloxacin, was effective for several years, but resistance to this antibiotic also emerged, and in 2007, fluoroquinolones were also removed from the recommended list of antibiotics to treat gonococcal infections in the U.S. (6).

The mechanisms of chromosomally mediated penicillin resistance in the gonococci are complex and multi-faceted. Early studies, most notably by Sparling and colleagues, demonstrated a step-wise transfer of resistance from a penicillin-resistant strain (e.g. FA6140) to

a penicillin-susceptible strain (e.g. FA19) via DNA uptake and homologous recombination (10, 12, 30). At least five resistance determinants, which are mutated alleles of normal genes, have been identified and characterized (16, 23, 25, 28, 31). Each of these determinants incrementally increases resistance on its own, but it is the cumulative effects of these determinants and their synergistic interactions that result in clinical levels of resistance. The first step in transfer of resistance is acquisition of mutated alleles of the *penA* gene, which encodes the penicillin-binding protein 2 (PBP 2), the primary lethal target of penicillin. The hallmark of these *penA* alleles is a single codon insertion, Asp345a, and 4-8 mutations clustered in the C-terminus of the protein, which together lower the rate of acylation of PBP 2 with penicillin G by 16-fold (26). The second step of transformation results from mutations in the promoter region or coding sequence of *mtrR*, a transcriptional repressor that regulates expression of the MtrC-MtrD-MtrE efflux pump (and other genes; (13)). *mtrR* mutations result in overexpression of the efflux pump and high-level resistance to hydrophobic agents such as detergents and dyes, and small (~2-fold) increases in the MIC of penicillin (16). The third step in resistance is *penB*, which encodes mutated alleles of the major outer membrane porin PorB1b with amino acid substitutions in the constriction loop that presumably decrease the influx of the antibiotic into the periplasm (14, 23). Interestingly, the increase in resistance conferred by *penB* occurs only in strains harboring a co-resident *mtrR* mutation, but the molecular basis for this requirement is not completely understood (24).

Although the aforementioned determinants markedly increase the MIC of penicillin G when transformed into FA19 (from 0.01 to 0.75 μg/ml), the MIC is still well below the level of the clinical isolates that serve as DNA donors in transformation experiments (MIC ≅ 4 μg/ml). Moreover, DNA from these donors is not capable of further transforming the third-step

transformant to higher levels of resistance (3, 10, 28). We demonstrated that high-level penicillin-resistant strains contain a *ponA* allele (*ponA1*) encoding PBP 1 with a missense mutation (L421P) that lowers the rate of acylation with penicillin G by ~4-fold (28). Surprisingly, whereas reversion of the *ponA1* allele back to the wild type *ponA* gene in high-level penicillin-resistant clinical strains decreased the MIC of penicillin G 2-fold, replacement of the wild type *ponA* allele with *ponA1* in the third step transformant did not increase the MIC of penicillin G. These data suggest that *ponA1* does have a role in penicillin resistance, but requires an additional unknown gene(s) to exert its phenotypic effect on resistance. All attempts thus far to identify this gene(s) have been unsuccessful.

Gonococcal infections are now mostly treated with 3rd generation expanded-spectrum cephalosporins such as ceftriaxone and cefixime. Recently, however, strains of *N. gonorrhoeae* showing intermediate-level resistance to these antibiotics have emerged, which, if (when) they develop high-level resistance, pose a major threat to public health. Thus far, treatment failures have been reported for cefixime but not ceftriaxone (9, 34). Cephalosporin-intermediate resistance (ceph^{I}) is chromosomally mediated, and the mechanisms bear resemblance to those for chromosomally mediated penicillin resistance. The hallmark of ceph^{I} strains is the presence of mosaic *penA* alleles, which encode PBP 2 variants with 50-60 amino acid changes compared to a wild-type allele. These mosaic alleles were generated by recombination of regions of the *penA* genes from *N. sicca*, *N. perflava*, *N. cinerea*, and/or *N. flavescens* into the gonococcal *penA* gene (1). Aside from *penA*, however, the genetics of ceph^{I} resistance have not been comprehensively studied and are mostly unknown. In the present study, we examined the roles of *mtrR*, *penB*, and *ponA1* alleles in increasing resistance to ceftriaxone and cefixime. Our data reveal that the mechanisms by which strains increase resistance to the two cephalosporins are different, but that

both require the presence of the unknown determinant(s) necessary for high-level penicillin resistance.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are shown in Table 1. FA19 (a penicillin-and cephalosporin-susceptible laboratory strain) and FA6140 (a high-level penicillin-resistant isolate) were obtained from Dr. Fred Sparling, University of North Carolina at Chapel Hill. Strain 35/02 (a penicillin-resistant and ceph^{I} strain) was isolated in Sweden in 2002 (19). pUC18us-*penA35* contains the *penA* gene from 35/02, along with 300 bp of downstream sequence and an uptake sequence (11) to facilitate homologous recombination. Transformants harboring the mosaic *penA* allele were selected with 0.02 μg/ml (FA19) or 0.08 μg/ml (FA6140) cefixime. Transfer of *mtrR* from strain 35/02 was accomplished by selecting transformants on 1 mg/ml Triton X-100, and *penB35* transformants were selected on 0.37 μg/ml penicillin G; both of these transformations were accomplished using PCR-amplified fragments. The *mtrR* gene and promoter from strain 35/02 contains both a G45D mutation in the coding sequence of MtrR, which disrupts the DNA-binding domain of the MtrR repressor, and a –A deletion in the 13-bp inverted repeat in the *mtrR* promoter (19). The –A deletion results in maximal transcription of *mtrCDE*, whereas the G45D mutation, when present by itself, also increases *mtrCDE* transcription, albeit to a lower level than the promoter mutation (35). pMO-$porB_{1b}^{FA1090}$, which was used to revert *penB* back to $porB_{1b}$, contains a 2 kb fragment of the $porB_{1b}$ gene from FA1090 with an erythromycin resistance cassette downstream of the gene to aid in selection. Plasmids pPR16 and pPR17 harbor the coding regions of the wild-type and mutant *ponA* genes, respectively, with an extra 546 bp of downstream sequence to facilitate homologous recombination and the Ω fragment encoding spectinomycin and streptomycin resistance (27) inserted 68 bp downstream of the *ponA* stop codon to aid in selection (28).

Transformation. Transformation experiments were carried out essentially as described by Ropp et al. (28). Briefly, piliated colonies of the recipient strain were passaged on a fresh GC broth (GCB) agar plate and grown for 16-20 hrs. The cells were swabbed from the plate, resuspended in GCB with Supplements I and II (18), 20 mM bicarbonate, and 10 mM $MgCl_2$, and diluted to an OD_{560} of 0.18. Cells (0.9 ml) were incubated for 5 hrs at 37°C in a CO_2 incubator with 10 µL of a PCR product or plasmid (~3-5 µg), and aliquots of the cells were plated on GCB agar plates containing the appropriate antibiotics and allowed to grow overnight. Transformants were passaged on GCB agar plates, and the following day colonies were boiled in 30 µL of water and spun briefly to pellet debris. Two µL of the supernatants were used as templates in a PCR reaction, and transformants showing correct recombination were identified by sequencing.

MIC measurements. The MICs of penicillin G, ceftriaxone, and cefixime were determined as described previously (28). Briefly, non-piliated colonies were streaked on a GCB agar plate and allowed to grow for 18 hr. The cells were swabbed into 2 ml of GCB with Supplements I and II (16), 20 mM sodium bicarbonate, and 10 mM $MgCl_2$, and diluted to an OD560 of 0.18. Five µl of cell suspension (~50,000 cells) were spotted on a series of plates containing the following concentration ranges of antibiotics in approximately 1.5-fold increments: penicillin G, 0.008-8 µg/ml; ceftriaxone, 0.0004-0.4 µg/ml; and cefixime, 0.0004-0.8 µg/ml. The < 2–fold increases for most concentrations in the series allowed for a more accurate evaluation of MICs. At least 2 colonies (and often up to 4) from each transformation, verified by PCR amplification and sequencing, were examined for growth on antibiotic plates. At least three independent MIC experiments were carried out, and reported MICs represented the average of all experiments. Error bars in the figures represent the variation in the three determinations.

RESULTS

The role of the mosaic *penA* allele in ceph^{I} resistance. To examine directly the contribution of the mosaic *penA* allele to intermediate-level resistance to ceftriaxone and cefixime, we transformed FA19, a β-lactam antibiotic-susceptible strain, and FA6140, a high-level penicillin-resistant clinical isolate, with the mosaic *penA* gene (*penA35*) from the ceph^{I} strain 35/02, which was isolated in Sweden in 2002 (19). Upon recombination of *penA35* into FA19, the MIC of penicillin G increased from 0.012 μg/ml to 0.13 μg/ml, a 10-fold increase (Fig. 1). The MIC of FA19 *penA35* was 2-fold higher than the MIC for FA19 transformed with the *penA* gene (*penA4*) from FA6140 (MIC = 0.06 μg/ml), which harbors the Asp-345a insertion and 4 C-terminal mutations (24, 26). When the *penA35* allele was transformed into FA6140, the MIC of penicillin G increased 2-fold, from 3 to 6 μg/ml, consistent with the 2-fold difference in MIC between FA19 *penA4* and FA19 *penA35*. Upon acquisition of *penA35*, the MIC of ceftriaxone increased 20-fold in FA19 (from 0.0006 to 0.012 μg/ml) and 3.5-fold (from 0.06 to 0.21 μg/ml) in FA6140. The *penA35* allele conferred even larger increases in the MIC of cefixime: 100-fold in FA19 (from 0.001 to 0.1 μg/ml) and 10-fold in FA6140 (from 0.048 to 0.5 μg/ml). Importantly, the MICs of ceftriaxone and cefixime for FA19 *penA35*, although markedly increased over those for FA19, were still substantially lower than their respective MICs for 35/02 (especially for ceftriaxone), demonstrating that other resistance determinants play a role in ceph^{I} resistance.

Strain 35/02 has all of the known resistance determinants found in FA6140, i.e. *penA*, *mtrR*, *penB*, and *ponA1* (19). Although the sequences of their *penA* genes are markedly different, the sequences of the *mtrR* and *ponA1* determinants in the two strains are 100% identical, and the *penB* genes are over 95% identical, with 100% identity within loop 3 that contains the amino

184 acids most relevant for conferring resistance (23). Thus, we expected that FA6140 *penA35* and
185 35/02, which also have the same *penA* allele, would have the same level of resistance to the β-
186 lactam antibiotics; however, the MICs of all three antibiotics for FA6140 *penA35* were ~2-fold
187 higher than those for 35/02 (Fig. 1). These data suggest that either FA6140 contains an additional
188 determinant(s) not present in 35/02 or that the unknown resistance determinant(s) in FA6140
189 has(have) a stronger phenotype than in 35/02.

190 **The role of the *mtrR* and *penB* in ceph^{I} resistance.** The synergistic effects of *mtrR* and
191 *penB* on penicillin resistance have been well documented (24, 33, 37). When present individually
192 in FA19 *penA4*, these determinants have either a small (*mtrR*) or no (*penB*) effect on the MIC of
193 penicillin G, whereas together they increase the MIC by ~8-10-fold. To examine the role of *mtrR*
194 and *penB* in ceph^{I} resistance, we carried out a step-wise transfer of the *mtrR* and *penB* alleles
195 from 35/02 into FA19 *penA35* (Table 1), and determined the MICs of penicillin G, ceftriaxone,
196 and cefixime for the resulting transformants (Fig. 2). Consistent with previous results,
197 transformation of *mtrR* into FA19 *penA35* increased the MIC of penicillin G ~1.5-fold, and
198 further acquisition of *penB35* resulted in an additional 4-fold increase. Transfer of the *mtrR* gene
199 into FA19 *penA35* had little to no effect on the MIC of ceftriaxone, but, upon further transfer of
200 *penB35*, resistance increased about 2.5-fold. In marked contrast to the other antibiotics, neither
201 *mtrR* nor *penB* increased cefixime resistance above that of FA19 *penA35*.

202 Although overexpression of the MtrC-MtrD-MtrE efflux pump (as a result of the *mtrR*
203 promoter mutation) has only minimal effects on penicillin G resistance of FA19 *penA35* (Fig. 2),
204 the resistance to penicillin G is markedly decreased when the MtrC-MtrD-MtrE efflux pump is
205 inactivated in a high-level penicillin-resistant clinical strain, indicating that the efflux pump is a
206 critical element of high-level penicillin resistance (33). To investigate further the role of the

overexpressed MtrC-MtrD-MtrE efflux pump in ceph^{I} resistance, we knocked out the efflux pump by transforming FA6140, FA6140 *penA35* and 35/02 with a construct of the *mtrD* gene (16) disrupted by insertion of the *kpt* resistance gene (*mtrD*::kan) (Table 1). Inactivation of *mtrD* in all three strains decreased the MIC of penicillin G nearly 16-fold (Fig. 3, Table 2), highlighting the importance of the MtrC-MtrD-MtrE efflux pump in high-level penicillin resistance. Ceftriaxone intermediate resistance was slightly less dependent on the efflux pump, with an ~8-fold decrease in the MIC for all three strains when the pump was disrupted. In contrast, disruption of *mtrD* conferred a much smaller decrease (2- to 3-fold) in cefixime resistance, which was consistent with the apparent lack of effect of both *mtrR* and *penB* on cefixime resistance described above (Fig. 2).

In contrast to *mtrR*, the role of *penB* in high-level penicillin resistance is less clear. The synergism between *mtrR* and *penB* suggests that at least part of the role of *mtrR*, in addition to increasing efflux of antibiotics, is to promote the phenotypic effect of *penB*, which is silent in the absence of *mtrR* (24). To distinguish between its role in efflux and "activation" of PenB, we reverted the *penB* gene of FA6140 *penA35* back to the wild-type $porB_{1B}^{FA1090}$ gene by allelic exchange and determined the MICs of penicillin G, ceftriaxone, and cefixime for the resulting transformants. Reversion of the *penB* allele back to wild-type conferred a consistent 2- to 3-fold decrease in the MICs of each of the three antibiotics (Fig. 3 and Table 2). For both penicillin G and ceftriaxone, reversion of *penB* to wild-type $porB_{1B}$ did not decrease the MIC as much as a deletion of the MtrC-MtrD-MtrE efflux pump, whereas for cefixime, both had similar effects on the MICs.

The role of the *ponA1* in ceph^{I} resistance. We also investigated the role of *ponA1*, which encodes PBP 1 containing a single missense mutation (L421P), in ceph^{I} resistance by

either transforming a third-level transformant with *ponA1* or by reverting the *ponA1* allele in FA6140 *penA35* and 35/02 back to the wild-type sequence. As previously reported for penicillin resistance, transformation of *ponA1* into a third-level transformant had no appreciable effect on resistance to any of the three antibiotics tested (Fig. 2). When the *ponA1* allele was reverted back to wild-type in FA6140 *penA35* and 35/02, penicillin G resistance decreased 2-fold, consistent with our previous data (Fig. 4 and (28)). However, reversion of the *ponA* allele had little to no effect on ceftriaxone or cefixime resistance. Thus, in contrast to high-level resistance to penicillin, *ponA1* appears to have little or no role in ceph^{I} resistance (Fig. 4).

DISCUSSION

The data presented in this study provide insight into the individual contributions of the mosaic *penA35* allele and the *mtrR*, *penB*, and *ponA1* resistance determinants to ceph^{I} resistance. We showed that mosaic *penA* alleles (e.g. *penA35*) play an important role in ceph^{I} resistance, but that the other determinants, with exception of *ponA1*, are also required. A useful way to examine the individual contributions of the *penA35* allele versus the entire set of other determinants is to compare the MICs of ceftriaxone and cefixime for three strains: FA19, FA19 *penA35*, and FA6140 *penA35* (Table 3). FA19 is an antibiotic-susceptible strain that serves as the starting point for transformation studies, FA19 *penA35* shows the increase in resistance due solely to the *penA35* allele, and FA6140 *penA35* shows the sum effects of *penA35* in the presence of the complete set of determinants critical for high-level penicillin resistance.

The MICs of both antibiotics increase ~400-fold from FA19 to FA6140 *penA35* (Table 3), but how this 400-fold increase is achieved is markedly different in the two antibiotics. For ceftriaxone, transfer of the *penA35* allele into FA19 confers a 20-fold increase in the MIC, while the additional determinants in FA6140 cause another 20-fold increase in MIC, for a total increase of 400-fold. Thus, the mosaic *penA* allele and the other resistance determinants contribute equally to the increase in MIC for this antibiotic. In contrast, transfer of *penA35* into FA19 increases the MIC of cefixime by 100-fold, while the other determinants in FA6140 cause only an additional 4-fold increase in the MIC. Thus, while the overall fold increases in the MICs of the two antibiotics are the same, it is clear that for cefixime the *penA35* allele is the major factor in resistance, while for ceftriaxone, the additional determinants, which function at least in part by limiting the concentration of the antibiotic in the periplasm, are as important as the mosaic *penA*

allele in increasing its MIC. These data demonstrate the different modes by which these two cephalosporins inhibit the growth of gonococci.

Our data can be compared to those reported by Takahata et al. (32), who transformed FA1090 with mosaic *penA* constructs and determined the MICs of a range of β-lactam antibiotics. In that study, the MICs of ceftriaxone and cefixime were increased 4- and 15-fold, respectively, upon transfer of the entire mosaic *penA* allele into FA1090, which are significantly lower than the increases reported here in FA19 (20- and 100-fold). The differences in the two studies in part may reflect the fact that FA1090 contains a *penA* gene (NGO1542 of Accession #AE004969) with an Asp345a codon insertion that decreases the rate of acylation of PBP 2 with β-lactams (29), which would increase the MIC for the starting strain and thus lower the fold increase of the resulting mosaic *penA* transformant.

Ochiai et al. (22) determined the IC_{50} values of a range of antibiotics for inhibition of bocillin FL (a fluorescent β-lactam) binding to purified PBP 2 variants from two antibiotic-resistant strains, one with a *penA* allele similar to FA6140 and the other with a *penA* allele similar to 35/02. They reported that the IC_{50} values of the two PBP 2 variants for ceftriaxone were not different, inferring that the mosaic *penA* allele plays little to no role in ceftriaxone intermediate resistance. In contrast, our data show that isogenic replacement of the non-mosaic *penA* allele in FA6140 with the mosaic *penA35* allele increases the MIC of ceftriaxone 4-fold, from 0.05 to 0.2 μg/ml (Fig. 1). These results clearly indicate that the mosaic *penA35* confers an increase in the ceftriaxone MIC over and above that conferred by *penA4*. The reasons for this discrepancy are not clear, but may be due to utilizing inhibition curves to define inhibition instead of deriving k_2/K_S acylation constants for the various antibiotics. Although in vitro binding data are useful in comparing the effects of mutations on PBPs, we believe that analysis

283 of isogenic strains created with resistance alleles of known sequence is the most valid and
284 straightforward approach to examine the contributions of specific alleles in conferring resistance
285 to antibiotics.

286 Comparison of the MICs of the three antibiotics for FA19 *penA35* with or without *mtrR*
287 and *penB* mutations reveals that the contributions of these two determinants to resistance vary
288 depending upon the antibiotic (Fig. 2). Thus, transfer of both *mtrR* and *penB* into FA19 *penA35*
289 increased the MIC of penicillin G 6-fold, and ceftriaxone 3.3-fold, but had little to no effect on
290 the MIC of cefixime (Fig. 1). The latter result was unexpected, as these determinants would be
291 predicted to decrease permeation of any antibiotic that enters the periplasm through porin
292 channels. Thus, these data suggest that either cefixime does not diffuse into the periplasm
293 through porin channels or that such diffusion is not altered by *penB* mutations. One obvious
294 distinction of cefixime compared to penicillin G and ceftriaxone is that the former antibiotic has
295 a −2 net charge at neutral pH, whereas the latter two have a −1 net charge, which may affect its
296 permeation.

297 Our data also shed light on the dual roles of the *mtrR* determinant on overall ceph^{I}
298 resistance and highlight the different modes of action of the three antibiotics. *mtrR* mutations
299 affect resistance in two ways—increased efflux of the antibiotic and activation of *penB*
300 mutations that decrease diffusion through porin channels—while *penB* mutations have a
301 phenotype only when an *mtrR* mutation is present. Deletion of *mtrD* in FA6140 *penA35* or 35/02
302 caused marked 16- and 8-fold decreases in the MICs of penicillin G and ceftriaxone respectively,
303 whereas the MIC of cefixime was less affected (3-fold decrease; Fig. 3 and Table 2). Conversely,
304 reversion of *penB* back to wild type *porB*$_{1b}$ decreased the MICs of all three antibiotics about the
305 same, between 2- and 3-fold. These data suggest that increases in resistance to penicillin and

ceftriaxone upon acquisition of the *mtrR* and *penB* determinants are due to a combination of efflux by the MtrC-MtrD-MtrE pump and a decrease in permeation through porins. In contrast, the *mtrR* mutation appears to contribute to cefixime resistance solely by activating *penB*, since the decreases in MIC following inactivation of *mtrD* and reversion of *penB* to wild type were highly similar. However, the fact that these genetic manipulations had any effect at all was surprising, since we were unable to observe a change in the MIC of cefixime when *mtrR* and *penB* were transferred into FA19 *penA35* (Fig. 3). The reason for this discrepancy is not known, but may reflect the influence of the unknown determinant in FA6140 on both the efflux pump and/or PenB.

Unlike previous studies, which associated *penA35*, *mtrR*, *penB*, and *ponA1* with ceph^{I} resistance through epidemiological and sequencing studies (19, 36), this study directly establishes the contributions of these resistance determinants in ceph^{I} resistance. However, these determinants were not sufficient to increase the MICs of any of the three antibiotics to donor levels when transformed into FA19. Attempts to transform FA19 *penA35 mtrR penB ponA1* to donor levels of resistance with 35/02 or FA6140 genomic DNA were unsuccessful (data not shown). These data are identical to those observed previously in this laboratory and others for penicillin resistance, and suggest the presence of an unknown determinant(s), non-transferrable by transformation, that modulates increased levels of resistance (10, 28).

The role of *ponA1* in high-level penicillin resistance is well established, as shown by the 2-fold decrease in MIC upon reversion of *ponA1* to *ponA*^{wt} (Fig. 4 and (28)). However, no such decrease in MIC was observed for ceftriaxone or cefixime, indicating that *ponA1* plays little to no role in resistance for these two antibiotics. This distinction may reflect the differences in the rates of acylation of the two essential PBPs, PBP 1 and PBP 2, by the three antibiotics. For the

wild type PBPs, PBP 2 has an ~16-fold higher acylation rate with penicillin G than PBP 1 (2, 28), making PBP 2 the primary lethal target for this antibiotic in wild type strains. Upon acquisition of the *penA* allele, the rate of acylation of PBP 2 decreases by ~16-fold, bringing it much closer to that of PBP 1 (although PBP 2 is still the lethal target, since introduction of an altered PBP 1 with a lower rate of acylation for the antibiotic does not increase the MIC of penicillin). Upon acquisition of the other resistance determinants, the lethal target of penicillin must shift to include PBP 1, and thus acquisition of an altered PBP 1 increases the MIC. For the cephalosporins, we suspect that there may be much larger differences in their rates of acylation of PBP 2 compared to PBP 1, such that even with the decrease in the acylation rate of mosaic PBP 2 with the cephalosporins and the presence of the other resistance determinants, PBP 1 never becomes a lethal target, and accordingly alterations in PBP 1 have no effect on resistance. Experiments to examine the acylation rates of the various wild type and mutant PBPs with the three antibiotics to test this possibility are in progress.

In conclusion, the present study clearly emphasizes that the mosaic *penA* allele, *mtrR*, *penB*, and an unknown mutation, but not *ponA1*, are important determinants for conferring intermediate resistance to the expanded spectrum cephalosporins, ceftriaxone and cefixime. Together, these determinants confer a 400-fold increase in the MIC of both antibiotics, but do so in different ways: the mosaic *penA* allele and the set of other resistance determinants contribute equally to resistance to ceftriaxone, while the mosaic *penA* allele confers the greatest increase in resistance to cefixime. The inability of *ponA1* to confer resistance to 35/02 or FA6140 *penA35*, even though it is found in a wide range of ceph^{I} strains (19), suggests that these strains did not arise de novo from antibiotic-sensitive strains, but likely arose by transfer of mosaic *penA* alleles into pre-existing chromosomally mediated penicillin-resistant strains, which persist in the

bacterial population even though penicillin has not been recommended for treatment of gonorrhea for nearly 25 years (4). This would explain the presence of the *ponA1* allele, which is required for high-level penicillin-resistance, in almost all ceph^{I} strains even though it does not contribute to ceph^{I} resistance. Such a mechanism would also account for the rapid emergence of these strains over the last several years. The current MIC breakpoint for strains considered resistant to either ceftriaxone or cefixime is > 0.5 μg/ml. Given the documented ability of *N. gonorrhoeae* to acquire additional resistance mechanisms to β-lactam antibiotics, it appears to be a matter of when, not if, strains resistant to these antibiotics emerge, and when they do, the dearth of other antibiotics useful for treating gonococcal infections portends a looming public health problem.

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477 TABLE 1. Bacterial strains used in this study

Plasmid or Strain	Description	Reference
pUC18us-*penA35*	Plasmid containing the *penA35* gene from strain 35/02	(7)
pPR16	Plasmid containing the wild type *ponA* gene and a downstream Ω resistance cassette conferring spectinomycin/streptomycin resistance	(28)
pPR17	Same as above but with the *ponA1* gene harboring an L421P mutation	(28)
pMO-$porB_{FA1090}$-erm	Plasmid containing the $porB_{1B}$ gene from FA1090 (i.e. wild type $porB_{1B}$) with a downstream erm cassette conferring erythromycin resistance	(23)
pBS-*mtrD*::kan	Plasmid containing the *mtrD* gene insertionally inactivated with a kan resistance cassette	(15)
FA19	clinical isolate	(20)
FA6140	clinical isolate	(8)
35/02	clinical isolate	(19)
FA19 *penA35*	FA19 X pUC18us-*penA35*	This study
FA6140 *penA35*	FA6140 X pUC18us-*penA35*	This study
FA19 *penA35 mtrR*	FA19 *penA35* X $mtrR^{35/02}$ PCR prod	This study
FA19 *penA35 mtrR penB35*	FA19 *penA35 mtrR* X $porB_{1B}{}^{35/02}$ PCR prod	This study
FA19 *penA35 mtrR penB35 ponA1*	FA19 *penA35 mtrR penB35* X pPR17	This study
FA6140 *mtrD*::kan	FA6140 X pBS-*mtrD*::kan	This study
FA6140 *penA35 mtrD*::kan	FA6140 *penA35* X pBS-*mtrD*::kan	This study
35/02 *mtrD*::kan	35/02 X pBS-*mtrD*::kan	This study
FA6140 *penA35 mtrD*::D405N	FA6140 *penA35* X pBS-*mtrD*-D405N	This study
FA6140 *penA35* $porB_{1B}{}^{FA1090}$	FA6140 *penA35* X pMO-$porB_{1B}{}^{FA1090}$	This study
FA6140 *penA35* $ponA^{wt}$	FA6140 *penA35* X pPR16	This study
35/02 $ponA^{wt}$	35/02 X pPR16	This study

478

479 TABLE 2. Fold decrease in the MIC of penicillin G, ceftriaxone and cefixime upon reversion of
480 *penB* or deletion of *mtrD*. The average fold decreases were surprising consistent in the three
481 strains. Par/wtPIB, ratio of MIC of parental strain vs. *penB* reversion strain; Par/mtrD, ratio of
482 parental strain vs. *mtrD* deletion strain.

	Penicillin G		Ceftriaxone		Cefixime	
Strains	Par/wtPIB	Par/mtrD	Par/wtPIB	Par/mtrD	Par/wtPIB	Par/mtrD
FA6140	2.3	15.7	4.4	10.7	2.3	4.5
FA6140 *penA35*	2.6	16.0	3.6	9.6	2.2	2.9
35/02	4.0	15.7	2.3	6.0	1.7	2.8
Average fold decrease	2.9 ± 0.9	15.8 ± 0.2	3.4 ± 1.1	8.8 ± 2.4	2.1 ± 0.3	3.4 ± 0.9

483

TABLE 3. Fold increases in ceftriaxone and cefixime resistance. The MICs of ceftriaxone and cefixime for FA19 (an antibiotic susceptible strain), FA19 *penA35* (increase in resistance due solely to the *penA35* allele), and FA6140 *penA35* (increase in resistance due to both *penA35* and other resistance determinants) are shown. [#]Fold increase was determined by dividing the MIC for the indicated strain by the MIC for FA19.

	Ceftriaxone		**Cefixime**	
Strain	**MIC (μg/ml)**	**Fold increase[#]**	**MIC (μg/ml)**	**Fold Increase[#]**
FA19	0.0006	---	0.0012	---
FA19 *penA35*	0.012	20	0.12	100
FA6140 *penA35*	0.24	20	0.5	4.2
FA19 → FA6140 *penA35*	---	400	---	420

Figure Legends

FIG. 1. MICs of penicillin G, ceftriaxone, and cefixime for FA19 and FA6140 transformed with the mosaic *penA35* gene from strain 35/02. The *penA35* allele from the ceph^{I} strain 35/02 was transformed into FA19 or FA6140 and MICs of penicillin G, ceftriaxone, and cefixime for the resulting strains were determined as described in Materials and Methods. The MIC values represent the average ± standard deviation for at least 2 transformants in a minimum of 3 independent experiments.

FIG. 2. MICs of penicillin G, ceftriaxone, and cefixime for stepwise transformants of *penA35*, *mtrR*, *penB35*, and *ponA1* in FA19. To create these strains, FA19 was transformed in a stepwise manner using PCR amplification products or plasmids of the indicated resistance determinants from the ceph^{I} strain 35/02. MICs of penicillin G, ceftriaxone, and cefixime for the resulting strains were determined as described in Materials and Methods and represent the average ± standard deviation for at least 2 transformants in a minimum of 3 independent experiments.

FIG. 3. Effects of deletion of the MtrC-MtrD-MtrE efflux pump or reversion of *penB* to wild-type in FA6140, FA6140 *penA35*, and 35/02 on the MICs of penicillin G, ceftriaxone, and cefixime. To investigate the individual contributions of *mtrR* and *penB* to ceph^{I} resistance, FA6140, FA6140 *penA35*, and 35/02 were transformed with plasmids harboring *mtrD* insertionally inactivated with a kanamycin resistance cassette or $porB_{1b}$ with a downstream erythromycin resistance cassette, and the MICs of the resulting transformants were determined as

described in Materials and Methods. The MIC values represent the average ± standard deviation for at least 2 transformants in a minimum of 3 independent experiments.

FIG. 4. Effects of reverting *ponA1* to the wild-type *ponA* sequence in FA6140 *penA35* and 35/02 on the MICs of penicillin G, ceftriaxone, and cefixime. The *ponA1* gene was reverted to wild-type *ponA* by transformation with a plasmid harboring the wild type *ponA* with a downstream spectinomycin/streptomycin resistance cassette, and the MICs of the resulting transformants were determined as described in Materials and Methods. The MIC values represent the average ± standard deviation for at least 2 transformants in a minimum of 3 independent experiments.