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Genetics of Chromosomally Mediated Intermediate Resistance to Ceftriaxone and Cefixime in *Neisseria gonorrhoeae*▽

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All strains of *Neisseria gonorrhoeae* with reduced susceptibility to ceftriaxone and cefixime (cephalosporin-intermediate-resistant [Ceph^i] strains) contain a mosaic *penA* allele encoding penicillin-binding protein 2 (PBP 2) with nearly 60 amino acid differences compared to the sequence of 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 coming from *mtrR* and *penB*, whereas ceftriaxone resistance is nearly equally dependent upon *mtrR* and *penB*. Unlike high-level penicillin resistance, the *ponA1* allele does not appear to be important for Ceph^i. A strain containing all four determinants has increased resistance to ceftriaxone and cefixime but not to the levels that the donor Ceph^i strain does, 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 the 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.

Neisseria gonorrhoeae is an obligate human pathogen that is the etiologic agent of the sexually transmitted infection gonorrhea. In 2007, over 350,000 cases of gonorrhea were reported in the United States, 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 it is left untreated, gonorrhea can cause more serious infections, such as pelvic inflammatory disease and disseminated gonococcal infections, leading to sterility and even death. Since no vaccine is currently available for *N. gonorrhoeae*, antibiotic therapy is the mainstay for the treatment of infections.

For nearly 40 years, penicillin was the antibiotic of choice for the treatment of 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* with either plasmid-mediated expression of the protective TetM determinant (21) or chromosomally mediated mutations that conferred resistance to the antibiotic (17) had emerged. One of the antibiotics used in lieu of penicillin and tetracycline, ciprofloxacin, was effective for several years, but resistance to this antibiotic also emerged. In 2007, fluoroquinolones were also removed from the recommended list of antibiotics to be used for the treatment of gonococcal infections in the United States (6).

The mechanisms of chromosomally mediated penicillin resistance in the gonococci are complex and multifaceted. Early studies, most notably by Sparling and colleagues (12, 30) and Dougherty (10), demonstrated a stepwise transfer of resistance from a penicillin-resistant strain (e.g., strain FA6140) to a penicillin-susceptible strain (e.g., strain FA19) via DNA uptake and homologous recombination. 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 the transfer of resistance is the acquisition of mutated alleles of the *penA* gene, which encodes penicillin-binding protein 2 (PBP 2), the primary target of penicillin for lethality. The hallmark of these *penA* alleles is a single codon insertion, Asp345a, and four to eight 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

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TABLE 1. Bacterial strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
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 and streptomycin resistance	28
pPR17	pPR16 with the *ponA* gene replaced by the *ponA1* gene harboring an L421P mutation	28
pMO-$porB_{FA1090}$-erm	Plasmid containing the *porB1b* gene from FA1090 (i.e., wild-type *porB1b*) 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
Strains		
FA19	Clinical isolate	20
FA6140	Clinical isolate	8
35/02	Clinical isolate	19
FA19 *penA35*	FA19 transformed with pUC18us-*penA35*	This study
FA6140 *penA35*	FA6140 transformed with pUC18us-*penA35*	This study
FA19 *penA35 mtrR*	FA19 *penA35* transformed with $mtrR_{35/02}$ PCR product	This study
FA19 *penA35 mtrR penB35*	FA19 *penA35 mtrR* transformed with $porB1b_{35/02}$ PCR product	This study
FA19 *penA35 mtrR penB35 ponA1*	FA19 *penA35 mtrR penB35* transformed with pPR17	This study
FA6140 *mtrD*::Kan	FA6140 transformed with pBS-*mtrD*::Kan	This study
FA6140 *penA35 mtrD*::Kan	FA6140 *penA35* transformed with pBS-*mtrD*::Kan	This study
35/02 *mtrD*::Kan	35/02 transformed with pBS-*mtrD*::Kan	This study
FA6140 *penA35* $porB1b_{FA1090}$	FA6140 *penA35* transformed with pMO-$porB1b_{FA1090}$	This study
FA6140 *penA35* $ponA_{wild\ type}$	FA6140 *penA35* transformed with pPR16	This study
35/02 $ponA_{wild\ type}$	35/02 transformed with pPR16	This study

(26). The second step of transformation results from mutations in the promoter region or the coding sequence of *mtrR*, a transcriptional repressor that regulates the expression of the MtrC-MtrD-MtrE efflux pump (and other genes [13]). *mtrR* mutations result in overexpression of the efflux pump, high-level resistance to hydrophobic agents (such as detergents and dyes), and small (approximately twofold) increases in the MIC of penicillin (16). The third step in resistance involves *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 coresident *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 (from 0.01 to 0.75 μg/ml) when they are transformed into FA19, the MIC is still well below the level for 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 approximately fourfold (28). Surprisingly, whereas the reversion of the sequence of the *ponA1* allele back to the sequence of the wild-type *ponA* gene in high-level-penicillin-resistant clinical strains decreased the MIC of penicillin G twofold, 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 expanded-spectrum cephalosporins, such as ceftriaxone and cefixime. Recently, however, strains of *N. gonorrhoeae* showing intermediate-level resistance to these antibiotics have emerged, and if (or when) they develop high-level resistance, they will pose a major threat to public health. Thus far, treatment failures have been reported with cefixime but not with ceftriaxone (9, 34). Cephalosporin-intermediate resistance ($Ceph^i$) is chromosomally mediated, and the mechanisms resemble 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 to 60 amino acid changes compared to the sequence of the 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 $Ceph^i$ have not been comprehensively studied and are mostly unknown. In the present study, we examined the roles of the *mtrR*, *penB*, and *ponA1* alleles in increasing resistance to ceftriaxone and cefixime. Our data reveal that the mechanisms by which strains have increased 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 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*

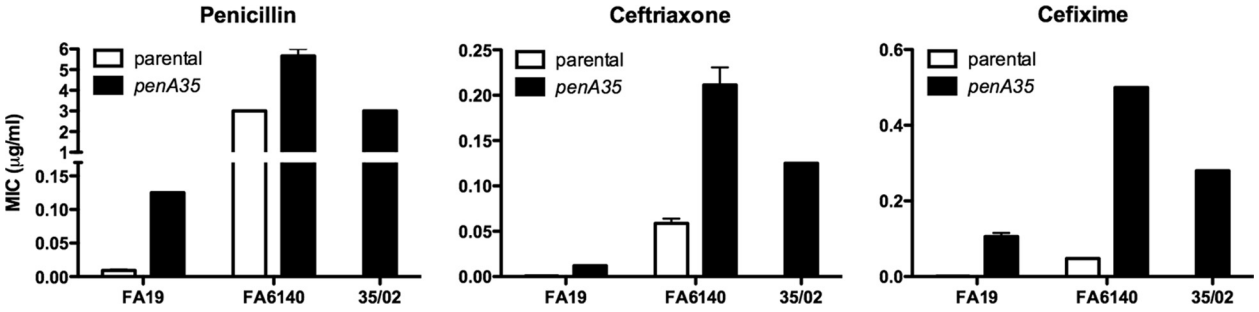

FIG. 1. MICs of penicillin G, ceftriaxone, and cefixime for strains FA19 and FA6140 transformed with the mosaic *penA35* gene from strain 35/02. The *penA35* allele from the Ceph^i strain 35/02 was transformed into FA19 or FA6140, and the MICs of penicillin G, ceftriaxone, and cefixime for the resulting strains were determined as described in Materials and Methods. The MICs represent the averages ± standard deviations for at least two transformants in a minimum of three independent experiments.

contains the *penA* gene from strain 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 (strain FA19) or 0.08 μg/ml (strain FA6140) cefixime. The 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 with PCR-amplified fragments. The *mtrR* gene and promoter from strain 35/02 contain both a G45D mutation in the coding sequence of MtrR, which disrupts the DNA-binding domain of the MtrR repressor, and an A deletion (−A) 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 it is present by itself, also increases the level of *mtrCDE* transcription, albeit to a lower level than the promoter mutation (35). pMO-*porB1b*$_{FA1090}$, which was used to revert *penB* back to *porB1b*, contains a 2-kb fragment of the *porB1b* gene from strain FA1090 (*porB1b*$_{FA1090}$) with an erythromycin resistance cassette downstream of the gene to aid with selection. Plasmids pPR16 and pPR17, which harbor the coding regions of the wild-type and mutant *ponA* genes, respectively, have 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 with 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 medium base (GCB) agar plate and grown for 16 to 20 h. The cells were swabbed from the plate; resuspended in GCB broth with supplements I and II (18), 20 mM bicarbonate, and 10 mM $MgCl_2$; and diluted to an optical density at 560 nm of 0.18. Cells (0.9 ml) were incubated for 5 h at 37°C in a CO_2 incubator with 10 μl of a PCR product or plasmid (~3 to 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 next day, colonies were boiled in 30 μl of water and spun briefly to pellet the debris. Two microliters of the supernatants was used as the template in a PCR, 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, nonpiliated colonies were streaked on a GCB agar plate and allowed to grow for 18 h. The cells were swabbed into 2 ml of GCB broth with supplements I and II (16), 20 mM sodium bicarbonate, and 10 mM $MgCl_2$ and diluted to an optical density at 560 nm of 0.18. Five microliters of the cell suspension (~50,000 cells) was spotted on a series of plates containing the following ranges of concentrations of antibiotics in approximately 1.5-fold increments: penicillin G, 0.008 to 8 μg/ml; ceftriaxone, 0.0004 to 0.4 μg/ml; and cefixime, 0.0004 to 0.8 μg/ml. The less than twofold increases for most concentrations in the series allowed a more accurate evaluation of the MICs. At least two colonies (and often up to four colonies) 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 the MICs reported here represent the averages of all experiments. Error bars in the figures represent the variations of the three determinations.

RESULTS

Role of mosaic *penA* allele in Ceph^i. 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 twofold higher than the MIC for FA19 transformed with the *penA* gene (*penA4*) from FA6140 (MIC = 0.06 μg/ml), which harbors the Asp345a insertion and four C-terminal mutations (24, 26). When the *penA35* allele was transformed into FA6140, the MIC of penicillin G increased twofold, from 3 to 6 μg/ml, consistent with the twofold difference in MICs 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 they were 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.

Strain 35/02 has all of the known resistance determinants found in strain FA6140, i.e., *penA*, *mtrR*, *penB*, and *ponA1* (19). Although the sequences of the *penA* genes are markedly different, the sequences of the *mtrR* and *ponA1* determinants in the two strains are 100% identical, and the sequences of the *penB* genes are over 95% identical, with 100% identity within loop 3, which contains the amino acids most relevant for conferring resistance (23). Thus, we expected that FA6140 *penA35* and 35/02, which also have the same *penA* allele, would have the same level of resistance to the β-lactam antibiotics; however, the MICs of all three antibiotics for FA6140 *penA35* were approximately twofold greater than those for 35/02 (Fig. 1). These data suggest either that FA6140 contains an additional determinant(s) not present in 35/02 or that the unknown re-

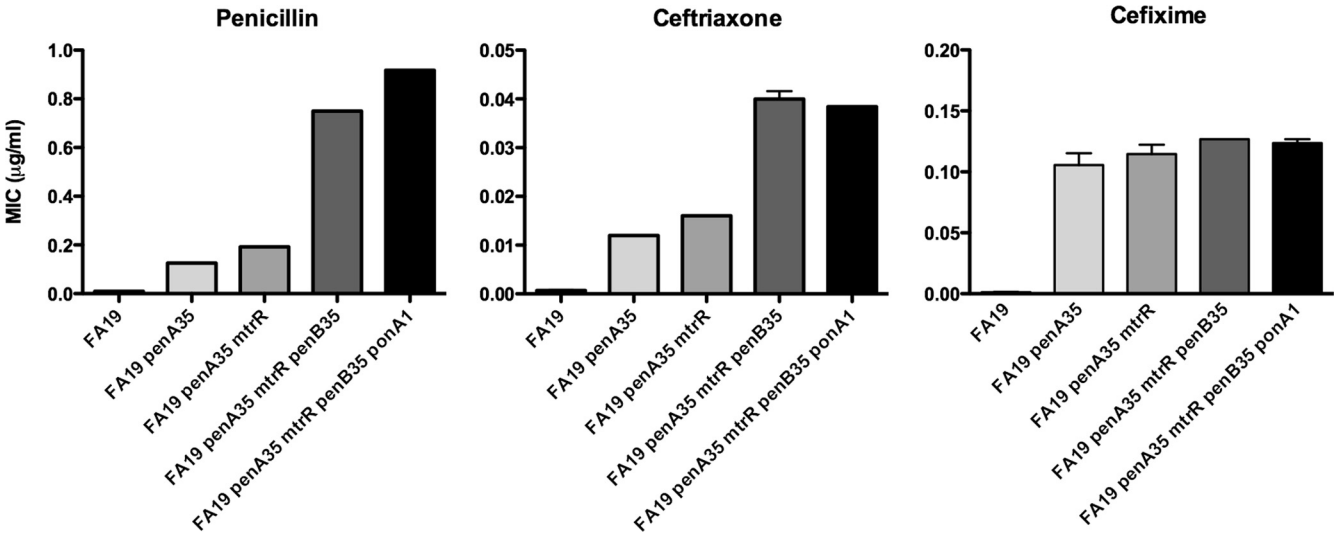

FIG. 2. MICs of penicillin G, ceftriaxone, and cefixime for stepwise transformants of *penA35*, *mtrR*, *penB35*, and *ponA1* in strain FA19. To create these strains, FA19 was transformed in a stepwise manner with PCR amplification products or plasmids with the indicated resistance determinants from Ceph^i strain 35/02. The MICs of penicillin G, ceftriaxone, and cefixime for the resulting strains were determined as described in Materials and Methods and represent the averages ± standard deviations for at least two transformants in a minimum of three independent experiments.

sistance determinant(s) in FA6140 has a stronger phenotype than that in 35/02.

Role of *mtrR* and *penB* in Ceph^i. The synergistic effects of *mtrR* and *penB* on penicillin resistance have been well documented (24, 33, 37). When they are present individually in strain FA19 *penA4*, these determinants have either a small effect (*mtrR*) or no effect (*penB*) on the MIC of penicillin G, whereas together they increase the MIC by ~8- to 10-fold. To examine the role of *mtrR* and *penB* in Ceph^i, we carried out a stepwise transfer of the *mtrR* and *penB* alleles from strain 35/02 into strain FA19 *penA35* (Table 1) and determined the MICs of penicillin G, ceftriaxone, and cefixime for the resulting transformants (Fig. 2). Consistent with previous results, the transformation of *mtrR* into FA19 *penA35* increased the MIC of penicillin G ~1.5-fold, and further acquisition of *penB35* resulted in an additional fourfold increase in resistance. Transfer of the *mtrR* gene into FA19 *penA35* had little to no effect on the MIC of ceftriaxone, but upon the further transfer of *penB35*, the level of resistance increased about 2.5-fold. In marked contrast to the other antibiotics, neither *mtrR* nor *penB* increased the level of cefixime resistance above that for FA19 *penA35*.

Although overexpression of the MtrC-MtrD-MtrE efflux pump (as a result of the *mtrR* promoter mutation) has only minimal effects on the penicillin G resistance of strain FA19 *penA35* (Fig. 2), the resistance to penicillin G is markedly decreased when the MtrC-MtrD-MtrE efflux pump is inactivated in a high-level-penicillin-resistant clinical strain, indicating that the efflux pump is a critical element of high-level penicillin resistance (33). To investigate further the role of the overexpressed MtrC-MtrD-MtrE efflux pump in Ceph^i, we knocked out the efflux pump by transforming strains 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 approximately eightfold decrease in the MIC for all three strains being detected when the pump was disrupted. In contrast, the disruption of *mtrD* conferred a much smaller decrease (two- to threefold) in the level of cefixime resistance, which was consistent with the apparent lack of an effect of either *mtrR* or *penB* on cefixime resistance, as described above (Fig. 2).

In contrast to the role of *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 the efflux of antibiotics, is to promote the resistance phenotype of *penB*, which is silent in the absence of *mtrR* (24). To distinguish between its role in efflux and the activation of PenB, we reverted the *penB* gene of FA6140 *penA35* back to the wild-type $porB1b_{FA1090}$ 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 the wild-type conferred consistent two- to threefold decreases 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 *porB1b* did not decrease the MIC as much as deletion of the MtrC-MtrD-MtrE efflux pump, whereas for cefixime, both reversion to wild-type *porB1b* and deletion of the efflux pump had similar effects on the MICs.

Role of *ponA1* in Ceph^i. We also investigated the role of *ponA1*, which encodes PBP 1 containing a single missense mutation (L421P), in Ceph^i either by transforming a third-level transformant with *ponA1* or by reverting the sequence of the *ponA1* allele in FA6140 *penA35* and 35/02 back to the wild-type sequence. As previously reported for penicillin resistance,

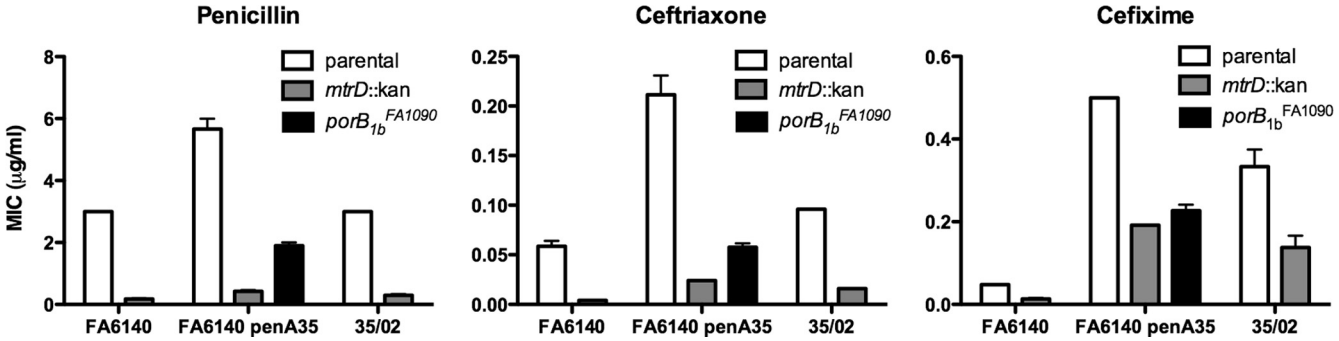

FIG. 3. Effects of deletion of the MtrC-MtrD-MtrE efflux pump or reversion of *penB* to the wild-type sequence in strains FA6140, FA6140 *penA35*, and 35/02 on the MICs of penicillin G, ceftriaxone, and cefixime. To investigate the individual contributions of *mtrR* and *penB* to Ceph^i, FA6140, FA6140 *penA35*, and 35/02 were transformed with plasmids harboring *mtrD* insertionally inactivated with a kanamycin resistance cassette or *porB1b* with a downstream erythromycin resistance cassette, and the MICs of the resulting transformants were determined as described in Materials and Methods. The MICs represent the averages ± standard deviations for at least two transformants in a minimum of three independent experiments.

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 sequence of the *ponA1* allele was reverted back to the wild-type sequence in FA6140 *penA35* and 35/02, the level of penicillin G resistance decreased twofold, consistent with our previous data (Fig. 4) (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 (Fig. 4).

DISCUSSION

The data presented in this report provide insight into the individual contributions of the mosaic *penA35* allele and the *mtrR*, *penB*, and *ponA1* resistance determinants to Ceph^i. We showed that mosaic *penA* alleles (e.g., *penA35*) play an important role in Ceph^i but that the other determinants, with the exception of *ponA1*, are also required. A useful way of examining the individual contributions of the *penA35* allele versus the contributions of 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 of the effects of *penA35* in the presence of the complete set of determinants critical for high-level penicillin resistance.

The MICs of both antibiotics increased ~400-fold from FA19 to FA6140 *penA35* (Table 3), but how this 400-fold increase was achieved was markedly different for the two antibiotics. For ceftriaxone, transfer of the *penA35* allele into FA19 conferred a 20-fold increase in the MIC, while the additional determinants in FA6140 caused 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 the MIC for this antibiotic. In contrast, transfer of *penA35* into FA19 increased the MIC of cefixime by 100-fold, while the other determinants in FA6140 caused only an additional 4-fold increase in the MIC. Thus, while the overall 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 the 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

TABLE 2. Decreases in MICs of penicillin G, ceftriaxone, and cefixime upon reversion of *penB* or deletion of *mtrD*

Strain	Fold decrease in MIC[a]					
	Penicillin G		Ceftriaxone		Cefixime	
	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
Avg 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

[a] The average decreases were surprisingly consistent among the three strains. Par/wtPIB, ratio of the MIC for the parental strain to the MIC for the *penB* reversion strain; Par/mtrD, ratio of the MIC for the parental strain to the MIC for the strain with the *mtrD* deletion.

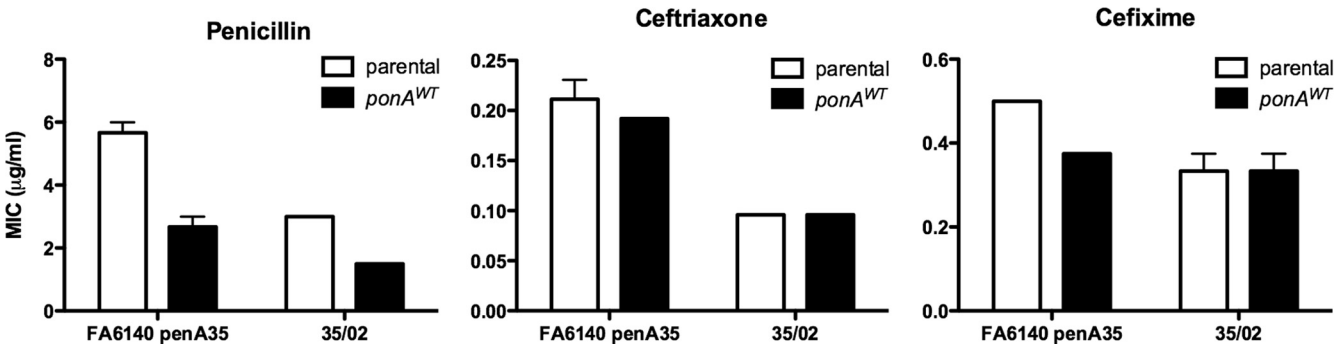

FIG. 4. Effects of reverting the *ponA1* sequence to the wild-type *ponA* sequence in strains FA6140 *penA35* and 35/02 on the MICs of penicillin G, ceftriaxone, and cefixime. The *ponA1* gene sequence was reverted to the wild-type *ponA* sequence by transformation with a plasmid harboring the wild-type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

mosaic *penA* allele into FA1090. Those increases 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 the sequence with GenBank accession no. 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 increase in the MIC for the resulting mosaic *penA* transformant.

Ochiai et al. (22) determined the 50% inhibitory concentrations 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 that of strain FA6140 and the other with a *penA* allele similar to that of strain 35/02. They reported that the 50% inhibitory concentrations of ceftriaxone for the two PBP 2 variants 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 nonmosaic *penA* allele in FA6140 with the mosaic *penA35* allele increases the MIC of ceftriaxone fourfold, 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 the utilization of inhibition curves to define inhibition instead of the derivation of k_2/K_s acylation constants for the various antibiotics. Although in vitro binding data are useful for comparison of the effects of mutations on PBPs, we believe that the analysis of isogenic strains created with resistance alleles whose sequences are known is the most valid and straightforward approach to examination of the contributions of specific alleles in conferring resistance to antibiotics.

TABLE 3. Increases in ceftriaxone and cefixime resistance[a]

Strain	Ceftriaxone		Cefixime	
	MIC (μg/ml)	Fold increase[b]	MIC (μg/ml)	Fold increase[b]
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

[a] The MICs of ceftriaxone and cefixime for FA19 (which is an antibiotic-susceptible strain), FA19 *penA35* (which has an increase in resistance solely because of the *penA35* allele), and FA6140 *penA35* (which has an increase in resistance because of both *penA35* and other resistance determinants) are shown.
[b] The increase was determined by dividing the MIC for the indicated strain by the MIC for FA19.

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

Our data also shed light on the dual roles of the *mtrR* determinant on Ceph^i and highlight the different modes of action of the three antibiotics. *mtrR* mutations affect resistance in two ways: increased efflux of the antibiotic and activation of *penB* mutations that decrease diffusion through porin channels. In contrast, *penB* mutations increase reistance only when an *mtrR* mutation is present. Deletion of *mtrD* in FA6140 *penA35* or 35/02 caused marked 16- and 8-fold decreases in the MICs of penicillin G and ceftriaxone, respectively, whereas the MIC of cefixime was less affected (three-fold decrease) (Fig. 3 and Table 2). Conversely, the reversion of the *penB* sequence back to the wild-type *porB1b* sequence decreased the MICs of all three antibiotics about the same: between two- and three-fold. These data suggest that the increases in resistance to penicillin and ceftriaxone upon the 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 MICs following the inactivation of *mtrD* and the reversion of the *penB* sequence to the wild-type sequence were highly similar. 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 it may reflect the influence of the unknown determinant in FA6140 on both the efflux pump and PenB.

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

The role of *ponA1* in high-level penicillin resistance is well established, as shown by the twofold decrease in the MIC upon the reversion of the *ponA1* sequence to the *ponA* wild-type sequence (Fig. 4) (28). However, no such decrease in the MIC was observed for ceftriaxone or cefixime, indicating that *ponA1* plays little to no role in resistance to 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 the 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 those of PBP 1, such that even with the decrease in the rate of acylation 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 rates of acylation 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 400-fold increases in the MICs 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 strain 35/02 or strain 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 preexisting chromosomally mediated penicillin-resistant strains, which persist in the bacterial population, even though penicillin has not been recommended for use for the 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. 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 >0.25 μg/ml. Given the documented ability of *N. gonorrhoeae* to acquire additional mechanisms of resistance to β-lactam antibiotics, it appears to be a matter of when and not if strains resistant to these antibiotics will emerge, and when they do, the dearth of other antibiotics useful for the treatment of gonococcal infections portends a looming public health problem.

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REFERENCES

1. **Ameyama, S., S. Onodera, M. Takahata, S. Minami, N. Maki, K. Endo, H. Goto, H. Suzuki, and Y. Oishi.** 2002. Mosaic-like structure of penicillin-binding protein 2 gene (*penA*) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. Antimicrob. Agents Chemother. **46:** 3744–3749.
2. **Barbour, A. G.** 1981. Properties of penicillin-binding proteins in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **19:**316–322.
3. **Cannon, J. G., and P. F. Sparling.** 1984. The genetics of the gonococcus. Annu. Rev. Genet. **38:**111–133.
4. **CDC.** 2008. Gonococcal Isolate Surveillance Project (GISP) annual report 2006. CDC, Atlanta, GA.
5. **CDC.** 2009. Sexually transmitted disease surveillance, 2007. CDC, Atlanta, GA.
6. **CDC.** 2007. Update to CDC's sexually transmitted diseases treatment guidelines, 2006: fluoroquinolones no longer recommended for treatment of gonococcal infections. MMWR Morb. Mortal. Wkly. Rep. **56:**332–336.
7. **Chen, C. J., D. M. Tobiason, C. E. Thomas, W. M. Shafer, H. S. Seifert, and P. F. Sparling.** 2004. A mutant form of the *Neisseria gonorrhoeae* pilus secretin protein PilQ allows increased entry of heme and antimicrobial compounds. J. Bacteriol. **186:**730–739.
8. **Danielsson, D., H. Faruki, D. Dyer, and P. F. Sparling.** 1986. Recombination near the antibiotic resistance locus *penB* results in antigenic variation of gonococcal outer membrane protein I. Infect. Immun. **52:**529–533.
9. **Deguchi, T., M. Yasuda, S. Yokoi, K. Ishida, M. Ito, S. Ishihara, K. Minamidate, Y. Harada, K. Tei, K. Kojima, M. Tamaki, and S. Maeda.** 2003. Treatment of uncomplicated gonococcal urethritis by double-dosing of 200 mg cefixime at a 6-h interval. J. Infect. Chemother. **9:**35–39.
10. **Dougherty, T. J.** 1986. Genetic analysis and penicillin-binding protein alterations in *Neisseria gonorrhoeae* with chromosomally mediated resistance. Antimicrob. Agents Chemother. **30:**649–652.
11. **Elkins, C., C. E. Thomas, H. S. Seifert, and P. F. Sparling.** 1991. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J. Bacteriol. **173:**3911–3913.
12. **Faruki, H., and P. F. Sparling.** 1986. Genetics of resistance in a non-ß-lactamase-producing gonococcus with relatively high-level penicillin resistance. Antimicrob. Agents Chemother. **30:**856–860.
13. **Folster, J. P., V. Dhulipala, R. A. Nicholas, and W. M. Shafer.** 2007. Differential regulation of *ponA* and *pilMNOPQ* expression by the MtrR transcriptional regulatory protein in *Neisseria gonorrhoeae*. J. Bacteriol. **189:**4569–4577.
14. **Gill, M. J., S. Simjee, K. Al-Hattawi, B. D. Robertson, C. S. Easmon, and**

C. A. Ison. 1998. Gonococcal resistance to beta-lactams and tetracycline involves mutation in loop 3 of the porin encoded at the *penB* locus. Antimicrob. Agents Chemother. **42:**2799–2803.
15. **Hagman, K. E., C. E. Lucas, J. T. Balthazar, L. Snyder, M. Nilles, R. C. Judd, and W. M. Shafer.** 1997. The MtrD protein of *Neisseria gonorrhoeae* is a member of the resistance/nodulation/division protein family constituting part of an efflux system. Microbiology **143:**2117–2125.
16. **Hagman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd, and W. M. Shafer.** 1995. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. Microbiology **141:** 611–622.
17. **Hu, M., S. Nandi, C. Davies, and R. A. Nicholas.** 2005. High-level chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* results from a point mutation in the *rpsJ* gene encoding ribosomal protein S10 in combination with the *mtrR* and *penB* resistance determinants. Antimicrob. Agents Chemother. **49:**4327–4334.
18. **Kellogg, D. S., W. L. Peacock, W. E. Deacon, L. Browh, and C. I. Perkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to colonial variation. J. Bacteriol. **85:**1274–1279.
19. **Lindberg, R., H. Fredlund, R. A. Nicholas, and M. Unemo.** 2007. *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA*, *mtrR*, *porB1b*, and *ponA*. Antimicrob. Agents Chemother. **51:**2117–2122.
20. **Maness, M. J., and P. F. Sparling.** 1973. Multiple antibiotic resistance due to a single mutation in Neisseria gonorrhoeae. J. Infect. Dis. **128:**321–330.
21. **Morse, S. A., S. R. Johnson, J. W. Biddle, and M. C. Roberts.** 1986. High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal *tetM* determinant. Antimicrob. Agents Chemother. **30:**664–670.
22. **Ochiai, S., S. Sekiguchi, A. Hayashi, M. Shimadzu, H. Ishiko, R. Matsushima-Nishiwaki, O. Kozawa, M. Yasuda, and T. Deguchi.** 2007. Decreased affinity of mosaic-structure recombinant penicillin-binding protein 2 for oral cephalosporins in *Neisseria gonorrhoeae*. J. Antimicrob. Chemother. **60:**54–60.
23. **Olesky, M., M. Hobbs, and R. A. Nicholas.** 2002. Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **46:**2811–2820.
24. **Olesky, M., R. L. Rosenberg, and R. A. Nicholas.** 2006. Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with *penB* mutations. J. Bacteriol. **188:**2300–2308.
25. **Pan, W., and B. G. Spratt.** 1994. Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. Mol. Microbiol. **11:**769–775.
26. **Powell, A. J., J. Tomberg, A. M. Deacon, R. A. Nicholas, and C. Davies.** 2009. Crystal structures of penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of *Neisseria gonorrhoeae* reveal an unexpectedly subtle mechanism for antibiotic resistance. J. Biol. Chem. **284:**1202–1212.
27. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
28. **Ropp, P. A., M. Hu, M. Olesky, and R. A. Nicholas.** 2002. Mutations in *ponA*, the gene encoding penicillin-binding protein 1, and a novel locus, *penC*, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **46:**769–777.
29. **Schultz, D. E., B. G. Spratt, and R. A. Nicholas.** 1991. Expression and purification of a soluble form of penicillin-binding protein 2 from both penicillin-susceptible and penicillin-resistant *Neisseria gonorrhoeae*. Prot. Express Purif. **2:**339–349.
30. **Sparling, P. F., F. A. J. Sarubbi, and E. Blackman.** 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. **124:**740–749.
31. **Spratt, B. G.** 1988. Hybrid penicillin-binding proteins in penicillin-resistant strains of *Neisseria gonorrhoeae*. Nature **332:**173–176.
32. **Takahata, S., N. Senju, Y. Osaki, T. Yoshida, and T. Ida.** 2006. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **50:**3638–3645.
33. **Veal, W. L., R. A. Nicholas, and W. M. Shafer.** 2002. Overexpression of the MtrC-MtrD-MtrE efflux pump due to an *mtrR* mutation is required for chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. J. Bacteriol. **184:**5619–5624.
34. **Wang, S. A., M. V. Lee, N. O'Connor, C. J. Iverson, R. G. Ohye, P. M. Whiticar, J. A. Hale, D. L. Trees, J. S. Knapp, P. V. Effler, and H. S. Weinstock.** 2003. Multidrug-resistant Neisseria gonorrhoeae with decreased susceptibility to cefixime—Hawaii, 2001. Clin. Infect. Dis. **37:**849–852.
35. **Warner, D. M., W. M. Shafer, and A. E. Jerse.** 2008. Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE Efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol. Microbiol. **70:**462–478.
36. **Whiley, D. M., E. A. Limnios, S. Ray, T. P. Sloots, and J. W. Tapsall.** 2007. Diversity of *penA* alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob. Agents Chemother. **51:**3111–3116.
37. **Zhao, S., D. M. Tobiason, M. Hu, H. S. Seifert, and R. A. Nicholas.** 2005. The *penC* mutation conferring antibiotic resistance in *Neisseria gonorrhoeae* arises from a mutation in the PilQ secretin that interferes with multimer stability. Mol. Microbiol. **57:**1238–1251.