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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[†]

Joshua Tomberg,[‡] Magnus Unemo,[§] Christopher Davies,[∥] and Robert A. Nicholas*,[‡]

[‡]*Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599-7365,* [§]*National Reference Laboratory for Pathogenic Neisseria, Department of Laboratory Medicine, Clinical Microbiology, Örebro University Hospital, Örebro, Sweden, and* [∥]*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425*

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ABSTRACT: Mutations in penicillin-binding protein 2 (PBP 2) encoded by mosaic *penA* alleles are crucial for intermediate resistance to the expanded-spectrum cephalosporins ceftriaxone and cefixime in *Neisseria gonorrhoeae*. Three of the ~60 mutations present in mosaic alleles of *penA*, G545S, I312M, and V316T, have been reported to be responsible for increased resistance, especially to cefixime [Takahata, S., et al. (2006) *Antimicrob. Agents Chemother. 50*, 3638−3645]. However, we observed that the minimum inhibitory concentrations (MICs) of penicillin, ceftriaxone, and cefixime for a wild-type strain (FA19) containing a *penA* gene with these three mutations increased only 1.5-, 1.5-, and 3.5-fold, respectively. In contrast, when these three mutations in a mosaic *penA* allele (*penA35*) were reverted back to the wild type and the gene was transformed into FA19, the MICs of the three antibiotics were reduced to near wild-type levels. Thus, these three mutations display epistasis, in that their capacity to increase resistance to β-lactam antibiotics is dependent on the presence of other mutations in the mosaic alleles. We also identified an additional mutation, N512Y, that contributes to the decreased susceptibility to expanded-spectrum cephalosporins. Finally, we investigated the effects of a mutation (A501V) currently found only in nonmosaic *penA* alleles on decreased susceptibility to ceftriaxone and cefixime, with the expectation that this mutation may arise in mosaic alleles. Transfer of the mosaic *penA35* allele containing an A501V mutation to FA6140, a chromosomally mediated penicillin-resistant isolate, increased the MICs of ceftriaxone (0.4 μg/mL) and cefixime (1.2 μg/mL) to levels above their respective break points. The proposed structural mechanisms of these mutations are discussed in light of the recently published structure of PBP 2.

Neisseria gonorrhoeae is the etiologic agent of the sexually transmitted infection gonorrhea. In 2007, there were more than 350000 infections reported in the United States (*1*). Gonococcal infections are often asymptomatic, especially in females, and if left untreated can cause pelvic inflammatory disease and disseminated infections, as well as contribute to the spread of the disease. Because of the lack of a vaccine, antibiotics are the primary treatment for gonococcal infections.

Traditionally, gonorrhea was treated with penicillin G, but in 1986, this antibiotic was discontinued in the United States because of the onset of resistance. For the same reason, tetracyclines were also withdrawn. More recently, high-level resistance to fluoroquinolones led to the withdrawal of these antibiotics from the recommended list in the United States, leaving only expanded-spectrum cephalosporins such as ceftriaxone and cefixime as reliable treatments for gonococcal infections (*2*). Importantly, strains with decreased susceptibility to these antibiotics (also called ceph[I] strains) are becoming widespread in the population (*3*, *4*), making it crucial that we understand the mechanisms that lead to ceph[I] resistance so that new antibiotics and new approaches can be found for the treatment of gonococcal infections before we are left with no effective antibiotics (*4*). This need is highlighted by recent reports of treatment failures using oral expanded-spectrum cephalosporins in Japan and Hong Kong (*5*, *6*), as well as two confirmed treatment failures of pharyngeal gonococcal infections with ceftriaxone (*7*), although the latter two cases probably reflect the well-recognized difficulties in eradicating pharyngeal gonorrhea (*4*).

The most common mechanism of penicillin resistance in *N. gonorrhoeae*, termed chromosomally mediated resistance, involves at least five resistance determinants (*8*, *9*). These determinants, which are mutated forms of normal genes and loci, can be transferred from a highly resistant donor to a susceptible strain by homologous recombination and selection, with transfer occurring in a defined order (*8*, *10*, *11*). The first step is transfer of *penA*, which encodes altered forms of penicillin-binding protein 2 (PBP 2),[1] the lethal target of penicillin G in *N. gonorrhoeae* (*12*). The second determinant transferred is *mtrR*,

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*To whom correspondence should be addressed: Department of Pharmacology, CB#7365, University of North Carolina, Chapel Hill, NC 27599-7365. Phone: (919) 966-6547. Fax: (919) 966-5640. E-mail: nicholas@med.unc.edu.

[1]Abbreviations: PBP, penicillin-binding protein; MIC, minimum inhibitory concentration; ceph[I], cephalosporin intermediate resistance; NTA, nitriloacetic acid; TEV, tobacco etch virus; STI, sexually transmitted infection.

Table 1: Strains and Plasmids Used in This Study

	description	ref
	Plasmids	
pUC18us-*penA35*[a]	plasmid containing the *penA35* gene from strain 35/02 and an uptake sequence	*16* and this study
pUC18us-*penA*[a]	plasmid containing the *penA* gene from strain FA19 and an uptake sequence	*16* and this study
pUC18us-*penA35*[a]-A501V	pUC18us-*penA35*[a] harboring an A501V mutation	this study
pUC18us-*penA4*	plasmid containing the *penA4* gene from strain FA6140 and an uptake sequence	*16*
pUC18us-*penA4*-A501V	pUC18us-*penA4* harboring an A501V mutation	this study
	Strains	
FA19	penicillin- and cephalosporin-susceptible recipient strain	*22*
FA6140	penicillin-resistant but cephalosporin-susceptible recipient strain	*23*
WT-3X	FA19 transformed with pUC18us-*penA* containing I312M, V316T, and G545S mutations	this study
-mod0, -mod1, etc.	FA19 transformed with pUC18us-*penA35*[a] in which the indicated module (see Figure 1) was replaced with the corresponding module from *penA*[a]	this study
-mod5 + G545S	FA19 transformed with pUC18us-*penA35*[a] in which mod5 was replaced with corresponding mod5 from the wild type; mod5 also contains the G545S mutation	this study
-m1,5 + G545S	FA19 transformed with pUC18us-*penA35*[a] in which mods 1 and 5 were replaced with corresponding mods from the wild type; mod5 also had a G545S mutation	this study
-m1,5 + I312M/V316T/G545S	FA19 transformed with pUC18us-*penA35*[a] in which mods 1 and 5 were replaced with corresponding mods from the wild type; mod1 additionally had I312M and V316T mutations, and mod5 had a G545S mutation	this study
FA19 *penA35*-S545G	FA19 containing the *penA35* gene with reversion of the G545S mutation	this study
FA19 *penA35*- M312I/T316V	FA19 containing the *penA35* gene with reversion of the I312M and V316T mutations	this study
FA19 *penA35*-S545G/M312I/T316V	FA19 containing the *penA35* gene with reversion of the G545S, I312M, and V316T mutations	this study

[a]Genes contain silent restriction sites incorporated into the coding sequence as depicted in Figure 1.

which leads to increased efflux pump expression and activity (*13*). The third determinant transferred is *penB*, which encodes altered forms of the major outer membrane porin, $PorB_{1B}$ (*14*, *15*). The final steps, which result in high-level penicillin resistance, include acquisition of *ponA*, encoding an altered form of PBP 1, and a resistance determinant whose identity is unknown (*8*). The sum effect of these determinants is to increase the minimum inhibitory concentration (MIC) of penicillin by 400-fold (from 0.01 to 4 μg/mL).

The major difference between penicillin-resistant and ceph[I] strains is the presence of mosaic *penA* alleles that encode PBP 2 variants containing up to 60 amino acid changes compared to PBP 2 from wild-type strains. We and others have proposed that the rapid emergence of ceph[I] strains has occurred by horizontal transfer of mosaic *penA* genes, which were generated by recombination events between *N. gonorrhoeae* and commensal *Neisseria* species, into chromosomally mediated penicillin-resistant strains already harboring the necessary determinants to increase resistance to intermediate levels (*16*, *17*).

Takahata et al. (*18*) have reported that three mutations in mosaic variants of PBP 2, G545S, I312M, and V316T, are responsible for decreased susceptibility to cefixime. However, when we incorporated these mutations into a wild-type *penA* gene and transformed the gene into FA19, resistance to penicillin, ceftriaxone, and cefixime increased only marginally (≤3-fold), putting the original conclusion in doubt (see Results). Therefore, we set out to identify important regions of PBP 2, and amino acids within them, that confer resistance to penicillin and expanded-spectrum cephalosporins. Our data indicate that more complex and subtle mechanisms are at play; that is, mosaic PBP 2 variants display epistasis, in which the three residues identified by Takahata et al. (*18*) are important in decreasing the rate of acylation, but only in the presence of other residues that have little to no apparent effect on their own. We also find that if an A501V mutation, which has been observed recently in nonmosaic *penA* alleles (*19*−*21*), were to emerge in mosaic *penA* alleles, the MICs of ceftriaxone and cefixime would increase to above the established break points of resistance.

MATERIALS AND METHODS

Strains and Plasmids. The strains and plasmids used in this study are listed in Table 1. FA19 is a penicillin- and cephalosporin-susceptible strain that served as the recipient strain for most of the studies described herein (*22*). In the experiments examining the effect of the A501V mutation, FA6140, a penicillin-resistant, cephalosporin-susceptible strain (*23*) that contains all of the known resistance determinants (i.e., *penA*, *mtrR*, *penB*, and *ponA*) as well as the unknown determinant, also served as a recipient strain. pUC18us-*penA* and pUC18us-*penA35* contain the wild-type *penA* gene from FA19 and the mosaic *penA* gene from ceph[I] strain 35/02 (*24*), respectively, along with 300 bp of downstream sequence and an uptake sequence to facilitate homologous recombination. To construct the different chimeric PBPs, silent restriction sites were introduced into the coding sequences of pUC18us-*penA* and pUC18us-*penA35* by the QuikChange method (Stratagene, Carlsbad, CA). These sites allowed us to swap out individual cassettes from *penA35* with the corresponding cassettes from *penA* to create the "-mod" constructs (Figure 1 and Figure 1 of the Supporting Information). Other point mutations were introduced into the appropriate constructs by overlap extension polymerase chain reaction (PCR) (*25*). All constructs were verified by sequencing before we proceeded with transformation experiments.

Transformation. Transformation of the chimeric and mutant constructs into FA19 and FA6140 was accomplished as described previously (*8*). Transformants were selected on GCB plates containing various amounts of antibiotics just above their respective MICs. To verify correct recombination, transformants

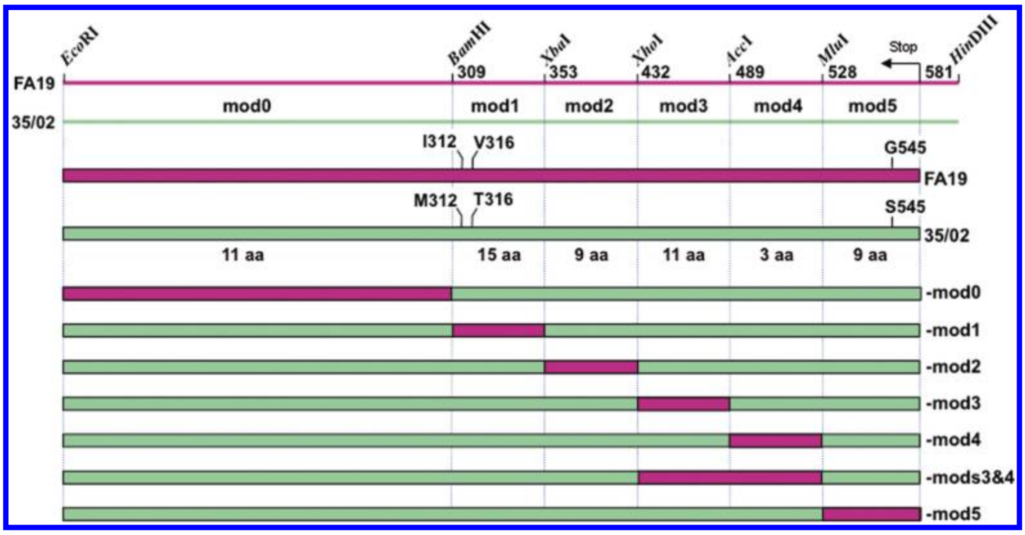

FIGURE 1: Chimeric *penA* genes used in this study. Silent restriction sites were incorporated into the coding sequences of the *penA* genes from FA19 (blue) and 35/02 (green). The modules, designated mod0−mod5, were used to create chimeric *penA* genes in which modules from *penA35* were replaced with the corresponding modules from wild-type *penA*. These chimeric constructs were then used to create the strains listed in Table 1. The number of amino acid alterations in *penA35* relative to *penA* for each module is shown below 35/02. The lines represent DNA, and the rectangles represent the proteins encoded.

were passaged on GCB plates, and the next day, colonies were boiled in 30 μL of water and spun briefly to pellet cell debris and the supernatants were used as templates in PCR with the appropriate *penA* primers. PCR products were sequenced by the University of North Carolina sequencing facility or by Eton Bioscience Inc. (Research Triangle Park, NC).

MIC Measurements. The MICs for penicillin, ceftriaxone, and cefixime were determined exactly as described previously (*16*). The antibiotics were tested in ~1.5-fold increments to increase the accuracy of the MIC determination. At least two (and often up to four) colonies from each transformation were tested, each verified by PCR amplification and sequencing as described above. At least three independent MIC experiments were conducted, and the MICs reported represent the averages of all experiments. Error bars indicate the standard deviation of the determinations.

Purification of PBP 2 Variants. PBP 2, PBP $2^{35/02}$, and PBP $2^{35/02}$-A501V were purified as described previously (*26*). Briefly, the genes encoding each variant were cloned into the derivative of pMAL-C2 (New England Biolabs, Beverly, MA), pMAL-C2KV, which fuses His_6-maltose binding protein and an intervening tobacco etch virus (TEV) protease site to amino acid 44 of PBP 2, and the proteins were expressed in *Escherichia coli*. The fusion proteins were purified on a Ni^{2+}-NTA column (GE Healthcare, Piscataway, NJ) and cleaved with His_6-tagged tobacco etch virus (TEV) protease, and the digests were rechromatographed over a Ni^{2+}-NTA column. The purified proteins were eluted in buffer containing 15 mM imidazole, while uncleaved protein, His_6-TEV, and His_6-maltose binding protein were eluted with 250 mM imidazole. The proteins were dialyzed to remove imidazole, concentrated to ~6 mg/mL, and frozen at −80 °C.

k_2/K_s Measurements of the Rate of Acylation by β-Lactam Antibiotics. The reaction of β-lactam antibiotics with PBP 2 is denoted by the following equation:

$$E + S \overset{K_s}{\leftrightarrow} E{\cdot}S \xrightarrow{k_2} E{-}S' \xrightarrow{k_3} E + P$$

where E·S is the noncovalent enzyme−antibiotic complex, E−S′ is the acyl−enzyme complex, and P is the hydrolyzed antibiotic. k_2/K_s constants, which are a direct measure of the ability of an antibiotic to inhibit a PBP (*27*), were calculated from first-order rates of acylation of purified, soluble PBP 2 variants by [^{14}C]penicillin G (Moravek, Brea, CA) as previously described (*26*, *28*). Graphs of the level of formation of the PBP−2-[^{14}C]penicillin G complex versus time were obtained by incubation of 27 μg of protein with 1.0 μM [^{14}C]penicillin G; aliquots of ~4 μg were removed at 15 s intervals, precipitated with 5% trichloroacetic acid, and filtered over Whatman GC-A filters, and the filters were submitted to scintillation counting. The concentration of [^{14}C]penicillin G was increased to 25 and 50 μM for determination of k_2/K_s values with PBP $2^{35/02}$ and PBP $2^{35/02}$-A501V, respectively. The k_2/K_s values of nonradioactive cephalosporin antibiotics were obtained in competition experiments with [^{14}C]penicillin G using the following equation:

$$(k_2/K_s)_{\text{ceph}} = (k_2/K_s)_{\text{penG}}\left(\frac{[\text{penG}]}{[\text{ceph}]_{0.5}}\right)$$

where [penG] is the concentration of [^{14}C]penicillin G used in the reaction and $[\text{ceph}]_{0.5}$ is the concentration of cephalosporin antibiotic that inhibits the binding of [^{14}C]penicillin G by 50% (*27*).

RESULTS

Analysis of Mosaic PBP 2 Mutations I312M, V316T, and G545S in ceph^I Resistance following Incorporation into a Wild-Type penA Background. Takahata et al. (*18*) reported that three amino acid mutations found in mosaic PBP 2 variants, I312M, V316T, and G545S, are responsible for decreased susceptibility to cefixime in *N. gonorrhoeae*. To confirm these data, we introduced a single mutation (G545S), two mutations (I312M/V316T), or all three mutations (G545S/I312M/V316T) into the wild-type *penA* gene from FA19 and used these plasmids to transform FA19 to increased penicillin G or cefixime resistance. No transformants could be isolated with either the single or double mutant, presumably because they did not confer an increase in resistance. However, we were able to select for transformants harboring the triple mutant, but the MICs of

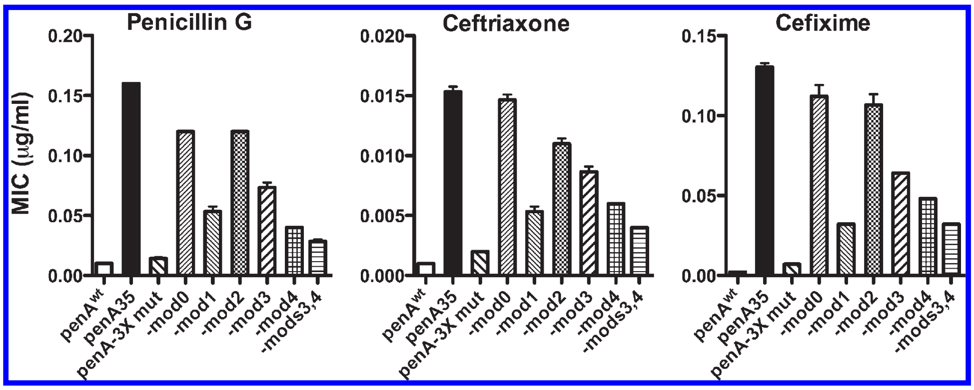

FIGURE 2: MICs of penicillin, ceftriaxone, and cefixime for FA19 harboring triple mutant and chimeric -mod *penA* genes. The MICs of penicillin, ceftriaxone, and cefixime for FA19 transformants containing the indicated triple mutant (*penA*-I312M/V316T/G545S) and chimeric (-mod) *penA* genes (see Figure 1 and Table 1) were determined as described in Materials and Methods. The MICs represent the averages for at least two transformants in a minimum of three independent experiments, and error bars represent the standard deviation of the values.

penicillin, ceftriaxone, and cefixime for these transformants increased by only 1.5-, 1.5-, and 3.5-fold, respectively, compared to 12-, 20-, and 100-fold increases, respectively, with the full *penA35* gene (*16*) (Figure 2). Thus, it appears that together these three mutations confer only minimal increases in resistance to these antibiotics when incorporated into the *penA* gene from FA19.

Construction and Analysis of PBP 2 Chimeras. These data indicated that the mechanisms leading to the emergence of ceph^{I} strains are more complex than originally reported, and thus, we constructed chimeras between wild-type and mosaic *penA* genes to identify the regions and amino acids that are important in decreasing the rate of acylation by the three antibiotics. As shown in Figure 1, six regions in *penA35* bounded by silent restriction sites, termed "modules" and denoted mod0−mod5, were replaced with their corresponding modules from *penA*, and vice versa, and these constructs were used to transform FA19 to increased cefixime or penicillin resistance. Each module contained between 3 and 15 amino acid changes, with mod0 comprising the entire N-terminal domain of PBP 2 and mods 1−5 covering the C-terminal transpeptidase/β-lactam-binding domain (Figure 1 and Figure S1 of the Supporting Information). No transformants could be selected when individual *penA35* modules were transferred into wild-type *penA* in an attempt to show gain of function, indicating that no single region contained changes that were capable of conferring increased resistance to the antibiotics.

In contrast, we were able to select for transformants of FA19 with *penA35* constructs in which individual mosaic *penA35* modules were replaced with their wild-type counterpart in *penA* (Figure 2). In all cases, the *penA35*/*penA* chimeras resulted in lower levels of resistance relative to *penA35*, and except for a few instances, the MICs of the three antibiotics decreased similarly for each chimera. For the sake of clarity, the different chimeric constructs are defined in terms of the fold decrease in MIC compared to that conferred by *penA35*.

Replacing module 0 or 2 of *penA35* with the wild-type module had little to no effect on the MICs of the three antibiotics, while replacing module 1, 3, or 4 of *penA35* with the corresponding wild-type module decreased MICs 2−3-fold compared to that of *penA35* (Figure 2). Replacing module 1 or 4 of *penA35* resulted in a greater decrease in the MICs than replacement of module 3, whereas replacement of modules 3 and 4 together decreased the MICs of the three antibiotics slightly more than replacement of the individual modules. Despite repeated attempts, we could not select transformants in which module 5 of *penA35* was replaced by the wild-type module, most likely because this chimera did not increase the MIC of FA19 above that conferred by wild-type *penA*. These data indicate that mutations within module 5 are the most critical for decreased susceptibility, whereas mutations in modules 1 and 4 are important but less so than those in module 5.

Role of the G545S Mutation and the I312M/V316T Double Mutations in ceph^{I} Resistance. Importantly, modules 1 and 5 contain the I312M/V316T double mutation and the G545S mutation, respectively, initially described by Takahata et al. (*18*). To examine the role of the G545S mutation in resistance, we incorporated this mutation back into the -mod5 chimera (i.e., -mod5 + G545S). Unlike the parent -mod5 construct, which did not confer resistance over wild-type levels, the resulting transformants were only slightly less resistant to penicillin and ceftriaxone than those containing the unmodified *penA35* gene (Figure 3), demonstrating the importance of this mutation. Resistance to cefixime, however, was nearly 3-fold lower, indicating that the G545S mutation is less important for cefixime resistance and that one or more additional mutations within module 5 appear to be required to reach the level of *penA35*. Overall, these data indicated that the G545S mutation is the most important mutation within mod5 for increasing resistance, but the degree to which the mutation is responsible for resistance depended on the antibiotic being examined.

To examine the role of the I312M and V316T mutations in resistance, we replaced module 1 within the -mod5 + G545S chimera with the wild-type sequence of *penA* (-m1,5 + G545S). The MICs of all three antibiotics for the resulting transformants decreased by ~2-fold compared to that of -mod5 + G545S (Figure 3). Incorporation of the I312M/V316T double mutation back into this construct (i.e., -m1,5 + I312M/V316T/G545S) restored the MICs of ceftriaxone and cefixime in these transformants to the same level as that of -mod5 + G545S, whereas the MIC of penicillin did not change. These data suggest that all of the decrease in the MICs of ceftriaxone and cefixime caused by replacement of module 1 of *penA35* with the wild-type sequence was due to the I312M/V316T double mutation, while the decrease in the penicillin MIC was due to other residue(s) within the module.

Reversion of the G545S Mutation and the I312M/V316T Double Mutation in Mosaic penA35. Reversion of the G545S mutation in *penA35* back to the wild-type residue decreased the MICs of penicillin, ceftriaxone, and cefixime for the resulting transformants 2-, ~6-, and 8-fold, respectively, compared to that of *penA35*, whereas reversion of the I312M/V316T double

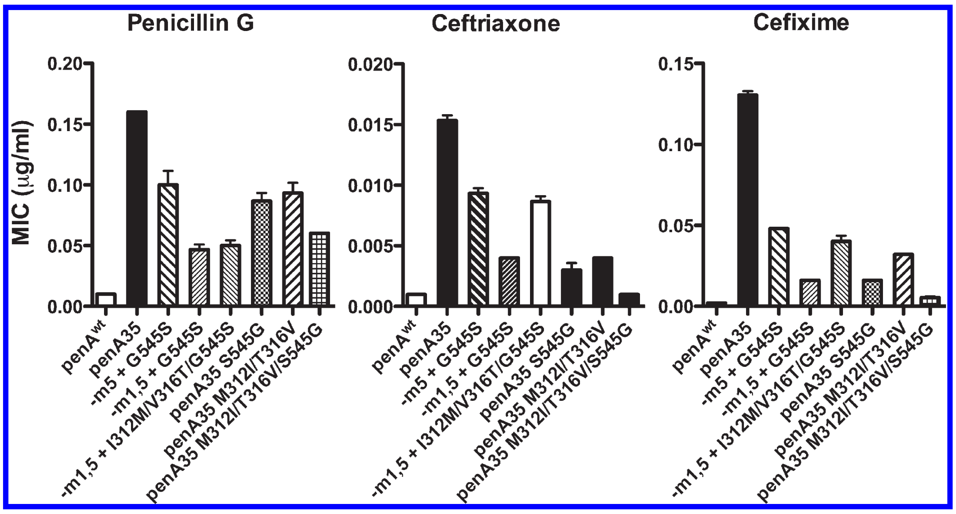

FIGURE 3: G545S and I312M/V316T mutations are critical for conferring resistance to penicillin and expanded-spectrum cephalosporins only when present in the *penA35* background. The contributions of the G545S mutation and the I312M/V316T double mutation within their respective modules were probed by either incorporating the mutations in the -mod1, -mod5, and -mod1,5 chimeric constructs or by reverting the mutations back to the wild-type form in the *penA35* gene. The indicated constructs were transformed into FA19, and the MICs of penicillin, ceftriaxone, and cefixime were determined.

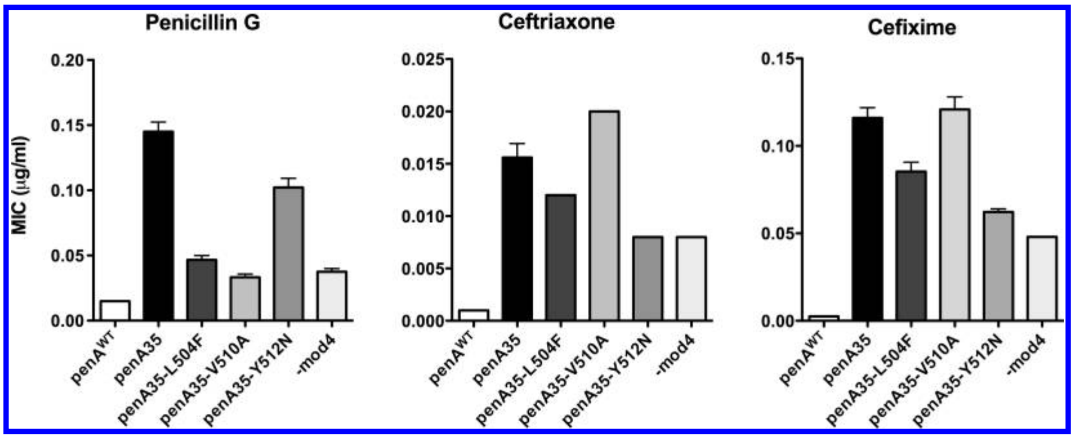

FIGURE 4: MICs of penicillin, ceftriaxone, and cefixime for FA19 harboring *penA35* containing reversion mutations from module 4. FA19 was transformed with the indicated *penA35* alleles in which the three mutations in module 4 (F504L, A510V, and N512Y) were reverted individually back to the wild-type residues. The MICs of penicillin G, ceftriaxone, and cefixime of the resulting transformants were determined as described in Materials and Methods.

mutation to the wild-type residues decreased the MICs of penicillin, ceftriaxone, and cefixime by 2-, 4-, and 4-fold, respectively. When all three mutations were reverted to the wild-type residues, the MICs of penicillin, ceftriaxone, and cefixime were decreased by 2.5-, 16-, and 25-fold, respectively, relative to that of *penA35*. Importantly, the MICs of ceftriaxone and cefixime for the triple reversion mutant were nearly identical to that of FA19. These data are consistent with the idea that the three mutations are important in resistance, but only in the context of some or all of the other 55 mutations found in mosaic *penA* alleles.

Role of Module 4 Amino Acids in Antibiotic Resistance. Replacement of module 4 from *penA35* with the wild-type sequence resulted in an ~3-fold decrease in the MIC of the three antibiotics when the chimera was transformed into FA19 (Figure 2). This result was intriguing, because module 4 has only three amino acid changes, F504L, A510V, and N512Y, the first two of which are also found in the nonmosaic *penA4* allele from FA6140 (*26*), a high-level, penicillin-resistant but cephalosporin-susceptible strain (*8*, *26*). These three mutations are clustered on the $\beta3-\beta4$ loop of PBP 2, which is just C-terminal to the KTG active site motif. We therefore reverted the three changes individually in *penA35* back to the wild-type sequence and determined the MICs of penicillin, ceftriaxone, and cefixime of the resulting transformants.

Surprisingly, the individual mutations had different effects on the MIC depending on the antibiotic being examined. For the expanded-spectrum cephalosporins, the most important reversion was Y512N, which was responsible for most if not all of the decrease in the MICs observed in strains containing *penA35* -mod4. For penicillin, the Y512N mutation had a minor effect on resistance; instead, both the L504F and V510A reversions decreased resistance to the same level as that of the -mod4 construct (Figure 4). These data highlight the importance of the $\beta3-\beta4$ loop for the reactivity of β-lactam antibiotics toward PBP 2 and demonstrate the differential effects of these mutations on the different antibiotics.

Effects of the A501V Mutation in PBP $2^{35/02}$. We also examined the effects of the A501V mutation in both mosaic and nonmosaic *penA* genes by incorporating this mutation into the *penA4* and *penA35* genes and transforming these genes into FA19 and FA6140 (Figure 5). When transformed into FA19, the *penA4*-A501V and *penA35*-A501V mutant alleles decreased the

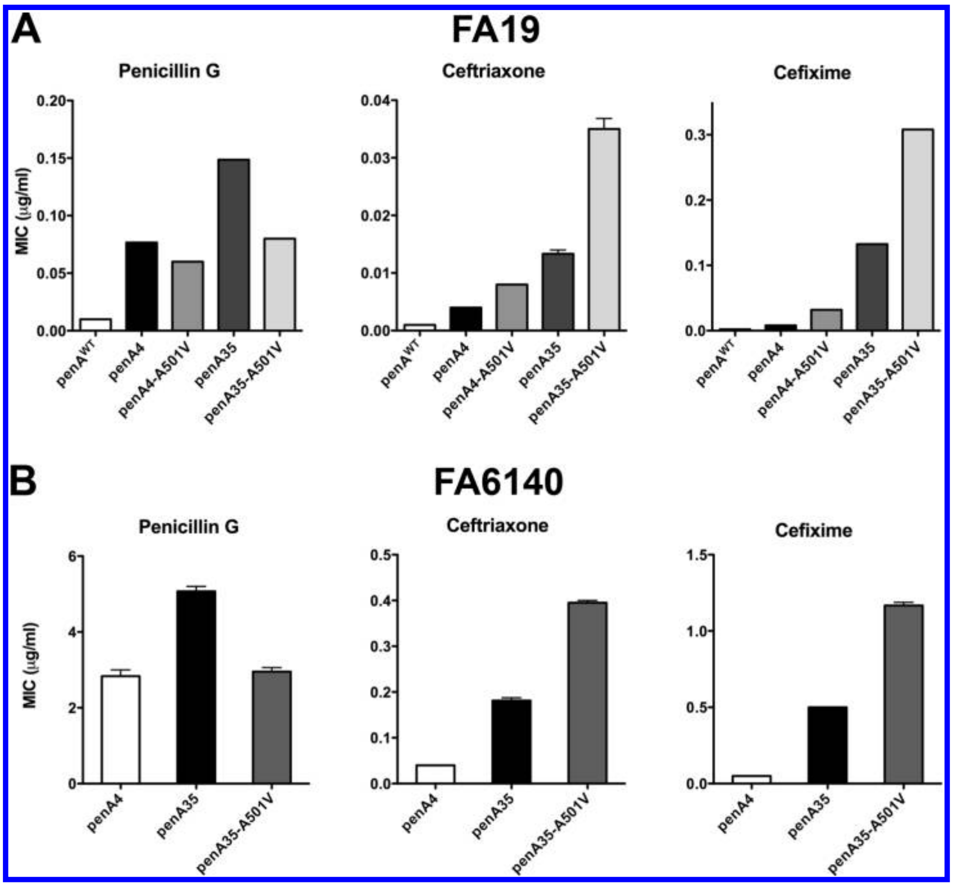

FIGURE 5: MICs of penicillin, ceftriaxone, and cefixime for FA19 and FA6140 harboring the *penA4* (from FA6140) or *penA35* (from 35/02) allele with or without an A501V mutation. FA19 (A) or FA6140 (B) was transformed with *penA4*, *penA4*-A501V, *penA35*, or *penA35*-A501V, and the MICs of penicillin, ceftriaxone, and cefixime for the resulting transformants were assessed as described in Materials and Methods.

MIC of penicillin by 20 and 50%, respectively, compared to levels conferred by the parental alleles (Figure 5A). In contrast, *penA4*-A501V and *penA35*-A501V both increased ceftriaxone and cefixime MICs between 2- and 4-fold versus those conferred by *penA4* and *penA35*, respectively. In general, the effects of the A501V mutation were greater in the *penA35* background than in the *penA4* background.

Consistent with our results in FA19, when the *penA35*-A501V gene was transformed into FA6140, the MIC of penicillin for FA6140 *penA35*-A501V was nearly half of that for FA6140 *penA35* (Figure 5B), whereas the MICs of both ceftriaxone and cefixime increased by more than 2-fold. Importantly, the MICs of ceftriaxone and cefixime for the resulting strains, 0.4 and 1.2 μg/mL, respectively, are well above the break points for "resistance" (>0.25 μg/mL for both antibiotics). These data suggest that the emergence of this mutation in mosaic *penA* genes, which to date has not occurred, could render both ceftriaxone and cefixime ineffective for treating gonococcal infections.

k_2/K_s Constants of Wild-Type PBP 2, PBP $2^{35/02}$, and PBP $2^{35/02}$-A501V. To complement our MIC data, we determined the acylation rate constants of penicillin, ceftriaxone, and cefixime for purified wild-type PBP 2, PBP $2^{35/02}$, and PBP $2^{35/02}$-A501V. For wild-type PBP 2, [^{14}C]penicillin G had a k_2/K_s of 76000 $M^{-1}\,s^{-1}$, whereas ceftriaxone and cefixime displayed k_2/K_s constants that were markedly higher [1.7×10^6 and 1.5×10^6 $M^{-1}\,s^{-1}$, respectively (Table 2)], consistent with their low MICs for wild-type gonococcal strains. The acylation rates of penicillin G, ceftriaxone, and cefixime for PBP $2^{35/02}$ decreased 150−200-fold compared to that of the wild type, underscoring the marked effect of the multiple substitutions in mosaic PBP 2 on the kinetics of β-lactam binding. The acylation rate of penicillin for PBP $2^{35/02}$-A501V increased ~2.7-fold compared to that for PBP $2^{35/02}$, while the k_2/K_s of cefixime decreased by 2.3-fold (Table 2). Surprisingly, the rate for ceftriaxone increased 1.8-fold, the opposite of what we expected on the basis of MIC experiments. Except for this latter result, these data are consistent with the changes observed in strains transformed with the mosaic *penA* allele.

DISCUSSION

Takahata et al. (*18*) reported that three amino acid mutations found in mosaic PBP 2 variants, I312M, V316T, and G545S, are responsible for decreased susceptibility to cefixime in *N. gonorrhoeae*. In that study, the authors determined the MICs of a range of expanded-spectrum cephalosporins for transformants of FA1090 harboring *penA*^{FA1090} alleles containing either the G545S mutation alone or the G545S mutation together with either the I312M or V316T mutation (a strain containing all three mutations could not be isolated). The MICs of ceftriaxone and cefixime increased 2-fold for the G545S transformant and 4- and 8-fold for the G545S/I312M and G545S/V316T double transformants, respectively. From these data, the authors concluded that these three mutations were responsible for the decreased MICs conferred by mosaic *penA* alleles.

However, when we incorporated the three mutations into the wild-type *penA* allele from FA19 and transformed it into FA19, the MICs of ceftriaxone and cefixime increased by only 1.5- and 3.5-fold, respectively, well below the levels obtained with the full

Table 2: k_2/K_s Acylation Rates for Wild-Type PBP 2, PBP $2^{35/02}$, and PBP $2^{35/02}$-A501V[a]

	k_2/K_s (M^{-1} s^{-1})		
PBP 2 protein	penicillin	ceftriaxone	cefixime
wild type	75700 ± 2300 (7)	1710000 ± 90000 (3)	1480000 ± 22000 (3)
35/02	510 ± 90 (12)	11300 ± 400 (3)	7200 ± 300 (4)
35/02-A501V	1400 ± 140 (6)	20000 ± 400 (3)	3100 ± 100 (4)

[a]Proteins were purified and the k_2/K_s values determined as described in Materials and Methods. The values shown are averages ± the standard deviation (number of determinations).

mosaic *penA35* gene (Figure 2). An important difference between our work and the study by Takahata et al. is the choice of the parental *penA* allele. Whereas we used the *penA* gene from FA19, which we consider to be a true "wild-type" allele (*22*), Takahata et al. (*18*) used the *penA* gene from FA1090, which contains an Asp345a insertion that decreases the k_2/K_s acylation rate constant of penicillin G by 6-fold (*26*). Presumably, the increased levels of resistance observed by Takahata et al. were due to the Asp345a insertion adding to or amplifying the effects of the mutations. Because the Asp345a insertion is not present in mosaic *penA* alleles, the *penA* gene from FA1090 is not an optimal background for examination of the effects of these mutations.

While the three mutations (I312M, V316T, and G545S) do not increase resistance markedly when incorporated into a wild-type *penA* gene, they have a striking effect on resistance when reverted back to the wild type in the mosaic *penA35* background, indicating that these mutations require at least some of the other amino acid changes found in 35/02 to decrease susceptibility. This phenomenon, termed epistasis, has been elegantly described by Thornton and colleagues (*29*, *30*), who identified several "permissive" mutations in steroid hormone receptors that, while having no functional importance on their own, stabilized function-switching mutations and facilitated the evolution of new steroid hormone binding activity. Weinreich et al. (*31*), studying the evolution of hydrolytic activity against cefotaxime in TEM β-lactamase, have described a similar epistatic phenomenon. These authors showed that only a few pathways to full resistance are permissible, because some mutations do not increase cefotaxime resistance in certain allelic backgrounds. In an example involving PBPs, Hedge and Spratt (*32*) defined the steps for acquisition of resistance of *E. coli* to a range of cephalosporins through serial mutagenesis of PBP 3 (the functional equivalent of PBP 2 in *N. gonorrheae*). The authors were able to obtain high-level resistance to cephalosporins in four steps. Importantly, two of the steps had little effect on resistance but increased the thermostability of PBP 3 and allowed for subsequent isolation of resistance-conferring mutations (*32*).

To suggest how the three mutations described in this study might impact the rate of acylation, we examined our recent crystal structure of PBP 2 from *N. gonorrhoeae*, determined in the apo form. G545S, which is the most important mutation for resistance in mosaic *penA* alleles, is present at the start of the α11 helix, one of the two helices that pack on top of the five antiparallel β-strands typical of penicillin-interacting proteins. The main chain amides of G545 and G546 are within hydrogen bonding distance of the side chain hydroxyls of Thr498 and Thr500, respectively, located within the KTG(T) active site motif (Figure 6A). By analogy with other PBPs such as *E. coli* PBP 5, the main chain amide of Thr500 is predicted to stabilize the

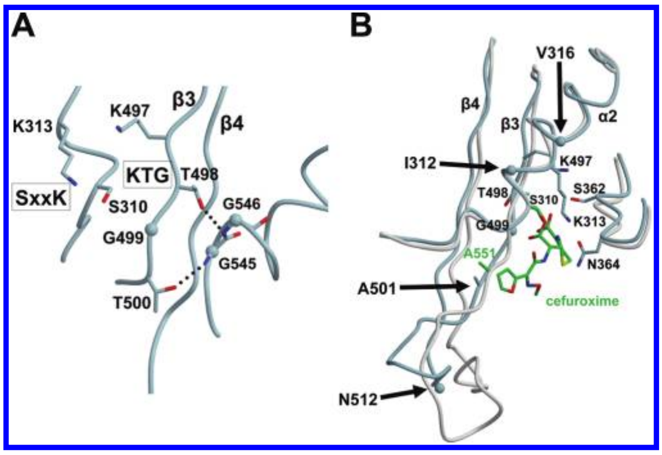

FIGURE 6: Structure of PBP 2 showing the location of important mutations and the active site sequence motifs. (A) Interaction of Thr498 and Thr500 within the KTG(T) active site motif with the main chain amides of Gly545 and Gly546. Mutation of Gly545 to Ser incorporates a hydroxylated side chain that potentially could perturb the interactions with the two Thr residues on β3 with the main chain of helix α11. (B) The crystal structure of apo-PBP 2 from strain CDC84-060418 of *N. gonorrhoeae* (2.1 Å, unpublished data) was superimposed onto that of *S. pneumoniae* PBP 2X in complex with cefuroxime (*34*) using the SUPERPOSE program of CCP4 (*35*). The structure of PBP 2 is from a construct containing the six C-terminal mutations (but missing the Asp345a insertion) of strain CDC84-060418 primarily because its $\beta3-\beta4$ loop is more ordered than other PBP 2 structures. In this calculation, the main chain atoms of 18 residues comprising the three conserved active site motifs superimposed with a root-mean-square difference of 0.97 Å. Note the proximity of the R1 furyl group of cefuroxime to the β3 strand nearest the A501V mutation. The view in this image is from underneath helix α2 containing the active site SxxK motif. Also shown are the locations of the I312M, V316T, and N512Y mutations discussed in the text.

oxyanion hole for the transition state, so one effect of the G545S mutation might be to lower the level of acylation by compromising the geometry of the transition state/tetrahedral intermediate. Alternatively, because the hydroxyl side chains of the residues equivalent to Thr498 and Thr500 in *Streptococcus pneumoniae* PBP 2x (Ser548 and Thr550) interact with the β-lactam carboxylate in the covalent complex (*34*), alteration of these contacts may be another mechanism that lowers the level of acylation. Ile312 and Val316 are located on the opposite side of the helix like Ser310 and Lys313 of the SxxK motif and pack into a hydrophobic pocket (Figure 6B). Mutation to larger (I312M) or more hydrophilic (V316T) side chains might disrupt these interactions such that the position of the SxxK motif is altered, leading to decreases in acylation rates with β-lactam antibiotics. A similar argument has been put forth for the M339F mutation in *S. pneumoniae* PBP 2X (*33*).

In addition to the three mutations discussed above, we also showed that reversion of the N512Y mutation in *penA35* decreases resistance to ceftriaxone and cefixime by 2-fold without affecting penicillin resistance. This result is consistent with its emergence in mosaic *penA* alleles and its relative absence in nonmosaic *penA* alleles found in penicillin-resistant strains (*18*, *19*, *21*). Asn512 is relatively distant from the active site (Figure 6B), and the exact impact of the N512Y mutation on acylation is difficult to predict; however, it is located on the $\beta3-\beta4$ loop that contains mutations that are known to be important for resistance to penicillin in nonmosaic forms of PBP 2 (*26*). One possibility is that such a mutation perturbs the architecture of the KTG motif of β3.

Our work has also highlighted the impending danger of the A501V mutation arising in mosaic *penA* alleles, because if it happens this would increase the MICs of ceftriaxone and cefixime above their break points for resistance. The alteration of A501 in PBP 2 appears to be a gonococcal-specific alteration, as it has not yet been observed in commensal *Neisseria* species, which suggests that it arose through mutation in response to selective pressure with expanded-spectrum cephalosporins instead of by transformation. Indeed, Takahata et al. (*18*) reported the isolation of a spontaneous A501V mutation during transformation experiments. Thus, it may be a matter of when and not if mutations in Ala501 arise in mosaic *penA* genes. Ala501 resides on the $\beta3-\beta4$ loop where mutation of the methyl side chain to the bulkier side chain of valine could clash with the R1 substituent of the cephalosporin (Figure 6B). Consistent with this hypothesis, an A501T mutation, which introduces a similar branched side chain, affects resistance in a manner similar to that of the A501V mutation (M. Unemo, unpublished observations).

The k_2/K_s acylation rate constants of the three antibiotics for wild-type PBP 2, PBP $2^{35/02}$, and PBP $2^{35/02}$-A501V were determined to assess directly the effects of the PBP mutations on reactivity with β-lactam antibiotics. The k_2/K_s rate constants for acylation are largely, but not entirely, consistent with MIC values. For example, acylation rates are dramatically impaired in PBP $2^{35/02}$ compared to that of the wild type, with 150−200-fold decreases in acylation rate for all three antibiotics. However, because the MICs increase 10-, 20-, and 100-fold for penicillin, ceftriaxone, and cefixime, respectively, when *penA35* is transformed into FA19, it is clear that other intrinsic factors, e.g., diffusion through wild-type porin channels, likely play an important role in defining the MIC. One anomaly in the acylation data is the ~2-fold increase in k_2/K_s of ceftriaxone for the PBP $2^{35/02}$-A501V variant, which is inconsistent with the ~2-fold increase in the MIC for transformants harboring this mutant.

In conclusion, the emergence of decreased susceptibility to expanded-spectrum cephalosporins through remodeling of the active site of PBP 2 is more complicated than originally envisioned, with important resistance-conferring mutations showing epistasis. The acquisition of further mutations, such as A501V, in mosaic *penA* alleles is likely to increase resistance to levels that render expanded-spectrum cephalosporins ineffective in treating gonococcal infections. The need to identify new antibiotics against this organism is therefore of prime importance in the treatment of STIs.

SUPPORTING INFORMATION AVAILABLE

Alignment of wild-type PBP 2 with PBP $2^{35/02}$ showing the location of mutations in each module. This material is available free of charge via the Internet at http://pubs.acs.org.

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