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

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*Neisseria gonorrhoeae*

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16 Running Title: Genetics of cephalosporin resistance in *N. gonorrhoeae*

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23 **ABSTRACT**

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

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

45

**INTRODUCTION**

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

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

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

68 a penicillin-susceptible strain (e.g. FA19) via DNA uptake and homologous recombination (10,  
69 12, 30). At least five resistance determinants, which are mutated alleles of normal genes, have  
70 been identified and characterized (16, 23, 25, 28, 31). Each of these determinants incrementally  
71 increases resistance on its own, but it is the cumulative effects of these determinants and their  
72 synergistic interactions that result in clinical levels of resistance. The first step in transfer of  
73 resistance is acquisition of mutated alleles of the *penA* gene, which encodes the penicillin-  
74 binding protein 2 (PBP 2), the primary lethal target of penicillin. The hallmark of these *penA*  
75 alleles is a single codon insertion, Asp345a, and 4-8 mutations clustered in the C-terminus of the  
76 protein, which together lower the rate of acylation of PBP 2 with penicillin G by 16-fold (26).  
77 The second step of transformation results from mutations in the promoter region or coding  
78 sequence of *mtrR*, a transcriptional repressor that regulates expression of the MtrC-MtrD-MtrE  
79 efflux pump (and other genes; (13)). *mtrR* mutations result in overexpression of the efflux pump  
80 and high-level resistance to hydrophobic agents such as detergents and dyes, and small (~2-fold)  
81 increases in the MIC of penicillin (16). The third step in resistance is *penB*, which encodes  
82 mutated alleles of the major outer membrane porin PorB1b with amino acid substitutions in the  
83 constriction loop that presumably decrease the influx of the antibiotic into the periplasm (14, 23).  
84 Interestingly, the increase in resistance conferred by *penB* occurs only in strains harboring a co-  
85 resident *mtrR* mutation, but the molecular basis for this requirement is not completely understood  
86 (24).

87         Although the aforementioned determinants markedly increase the MIC of penicillin G  
88 when transformed into FA19 (from 0.01 to 0.75  $\mu\text{g/ml}$ ), the MIC is still well below the level of  
89 the clinical isolates that serve as DNA donors in transformation experiments (MIC = 4  $\mu\text{g/ml}$ ).  
90 Moreover, DNA from these donors is not capable of further transforming the third-step

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

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

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

## 116 MATERIALS AND METHODS

117 **Strains and plasmids.** The strains and plasmids used in this study are shown in Table 1.  
118 FA19 (a penicillin-and cephalosporin-susceptible laboratory strain) and FA6140 (a high-level  
119 penicillin-resistant isolate) were obtained from Dr. Fred Sparling, University of North Carolina  
120 at Chapel Hill. Strain 35/02 (a penicillin-resistant and cep<sup>h</sup> strain) was isolated in Sweden in  
121 2002 (19). pUC18us-*penA35* contains the *penA* gene from 35/02, along with 300 bp of  
122 downstream sequence and an uptake sequence (11) to facilitate homologous recombination.  
123 Transformants harboring the mosaic *penA* allele were selected with 0.02 µg/ml (FA19) or 0.08  
124 µg/ml (FA6140) cefixime. Transfer of *mtrR* from strain 35/02 was accomplished by selecting  
125 transformants on 1 mg/ml Triton X-100, and *penB35* transformants were selected on 0.37 µg/ml  
126 penicillin G; both of these transformations were accomplished using PCR-amplified fragments.  
127 The *mtrR* gene and promoter from strain 35/02 contains both a G45D mutation in the coding  
128 sequence of MtrR, which disrupts the DNA-binding domain of the MtrR repressor, and a –A  
129 deletion in the 13-bp inverted repeat in the *mtrR* promoter (19). The –A deletion results in  
130 maximal transcription of *mtrCDE*, whereas the G45D mutation, when present by itself, also  
131 increases *mtrCDE* transcription, albeit to a lower level than the promoter mutation (35). pMO-  
132 *porB<sub>1b</sub><sup>FA1090</sup>*, which was used to revert *penB* back to *porB<sub>1b</sub>*, contains a 2 kb fragment of the  
133 *porB<sub>1b</sub>* gene from FA1090 with an erythromycin resistance cassette downstream of the gene to  
134 aid in selection. Plasmids pPR16 and pPR17 harbor the coding regions of the wild-type and  
135 mutant *ponA* genes, respectively, with an extra 546 bp of downstream sequence to facilitate  
136 homologous recombination and the Ω fragment encoding spectinomycin and streptomycin  
137 resistance (27) inserted 68 bp downstream of the *ponA* stop codon to aid in selection (28).

138           **Transformation.** Transformation experiments were carried out essentially as described  
139 by Ropp et al. (28). Briefly, piliated colonies of the recipient strain were passaged on a fresh GC  
140 broth (GCB) agar plate and grown for 16-20 hrs. The cells were swabbed from the plate,  
141 resuspended in GCB with Supplements I and II (18), 20 mM bicarbonate, and 10 mM MgCl<sub>2</sub>,  
142 and diluted to an OD<sub>560</sub> of 0.18. Cells (0.9 ml) were incubated for 5 hrs at 37°C in a CO<sub>2</sub>  
143 incubator with 10 µL of a PCR product or plasmid (~3-5 µg), and aliquots of the cells were  
144 plated on GCB agar plates containing the appropriate antibiotics and allowed to grow overnight.  
145 Transformants were passaged on GCB agar plates, and the following day colonies were boiled in  
146 30 µL of water and spun briefly to pellet debris. Two µL of the supernatants were used as  
147 templates in a PCR reaction, and transformants showing correct recombination were identified  
148 by sequencing.

149           **MIC measurements.** The MICs of penicillin G, ceftriaxone, and cefixime were  
150 determined as described previously (28). Briefly, non-piliated colonies were streaked on a GCB  
151 agar plate and allowed to grow for 18 hr. The cells were swabbed into 2 ml of GCB with  
152 Supplements I and II (16), 20 mM sodium bicarbonate, and 10 mM MgCl<sub>2</sub>, and diluted to an  
153 OD<sub>560</sub> of 0.18. Five µl of cell suspension (~50,000 cells) were spotted on a series of plates  
154 containing the following concentration ranges of antibiotics in approximately 1.5-fold  
155 increments: penicillin G, 0.008-8 µg/ml; ceftriaxone, 0.0004-0.4 µg/ml; and cefixime, 0.0004-0.8  
156 µg/ml. The < 2-fold increases for most concentrations in the series allowed for a more accurate  
157 evaluation of MICs. At least 2 colonies (and often up to 4) from each transformation, verified by  
158 PCR amplification and sequencing, were examined for growth on antibiotic plates. At least three  
159 independent MIC experiments were carried out, and reported MICs represented the average of all  
160 experiments. Error bars in the figures represent the variation in the three determinations.



161

**RESULTS**

162       **The role of the mosaic *penA* allele in cep<sup>I</sup> resistance.** To examine directly the  
163 contribution of the mosaic *penA* allele to intermediate-level resistance to ceftriaxone and  
164 cefixime, we transformed FA19, a  $\beta$ -lactam antibiotic-susceptible strain, and FA6140, a high-  
165 level penicillin-resistant clinical isolate, with the mosaic *penA* gene (*penA35*) from the cep<sup>I</sup>  
166 strain 35/02, which was isolated in Sweden in 2002 (19). Upon recombination of *penA35* into  
167 FA19, the MIC of penicillin G increased from 0.012  $\mu$ g/ml to 0.13  $\mu$ g/ml, a 10-fold increase  
168 (Fig. 1). The MIC of FA19 *penA35* was 2-fold higher than the MIC for FA19 transformed with  
169 the *penA* gene (*penA4*) from FA6140 (MIC = 0.06  $\mu$ g/ml), which harbors the Asp-345a insertion  
170 and 4 C-terminal mutations (24, 26). When the *penA35* allele was transformed into FA6140, the  
171 MIC of penicillin G increased 2-fold, from 3 to 6  $\mu$ g/ml, consistent with the 2-fold difference in  
172 MIC between FA19 *penA4* and FA19 *penA35*. Upon acquisition of *penA35*, the MIC of  
173 ceftriaxone increased 20-fold in FA19 (from 0.0006 to 0.012  $\mu$ g/ml) and 3.5-fold (from 0.06 to  
174 0.21  $\mu$ g/ml) in FA6140. The *penA35* allele conferred even larger increases in the MIC of  
175 cefixime: 100-fold in FA19 (from 0.001 to 0.1  $\mu$ g/ml) and 10-fold in FA6140 (from 0.048 to 0.5  
176  $\mu$ g/ml). Importantly, the MICs of ceftriaxone and cefixime for FA19 *penA35*, although markedly  
177 increased over those for FA19, were still substantially lower than their respective MICs for 35/02  
178 (especially for ceftriaxone), demonstrating that other resistance determinants play a role in cep<sup>I</sup>  
179 resistance.

180       Strain 35/02 has all of the known resistance determinants found in FA6140, i.e. *penA*,  
181 *mtrR*, *penB*, and *ponA1* (19). Although the sequences of their *penA* genes are markedly different,  
182 the sequences of the *mtrR* and *ponA1* determinants in the two strains are 100% identical, and the  
183 *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  $\beta$ -  
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<sup>I</sup> 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<sup>I</sup> 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

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

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

228 **The role of the *ponA1* in ceph<sup>I</sup> resistance.** We also investigated the role of *ponA1*,  
229 which encodes PBP 1 containing a single missense mutation (L421P), in ceph<sup>I</sup> resistance by

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

238 **DISCUSSION**

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

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

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

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

271 Ochiai et al. (22) determined the  $IC_{50}$  values of a range of antibiotics for inhibition of  
272 bocillin FL (a fluorescent  $\beta$ -lactam) binding to purified PBP 2 variants from two antibiotic-  
273 resistant strains, one with a *penA* allele similar to FA6140 and the other with a *penA* allele  
274 similar to 35/02. They reported that the  $IC_{50}$  values of the two PBP 2 variants for ceftriaxone  
275 were not different, inferring that the mosaic *penA* allele plays little to no role in ceftriaxone  
276 intermediate resistance. In contrast, our data show that isogenic replacement of the non-mosaic  
277 *penA* allele in FA6140 with the mosaic *penA35* allele increases the MIC of ceftriaxone 4-fold,  
278 from 0.05 to 0.2  $\mu$ g/ml (Fig. 1). These results clearly indicate that the mosaic *penA35* confers an  
279 increase in the ceftriaxone MIC over and above that conferred by *penA4*. The reasons for this  
280 discrepancy are not clear, but may be due to utilizing inhibition curves to define inhibition  
281 instead of deriving  $k_2/K_S$  acylation constants for the various antibiotics. Although in vitro  
282 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 cep<sup>I</sup>  
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<sub>1b</sub>* 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

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

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

324         The role of *ponA1* in high-level penicillin resistance is well established, as shown by the  
325 2-fold decrease in MIC upon reversion of *ponA1* to *ponA*<sup>wt</sup> (Fig. 4 and (28)). However, no such  
326 decrease in MIC was observed for ceftriaxone or cefixime, indicating that *ponA1* plays little to  
327 no role in resistance for these two antibiotics. This distinction may reflect the differences in the  
328 rates of acylation of the two essential PBPs, PBP 1 and PBP 2, by the three antibiotics. For the



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

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

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

362

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- 476



477 TABLE 1. Bacterial strains used in this study

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

Strains	Penicillin G		Ceftriaxone		Cefixime	
	Par/wtPIB	Par/mtrD	Par/wtPIB	Par/mtrD	Par/wtPIB	Par/mtrD
<b>FA6140</b>	2.3	15.7	4.4	10.7	2.3	4.5
<b>FA6140 <i>penA35</i></b>	2.6	16.0	3.6	9.6	2.2	2.9
<b>35/02</b>	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

484 TABLE 3. Fold increases in ceftriaxone and cefixime resistance. The MICs of ceftriaxone and  
 485 cefixime for FA19 (an antibiotic susceptible strain), FA19 *penA35* (increase in resistance due  
 486 solely to the *penA35* allele), and FA6140 *penA35* (increase in resistance due to both *penA35* and  
 487 other resistance determinants) are shown. <sup>#</sup>Fold increase was determined by dividing the MIC for  
 488 the indicated strain by the MIC for FA19.  
 489

Strain	Ceftriaxone		Cefixime	
	MIC (µg/ml)	Fold increase <sup>#</sup>	MIC (µg/ml)	Fold Increase <sup>#</sup>
<b>FA19</b>	0.0006	---	0.0012	---
<b>FA19 <i>penA35</i></b>	0.012	20	0.12	100
<b>FA6140 <i>penA35</i></b>	0.24	20	0.5	4.2
<b>FA19 → FA6140 <i>penA35</i></b>	---	400	---	420

490

491 **Figure Legends**

492

493 FIG. 1. MICs of penicillin G, ceftriaxone, and cefixime for FA19 and FA6140 transformed with  
494 the mosaic *penA35* gene from strain 35/02. The *penA35* allele from the cep<sup>I</sup> strain 35/02 was  
495 transformed into FA19 or FA6140 and MICs of penicillin G, ceftriaxone, and cefixime for the  
496 resulting strains were determined as described in Materials and Methods. The MIC values  
497 represent the average  $\pm$  standard deviation for at least 2 transformants in a minimum of 3  
498 independent experiments.

499

500 FIG. 2. MICs of penicillin G, ceftriaxone, and cefixime for stepwise transformants of *penA35*,  
501 *mtrR*, *penB35*, and *ponA1* in FA19. To create these strains, FA19 was transformed in a stepwise  
502 manner using PCR amplification products or plasmids of the indicated resistance determinants  
503 from the cep<sup>I</sup> strain 35/02. MICs of penicillin G, ceftriaxone, and cefixime for the resulting  
504 strains were determined as described in Materials and Methods and represent the average  $\pm$   
505 standard deviation for at least 2 transformants in a minimum of 3 independent experiments.

506

507 FIG. 3. Effects of deletion of the MtrC-MtrD-MtrE efflux pump or reversion of *penB* to wild-  
508 type in FA6140, FA6140 *penA35*, and 35/02 on the MICs of penicillin G, ceftriaxone, and  
509 cefixime. To investigate the individual contributions of *mtrR* and *penB* to cep<sup>I</sup> resistance,  
510 FA6140, FA6140 *penA35*, and 35/02 were transformed with plasmids harboring *mtrD*  
511 insertionally inactivated with a kanamycin resistance cassette or *porB<sub>1b</sub>* with a downstream  
512 erythromycin resistance cassette, and the MICs of the resulting transformants were determined as

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

515

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

522









