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*

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Chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae* occurs in part through alterations in penicillin-binding proteins (PBPs) and a decrease in outer membrane permeability. However, the genetic and molecular mechanisms of transformation of a penicillin-susceptible strain of *N. gonorrhoeae* to high-level penicillin resistance have not been clearly elucidated. Previous studies suggested that alterations in PBP 1 were involved in high-level penicillin resistance. In this study, we identified a single amino acid mutation in PBP 1 located 40 amino acids N terminal to the active-site serine residue that was present in all chromosomally mediated resistant *N. gonorrhoeae* (CMRNG) strains for which MICs of penicillin were ≥ 1 μg/ml. PBP 1 harboring this point mutation (PBP 1*) had a three- to fourfold lower rate of acylation (k_2/K') than wild-type PBP 1 with a variety of β-lactam antibiotics. Consistent with its involvement in high-level penicillin resistance, replacement of the altered *ponA* gene (*ponA1*) in several CMRNG strains with the wild-type *ponA* gene resulted in a twofold decrease in the MICs of penicillin. Surprisingly, transformation of an intermediate-level penicillin-resistant strain (PR100; FA19 *penA4 mtr penB5*) with the *ponA1* gene did not increase the MIC of penicillin for this strain. However, we identified an additional resistance locus, termed *penC*, which was required along with *ponA1* to increase penicillin resistance of PR100 to a high level (MIC = 4 μg/ml). The *penC* locus by itself, when present in PR100, increases the MICs of penicillin and tetracycline twofold each. These data indicate that an additional locus, *penC*, is required along with *ponA1* to achieve high-level penicillin resistance.

Up until 1987, *Neisseria gonorrhoeae* was treated with a single dose of penicillin, which kills the bacteria by inhibiting the penicillin-binding proteins, or PBPs, that synthesize the cell wall peptidoglycan. *N. gonorrhoeae* has four PBPs, designated PBP 1, 2, 3, and 4 (1; P. A. Ropp and R. A. Nicholas, unpublished data.). Of these, only PBPs 1 and 2 are essential for cell viability and thus are potential antibiotic killing targets in *N. gonorrhoeae*. Because penicillin G has an approximately 10-fold higher rate of acylation with PBP 2 than with PBP 1, penicillin G kills *N. gonorrhoeae* at its MIC by inactivation of PBP 2 (1).

While early isolates of *N. gonorrhoeae* were extremely sensitive to penicillin (MICs $\cong 0.004$ to 0.01 μg/ml), this sensitivity gradually decreased such that by the late 1970s strains emerged that were resistant to penicillin (MICs ≥ 2 μg/ml). Resistance to other antibiotics, including tetracycline and erythromycin, also increased during this time. Penicillin resistance in the gonococci arose by two independent mechanisms: the plasmid-mediated production of a penicillinase (TEM-1 β-lactamase) and the chromosomally mediated expression of multiple resistance genes. In 1998, 29.4% of the clinical gonococcal isolates collected throughout the United States by the Gonococcal Isolate Surveillance Project overseen by the Centers for Disease Control and Prevention (CDC) were resistant to either penicillin, tetracycline, or both (6). While the percentage of penicillinase-producing *N. gonorrhoeae* organisms (PPNG) declined significantly during the years 1991 to 1998, the percentage of chromosomally mediated resistant *N. gonorrhoeae* organisms (CMRNG) rose.

The genetic mechanisms of chromosomally mediated resistance to penicillin have been investigated in some detail (8, 11). Because *N. gonorrhoeae* is naturally transformable, penicillin-resistant strains are capable of transferring their resistance genes to susceptible strains in a stepwise manner via transformation and homologous recombination (Fig. 1). The first step in transformation to high-level penicillin resistance (MIC ≥ 2 μg/ml) is mediated by the *penA* gene, which encodes altered forms of PBP 2 that display 5- to 10-fold decreases in their rate of acylation by penicillin (28). The second step of transformation is mediated by the *mtr* locus, which confers nonspecific resistance to erythromycin, rifampin, and detergents through increased expression of the MtrC-MtrD-MtrE efflux pump (17). Transfer of the *penB* locus leads to further increases in both penicillin and tetracycline resistance (5). Recently, the *penB* phenotype has been correlated to mutations in the porin P1B allele that presumably decrease the porin-mediated flux of antibiotics across the outer membrane (15).

Unexpectedly, transformation of a *penA mtr penB* strain (for which the MIC of penicillin is 0.5 to 1.0 μg/ml) to a level of penicillin resistance equal to that of the donor strain (MIC = 4 μg/ml) has been difficult to achieve in the laboratory (8, 11).

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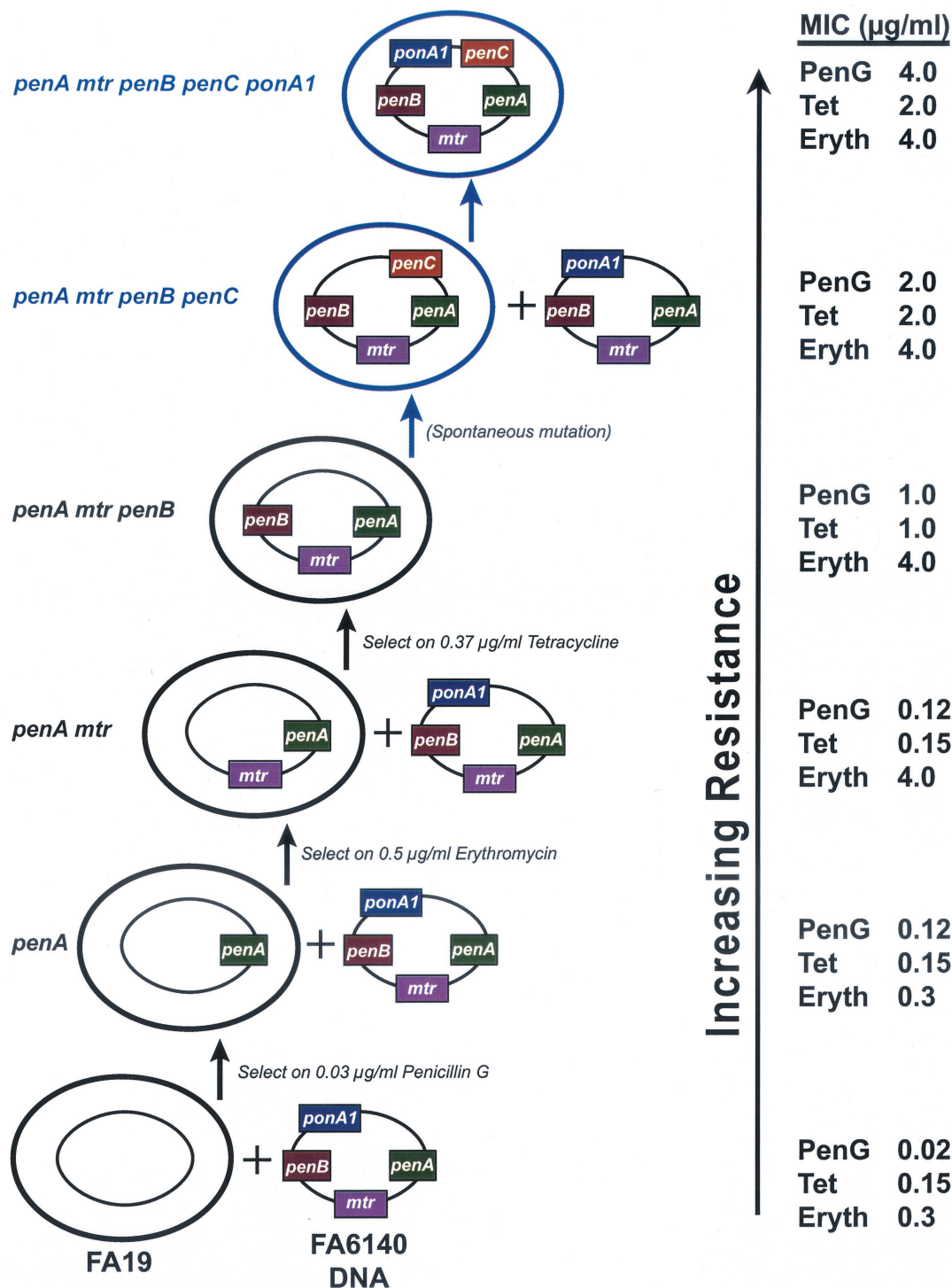
FIG. 1. Stepwise acquisition of resistance genes in gonococci by DNA uptake and homologous recombination. The stepwise transfer of resistance genes occurs in the order shown, and each step increases resistance to at least one of the three antibiotics shown on the right. The first three resistance genes (penA, mtr, and penB) are well characterized, and transformants with these genes are easy to obtain in the laboratory. In contrast, transformation of an FA19 penA mtr penB strain up to a level of resistance equal to that of the donor strain has been very hard to achieve in the laboratory. The last two steps of transformation to high-level resistance (in blue) are mediated by two resistance loci, penC and ponA1, that are the subjects of this study.

Because penicillin-resistant strains for which MICs are ≥ 2 μg/ml appear to express an altered form of PBP 1 that displays a lower rate of acylation by penicillin, the PBP 1 gene likely is involved in transformation to high-level penicillin resistance (7). Dougherty was able to obtain high-level penicillin-resistant transformants at low frequency by using a modified transformation procedure and by selecting transformants at penicillin concentrations two- to fourfold less than the MIC for the

donor strain (7). Assessment of the binding of [^{3}H]penicillin G to membranes prepared from these transformants revealed an apparent heterogeneity in the affinity of PBP 1 for penicillin. One group expressed a PBP 1 with an affinity for penicillin G equal to that of the donor strain, whereas another group expressed a PBP 1 with an intermediate affinity. These results suggested that PBP 1 plays a role in mediating high-level penicillin resistance in CMRNG.

We recently cloned the *ponA* gene encoding PBP 1 from a penicillin-susceptible strain of *N. gonorrhoeae* (24). In the present study, we isolated and sequenced the *ponA* gene from several clinical isolates of *N. gonorrhoeae* for which penicillin MICs were ≥ 1 µg/ml in order to determine the changes in the amino acid sequence that presumably lead to a decreased rate of acylation by penicillin. In addition, we investigated the role of the altered *ponA* gene in mediating transformation of intermediate-level penicillin-resistant transformants to high-level penicillin resistance. Our results indicate that both the *ponA1* gene encoding an altered form of PBP 1 and another resistance locus, termed *penC*, are required to achieve high-level chromosomally mediated penicillin resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. Clinical isolates of *N. gonorrhoeae* for which penicillin G MICs were ≥ 1 µg/ml were obtained from several sources. Strains 2227, 0387, 3391, 5611, and 9634 were kindly provided by Marcia Hobbs and Myron S. Cohen, University of North Carolina at Chapel Hill, from a Wilson county (North Carolina) surveillance program (12). FA19 (21) and FA6140 (for which the penicillin MIC was 4 µg/ml) (5) were obtained from Fred Sparling, University of North Carolina at Chapel Hill. Piliated FA6140 was obtained from William Shafer, Emory University. Strains 111, 114, 131, 151, and 154 were obtained from Joan Knapp at the CDC. Chromosomal DNA from CMRNG strains CDC77-124615 (8) and CDC84-060418 (7) was kindly provided by Brian Spratt, Imperial College, London.

The plasmids pPR16 and pPR17 harbor the coding regions of the wild-type and mutant *ponA* genes, respectively, with an extra 546 bp of downstream sequence to facilitate homologous recombination. To aid in selection, each plasmid also contained the Ω fragment encoding spectinomycin and streptomycin resistance (23), inserted 68 bp downstream of the *ponA* stop codon (see Fig. 2). pMutS-erm, which contained the gonococcal *mutS* gene inactivated by insertion of an erythromycin resistance cassette, was provided by Janne Cannon, University of North Carolina at Chapel Hill.

Media, growth conditions, and MIC determinations. *N. gonorrhoeae* strains were grown on GC medium base (GCB) agar (Difco Laboratories, Detroit, Mich.) with supplements I and II (20) at 37°C in 5% CO_2. Liquid cultures were grown in GCB broth containing supplements I and II, supplement B, and 10 mM $NaHCO_3$ in a shaking incubator at 37°C. MICs were determined according to the method of Sparling et al. (27) or by the spot method. Briefly, cells were resuspended in GCB broth, and 1,000 colonies were spread onto GCB agar plates containing increasing concentrations of the appropriate antibiotic. The MIC was defined as the minimal concentration of antibiotic at which no more than five colonies were observed after 24 h. Alternatively, colonies were suspended in GCB broth and 5×10^4 colonies in 5 µl were spotted onto GCB agar plates containing increasing twofold concentrations of the antibiotic. The MIC was defined as the minimal concentration of antibiotic that inhibited growth after 24 h. The two methods gave very similar results.

General methods. Chromosomal DNA was isolated by ethidium bromide-CsCl equilibrium centrifugation by a modification of the method of Dowson et al. (10). In some instances, chromosomal DNA was isolated with the DNAzol reagent (Gibco/BRL, Gaithersburg, Md.) following the manufacturer's instructions. p-Trimethylstannyl-penicillin V (a gift from Larry Blaszczak; Eli Lilly and Co.) was converted to [^{125}I]iodopenicillin ([^{125}I]IPV) as described previously (2). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, Calif.).

Isolation and sequencing of the *ponA* gene from penicillin-resistant strains. The *ponA1* gene was cloned initially from FA6140. The complete coding region of the *ponA* gene was amplified by PCR from FA6140 DNA with primers GC23

(5'-GTGAGTAACCGTTTCGGTATCC-3'), which hybridizes 124 bp upstream of the ATG start codon, and GC44 (5'-TTAAAACAGGGAATCCAACTGC-3'), an antisense oligonucleotide that hybridizes to the last 22 bp of the *ponA* gene. The amplified product was subcloned into pUC19-K (pUC19 containing the kanamycin resistance gene). Both strands of the plasmid harboring the *ponA* gene as well as the *ponA* PCR product were sequenced with the Amplicycle CS sequencing kit (Perkin-Elmer, Foster City, Calif.). The same sequence was obtained from multiple independent amplifications, verifying that the mutation was not a PCR artifact. Sequences of the *ponA* genes from the other penicillin-resistant strains examined in this study were amplified by PCR and sequenced directly as described above.
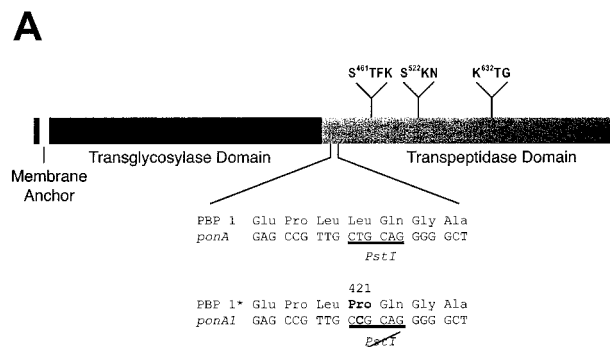
Genetic transformation. *N. gonorrhoeae* was transformed as described previously (25). Briefly, cells were passaged on GCB agar, and a single piliated colony was streaked onto a fresh GCB plate and allowed to grow overnight. Cells were scraped from the plate and gently resuspended in prewarmed GCB broth containing supplements I and II and 10 mM $MgCl_2$. Resuspended cells were diluted in prewarmed GCB broth to a cell density of 10^8/ml (optical density at 560 nm, 0.18), followed by the addition of $NaHCO_3$ to a final concentration of 10 mM. Aliquots (900 µl) of diluted cells were mixed gently with 100 µl of 20-µg/ml donor DNA (either plasmid or chromosomal DNA) and allowed to incubate for 5 h at 37°C in a humidified 5% CO_2 atmosphere. Various amounts of cells were then plated onto GCB agar containing the appropriate antibiotic concentrations (described below) and grown 24 to 48 h at 37°C in 5% CO_2.

PR100 (FA19 *penA4 mtrR penB5*; see Table 3) was constructed by transforming FA19 with FA6140 genomic DNA and selecting for each resistance gene at the following antibiotic concentrations: (i) *penA4*, 0.16 µg of penicillin G per ml; (ii) *mtr*, 0.5 µg of erythromycin per ml; and (iii) *penB5*, 0.37 µg of tetracycline per ml. Each of these genes was amplified by PCR from PR100 genomic DNA and sequenced; all three genes corresponded exactly to the sequences obtained from FA6140. Other transformants were selected on GCB agar containing antibiotic concentrations as indicated. Gonococci transformed with pPR16 or pPR17 plasmid DNA harboring the Ω fragment were selected on GCB agar containing 100 µg of spectinomycin per ml, whereas cells transformed with plasmid constructs containing the erythromycin resistance gene (i.e., for disruption of *mutS*) were selected on 10 µg of erythromycin per ml. Since pUC plasmids are not replicative in *N. gonorrhoeae*, transformation occurred via homologous recombination into the genome. To test for the presence of the *ponA1* mutation in transformation experiments, individual colonies were resuspended in 100 µl of 10 mM Tris-HCl–1 mM EDTA, pH 8.0, placed in a boiling water bath for 10 min, and then centrifuged. The *ponA* gene was then amplified from 1 µl of the boiled lysates with the following primers: 5'-CGCGGTGCGGAAAACTGATATCGAT-3' (bp 955 to 978 of *ponA* open reading frame) and 5'-AGCCCGGATCGGTTACCA TACGTT-3' (bp 2218 to 2195 of *ponA* open reading frame). Aliquots (5 µl) of the amplification reaction mixture were used for subsequent *Pst*I digestion without further purification.

To examine if the increased resistance in *penC* strains was due to an additional mutation(s) in one of the genes comprising the *mtr* operon, we amplified the *mtrR* + *mtrC*, *mtrD*, *mtrE*, and *mtrF* genes from the *mtr* operon by PCR from 1 ng of PR102 DNA and tested the ability of each fragment to transform PR100 to PR100 *penC*. The primers were designed such that each amplified fragment contained at least one uptake sequence. Following amplification, the fragments were purified from an agarose gel and mixed with piliated PR100 cells, and resistant transformants were selected on GCB plates containing 0.95 µg of penicillin per ml. As a positive control, the *mtrR* + *mtrC* fragment (which contains the original *mtr* mutation) was used to transform FA19 *penA4* to FA19 *penA4 mtr* (transformation frequency, $\approx 3 \times 10^{-5}$).

Purification of recombinant PBP 1 and PBP 1*. The coding sequences of the *ponA* genes from both FA19 and FA6140 were amplified by PCR with *Taq* DNA polymerase (Gibco/BRL), cloned into pET15b-K (pET15b containing the kanamycin resistance gene in place of the β-lactamase gene), and transformed into *Escherichia coli* BL21(DE3) as previously described (24). These constructs result in the fusion of 20 additional amino acids, including a hexahistidine tag, to the amino termini of PBP 1 and PBP 1* (PBP 1 containing the Leu-421→Pro mutation).

E. coli BL21(DE3) cells containing PBP 1 expression plasmids were grown in Luria-Bertani medium supplemented with kanamycin (50 µg/ml). Overnight growth resulted in significant expression of PBP 1 in the absence of induction. Cells were pelleted, resuspended in cold lysis buffer (50 mM sodium phosphate buffer [pH 8.0], 10% glycerol, 0.5 mM phenylmethylsulfonyl chloride), and lysed by three passes through an Aminco French pressure cell (Champaign, Ill.) at 16,000 lb/in². Membranes were isolated from the cell lysate by centrifugation at $225,000 \times g$, washed, resuspended, and stored at −20°C. Crude membranes were solubilized with an equal volume of 50 mM sodium phosphate buffer (pH 8.0)–
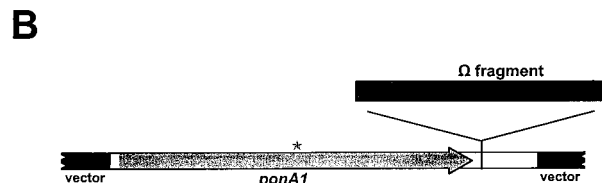
A

B

FIG. 2. Location of the Leu-421→Pro mutation in PBP 1*. (A) The *ponA* gene is shown along with the locations of the two functional domains, the transglycosylase domain and the transpeptidase domain. Locations of the three highly conserved sequence motifs found in all penicillin-interacting proteins are shown above the transpeptidase domain. The location of the Leu-421→Pro mutation is shown by an asterisk. Also shown are the DNA sequences of the *ponA* and *ponA1* genes. The mutation fortuitously disrupts a unique *Pst*I restriction site. (B) Schematic showing the construct (pPR17) used to create the strains containing the *ponA1* gene. The Ω fragment, which encodes resistance to both spectinomycin and streptomycin, was used to identify transformants containing the *ponA1* mutation (see text for details). pPR16 is identical to pPR17, except that it contains the wild-type *ponA* gene.

2 M NaCl–2% Triton X-100–40 mM imidazole for 1 h with stirring at room temperature.

Following centrifugation at 225,000 × *g*, the supernatants containing solubilized PBP 1 or PBP 1* were loaded directly onto Ni²⁺-NTA columns (Qiagen, Chatsworth, Calif.) and washed with 50 mM sodium phosphate (pH 8.0)–1 M NaCl–10% glycerol–0.1% Triton X-100–15 mM imidazole. PBP 1 and PBP 1* were eluted with an increasing linear gradient of imidazole (15 to 500 mM) in the above buffer. Fractions containing PBP 1 or PBP 1* were pooled, concentrated by ultrafiltration, and then dialyzed extensively against 50 mM sodium phosphate (pH 8.0)–0.5 M NaCl–10% glycerol–0.1% Triton X-100 to remove imidazole. The purified proteins were stored at −80°C at a concentration of 2 to 4 mg/ml.

Determination of the kinetic constants for the interaction of β-lactam antibiotics with PBP 1 and PBP 1*. k_2/K' constants of recombinant PBP 1 and PBP 1* for [^{125}I]IPV were determined from time courses of acyl-enzyme formation as described by Frere et al. (14). Briefly, purified proteins were incubated at 30°C with [^{125}I]IPV concentrations ranging from 0.625 to 5 mM in 50 mM sodium phosphate–1 mM EDTA–0.1% Triton X-100 (pH 7.4), and the reaction was stopped by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer at 0.5- to 1-min time intervals. The samples were run on a sodium dodecyl sulfate–8% polyacrylamide gel, and the dried gel was exposed to a phosphorimaging screen for 12 to 24 h. Levels of acyl-enzyme formed were quantitated with ImageQuant software following imaging on a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). The k_2/K' constants was derived from the slope of a plot of the apparent first-order rate constant k_a versus the concentration of [^{125}I]IPV. The k_2/K' values for penicillin, ceftriaxone, and cephaloridine were determined by the competition method as described previously (14).

k_3 values were determined by monitoring the formation of free PBP from samples of PBP-antibiotic complexes. PBPs were incubated with unlabeled antibiotics for 15 min at 30°C, and excess antibiotics were removed by dialysis at 4°C against the same buffer as above with the addition of 10% glycerol. The addition of glycerol was necessary to ensure the stability and activity of PBP 1 proteins during prolonged incubations. Following dialysis, the protein complex was incubated at 30°C, aliquots were removed at various times, and the amount of free PBP was assessed by incubation with a saturating concentration of [^{125}I]IPV. The relative amounts of labeled PBPs were derived by phosphorimager analysis as described above. No significant hydrolysis was observed over 3 days of incubation.

RESULTS

Identification of an altered form of the *ponA* gene from CMRNG strains. Previous studies have shown that high-level penicillin resistance in *N. gonorrhoeae* is correlated with expression of PBP 1 displaying an apparent decrease in its affinity for penicillin (7, 8). These data suggested that one or more mutations were present in the primary sequence of PBP 1 that decreased its rate of acylation with penicillin. To test this hypothesis, we isolated the *ponA* gene from the high-level penicillin-resistant strain FA6140 (5) (penicillin MIC = 4 μg/ml) by PCR amplification of genomic DNA and compared its nucleotide sequence to that of the *ponA* gene from the peni-

cillin-susceptible strain FA19 (24). Sequence analyses of several independent clones isolated from separate amplification reactions identified a single point mutation, a T-to-C transition, at nucleotide 1261 of the *ponA* coding region (hereafter designated *ponA1*) (Fig. 2A). This mutation, which results in the change of Leu-421→Pro, was the only difference observed in the entire 2,400-bp coding region.

To determine if this mutation occurs in the *ponA* gene from other penicillin-resistant strains, the *ponA* genes from 10 geographically and temporally distinct CMRNG isolates for which penicillin MICs were ≥1 μg/ml were amplified and sequenced. Serovars of these strains suggest that these strains were descended from different lineages (Table 1). Nine of these strains harbored the *ponA1* gene containing a point mutation identical to that observed in the *ponA* gene from FA6140 (Table 1). Two of these strains, CDC120177 and FA6140, were isolated in the late 1970s; strains 111, 114, and 131 were isolated in Cincinnati, Ohio, in 1994; and strains 0387, 3391, 5611, and 9634 were isolated in rural North Carolina in 1993 from a gonococcal

TABLE 1. Characteristics of the *N. gonorrhoeae* strains used in this study

Strain[a]	MIC (μg/ml)	Codon-421 in the *ponA* gene	Serovar	Reference or source
FA19	0.016	CTG	A10	21
0387	2	CCG	B3	12
3391	1	CCG	B2	12
5611	1	CCG	B3	12
9634	1	CCG	B2	12
111	4	CCG	B3	CDC
114	4	CCG	B3	CDC
131	4	CCG	B3	CDC
FA6140	4	CCG	B1	5
CDC77-124615	2[b]	CCG	B4	8
CDC84-060418	1[b]	CTG	B2	7

[a] Gonococcal isolates (with the exception of CDC77-124615 and CDC84-060418) were obtained from the indicated sources, and the MICs of penicillin for these strains were determined as described in Materials and Methods. Serovars of CDC77-124615 and CDC84-060418 were determined from the sequences of their porin genes (19).

[b] Only genomic DNA was available from these strains.

TABLE 2. Kinetic constants[a] of PBP 1 and PBP 1* for
interaction with β-lactam antibiotics

Antibiotic	PBP 1		PBP 1*	
	k_2/K' (M^{-1}s^{-1})	k_3 (s^{-1})	k_2/K' (M^{-1}s^{-1})	k_3 (s^{-1})
[^{125}I]IPV	1,171 ± 425	>10^{-5}	293 ± 143	>10^{-5}
Penicillin G	4,036 ± 1,316	>10^{-5}	1,156 ± 200	>10^{-5}
Ceftriaxone	20,649 ± 2,476	>10^{-5}	7,048 ± 1,512	>10^{-5}
Cephaloradine	217 ± 39	>10^{-5}	66 ± 16	>10^{-5}

[a] k_2/K' values of the purified PBPs for [^{125}I]IPV were derived from the slopes of the apparent rate constant, k_a, for formation of the acyl-enzyme complex versus concentration of [^{125}I]IPV, while the k_2/K' values for the other antibiotics were obtained from the competition of [^{125}I]IPV binding as described in Materials and Methods. k_3 values were obtained either by quantitating the loss of radioactivity from the protein over time ([^{125}I]IPV) or by quantitating the gain of [^{125}I]IPV binding over time (all other antibiotics).

surveillance program. As we observed with the *ponA1* gene from FA6140, there were no other differences in the *ponA* genes from these other strains. CDC84-060418, the only strain that did not contain the mutation, is known to be different from the majority of CMRNG strains in that it expresses an altered PBP 2 with an extremely low rate of acylation by penicillin, but with little to no change in the rate of acylation of PBP 1 (7). Thus, with the exception of CDC84-060418, all CMRNG strains for which the MIC of penicillin was ≥1 µg/ml showed the presence of the altered codon in the *ponA* gene.

Interaction of PBP 1 and PBP 1* with β-lactam antibiotics. The T-to-C transition in the *ponA1* gene results in a change of Leu-421→Pro in the PBP 1 amino acid sequence (hereafter referred to as PBP 1*; Fig. 2A). Leu-421 is 40 amino acids to the amino-terminal side of the active-site serine residue (Ser-461) and thus is near (but not necessarily within) the active-site cavity. To assess the functional consequences of the Leu-421→Pro amino acid mutation in PBP 1*, both the wild-type and mutant forms of PBP 1 were expressed in *E. coli* and purified, and the kinetic constants for their interaction with β-lactam antibiotics were determined. β-Lactam antibiotics interact with PBPs according to the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot S \overset{k_2}{\rightarrow} E - S' \overset{k_3}{\rightarrow} E + P$$
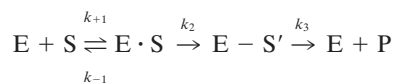
where E is the enzyme, S is a β-lactam antibiotic, and P is the inactive degradation product (13, 22). The effectiveness of an antibiotic is defined by two parameters: (i) the second-order specificity constant, k_2/K', where $K' = k_{-1}/k_{+1}$; and (ii) the rate constant for hydrolysis of the acyl-enzyme complex, k_3. The Leu-421→Pro mutation results in a fourfold decrease in the k_2/K' constant for [^{125}I]IPV and approximately three- to fourfold decreases in the k_2/K' constants for penicillin G, ceftriaxone, and cephaloridine (Table 2). No changes in the rates of hydrolysis of the acyl-enzyme complex were observed, indicating that resistance arises from a lower rate of acylation by β-lactam antibiotics and not by an increase in the rate of deacylation.

Role of the *ponA1* gene in transformation of an intermediate-level resistant strain to high-level penicillin resistance. The identification of a mutation in PBP 1 that decreases its rate of acylation with β-lactam antibiotics suggests that the *ponA1* gene is involved in conferring high-level penicillin resistance. To determine the role of PBP 1* in resistance, we first produced an intermediate-level penicillin-resistant strain (PR100; FA19 *penA4 mtr penB5*) as described in Materials and Methods. PCR amplification and sequencing of the *penA4*, *mtr*, and *penB5* genes from PR100 verified that these genes were identical to those in FA6140 (data not shown). For PR100, both penicillin and tetracycline have a MIC of 1.0 µg/ml, and this strain also shows increased resistance (relative to FA19) to both ceftriaxone and cephaloridine (Table 3).

PR100 was transformed with the plasmid pPR17, which contains the entire *ponA1* gene and 546 bp of the 3′ flanking sequence with the Ω fragment (encoding spectinomycin resistance) (23) inserted 68 bp downstream of the *ponA1* stop codon (Fig. 2B). The transformed cells were plated on GCB plates containing either 2 µg of penicillin per ml or 100 µg of spectinomycin per ml. No colonies grew on the GCB-penicillin plates, even though spectinomycin-resistant colonies were iso-

TABLE 3. Derivation of and MICs for strains of *N. gonorrhoeae* used in this study

Strain	Description	Recipient strain	Donor DNA	Selection agent (concn, µg/ml)	MIC[a] (µg/ml)				
					PenG	Tet	Erm	Ceftr	Ceph
FA19	Wild-type, antibiotic-susceptible isolate				0.016	0.15	0.5	0.0006	2
FA19 *ponA1*	FA19 *ponA1*∞Ω[b]	FA19	pPR17	Spectinomycin (100)	0.016	0.15	0.5	ND	ND
FA19 *penA4*	First-level penicillin-resistant transformant	FA19	FA6140	PenG (0.016)	0.128	0.15	ND	ND	ND
FA19 *penA4 ponA1*	FA19 *penA4 ponA1*	FA19 *penA4*	pPR17	Spectinomycin (100)	0.128	0.15	ND	ND	ND
PR100	FA19 *penA4 mtrR penB5*	FA19	FA6140	–[c]	1.0	1.0	4.0	0.01	8
PR101	PR100 *ponA1*∞Ω	PR100	pPR17	Spectinomycin (100)	1.0	1.0	4.0	ND	ND
PR102	PR100 *penC*	PR100		PenG (0.95)	2.0	2.0	4.0	0.04	12
PR103	PR100 *mutS::erm*[d]	PR100	pMutS-erm	Erythromycin (10)	ND	ND	ND	ND	ND
PR105	PR100 *penC ponA1*∞Ω	PR102	pPR17	Spectinomycin (100)	4.0	2.0	4.0	0.04	24
FA6140	High-level penicillin-resistant clinical isolate				4.0	4.0	4.0	0.08	24
FA6140 *ponA*-wt	FA6140 *ponA*^{WT}∞Ω	FA6140	pPR16	Spectinomycin (100)	2.0	4.0	ND	ND	ND

[a] PenG, penicillin G; Tet, tetracycline; Erm, erythromycin; Ceftr, ceftriaxone; Ceph, cephaloridine; ND, not determined.
[b] The linked spectinomycin-streptomycin resistance gene (Ω) is located 40 bp downstream of the *ponA1* stop codon (Fig. 2B).
[c] Construction of PR100 is described in Materials and Methods.
[d] The *mutS* gene was disrupted by insertion of the erythromycin resistance gene.

lated at a frequency of $\approx 10^{-5}$. We screened the spectinomycin-resistant transformants for the successful recombination of the linked *ponA1* mutation by PCR amplification of the *ponA* gene and subsequent digestion of the PCR products with *Pst*I. Fortuitously, the *ponA1* mutation destroys a unique *Pst*I site within the *ponA* gene, and thus loss of the *Pst*I site in the amplified fragments is diagnostic for the presence of *ponA1* (Fig. 2A). Approximately half of the spectinomycin-resistant colonies had incorporated the *ponA1* mutation (data not shown). Surprisingly, there was no increase in the MIC of penicillin for spectinomycin-resistant transformants containing the *ponA1* mutation (PR101; Table 3). Thus, acquisition of the *ponA1* gene by an intermediate-level penicillin-resistant strain does not increase the MIC of penicillin to the level seen with FA6140.

To determine whether PBP 1* is involved in high-level penicillin resistance at all, we replaced the *ponA1* gene in FA6140 with the wild-type *ponA* gene and determined the MIC of penicillin for the resulting isogenic strain. FA6140 was transformed with pPR16, a plasmid containing the wild-type *ponA* gene and the linked Ω fragment, and spectinomycin-resistant colonies were screened for the presence of the wild-type gene by *Pst*I digestion of the amplified *ponA* gene. For several such transformants, designated FA6140 *ponA*-wt, consistent twofold decreases in the MIC of penicillin (2 μg/ml; Table 3) were observed, indicating that the *ponA1* gene is involved in high-level penicillin resistance in FA6140. The MIC of tetracycline was unchanged. Identical results were obtained with the CMRNG strains 111, 114, and 131.

Identification of *penC*, a previously uncharacterized resistance gene. In our attempts to obtain penicillin-resistant colonies following transformation of PR100 with either pPR17 plasmid DNA or FA6140 chromosomal DNA, we observed the growth of a few (5 to 25) colonies following plating on GCB plates containing 0.95 μg of penicillin per ml. For all of these colonies, twofold increases in the MICs of both penicillin (2.0 μg/ml) and tetracycline (2.0 μg/ml) and similar increases in the MICs of ceftriaxone and cephaloridine (Table 3) were observed. PCR amplification of the *ponA* gene and digestion with *Pst*I showed that none of these colonies had recombined the *ponA1* gene (data not shown), which was consistent with our previous data showing that the *ponA1* gene does not increase the MIC of penicillin when transformed into PR100. Moreover, we were able to isolate penicillin- and tetracycline-resistant colonies for which the MICs showed twofold increases simply by plating PR100 (FA19 *penA mtr penB*) on GCB-penicillin or GCB-tetracycline plates (i.e., in the absence of DNA transformation). The increase in MICs observed with these colonies (designated PR102; Table 3) was stable to repeated passages on GCB agar over several days with no antibiotics present, strongly suggesting that resistance was due to a genetic event and not to an induction of an enzyme in response to antibiotic challenge.

These data suggest that the twofold increase in penicillin and tetracycline resistance is due to a low-frequency, spontaneous mutation within the population of PR100. If true, then plating PR100 containing a disrupted *mutS* gene (which increases mutation frequency) on selective media should result in a large increase in the numbers of penicillin- and tetracycline-resistant colonies. Consistent with this hypothesis, plating

PR103 (PR100 *mutS*) on selective media produced colonies at a frequency 17-fold higher on average than for PR100 ($n = 3$; data not shown). Moreover, the MICs of both penicillin and tetracycline for all of the resistant colonies derived from PR103 increased twofold. This experiment provides additional evidence that the increased resistance is due to a spontaneously arising genetic mutation in an as yet unidentified locus. This locus has been designated *penC*.

Repeated attempts to transform PR100 to PR100 *penC* with DNA from the CMRNG strains FA6140, 114, and 131 were unsuccessful. However, we were able to transform *penC* into PR100 with chromosomal DNA isolated from PR102 (PR100 *penC*; Table 3) at a frequency of $\approx 10^{-5}$. (While this transformation frequency is lower than that obtained when *penA4* is transformed into FA19 [$\approx 3 \times 10^{-4}$], similar transformation frequencies were obtained when PR100 was transformed with either plasmid or chromosomal DNA containing different resistance markers. These results suggest that the lower transformation frequency of PR100 is a characteristic of the strain and is not related to the *penC* locus.) The inability to transform the *penC* locus into PR100 from FA6140, 114, and 131 indicates that these CMRNG strains do not contain the *penC* locus and suggests that a novel mechanism or another as yet unidentified mutation is involved in high-level penicillin resistance in these strains. However, given that *penC* can arise spontaneously, we suspect that the *penC* locus will be found in other CMRNG strains when a more extensive search is conducted.

Since both *ponA1* and *penC* are required to mediate high-level penicillin resistance in FA19, we attempted to identify a similar resistance locus in FA6140 by transforming PR101 (PR100 *ponA1*) with FA6140 DNA to high-level penicillin resistance. However, these attempts were unsuccessful, suggesting that in FA6140 an additional resistance locus that is not easily transformable or a novel genetic mechanism is involved in high-level resistance.

The *penC* locus does not encode additional mutations in previously characterized resistance genes. We also considered the possibility that additional mutations in the three known resistance loci (*penA*, *mtr*, and *penB*) might underlie the increased resistance observed in *penC* strains. To test this possibility, we sequenced the *penA* and *penB* genes from both PR100 and PR102 (PR100 *penC*). No differences were noted in either gene obtained from the two strains. To determine if mutation(s) in one or more of the genes encoding the Mtr efflux pump might be responsible for the increased resistance observed in *penC* strains, we amplified the *mtrR* + *mtrC*, *mtrD*, *mtrE*, and *mtrF* genes from PR102 by PCR and used these fragments in an attempt to transform PR100 into PR100 *penC*. None of the fragments gave rise to penicillin- or tetracycline-resistant colonies at a frequency higher than that of a no-DNA control, while DNA from PR102 transformed PR100 into PR102 with a frequency of $\approx 10^{-5}$. These data are consistent with the idea that *penC* is a novel resistance locus and not an already-characterized resistance gene that has acquired an additional mutation.

Transformation of a *penC* strain to high-level penicillin resistance with *ponA1*. Even though acquisition of the *ponA1* gene by PR100 by itself did not increase penicillin resistance, acquisition of *penC* may be required before the *ponA1* gene can increase penicillin resistance. To test this hypothesis, we

transformed the *penC* strain PR102 with pPR17 and screened spectinomycin-resistant transformants for the presence of the linked *ponA1* gene as described above. For the resulting transformants, designated PR105, the MIC of penicillin was 4 μg/ml, the same as for FA6140 (Table 3). The MIC of cephaloridine increased following acquisition of *ponA1*, whereas no increases were observed for ceftriaxone and tetracycline. We were unable to transform PR102 to higher penicillin resistance with FA6140 chromosomal DNA or pPR17 plasmid DNA by selecting on GCB-penicillin plates, but this may simply reflect the difficulty in selecting transformants having such a small increase in penicillin resistance (7). These data demonstrate that both *ponA1* and *penC* are necessary to transform an intermediate-level resistant recipient strain to high-level penicillin resistance. Moreover, the *penC* locus must already be present in order for *ponA1* to increase resistance. Thus, increasing the penicillin resistance of FA19 to a high level (MIC = 4 μg/ml) requires mutations in five genes or loci (*penA*, *mtr*, *penB*, *penC*, and *ponA1*), including both essential PBPs (Fig. 1).

DISCUSSION

The mechanism by which chromosomally mediated penicillin-resistant strains arise in *N. gonorrhoeae* is different from that utilized by most other bacteria. Susceptible strains of *N. gonorrhoeae* become resistant to penicillin by acquiring multiple resistance genes in a stepwise fashion. As each gene is acquired, penicillin resistance increases until treatment failure occurs. While the genes involved in transforming *N. gonorrhoeae* to an intermediate level of penicillin resistance are well established, those that mediate high-level resistance have not been identified. In this study, we show that two loci, the *ponA1* gene encoding an altered form of PBP 1 and a newly identified locus, *penC*, are required to transform an intermediate-level penicillin-resistant strain to high-level resistance.

The *ponA1* gene encodes PBP 1 containing a single amino acid mutation, Leu-421→Pro, which decreases the rate of acylation with β-lactam antibiotics three- to fourfold compared to the wild type. An identical mutation was observed in the *ponA* genes from penicillin-resistant strains isolated 17 years apart, from geographically distinct regions, and with different serovars. Transformation of an intermediate-level penicillin-resistant strain with the *ponA1* gene increased penicillin resistance only when an additional locus, *penC*, was already present. These data demonstrate that a penicillin-susceptible strain must acquire five resistance genes, *penA*, *mtr*, *penB*, *penC*, and *ponA1*, to reach the level of resistance of a high-level, chromosomally mediated penicillin-resistant strain such as FA6140.

The mechanism of the emergence and propagation of the *ponA1* mutation in resistant strains of *N. gonorrhoeae* is in marked contrast to that described for the *penA* gene encoding PBP 2. *PenA* is the first resistance gene in the stepwise transfer of resistance genes from a resistant donor strain to a susceptible recipient strain (11). Analysis of the *penA* genes from penicillin-resistant strains of *N. gonorrhoeae* has shown that the coding sequences contain blocks of DNA with high sequence divergence from *penA* genes of susceptible strains (28). Further studies showed that in the closely related *Neisseria* species

N. meningitidis, the divergent blocks of DNA in altered *penA* genes arose by horizontal transfer of resistant *penA* genes from commensal strains such as *N. flavescens* or *N. cinerea* (3, 29, 30). These alterations lead to multiple amino acid changes in PBP 2, with the most important change being an amino acid insertion (Asp-345a) that lowers the rate of acylation of PBP 2 with penicillin four- to fivefold (4, 26).

In contrast to the high sequence divergence observed in altered forms of PBP 2, alteration of PBP 1 is due to a single base change in the *ponA* gene that results in mutation of Leu-421 to proline. We have sequenced the *ponA* genes from at least 10 different CMRNG isolates, and the only difference within the entire 2,400 bp of coding sequence is at codon 421. These isolates include one of the original CMRNG isolates from 1977, CDC77-124615, and resistant strains isolated nearly 20 years later in both Cincinnati and North Carolina. This mutation evidently arose in *N. gonorrhoeae*, since the *ponA* genes from several *Neisseria* commensal species show significant sequence divergence from the *ponA* gene of *N. gonorrhoeae*, with overall DNA sequence identities of 88% (*N. lactamica*; accession no. AF085689), 85% (*N. cinerea*; AF085340), and 73% (*N. flavescens*; AF087677) (Ropp and Nicholas, unpublished). Although it is possible that the mutation arose in *N. meningitidis*, whose *ponA* gene is 100% identical to its gonococcal homologue in the region of the mutation (24), it seems unlikely since high-level penicillin resistance in this species has not yet been noted.

Substitution of Leu-421 with proline causes a three- to fourfold decrease in the acylation rate of the protein when assessed with a variety of antibiotics. One of the most unusual aspects of this mutation is that the alteration is located on the amino-terminal side of the active-site serine residue. In other PBPs whose acylation rates have been altered by amino acid mutations, these mutations occur on the C-terminal side of the active-site serine residue. For example, mutations in altered forms of PBP 2 are located near two conserved active-site sequence motifs, the SXN triad and the KTG triad (28), and a similar location of alterations is observed in the PBPs from penicillin-resistant strains of *Streptococcus pneumoniae* (9, 16, 18). Because no high-resolution crystal structure of a class A PBP is available, it is not possible at this time to understand at a molecular level how this mutation causes a decrease in the rate of acylation. Although it is possible that Leu-421 is near the active site, mutation to proline may simply impart a structural perturbation (due to the structural effects of proline) that is propagated from a distance and disturbs the active-site architecture. However, in the absence of structural information for PBP 1 or one of its homologues, the mechanism by which the mutation decreases acylation remains highly speculative. Because the fold decrease in k_2/K' for each of the antibiotics tested were very similar, i.e., approximately three- to fourfold, it appears that the structural alteration is general in nature and not specific to penicillin. However, a more extensive analysis with additional antibiotics is necessary to confirm this hypothesis.

The original reports detailing the stepwise transformation of susceptible strains of *N. gonorrhoeae* by donor DNA from high-level penicillin-resistant strains noted that transformation of recipient strains to a level of penicillin resistance equivalent to that of the donor strain could not be achieved (8, 11). How-

ever, Dougherty was able to isolate transformants at low frequency for which the MICs of penicillin were equal to those observed with the donor strain by using high concentrations of DNA and a modified transformation protocol and by selecting at penicillin concentrations fourfold below the MIC (7). Given the low frequency of transformation, Dougherty concluded that possibly two genes were involved in high-level penicillin resistance. Our study confirms and extends this hypothesis. We have shown that acquisition of *ponA1* increases the MIC only when the *penC* mutation is present. It is probable that Dougherty also isolated colonies containing spontaneous mutations in *penC* in his experiments, since well over half of the transformants isolated were cross resistant to tetracycline, even though no selection for tetracycline was carried out (7).

Despite multiple attempts, we could not obtain colonies containing the *penC* locus (with frequencies greater than those of no-DNA controls) by transforming PR100 with donor DNA from several high-level penicillin-resistant strains. Our inability to obtain *penC* transformants was not due to *penC* being a nontransformable locus, since we were able to transform PR100 to PR100 *penC* with PR102 DNA at reasonably high frequencies (i.e., $\approx 10^{-5}$). This result indicates that the *penC* mutation is not present in the CMRNG isolates that we tested and suggests that a novel mechanism or perhaps a different genetic background is responsible for resistance in these strains. It is interesting that the penicillin MIC for FA6140 *ponA*-wt is 2 µg/ml, twofold higher than that of FA19 containing the first three resistance genes from FA6140 (PR100; FA19 *penA4 mtr penB5*). The fact that the MIC for FA6140 *ponA*-wt is higher than for PR100 implies that the novel mechanism or genetic background involved in high-level penicillin resistance in FA6140 increases the MIC of penicillin above that which can be accounted for by the *penA4*, *mtr*, and *penB5* genes. This scenario is reminiscent of the effects of the *penC* locus.

Introduction of the *ponA1* gene into FA19, FA19 *penA4*, and FA19 *penA4 mtr penB5* (PR100) had no effect on the MIC of penicillin (Table 3), indicating that penicillin kills these strains by inhibition of PBP 2. These data also show that PBP 2 remains the killing target even after its rate of acylation is markedly reduced by alterations in its coding sequence (i.e., *penA2*). However, PBP 1* clearly has a role in clinically relevant high-level penicillin resistance, since replacing the *ponA1* gene with the wild-type gene in FA6140 (Table 3) and three other CMRNG strains resulted in twofold decreases in the penicillin MICs for these strains. The fact that introduction of the *ponA1* gene into PR102 (PR100 *penC*) increases the MIC of penicillin indicates that the *penC* gene must in some way decrease the rate of acylation of PBP 2 such that PBP 1 becomes the killing target. A similar argument can be made for CMRNG strains such as FA6140, although the modifying gene(s) in these strains is not *penC* but some other as yet unknown gene or genetic mechanism. It is tempting to speculate that in both cases the membrane surface is altered in such a way that the rate of acylation of PBP 2 within the cell division complex is decreased below that of PBP 1, allowing PBP 1* to increase the MIC of penicillin.

In conclusion, this study is the first to show unequivocally that PBP 1 is involved in penicillin resistance. However, transformation experiments demonstrate that a decrease in the rate of acylation of PBP 1 is still not sufficient to impart high-level

penicillin resistance (MIC = 2 to 4 µg/ml) to an intermediate-level resistant strain, implicating the involvement of an additional resistance locus in the transformation of gonococci to high-level resistance. In our studies, that additional locus is *penC*; however, in several clinical isolates, the identity of this locus is not *penC* but an as yet unidentified locus. Studies are in progress to identify the *penC* locus and to define the mechanism by which it increases both penicillin and tetracycline resistance.

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