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Genetic Analysis and Penicillin-Binding Protein Alterations in *Neisseria gonorrhoeae* with Chromosomally Mediated Resistance

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Eight recent isolates of *Neisseria gonorrhoeae* with high-level non-β-lactamase-mediated penicillin resistance were investigated. The penicillin-binding proteins of these strains were found to have reduced affinity for radiolabeled penicillin. Testing for known resistance genes showed that these were present in the resistant isolates. Genetic transformation was used to construct strains with increasing levels of antibiotic resistance. Modification of the transformation protocol made it possible to isolate transformants at the highest (penicillin-resistant DNA donor) level of resistance. These transformants unexpectedly yielded two distinct penicillin-binding protein patterns when tested.

In recent years there have been increasing reports of outbreaks of non-penicillinase-producing, resistant strains of *Neisseria gonorrhoeae*. These strains, which have been termed chromosomally mediated resistance (CMR) strains of *N. gonorrhoeae*, are often not recognized in routine surveillance (5). Several groups have documented the increased incidence of these isolates (5, 7, 11, 12). A major outbreak in North Carolina in 1983 has been attributed to a single strain which spread through the population (5). Alarmingly, 15 of 16 patients initially treated with the recommended penicillin regimen experienced treatment failures in this outbreak. In another report, 15% of isolates in a survey were classified as having chromosomally mediated resistance to penicillin (7). In contrast to the North Carolina epidemic, these strains were found to belong to different auxotype or serovar classes.

In the present report, several recently isolated CMR *N. gonorrhoeae* strains obtained from the Centers for Disease Control (CDC) were characterized for possible antibiotic resistance mechanisms. As was the case in an earlier study with a highly resistant gonococcal strain (1, 3), alterations in the affinity of the penicillin-binding proteins (PBPs) were found in these recent isolates. Genetic transformation was used to determine whether previously characterized resistance loci were present. In addition, it was possible under certain conditions to transform susceptible gonococci in several steps to the resistance level of the CMR isolates. These high-level transformants had PBPs with reduced affinity for penicillin.

MATERIALS AND METHODS

Strains and culture conditions. The penicillin-susceptible strain FA19, the highly resistant strain CDC 77-124615, and intermediately resistant genetic transformants have all been described previously (3, 4). The CMR *N. gonorrhoeae* strains, all isolated in 1984, were obtained from J. Biddle, CDC, Atlanta, Ga. These strains (Table 1) were isolated in several geographic locations. The North Carolina strains were found as part of surveillance following the 1983 outbreak. Their antibiotic susceptibility patterns are listed in Table 1. All were β-lactamase negative with nitrocefin (Glaxo Research Ltd., Greenford, Middlesex, England). Strains were maintained by daily passage on GCBA agar plates (BBL Microbiology Systems, Cockeysville, Md.) supplemented with IsoVitaLex (BBL Microbiology Systems) and incubated at 36°C in 5% CO_2. Liquid cultures were grown in gonococcal broth with IsoVitaLex and $NaHCO_3$ (420 μg/ml) (3). Growth was monitored by absorbance measurement in a Sequoia-Turner model 340 spectrophotometer at 610 nm.

PBP assay. PBPs were assayed by incubating logarithmic-phase gonococci with [^{3}H]penicillin (24.4 Ci/mmol; Merck Institute, Rahway, N.J.). Cells were chilled, collected by centrifugation, and prepared for electrophoresis as described previously (3). Polyacrylamide gel electrophoresis and fluorography were used for PBP separation and detection (3).

Genetic transformation. Chromosomal DNA was extracted with chloroform and purified by ethanol precipitation (2). Saturating concentrations (2 μg) of DNA were incubated with 1 ml of piliated gonococci suspended in gonococcal broth, IsoVitaLex, and 4 mM $MgCl_2$. After 10 min of incubation, 10 μg of DNase was added, and the cells were diluted and mixed with 5 ml of warm (45°C) GCBA agar base overlay. After 6 h of incubation for expression, a 7-ml agar overlay with sufficient antibiotic to give the indicated final concentration of antibiotic per 30 ml of agar was added.

To successfully transform gonococci to the highest level of resistance (i.e., CMR *N. gonorrhoeae* donor DNA level), several modifications of the transformation protocol were necessary. The DNA was prepared by lysing gonococci in proteinase K-sodium dodecyl sulfate, followed by phenol extraction. The DNA was recovered by ethanol precipitation (10). Transformation of the intermediate-level transformant (*penA mtr penB*) to the highest level was accomplished with phenol-extracted DNA in the presence of 2 mM $CaCl_2$ (17). A key to obtaining high-level transformants was to select resistant cells only at penicillin concentrations fourfold below the MIC. Transformants that were subsequently shown to have penicillin MICs of 4.0 μg/ml could not be initially selected if the penicillin concentration exceeded 1 μg/ml. Typically, 10^7 CFU yielded only between 30 and 200

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TABLE 1. Properties of CMR gonococci

Strain[a]	Site of original isolation	MIC (μg/ml)[b]							
		Penicillin	Erythromycin	Rifampin	Cefotaxime	Piperacillin	Cefoxitin	Aztreonam	Tetracycline
FA19	Lab strain	0.01	0.30	0.035	0.0018	0.015	0.3	0.5	0.15
84-060-383	North Carolina	2.0	0.60	0.07	0.03	1.0	1.2	8.0	2.0
84-060-384	North Carolina	4.0	2.50	0.30	0.06	2.0	2.5	8.0	4.0
84-060-385	North Carolina	4.0	2.50	0.60	0.5	4.0	5.0	8.0	4.0
84-060-388	North Carolina	4.0	2.50	1.20	0.5	4.0	5.0	8.0	4.0
84-060-393	New Mexico	4.0	2.50	1.20	0.25	2.0	5.0	4.0	4.0
84-060-394	New Mexico	4.0	2.50	1.20	0.25	4.0	2.5	8.0	4.0
84-060-409	North Carolina	4.0	2.50	0.60	0.12	2.0	5.0	4.0	2.0
84-060-418	Massachusetts	1.0	0.60	0.07	0.06	1.0	1.2	4.0	2.0
77-124615	Lab strain	2.0	1.25	0.30	0.12	2.0	2.5	4.0	2.0

[a] Except for the FA19 penicillin-susceptible strain and the previously characterized 77-124615 (3,4), all isolates were obtained from CDC.
[b] MICs were determined by agar dilution as described previously (3).

transformants, which was below the efficiencies normally observed in gonococcal transformation (15, 16). Pretreatment of DNA with 10 μg of DNase for 5 min reduced the transformant count essentially to zero.

RESULTS

PBPs. To date, studies on the recent CMR *N. gonorrhoeae* strains have concentrated primarily on epidemiology, and very little has been reported on possible mechanisms of resistance. To test the possibility that reduced affinity for penicillin of the target proteins contributes to the observed resistance, an in vivo PBP assay was used (Fig. 1). Seven of the eight isolates had PBP profiles that were identical to those previously seen in earlier resistant isolates (1, 3). One strain isolated in Boston, Mass., CDC 84-060-418, had a PBP pattern not observed previously in the few resistant strains examined by this technique. In this case, very low PBP 2 affinity and an intermediate binding (ca. 60% of strain FA19 PBP 1 binding) of [^{3}H]penicillin to PBP 1 was found. Comparison of the β-lactam MICs (Table 1) showed the considerable degree of cross resistance that the PBP alterations engendered.

Genetic analysis. Characterization of the strains for the genes previously reported to be associated with antibiotic resistance in gonococci (8, 9, 13, 16, 17) was performed. Four of the isolates were selected for this analysis, two of which (84-060-383 and 84-060-418) had somewhat low resistance levels (Table 1). Chromosomal DNA was prepared from all four isolates, and standard genetic transformations with strain FA19 as recipient were carried out. The results (Table 2) indicated that *penA*, which results in increased resistance to β-lactam antibiotics, was present in all the isolates tested. Likewise, it was possible to introduce in stepwise fashion *mtr*, a gene involved with nonspecific resistance to antibiotics and detergents, by selecting for erythromycin resistance. Subsequently, *penB*, which increases penicillin and tetracycline resistance, was introduced by selecting for an additional fourfold increase in the MIC of penicillin and testing the transformants for tetracycline resistance (3, 6).

The ability of the two more susceptible strains to donate the *mtr* locus was confirmed by transformation to erythromycin resistance (transformation efficiency, 0.4%). Ten of the resulting transformant colonies were selected and tested for increased cross resistance to rifampin and Triton X-100 (15, 16). All the transformants tested exhibited levels of resistance to both agents that were somewhat higher than those of the parental DNA donors. This suggests that the two relatively susceptible parent strains contained *mtr* and that the locus may be phenotypically suppressed in these strains.

Transformation to high-level resistance. Past efforts to transform gonococci to a resistance level equivalent to that of the highly resistant donor strain have not been successful (3, 6). Modification of the transformation protocol by using phenol-extracted DNA and Ca^{2+} as the divalent cation permitted transformation of strain FA19 (*penA mtr penB*) to high-level penicillin resistance. Two donor strains, 84-060-385 and 84-060-388, were used as the DNA sources. Transformation frequencies were considerably below that observed for the *penA* locus (ca. 0.1 to 0.5% of *penA* frequency). To select for resistant transformants, it was essential to use penicillin concentrations of 1.0 μg/ml, although the resultant transformants had MICs two- to fourfold higher.

Transformants were tested by stabbing the colonies with a sterile wire and placing the cells on fresh GCBA medium. After growth, the independent transformants were subjected to penicillin susceptibility testing. Thirty transformants (16 from one experiment, 14 from a second transformation) were tested. All had an MIC of penicillin of 1.0 μg/ml or greater, and 27 of 30 had an MIC of 2.0 μg/ml or greater; 17 of 30 had an MIC of 4.0 μg/ml for penicillin. A test of tetracycline resistance indicated that resistance to this unselected marker

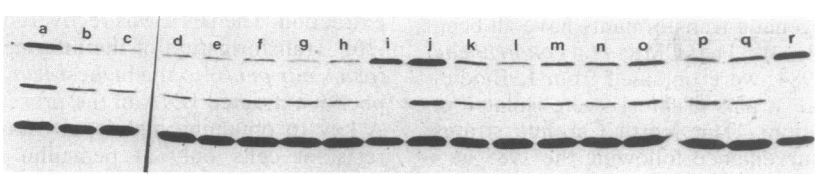

FIG. 1. Titration of resistant gonococcal strains with ^{3}H-labeled benzylpenicillin. Lanes: a and j, FA19 (penicillin susceptible); b and k, 84-060-383; c and l, 84-060-384; d and m, 84-060-385; e and n, 84-060-388; f and o, 84-060-393; g and p, 84-060-394; h and q, 84-060-409; i and r, 84-060-418. Samples were incubated with 0.5 (lanes a through i) or 5.0 (lanes j through r) μg of [^{3}H]penicillin per ml. As previously designated (3), PBPs are numbered from top (PBP 1) to bottom (PBP 3), in order of decreasing molecular weight.

had also increased in 18 of 30 of the transformants, from 2.0 to 4.0 μg/ml.

The PBP profile of the high-level penicillin-resistant transformants was determined. The results indicated an unexpected heterogeneity of PBP patterns (Fig. 2). Two classes of PBP 1 changes were observed. In one set, PBP 1 affinity was very low and resembled that of the parental DNA donor (Fig. 2, lanes A and C). In the second class, PBP 1 had an intermediate affinity for labeled penicillin, which was lower than the *penA mtr penB* recipient strain and higher than the DNA donor strain (Fig. 2, lane B). There were more (14 of 20 tested) of the latter class, and there was no obvious relationship between MIC of penicillin (2.0 versus 4.0 μg/ml) and PBP affinity pattern.

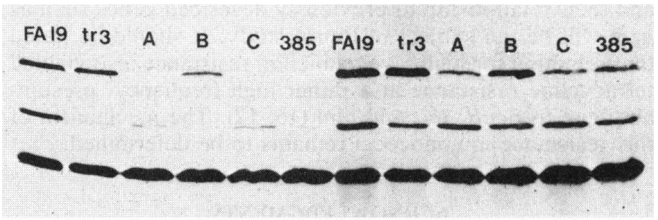

FIG. 2. PBP profile of high-level-resistant genetic transformants. Illustrated are the penicillin-susceptible (MIC, 0.01 μg/ml) strain FA19, the *penA mtr penB* third-level (MIC, 0.5 μg/ml) FA19 transformant tr3, three high-level-resistant (MIC, 4.0 μg/ml) FA19 transformants A, B, and C, and the CMR DNA donor, 84-060-385, used in transformant construction. Two concentrations of [^{3}H]penicillin were used, 0.1 μg/ml (left-hand set) and 1.0 μg/ml (right-hand set).

DISCUSSION

Several groups have recently reported data concerning outbreaks of CMR *N. gonorrhoeae* infections. The picture which emerges from these studies is one of increased incidence of such isolates over the past few years (5, 7, 12). All the isolates from the North Carolina outbreak, as well as all the isolates from CDC in 1983 to 1984, were serogroup 1B (5). The CDC group reported that while the Pro$^-$/1B-1 auxotype/serovar predominated, other auxotype/serovars were found among CMR *N. gonorrhoeae* isolates (11). It has been suggested that the current outbreak of CMR *N. gonorrhoeae* strains is due to multiple strain introductions and selection of the more resistant phenotypes in the community (7, 11). Clearly these strains present a potentially serious public health threat.

In the present study, the biochemical and genetic basis of resistance was addressed. Eight isolates were obtained from the CDC, and binding of labeled penicillin was assessed. As was the case in studies with earlier isolates (1, 3), these intrinsically resistant gonococci had reduced penicillin affinity for two of the three penicillin target proteins. In addition, one of the isolates (84-060-418) had a PBP pattern which has not been observed to date in this laboratory. Two possibilities are suggested for this observation. One is that this strain represents a different pathway for development of PBP affinity changes leading to resistance. A second explanation is that this strain may have evolved from an already existing resistant strain as a response to changing antibiotic use, e.g., β-lactam antibiotics with increased PBP 2 affinity (4).

TABLE 2. Genetic transformation of resistance genes

Mutation selection[a]	MIC (μg/ml)[b] for cross:			
	FA19 × 84-060-383	FA19 × 84-060-385	FA10 × 84-060-388	FA19 × 84-060-418
penA				
Penicillin	0.06	0.06	0.06	0.05
Erythromycin	0.30	0.34	0.30	0.26
Rifampin	0.035	0.035	0.031	0.035
Tetracycline	0.15	0.17	0.15	0.17
mtr				
Penicillin	0.02	0.01	0.01	0.01
Erythromycin	2.5	2.3	2.5	1.25
Rifampin	0.28	0.34	0.30	0.15
Tetracycline	0.15	0.15	0.14	0.15
penA mtr penB				
Penicillin	0.5	0.5	0.5	0.25
Erythromycin	2.50	2.34	2.50	1.56
Rifampin	0.3	0.3	0.3	0.15
Tetracycline	1.40	1.40	1.25	1.25

[a] *penA* defined on basis of selection at 0.03 μg of penicillin per ml and no cross-resistance; *mtr* defined on basis of erythromycin selection at 0.6 μg/ml and cross-resistance to rifampin and Triton X-100 (not shown); *penB* selected with 0.25 μg of penicillin per ml after transformation of a *penA mtr* strain (15) and tested for cross resistance to tetracycline

[b] MICs were determined for eight independent transformants isolated from the primary selection plate and represent average values.

As was the case in the 1983 North Carolina strain (6), the strains which were tested in the present study were all found to contain *penA*, *mtr*, and *penB*. Two of the strains, 84-060-383 and 84-060-418, were able to donate the *mtr* locus even though they were both phenotypically Mtr$^-$. This suggests that these strains may contain one of the *env* loci, which are dominant over *mtr* and suppress the Mtr phenotype (14).

Previous attempts to transform gonococci to the highest resistance level have been unsuccessful in this laboratory (3) and in others (6). One report of successful transformation to high-level penicillin resistance claimed that use of phenol-extracted DNA and $CaCl_2$ allowed detection of additional resistance loci (17). Adoption of this protocol led to isolation of high-level resistant transformants at low frequencies (ca. 0.001%). The inability to select high-level transformants at concentrations of penicillin that were subsequently shown to be below the MIC may be due to two possibilities. It is conceivable that full expression of resistance may take longer than the 6- to 8-h expression time allowed before antibiotic challenge (15, 16). Alternatively, the use of the double agar overlay technique requires a high concentration of antibiotic in the overlay, which subsequently diffuses throughout the agar (2, 3, 16). It is possible that at these high antibiotic concentrations, the gonococci are subjected to a rapid high-level antibiotic shock.

Rather surprisingly, two classes of high-level resistance transformants were isolated. Both classes had comparable penicillin MICs, 2.0 to 4.0 μg/ml. Tests with additional β-lactam compounds (ampicillin, amoxicillin, cephaloridine, and cefotaxime) failed to show susceptibility differences between the two transformant classes. Inasmuchas almost all clinical isolates of resistant gonococci tested resemble the transformant class with low PBP 1 binding, there may be an advantage in the infected host in having this PBP profile. It may, for example, allow the gonococci to survive transient high levels of penicillin in the blood, which can reach 25 μg/ml after parenteral administration (15).

The existence of two classes of high-level penicillin-resistant transformants based on PBPs also implies that two genes are involved. The possible interactions of these genes

and their relationship to previously described genes such as *pem* will be the subject of future study. It should be noted that selection for high-level penicillin resistance also yielded tetracycline resistance at a rather high frequency, presumably due to *penB*, *tet*, and *tem* (16, 17). The mechanism of this resistance in gonococci remains to be determined.

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