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Genetics of Resistance in a Non-β-Lactamase-Producing Gonococcus with Relatively High-Level Penicillin Resistance

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A penicillin-resistant (Pen^r) non-penicillinase-producing *Neisseria gonorrhoeae* strain responsible for an outbreak affecting 199 persons in Durham, N.C., in 1983 was studied to determine the genetic basis of its unusually high-level (MIC, 2.0 μg/ml) Pen^r. Plasmid screening of the strain revealed no plasmids other than the 2.6-megadalton cryptic plasmid. Pen^r was found to be partially due to mutations genotypically and phenotypically similar to the previously characterized chromosomal loci *penA*, *mtr*, and *penB*. Resistance loci from the epidemic donor strain were transformed into susceptible recipients FA19 and F62 in a stepwise fashion; the combination of the three loci resulted in moderate levels of penicillin resistance (MIC, 0.5 μg/ml), but donor levels of resistance were not obtainable in either recipient, for uncertain reasons. Occurrence of an antibiotic-susceptible (*env*) mutation in a clinical isolate of the Pen^r epidemic strain also was documented.

A relatively large outbreak (199 cases) of non-penicillinase-producing penicillin-resistant (Pen^r) gonococci occurred in Durham, N.C., in 1983 (3, 7). The resistant isolates were unusual in that they did not produce detectable beta-lactamase and yet showed relatively high resistance to penicillin (MIC, 2 to 4 μg/ml). Patients infected with this strain nearly always failed therapy with penicillin G (7) or tetracycline (unpublished data). Epidemiologic analysis of resistant isolates by using auxotyping, serotyping, and antibiotic susceptibility profiles indicated that they all belonged to a single strain of *Neisseria gonorrhoeae*. The strain was relatively resistant to erythromycin (MIC, 2.0 μg/ml) and tetracycline (MIC, 4.0 μg/ml) in addition to penicillin but was spectinomycin and streptomycin susceptible. Such isolates are relatively common in the Far East but rare in the United States (1).

The genetics and biochemistry of chromosomally mediated, low-level resistance of gonococci to penicillin and tetracycline has been studied extensively (5, 9, 13, 19, 22, 26). However, the Durham epidemic strain was more resistant to penicillin than any of the previously studied isolates, and it seemed possible that a novel genetic basis for resistance might account for its high levels of resistance. Previously studied resistant strains such as FA5 were reported to have an MIC (penicillin) of 2.0 μg/ml (22), but by present tests have an MIC (penicillin) of between 0.5 and 1.0 μg/ml (unpublished data). In this paper, we elucidate the genetics of resistance in this recent epidemic strain and compare it with previously studied chromosomally resistant gonococci.

(A preliminary report of some of these results appeared previously [8].)

MATERIALS AND METHODS

Media and culture techniques were as described previously (19). Gonococcal strains used in this study are listed in Table 1. Auxotyping of the epidemic strain (FA6140) and selected penicillin-susceptible isolates from Durham, N.C., was performed by the method of LaScolea and Young (11). The coagglutination technique with outer membrane protein I (P.I) monoclonal antibodies (24) was used to serotype all strains in this study. MICs of selected antibiotics were determined by an agar dilution procedure as described previously (22). Transformation experiments were carried out with DNA prepared by the Marmur method (15). Piliated recipients were transformed with limiting concentrations of DNA (≤0.1 μg/ml) by methods described earlier (19, 20). Phenotypic levels of resistance are indicated when appropriate with a number in parentheses, indicating MIC (micrograms per milliliter) for the strain. Thus Pen^r (0.5) indicates an MIC of penicillin of 0.5 μg/ml.

Outer membrane preparations. Cells suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4) (HEPES buffer) were ruptured by two passages through a cold French pressure cell at 16,000 lb/in^2. After one 10-min centrifugation at 5,000 × *g*, the clear supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet was washed one time by suspension and centrifugation at 100,000 × *g* for 1 h. The final pellet represented total membranes from which outer membranes were isolated by selectively solubilizing cytoplasmic membranes with 0.2% sodium lauryl sarcosinate detergent (Sarkosyl) essentially as described by Walstad et al. (25). Protein concentrations of membrane fractions were determined by the Lowry method (12), with bovine serum albumin as the standard. Outer membrane samples containing 20 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel by the method of Laemmli (10). Proteins were visualized by staining with Coomassie blue (23).

Plasmid analysis. Epidemic isolates were screened for their plasmid content by the method of Meyers et al (16). Penicillin-resistant isolates were also checked for beta-lactamase production by the chromogenic cephalosporin (nitrocefin; Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom) assay (17).

RESULTS

Several resistant isolates from the Durham epidemic (7) were screened for their plasmid content and revealed no plasmids other than the 2.6-megadalton cryptic plasmid (18). Beta-lactamase production was not detected in any of the

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TABLE 1. *N. gonorrhoeae* strains used

Strain	Description
FA19	Antibiotic-susceptible wild type (14)
F62	Antibiotic-susceptible wild type (14)
FA913	Spc^r Str^r transformant of FA19
FA6140	Durham Pen^r epidemic strain
FA6141	FA6140 × FA19 first-step Pen^r (*penA4*) transformant
FA6142	FA6140 × FA6141 second-step Pen^r (*penA4 mtr-4*) transformant
FA6143	FA6140 × FA6142 third-step Pen^r (*penA4 mtr-4 penB5*) transformant
FA6144	FA6140 × FA6143 Tet^r (*penA4 mtr-4 penB5 tet-4*) transformant
FA6145	FA6140 × FA19 Ery^r (*mtr-4*) transformant
FA6146	Spontaneous clinical *env-12* mutant of FA6140
FA6147	Nal^r mutant of FA6146
FA6148	FA19 × FA6146 Ery^r (*env*$^+$) transformant
FA6217	FA6140 × FA19 Tet^r (*tet-4*) transformant
BR54	*env-3* (20)
BR84	*env-1* (20)
BR87	*env-2* (20)
FA963	Nal^r *env-1* (21)
FA102	FA19 *penA2* (22)
FA140	FA19 *penA2 mtr-2 penB2* (22)
FA171	FA19 *mtr-2* (22)

epidemic isolates. This indicated that resistance in the epidemic strain FA6140 probably was due to chromosomal mutations, although the methods used might not have detected very large plasmids. DNA from the Pen^r (2.0) donor strain FA6140 was used to transform a laboratory strain FA19 (MIC of penicillin, ≤0.015 μg/ml) to higher levels of resistance. In three sequential transformations, FA19 assumed increasing levels of resistance, from an initial MIC (of penicillin) of 0.015 μg/ml to 0.5 μg/ml (strains FA6141, -6142, and -6143, respectively; Table 2). The levels of Pen^r at each transformation step, and resistance to erythromycin, tetracycline, and other antimicrobial agents at the second and third Pen^r transformation steps, were nearly identical phenotypically to those observed when the previously characterized *penA2*, *mtr-2*, and *penB2* loci were introduced into FA19 (13, 22). Second-step transformants were selected with equal efficiency with either penicillin or erythromycin; the phenotype of second-step transformants was identical regardless of the method of initial selection (data not shown).

TABLE 2. Polygenic, additive inheritance of low-level antibiotic resistance from donor strain FA6140

Strain	MIC (μg/ml)[a]			Serovar
	Pen	Tet	Ery	
FA19	≤0.015	≤0.25	≤0.25	P.IA-1
FA6140	2.0	4.0	2.0	P.IB-1
FA6141[b]	0.12	≤0.25	≤0.25	P.IA-1
FA6142[c]	0.25	1.0	2.0	P.IA-1
FA6143[d]	0.5	2.0	2.0	P.IB-1
FA6144[e]	0.5	4.0	2.0	P.IB-1
FA6145[f]	0.03	1.0	2.0	P.IA-1
FA6217[g]	≤0.015	1.0	≤0.25	P.IA-1

[a] Pen, Penicillin; Tet, tetracycline; Ery, erythromycin.
[b] First-step Pen^r transformant (FA6140 × FA19) selected with 0.03 μg of penicillin per ml.
[c] Second-step Pen^r transformant (FA6140 × FA6141) selected with 0.5 μg of erythromycin per ml.
[d] Third-step Pen^r transformant (FA6140 × FA6142) selected with 0.37 μg of penicillin per ml.
[e] Tet^r transformant (FA6140 × FA6143) selected with 2.5 μg of tetracycline per ml.
[f] Ery^r transformant (FA6140 × FA19) selected with 0.5 μg of erythromycin per ml.
[g] Tet^r transformant (FA6140 × FA19) selected with 0.25 μg tetracycline per ml.

TABLE 3. Lack of evident recombination between the penicillin resistance loci in FA6140 and *penA2*, *mtr-2*, and *penB2*

Donor (putative genotype)	Recipient (genotype)	Transformants to increased resistance[a]
FA6141 (*penA4*)	FA102 (*penA2*)	$<10^{-7}$[b]
FA6145 (*mtr-4*)	FA171 (*mtr-2*)	$<10^{-7}$[c]
FA6143 (*penA4 mtr-4 penB5*)	FA140 (*penA2 mtr-2 penB2*)	$<10^{-7}$[d]

[a] Control experiments with Nal^r DNA showed that the recipients were competent: transformation frequency (proportion of exposed cells transformed) to Nal^r = 3×10^{-4}.
[b] Transformants (FA6141 × FA102) were selected with 0.06 and 0.12 μg of penicillin per ml.
[c] Transformants (FA6145 × FA171) were selected with 2.0 to 3.0 μg of erythromycin per ml.
[d] Transformants (FA6143 × FA140) were selected with 0.5 to 1.0 μg of penicillin per ml.

To determine whether the resistance loci in FA6140 were similar genotypically to *penA*, *mtr*, and *penB*, transformants of FA19 containing resistance loci from FA6140 were crossed against reference strains derived previously in FA19 containing the *penA2*, *mtr-2*, or *penB2* markers (Table 3). In no instance was a recombinant obtained with higher levels of resistance. On the basis of these data, we concluded that resistance to penicillin in FA6140 was due to the additive effect of multiple mutations and that some of the mutations were phenotypically and genotypically similar to previously characterized *penA*, *mtr*, and *penB* loci. We designated the respective loci in FA6140 *penA4*, *mtr-4*, and *penB5*.

Many efforts were made to transform either FA6143 or FA6144 or similar derivatives of F62 to donor-level (penicillin MIC, ≥2.0 μg/ml) penicillin resistance, using saturating concentrations of FA6140 DNA. No such transformant was obtained, despite using small increments of penicillin in the selection down to a concentration that allowed the recipient strain to grow on control plates, and despite prolonged incubations (10 h) before addition of penicillin to plates containing recipient cells and FA6140 DNA.

In earlier studies of outer membranes of *mtr-2* and *penB2* transformants, the *mtr-2* locus resulted in increased amounts of a 52-kilodalton (kDa) protein (9), and *penB2* was closely linked to loci designated *nmp* that affected the M_r of the principal outer membrane P.I (2). When outer membranes of transformants containing *mtr-4* were examined, increased amounts of an approximately 52-kDa protein were observed (Fig. 1). In other experiments, the 52-kDa protein of FA171, an *mtr-2* derivative of FA19, migrated identically with the 52-kDa protein shown in Fig. 1 (data not shown). There was a change in recipient outer membrane P.IA-1 serovar to the donor P.IB-1 serovar with introduction of *penB5* in 119 of 120 tested transformants. Thus, by these criteria the *mtr* and *penB* loci in FA6140 were very similar to those characterized earlier in other somewhat less Pen^r strains (2, 9).

A transformant of FA6143 selected for increased tetracycline resistance (Tet^r) (FA6144) exhibited increased resistance to tetracycline only. It was not clear whether this was due to a locus similar to *tet* (18), *tem* (26), or another Tet^r locus. The *tet* locus was reported to increase resistance to

tetracycline in the absence of other resistance loci, whereas *tem* acts as a modifier of resistance mediated by other loci but by itself does not lead to Tet^r (26). Since introduction of the Tet^r locus from FA6140 into FA19 resulted in increased Tet^r (FA6217, Table 2) in the apparent absence of other resistance markers, the Tet^r locus in FA6144 was phenotypically more like the previously characterized *tet-2* than *tem* locus. We could not exclude the possibility that other linked resistance markers could have been introduced with the Tet^r locus, however.

To further identify the Tet^r locus, we investigated linkage with loci for spectinomycin resistance (Spc^r) or streptomycin resistance (Str^r). DNA from FA6217 (Tet^r Spc^s Str^s) was introduced into FA913 (Tet^s, Spc^r, Str^r). Tet^r transformants were selected and scored for acquisition of Spc^s or Str^s from the donor. Among Tet^r transformants, 77 of 80 became Str^s, and 63 of 80 became Spc^s; 61 of 80 Tet^r transformants acquired both Spc^s and Str^s from the donor. Although the linkage between the Tet^r locus and the *spc* and *str* loci was considerably greater than observed in previous experiments with DNA containing *tet-2* (19), the data suggested that the Tet^r locus in FA6140 was analogous to *tet-2*. We designated the Tet^r locus in FA6140 *tet-4*.

Although neither FA19 nor F62 could be transformed to donor-level (penicillin MIC, ≥2.0 μg/ml) penicillin resistance, FA6146, a clinical strain isolated in Durham in 1983 during the epidemic of Pen^r gonococcal infections, was readily transformed to such levels of Pen^r. FA6146 has MICs of 0.25 μg of penicillin per ml, 1.0 μg of tetracycline per ml, and <0.25 μg of erythromycin per ml; it also has an auxotype and serotype identical to the Pen^r epidemic strain FA6140 (proline-requiring auxotroph, P.IB-1 serovar). Using limiting concentrations of donor DNA from FA6140, we obtained single-step transformants of FA6146 with levels of resistance equal to that seen in FA6140 at high frequency (about 10^{-3}).

This result was compatible with the idea that FA6140 might donate single-step Pen^r (2.0) only to certain recipients. However, it also seemed plausible that FA6146 was a single-step antibiotic-susceptible mutant of FA6140 that had

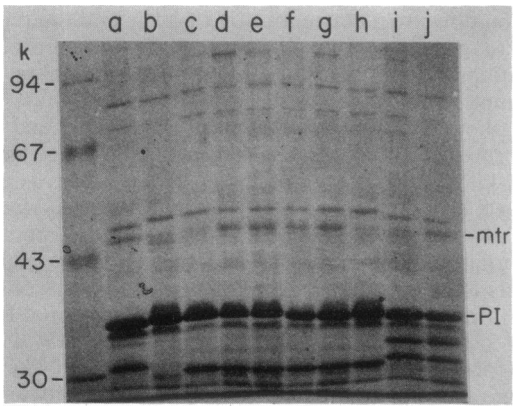

FIG. 1. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis of outer membrane proteins of various strains of *N. gonorrhoeae*. Lane a contains the donor Pen^r strain FA6140, and lane b contains the recipient sensitive strain FA19. Lanes c, d, e, f, g, and h contain transformants FA6141 (*penA4*), FA6142 (*penA4 mtr-4*), FA6145 (*mtr-4*), FA6143 (*penA4 mtr-4 penB5*), FA6144 (*penA4 mtr-4 penB5 tet-4*), and FA6217 (*tet-4*), respectively. Lane i contains the *env-12* mutant FA6146, and lane j contains FA6148, an *env*$^+$ transformant of FA6146. Numbers on left are molecular size in kilodaltons.

TABLE 4. Demonstration that the *env-12* locus recombines with *env-3* and *env-10* but not with *env-1* and *env-2*

Donor DNA	Recipient	Transformants (per ml)		Recombination index (Ery^r/Nal^r)
		Ery^r[a]	Nal^r[b]	
FA6147 (Nal^r *env-12*)	BR54 (*env-3* *nal*$^+$)	1,700	4,400	0.39
FA6147 (Nal^r *env-12*)	BR84 (*env-1* *nal*$^+$)	0	4,500	0
FA6147 (Nal^r *env-12*)	BR87 (*env-2* *nal*$^+$)	0	22,000	0
FA963 (Nal^r *env-10*)	FA6146 (*env-12* *nal*$^+$)	1,550	6,500	0.24

[a] Transformants were selected with 0.5 μg of erythromycin per ml.
[b] Transformants were selected with 5.0 μg of nalidixic acid per ml.

arisen during the 1983 epidemic in Durham; several mutations designated *env* have been characterized that phenotypically suppress multiple chromosomal antibiotic resistance mutations (6, 20, 21). If this were the case, donor DNA from FA6146 should be able to transfer antibiotic resistance to susceptible recipients such as FA19, and an antibiotic-susceptible (*env*$^+$) strain such as FA19 also should be able to transform FA6146 to multiple antibiotic resistance. Results showed that FA6146 was able to transform FA19 to Pen^r (0.5) in three steps exactly as FA6140 did and that FA6146 (erythromycin MIC, 0.25 μg/ml) was able to transform FA19 (erythromycin MIC, 0.25 μg/ml) to Ery^r (erythromycin MIC, 2.0 μg/ml) (data not shown). Moreover, FA19 was able to transform FA6146 to multiple drug resistance in a single step, and the transformants were phenotypically indistinguishable from FA6140 (data not shown).

These observations strongly suggested that FA6146 was an antibiotic-susceptible mutant of FA6140, and we designated the locus for antibiotic susceptibility *env-12*. To further confirm the identity of the *env-12* mutation, a spontaneous nalidixic acid-resistant (Nal^r) mutant of FA6146 (FA6147) was selected and crossed against strains with previously characterized *env* mutations (20, 21). The *env-12* mutation in FA6147 did not recombine with the previously characterized *env-1* and *env-2* mutations but did recombine readily with *env-3* and *env-10* (Table 4). Thus, the *env-12* mutation in FA6146 is very similar to *env-1* and *env-2*.

Outer membrane proteins of FA6140, *env-12* mutant FA6146, and FA6148, an *env*$^+$ transformant of FA6146, are shown in Fig. 1. FA6140 (lane a) and *mtr-4* transformants of FA19 (lanes d to g) demonstrate a prominent, approximately 52-kDa protein, as expected for strains containing *mtr* (9). Lane i contains FA6146 (*env-12*) in which the *mtr* phenotype is suppressed: the 52-kDa protein is decreased. Lane j contains the *env*$^+$ transformant FA6148 and demonstrates increased amounts of the *mtr-4*-determined 52-kDa protein again. These results are very similar to those demonstrated previously for *mtr-2* and *env-2* (9).

DISCUSSION

Resistance to penicillin and other antibiotics in FA6140 was shown to be due to the additive effects of several mutations. Multistep acquisition of Pen^r from FA6140 was demonstrated in two susceptible recipients (FA19, F62). The resistance loci in FA6140 were designated *penA4*, *mtr-4*, and *penB5* and were indistinguishable from the *penA2*, *mtr-2*, and *penB2* loci characterized a decade ago (22). Since no unusual plasmids were found in FA6140, and since *penA4*,

mtr-4, and *penB5* failed to recombine with loci (*penA2*, *mtr-2*, and *penB2*) that are known to be chromosomal (19, 22), the resistance loci in FA6140 almost certainly are chromosomal as well. The designation of strains such as FA6140 as CMRNG (4) (chromosomally mediated resistance *N. gonorrhoeae*) therefore is apt.

Comparison of the present results with those obtained earlier by several laboratories with a number of presumably unrelated slightly less resistant gonococcal isolates (5, 13, 22) reveals remarkably similar inheritance of low-level chromosomally mediated Pen^r. The genetic basis for the slightly higher Pen^r in FA6140 (Pen^r [2.0]) compared with that of previously studied isolates (Pen^r [0.5 to 1.0]) proved elusive. Despite multiple attempts, we were unable to transform FA19 or F62 to levels of resistance seen in the epidemic strain. It may be that the *penA4*, *mtr-4*, and *penB5* mutations in FA6140 result in higher phenotypic levels of resistance in certain strains owing to background differences in membrane or cell wall structure; we may not have used the proper recipient. It may also be that there are additional resistance markers which have not yet been identified which either are incompatible for some reason with the background of the FA19 and F62 strains we used as recipients or are too numerous and too widely separated to be transformed in a single step.

To eliminate some of the background differences which might have interfered with expression of the higher level of resistance, we screened Pen^s isolates from Durham looking for one with the same auxotype and serotype as the epidemic strain. FA6146 was then selected for transformation with the epidemic strain (FA6140) DNA. We were able to transform FA6146 to levels of resistance identical to those of FA6140. However, further analysis revealed that FA6146 probably was identical to FA6140, containing very similar to identical resistance loci but in addition an *env-12* mutation. *env* mutations can occur spontaneously and are responsible for membrane permeability changes which cause nonspecific increased susceptibility to a variety of drugs, dyes, and detergents, overcoming the phenotypic resistance determined by *mtr*, *penB*, and possibly other loci (6, 20, 21).

It has been noticed, although we have not seen it in our laboratory, that Pen^r non-penicillinase-producing gonococci such as FA6140 occasionally lose their high level of penicillin resistance on repeated subculture in vitro, with decreases in penicillin MIC to 0.25 to 0.5 μg/ml (J. Biddle, personal communication). The spontaneous acquisition of *env* mutations may explain the phenotypic loss of resistance seen occasionally with serial passage of these strains. FA6146 isolated from Durham during the 1983 epidemic apparently acquired the *env* mutation in vivo. Membrane changes associated with increased permeability may offer selective advantage to relatively resistant gonococci in the absence of selective pressures of antibiotics (6).

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