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Plasmid-Mediated Beta-Lactamase Production in *Neisseria gonorrhoeae*

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Several β-lactamase-producing, penicillin-resistant strains of *Neisseria gonorrhoeae* were examined for R plasmids. Penicillin-resistant strains isolated from men returning from the Far East and their contacts contained a 4.4×10^6-dalton plasmid in common. Transformation studies and the isolation of a spontaneous penicillin-susceptible segregant showed that the structural gene for β-lactamase was part of the 4.4×10^6-dalton plasmid. An additional penicillin-resistant gonococcal strain isolated in London was found to harbor a 3.2×10^6-dalton R plasmid. Deoxyribonucleic acid (DNA)-DNA duplex studies revealed that the penicillin-resistant gonococcal isolates contained a significant portion (about 40%) of the transposable DNA sequence, TnA, which includes the β-lactamase gene commonly found on R plasmids of the *Enterobacteriaceae* and *Haemophilus influenzae*.

It has been known for some time that the R plasmids of enteric species enjoy a very broad host range (1, 14). The recent finding (reviewed in 4, 6, 28) that certain plasmid-mediated antibiotic resistance genes reside upon discrete sequences of deoxyribonucleic acid (DNA) that have the capacity to transpose from plasmid to plasmid has further demonstrated that the range of plasmid-mediated antibiotic resistance genes might be even broader than that of the whole plasmid genome. Therefore, plausible mechanisms exist by which pathogenic microorganisms, previously antibiotic susceptible, may be converted to stable antibiotic resistance by the direct extension of an entire R plasmid or by the simple addition of only a segment of DNA to a preexisting indigenous plasmid gene pool (6, 15, 19).

Previous studies from this laboratory (21) and others (3, 24, 30) have established that most strains of *Neisseria gonorrhoeae* contain plasmid species that are cryptic in the phenotypic sense. For example, our examination of 30 antibiotic-susceptible gonococcal isolates showed that 28 strains harbored a multicopy (2.6 ± 0.2) $\times 10^6$-dalton (2.6 MDal) plasmid, and 1 strain contained a 24.5-MDal plasmid species, whereas 1 strain was plasmid free (13; L. P. Elwell and S. Falkow, *in* R. Roberts, ed., *The Gonococcus*, in press). The demonstration of a widespread plasmid gene pool in gonococci together with the emergence, epidemiology, and molecular nature of R plasmids mediating ampicillin resistance in *Haemophilus influenzae* (11, 12) led us to speculate (15) that gonococci were likely candidates to acquire plasmid-mediated penicillin resistance specified by a β-lactamase. Consequently, the recent isolation of β-lactamase-producing strains of *N. gonorrhoeae* (2, 25, 26) prompted us to examine them for the presence of a novel DNA species that might represent an R plasmid.

In this study we report the detection of plasmid species from three penicillin-resistant clinical isolates of *N. gonorrhoeae*. We further demonstrate that these plasmids probably represent a direct or indirect extension of the plasmid gene pool of enteric species to gonococci.

MATERIALS AND METHODS

Bacterial strains. Penicillin-resistant strains of *N. gonorrhoeae* were kindly supplied by Clyde Thornsberry, Center for Disease Control, Atlanta, Ga. The strains, CDC01, CDC66, CDC67, and CDC36N, were recent representative isolates from United States military personnel recently returned from the Far East as well as a sexual contact (3, 25). The IPL strain was originally isolated in London from the vagina of a woman with a pelvic inflammatory disease (26). The gonococcal strains F62 and KH45 were antibiotic-susceptible laboratory strains whose plasmid complement have been previously characterized (21; Elwell and Falkow, in press). Penicillinase production of the parental strains as well as selected transformants was detected by using the chromogenic cephalosporin method (23). Resistant strains were maintained on modified Kellogg agar (21) supplemented with 1 μg of penicillin G per ml.

Agarose gel electrophoresis of DNA. Plasmid

DNA was initially detected and characterized by the agarose gel electrophoretic method (22). The strains of *N. gonorrhoeae* were grown overnight in 30 ml of modified GC medium (Difco) supplemented as described by Mayer et al. (21). Cleared lysates were prepared (16, 22) and, after ribonuclease digestion and phenol extraction, the clear aqueous phase was adjusted to 0.2 M sodium acetate (final concentration) and precipitated with ethanol. The precipitate was dissolved in 0.2 ml of TES buffer [50 mM NaCl-5mM ethylenediaminetetraacetic acid (EDTA)-30 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8)] and electrophoresed in a 0.7% agarose gel dissolved in Tris-borate buffer (pH 8.2) (89 mM Tris base-2.5 mM sodium EDTA-89 mM boric acid). DNA was visualized by staining with ethidium bromide and viewing the gel under long-wave ultraviolet light. Plasmid DNA of known molecular weight was included in each gel as a standard (22).

Preparation of plasmid DNA. Unlabeled covalently closed circular (CCC) plasmid DNA was prepared as previously described (19). [^{3}H]thymine-labeled RSF1050 plasmid DNA was prepared from *Escherichia coli* 1485 as described by Heffron et al. (18, 19).

DNA contour length. Plasmid DNA was spread onto parlodion-coated electron microscope grids by the Kleinschmidt technique (10). Molecular weights of the plasmids were calculated as previously described (18, 19).

DNA-DNA hybridization studies. Unlabeled whole-cell DNA from penicillin-resistant and -susceptible strains of *N. gonorrhoeae* was prepared by the method of So et al. (29). For DNA-DNA duplex studies, approximately 0.01 μg of ^{3}H-labeled, sheared, denatured RSF1050 plasmid DNA was incubated with 150 μg of unlabeled, sheared, denatured DNA from each of the indicated organisms. DNA reassociation was performed in 0.21 M NaCl at 68°C for 240 min. At the end of the incubation period, samples were assayed by the S_1 endonuclease technique (7).

RESULTS

Detection of R plasmid in *N. gonorrhoeae*. Figure 1 shows the agarose gel electrophoresis of ethanol-precipitated plasmid DNA from cleared lysates of selected *N. gonorrhoeae* strains. The prominent band of linear chromosomal DNA migrates to a characteristic position in such gels and may obscure CCC plasmid species of about 10×10^6 to 15×10^6 daltons, depending upon its width (22). Figures 1A and B contain lysates extracted from the antibiotic-susceptible laboratory strains KH45 and F62 and show the presence of previously characterized CCC plasmids of 24.5 and 2.6 MDal, respectively. The faint DNA band in Fig. 1B, appearing between the 2.6-MDal CCC plasmid species and the chromosomal band, represents the open circular (OC) form of the 2.6-MDal plasmid. The identification of this intermediate band as the OC form of the 2.6-MDal plasmid species was deducted from the direct electron microscopic examination of molecules extracted from this band as well as the direct observation of the banding patterns of known 2.6-MDal CCC and OC species (see also 22). Figures 1C and D contain lysates extracted from independently isolated penicillin-resistant strains CDC66 and CDC67 contracted by military personnel in the Far East. The lysate of strain CDC66 (Fig. 1C) shows the CCC and OC forms of the 2.6-MDal indigenous plasmid and an additional plasmid species that migrates in the agarose gel at a position (relative to the molecular weight standard [Fig. 1H]) characteristic of a CCC molecule with a molecular mass of 4.4 MDal. The lysate of strain CDC67 (Fig. 1D) contains both the 2.6- and 24.5-MDal plasmids typical of gonococci, but also contains a 4.4-MDal plasmid species. The association of the 4.4-MDal molecular species with the penicillin-resistant phenotype was materially strengthened by examining the lysate extracted from a penicillin-resistant strain, CDC01 (Fig. 1F), isolated from a case contact and a spontaneous pencillin-susceptible derivative of CDC01 (Fig. 1E). It may be seen that the lysates from these two strains differ in only one aspect, namely, the 4.4-MDal plasmid species.

The lysate extracted from the penicillin-resistant IPL strain isolated in London from a patient with pelvic inflammatory disease is shown in Fig. 1G. The IPL strain was found to contain the 2.6-MDal plasmid and a unique plasmid species that, migrated at a position, relative to the molecular weight standards (Fig. 1H), for a CCC molecule with a molecular mass of 3.2 MDal.

The number and molecular mass of the plasmid species in the gonococcal isolates were verified by isolating purified CCC DNA from each strain and measuring the contour lengths of OC species seen in the electron microscope. The results were in excellent agreement with the agarose gel data. Relative to the indigenous gonococcal plasmid, which was taken as a molecular mass of 2.6 MDal (21, 24, 30), the molecular masses of the unique plasmid species present in the Far East and London strains were calculated to be 4.3 ± 0.15 and 3.2 ± 0.2 MDal, respectively, based on measurements of at least 10 molecules. In subsequent studies we have been able to transform, by using the method of Cohen et al. (5), a suitable *E. coli* recipient to ampicillin resistance (Ap^r) with purified plasmid DNA from the penicillin-resistant strains CDC36N and IPL. The resulting *E. coli* Ap^r transformants derived from CDC36N DNA contained a single plasmid species corresponding

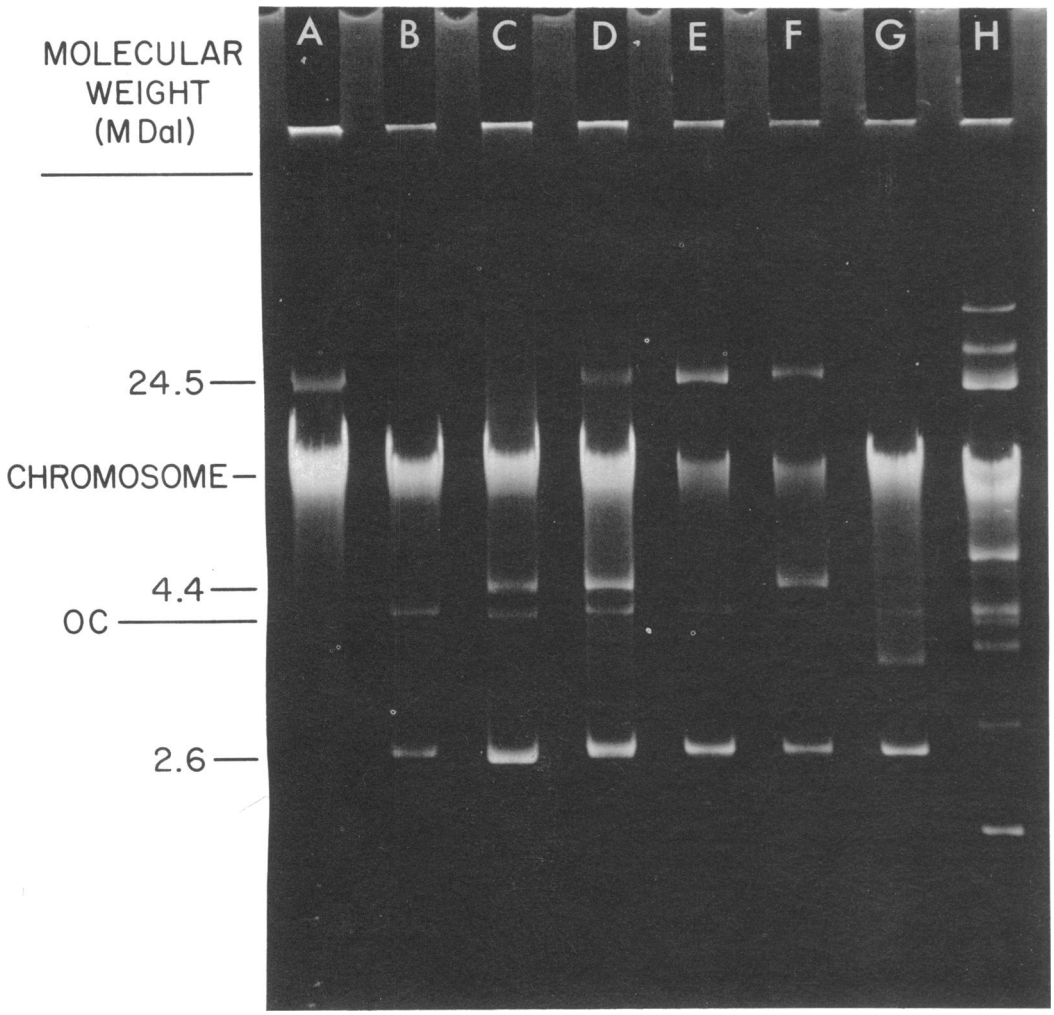

FIG. 1. *Agarose gel electrophoresis of ethanol-precipitated DNA from cleared lysates of penicillin-susceptible (Pen^s) and penicillin-resistant (Pen^r) N. gonorrhoeae strains. (A) Strain KH45 (Pen^s) containing the 24.5 × 10^6-dalton cryptic plasmid; (B) strain F62 (pen^s) containing the 2.6 × 10^6-dalton cryptic plasmid; (C) strain CDC66 (Pen^r), Far East isolate; (D) strain CDC67 (Pen^r), Far East isolate; (E) spontaneous Pen^s derivative of strain CDC01; (F) strain CDC01 (Pen^r), from case contact of patient infected in the Far East; (G) strain IPL (Pen^r), London, England, isolate; (H) standard plasmid DNAs, ranging in size from 62 × 10^6 (uppermost band) to 1.9 × 10^6 daltons (lowest band). OC refers to the open circular form of the 2.6 × 10^6-dalton cryptic plasmid.*

to 4.3 MDal, whereas the *E. coli* Ap^r transformants obtained with IPL DNA contained a single plasmid species of 3.2 MDal. The complete molecular characterization of these plasmids is currently in progress (M. Roberts, L. P. Elwell, and S. Falkow, manuscript in preparation). Nevertheless, the preliminary data presented here provide sufficient evidence to conclude that the penicillin resistance of the Far East and London gonococcal isolates is plasmid mediated.

Origin of β-lactamase genes residing on gonococcal plasmids. Although gonococci relatively resistant to penicillin have been known for a number of years (reviewed in 15), the penicillin-resistant gonococci isolated from the Far East and London were unlike any previously described strains in that they elaborated a β-lactamase that conferred resistance to penicillin, ampicillin, and cephaloridine, similar to the TEM β-lactamase of enteric species (2, 26, C. Thornsberry, personal communication; un-

published data from this laboratory). It is well established that among R plasmids within species of the family *Enterobacteriaceae*, the gene specifying the TEM β-lactamase resides upon a common transposable 3.2×10^6-dalton sequence of DNA (3, 4, 6, 17-19). The ability of this DNA sequence, TnA, to be excised from one plasmid and become inserted into a recipient plasmid has been suggested as an explanation for the ubiquity of the TEM β-lactamase gene among R plasmids of the *Enterobacteriaceae* and other bacterial species (15, 17-19). It seemed of considerable interest, therefore, to determine whether any, or all, of the TnA DNA sequence was present in the penicillin-resistant strains of *N. gonorrhoeae*. To study this possibility, DNA-DNA duplex studies were performed.

Whole-cell DNA was prepared from the *N. gonorrhoeae* Far East isolates CDC66 and CDC67, the London isolate IPL, as well as the laboratory strains F62 and KH45. [^{3}H]thymine-labeled RSF1050, a 4.9-MDal ColE1 plasmid derivative containing the entire TnA sequence (29), was hybridized against the unlabeled gonococcal whole-cell DNA. RSF1050 served as a particularly convenient probe for TnA since this sequence comprised about 50% of the entire plasmid genome (F. Heffron, P. Bedinger, J. Champoux, and S. Falkow, submitted for publication). Table 1 shows the results of the hybridization experiments. Labeled RSF1050 plasmid DNA shared no DNA sequences in common with the drug-susceptible strains of *N. gonorrhoeae* harboring either the 24.5- or 2.6-MDal indigenous plasmids. In contrast, all of the penicillin-resistant strains tested contained DNA that bound 20 to 22% of the RSF1050 nucleotide sequence. As indicated in Table 1, ^{3}H-labeled RSF1050 was hybridized against whole-cell DNA extracted from *H. influenzae* (RSF007). The plasmid RSF007 has previously been shown to contain the entire TnA sequence by heteroduplex analysis (11); therefore, the reaction between RSF1050 and *H. influenzae* (RSF007) was assumed to reflect only TnA-TnA interaction. On the basis of a relative DNA sequence homology of 51% in the case of an R plasmid containing the entire TnA segment, it can be estimated that both the 4.4- and 3.2-MDal gonococcal R plasmids contain 39 to 43% of the TnA sequence. To determine whether the homology shared by RSF1050 and the gonococcal R plasmids was specifically in the TnA segment and not another portion of RSF1050, hybridization experiments were carried out with ^{3}H-labeled PMB8 plasmid DNA. PMB8, which is RSF1050 minus TnA, showed no significant nucleotide base sequence homology with unlabeled DNA derived from the penicillin-resistant *N. gonorrhoeae* isolates. Heteroduplex studies are in progress to substantiate these hybridization data.

TABLE 1. *Hybridization between ^{3}H-labeled RSF1050 plasmid DNA and whole-cell DNA*

Source of unlabeled DNA	Relative DNA sequence homology with ^{3}H-labeled RSF1050 plasmid DNA (%)[a]
E. coli W1485 (RSF1050)	100
H. influenzae (RSF007)	51
N. gonorrhoeae F62 (Pen^s)[b]	1
N. gonorrhoeae 45 (Pen^s)	1
N. gonorrhoeae 67 (Pen^r)	22
N. gonorrhoeae 66 (Pen^r)	20
N. gonorrhoeae IPL (Pen^r)	20

[a] The degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method (7). In every case, the degree of duplex formation was calculated relative to the homologous reaction. The actual extent of binding in the homologous reaction was 75 to 78%. All other reactions were normalized to this value set at 100%. Each value shown is an average of three separate determinations. The RSF007 plasmid resident in *H. influenzae* has been previously shown to contain the entire TnA DNA sequence (11). The reaction between RSF1050 and *H. influenzae* (RSF007) was assumed to reflect only TnA-TnA interaction. The reaction between RSF1050 and the gonococcal isolates was also assumed to reflect only TnA-TnA reaction.

[b] Pen^s, Penicillin susceptible; Pen^r, penicillin resistant.

DISCUSSION

The sudden appearance of plasmid-mediated penicillin resistance among strains of *N. gonorrhoeae* in both the Far East and London bears a striking similarity to the earlier emergence of ampicillin-resistant strains of *H. influenzae* (20). Antibiotic resistance in *Haemophilus* strains has occurred by virtue of several independent events, as evidenced by the characterization of two genetically distinct R plasmids from ampicillin-resistant isolates (11, 12, 15). Similarly, antibiotic resistance in *N. gonorrhoeae* appears to be more complex than merely the dissemination of a single R plasmid-containing strain. This conclusion is suggested by the plasmid pattern observed in the lysates of the Far East strains CDC66 and CDC67 since one harbors the 24.5-MDal indigenous plasmid and the other does not (Fig. 1C and D). Both of these isolates carry the 4.4-MDal R plasmid as well as the 2.6-MDal indigenous plasmid. Furthermore, the IPL strain isolated in London

presumably represents a third independent resistant strain. Preliminary auxotyping of selected penicillin-resistant isolates (D. Rose, and M. Roberts, unpublished data) supports the conclusion that many of the strains are unrelated and that resistance has occurred by independent genetic events.

A number of investigators (reviewed in 1, 14, 31) have noted that the R plasmid pool of enteric bacteria has been increasing sharply, particularly over the past decade. As the pool of R plasmid-containing enteric bacteria has increased, so has the possibility that R plasmids might emerge that could better cross biological barriers and so, perhaps, enter bacterial species and genera apparently widely different from their original enterobacterial hosts (1, 31). We believe the emergence of penicillin resistance in gonococci provides yet another example of the direct or indirect extension of the enteric R plasmid pool. The R plasmids specifying β-lactamase characterized in *H. influenzae* were shown to contain, in one case, the entire TnA segment and, in another case, at least one-third of the transposable TnA sequence that is found on a wide variety of enteric R plasmids (11, 12, 15). Similarly, in this study we have shown that R plasmids of gonococci likewise contain a significant portion (about 40%) of the TnA sequence. The possibility that a relationship exists between the emergence of plasmid-mediated resistance in *N. gonorrhoeae* and *H. influenzae* is currently under study. Whatever the relationship, the evidence that both of these formerly antibiotic-susceptible pathogens have been recently penetrated by R plasmids, perhaps of enteric origin, has profound implications. For example, a survey of selected strains of *N. meningitidis* for the presence of extrachromosomal DNA has shown that this bacterial species does not ordinarily contain plasmid DNA (Elwell and Falkow, in press). Since *N. gonorrhoeae* and *N. meningitidis* are known to be closely related, we believe that there is cause for concern that meningococci may soon acquire plasmid-mediated drug resistance.

Whether the plasmids carrying a portion of TnA now residing in *N. gonorrhoeae* were directly acquired from some unknown enteric species by conjugation or whether the R plasmids were, in effect, generated by the translocation of the ubiquitous TnA sequence into resident neisserial plasmid species is not clear from currently available data. The relationship between indigenous gonococcal plasmids and gonococcal R plasmids is currently being investigated. A 4.4-MDal plasmid is probably not self-transmissible (14); however, it may be more than fortuitous that the 24.5-MDal cryptic plasmid co-resides with the R plasmid in three of the seven ampicillin-resistant strains we have examined thus far. This large indigenous plasmid was found in only 1 of over 30 antibiotic-susceptible gonococcal isolates that we had previously examined (Elwell and Falkow, in press), and it is tempting to speculate that this large plasmid is mobilizing the smaller R plasmid during conjugation. This possibility is currently being investigated.

Continued surveillance of resistance in *H. influenzae* has provided suggestive evidence that, after the initial breach of some sort of genetic barrier leading to the emergence of R plasmids bearing ampicillin resistance, there have followed in rapid succession R plasmids specifying resistance to tetracycline (8), kanamycin (9), and linked tetracycline and chloramphenicol resistance (B. Van Klingeren, J. Van Embden, and M. Dessens-Kroon, in press). If we look to the future of antimicrobial therapy of gonococcal infections, the experience with *H. influenzae* provides a disquieting precedent.

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