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Molecular Epidemiology of Beta-Lactamase-Specifying Plasmids of *Haemophilus ducreyi*

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We have studied the genetic basis of beta-lactamase production in eight strains of *Haemophilus ducreyi* isolated in diverse areas of the world. Beta-lactamase production in all strains was mediated by plasmids having a molecular mass of either 5.7 or 7.0 megadaltons. Plasmids of 5.7 megadaltons were shown to carry the entire sequence of pFA7, the beta-lactamase specifying plasmid found in isolates of *Neisseria gonorrhoeae* epidemiologically linked to West Africa. Plasmids of 7.0 megadaltons were shown to carry the entire sequence of pFA3, the beta-lactamase specifying plasmid found in Far Eastern isolates of *N. gonorrhoeae*. Both groups of *H. ducreyi* plasmids were shown to carry physically complete and functional Tn*A* sequences. Thus we have identified two types of *H. ducreyi* beta-lactamase plasmid which are identical to the two types of *N. gonorrhoeae* beta-lactamase plasmid, except that they carry complete Tn*A* sequences.

We have previously characterized the plasmid pJB1, which was found to mediate TEM-1 beta-lactamase production in a strain of *Haemophilus ducreyi* isolated in Winnipeg, Canada (7, 20). This plasmid carries 75% of the sequences present on pMRO360, the 4.7-megadalton (Mdal) plasmid which mediates beta-lactamase production in isolates of *Neisseria gonorrhoeae* epidemiologically linked to the Far East (6, 7, 24, 26, 30). In addition, pJB1 was found to carry a complete and functional Tn*A* sequence which appeared identical to Tn*2* on the basis of restriction enzyme analysis (6). Studies of the genetic basis of beta-lactamase production in *N. gonorrhoeae* strains isolated from diverse geographic areas have shown that it is mediated either by 4.7-Mdal plasmid species identical to pFA3 (pMRO360) or by a 3.4-Mdal species identical to pFA7 (pMRO200), the plasmid found in isolates originally epidemiologically associated with West Africa (24, 26, 30). We wondered whether a similar situation existed for the beta-lactamase specifying plasmids of *H. ducreyi*. In this report, we show that the beta-lactamase specifying plasmids found in eight strains of *H. ducreyi* isolated in widely separated regions have molecular masses of 7.0 or 5.7 Mdal. These two types of *H. ducreyi* plasmid are identical to the two types of *N. gonorrhoeae* beta-lactamase plasmid, except that they carry complete Tn*A* sequences.

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MATERIALS AND METHODS

The *Escherichia coli* strains and the plasmids used in this study are listed in Table 1. The *H. ducreyi* strains are listed in Table 2. Nutrient agar supplemented with the appropriate combination of carbenicillin (20 μg/ml), kanamycin (12 μg/ml), and nalidixic acid (30 μg/ml) was used for selection in mating experiments with *E. coli*. Mating experiments with *Haemophilus influenzae* and *H. ducreyi* were performed as previously described (7). In this study, we have used the gonococcal beta-lactamase plasmids pFA3 and pFA7 for comparison with *H. ducreyi* plasmids. These are identical to pMRO360 and pMRO200, respectively (31).

Transformation of *Escherichia coli* with purified plasmid DNA was performed by the method of Cohen et al. (8). Transformation of *H. influenzae* Rd was performed as previously described (3). Cleared lysates of *E. coli* were prepared as described by Grinsted et al. (16). Cleared lysates of *H. ducreyi* were prepared as described by Elwell et al. (14), except that a final concentration of 0.15% Sarkosyl was added to enhance lysis. Plasmid DNA was purified from cleared lysates in CsCl-ethidium bromide density gradients. Restriction endonucleases were purchased from Bethesda Research Laboratories. Reactions were carried out in the conditions recommended by the supplier. Electrophoresis was performed with Tris-borate buffer (23) in 0.7 to 1.5% agarose or 7.5% polyacrylamide gels. The molecular weight of fragments was estimated by comparison with ϕX174 RFII DNA cleaved with *Hin*cII or phage lambda DNA cleaved with *Hin*dIII or *Bgl*I (J. Grinsted, unpublished data). Mapping was performed by simultaneous digestion with one, two, or

TABLE 1. *E. coli* strains and plasmids used

Strain	Genotype	Derivation	Molecular mass (Mdal)	Phenotype[a]	Reference or source
E. coli					
JC6310	*his lys trp recA56 rpsL*				A. J. Clark (1, 6)
SF800	*thy polA nalA*				S. Falkow (6, 11)
Plasmids					
pFA3 (identical to pMR0360)		*N. gonorrhoeae*	4.7	Ap^r	P. Sparling (24, 29, 30)
pFA7 (identical to pMR0200)		*N. gonorrhoeae*	3.4	Ap^r	P. Sparling (24, 29, 30)
pJB1		*H. ducreyi* (54198)	5.7	Ap^r	(6, 7)
pHD131		*H. ducreyi* (HD131)	7.0	Ap^r	This study
pHD147		*H. ducreyi* (HD147)	23	Conjugative; mobilizes pHD131, pFA3 and pJB1	Deneer *et al.* (12)
pHD148		*H. ducreyi* (HD148)	4.9	Su^r	Albritton *et al.* (2)
pUB307		Carbenicillin-susceptible derivative of RP1		Tc^r, Km^r	G. Howe (5, 6)

[a] Nomenclature as described by Bachmann and Low (4): Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Su^r, sulfonamide resistant; Km^r, kanamycin resistant.

three enzymes. When more than one plasmid was present in the *H. ducreyi* isolate, the beta-lactamase plasmid for restriction endonuclease analysis was isolated from *H. influenzae* or *E. coli* transformants.

Electron microscope heteroduplex analysis was performed as previously described (6, 10). The preparations were reannealed in 50% formamide–0.05 M NaOH–0.1 M Tris-hydrochloride (pH 8.5) at 30°C for 5 h. These conditions are about 20°C below the melting temperature of DNA having a guanine-plus-cytosine content of 41% (22, 27). Nicked φX174 RFII DNA was included as an internal contour length standard for double-stranded DNA. Alkali-denatured φX174 RFII served as an internal single-stranded standard.

Southern blotting. Probe DNA was purified by rebanding twice in CsCl-ethidium bromide density gradients, before being nick translated as described by Maniatis et al. (21) with ^{32}P-labeled dCTP. Plasmid DNA was transferred to nitrocellulose from agarose gels by the method of Southern (28) after being depurinated with 0.25 N HCl. Filters were preincubated in Denhardt mix (13) before hybridization in 2× SSC (1×

TABLE 2. *H. ducreyi* strains used

Strain	Molecular mass of plasmid complement (Mdal)	Phenotype[a]	Area of isolation	Reference or source
54198	5.7	Ap^r, Tc^r	Winnipeg	G. Hammond (17)
HD4391	5.7	Ap^r, Tc^r	Sweden	E. Falsen
HD115	5.7	Ap^r, Tc^r	Kenya	A. Ronald
109	5.7, 4.9	Ap^r, Tc^r, Su^r	Atlanta	R. Weaver
HD643	23, 5.7	Ap^r, Tc^r	Kenya	A. Ronald
HD551	30, 5.7, 4.9	Ap^r, Tc^r, Su^r	Kenya	A. Ronald
HD131	7.0	Ap^r, Tc^r	Kenya	A. Ronald
9468	7.0, 4.9	Ap^r, Tc^r, Su^r	Kenya	A. Ronald
9265	30, 7.0	Tc^r, Ap^r	Kenya	A. Ronald
HD147	23, 7.0, 4.9	Ap^r, Su^r	Kenya	A. Ronald
V1159	34, 7.0	Cm^r, Tc^r, Ap^r	Seattle	K. Holmes
HD148	23, 7.0, 4.9	Ap^r, Tc^r, Su^r	Kenya	Albritton et al. (2)
HD696	23, 7.0	Ap^r, Tc^r	Kenya	A. Ronald

[a] See footnote *a* of Table 1.

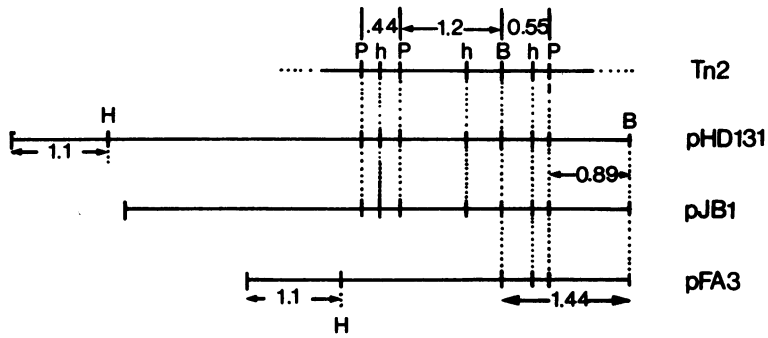

FIG. 1. Restriction endonuclease map of pHD131, pJB1, and pFA3 compared with a map of Tn*2*. Molecular weights of important fragments are indicated in megadaltons. Abbreviations: B, *Bam*HI; h, *Hin*cII; P, *Pst*I; H, *Hin*dIII.

SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–0.1% sodium dodecyl sulfate with 10^6 cpm of probe DNA at 37°C for 18 h. Assuming a guanine-plus-cytosine content of 41%, these conditions are 20°C below the melting temperature (22, 27). After hybridization, filters were washed in 5× SSC at 60°C for 1 h before being dried and subjected to autoradiography.

Plasmid molecular weight was estimated as described by Meyers et al. (23) with R1-*drd*19, RP4, S-a, RSF1010, and pMB8 as standards. Molecular weights were also determined by electron microscopy with φX174 RFII DNA as an internal standard. Gel electrophoresis data gave a small but significant overestimate of the mass of the 7.0-Mdal plasmids; hence electron microscope data were used exclusively to determine molecular mass.

RESULTS

Association of plasmid species with antibiotic resistance. The data in Table 2 show that all ampicillin-resistant isolates of *H. ducreyi* contain either a 5.7-Mdal or a 7.0-Mdal plasmid species. In addition, some strains also contained a 30-Mdal or a 23-Mdal plasmid and/or a 4.9-Mdal plasmid species. We have recently shown that the 4.9-Mdal plasmids specify sulfonamide resistance and are 79% related to RSF1010 (2). Conjugation experiments showed that the 30-Mdal plasmids specified tetracycline resistance. Strain V1159 transferred linked resistance to tetracycline and chloramphenicol which was specified by a 34-Mdal plasmid which could only be visualized in cleared lysates of the transconjugant. The 23-Mdal plasmid pHD147 carried no antibiotic resistance determinants (12). Conjugative transfer of ampicillin resistance in our strains of *H. ducreyi* was found to occur only by mobilization of 5.7- or 7.0-Mdal plasmids by pHD147 or other 23-Mdal plasmids. Rarely, however, strains containing the 23-Mdal plasmid and a 5.7- or 7.0-Mdal beta-lactamase plasmid conjugally transferred a 26-Mdal beta-lactamase specifying plasmid. Although this recombinant plasmid was not analyzed in detail, it was presumed to represent a transposition of Tn*A* onto the 23-Mdal plasmid because of the 3-Mdal difference in molecular weight. It was of some interest to note that we were unable to detect beta-lactamase specifying recombinants derived from the 30-Mdal conjugative tetracycline resistance plasmid. This plasmid was incapable of mobilizing 5.7- or 7.0-Mdal beta-lactamase plasmids at a detectable frequency.

Transformation of plasmid DNA into *H. influenzae* Rd showed that beta-lactamase production was mediated exclusively by 5.7- and 7.0-Mdal plasmids, and that these plasmids were not responsible for tetracycline resistance. Southern blotting of whole plasmid DNA subjected to electrophoresis with pJB1 as a probe showed that pJB1 was homologous to the 5.7- and 7.0-Mdal plasmids, but not to the 4.9- or 30-Mdal plasmids (data not shown).

Comparison of plasmids by restriction endonuclease digestion. Figure 1 shows a comparison of the restriction endonuclease maps of pHD131, pJB1, pFA3, and Tn*2*. Brunton et al. (6, 25; unpublished data) showed that the map of Tn*2* was identical to a portion of that of pJB1 for the enzymes *Pst*I, *Hin*cII, *Bgl*I, and *Bam*HI, whereas pMRO360 (and pFA3) possessed only those sites rightward of the *Bam*HI site in Tn*A*. It is apparent that all of the *Pst*I, *Hin*cII, and *Bam*HI sites within Tn*A* are present in pHD131, suggesting that this plasmid contains the entire Tn*A* sequence. All three plasmids have a common *Bam*HI fragment of 1.44 Mdal. pHD131 differs from pJB1 by having a *Hin*dIII site which maps in a position with respect to the *Bam*HI site which is identical to the *Hin*dIII site in pFA3. Thus, restriction mapping suggests that pHD131 carries the whole Tn*A* sequence and the non-Tn*A* sequences which are present on pFA3. Restriction digests of the 5.7-Mdal plasmids

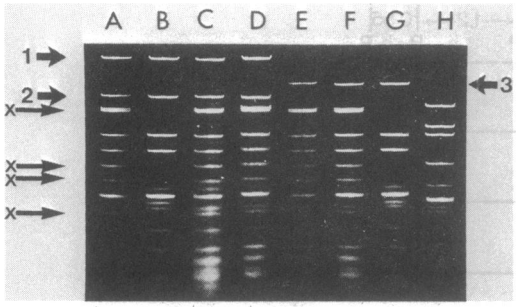

FIG. 2. Polyacrylamide gel electrophoresis of *Alu*I digests of β-lactamase specifying plasmid DNA. Lanes A, pHD131; B, pFA3; C, 9265 (7.0 Mdal); D, V1159 (7.0 Mdal); E, HD115 (5.7 Mdal); F, pJB1; G, pFA7; H, φX174 RFII cleaved with *Hin*cII. The numbers 1 and 2 denote the two largest *Alu*I fragments of pFA3 which are seen to comigrate with the corresponding fragments of the 7.0-Mdal plasmids in lanes A, C, and D; 3 denotes the largest *Alu* fragment of the 5.7-Mdal plasmids which comigrates with that of pFA7. X denotes fragments found in *H. ducreyi* plasmids but not in the gonococcal plasmids. The molecular masses of the largest five *Hin*cII fragments of φX174 are 0.68, 0.50, 0.39, 0.32, and 0.25 Mdal.

from strains HD115, HD4391, and 109 and the 7.0-Mdal plasmids from strains V1159, 9468, and 9265 contained the 1.44-Mdal *Bam*HI fragment. All of the plasmids contained all of the *Hin*cII, and *Pst*I sites expected in Tn*2*. All of the 7.0-Mdal plasmids had a *Hin*dIII site which mapped in the same position as that found in pHD131. The enzyme *Alu*I was next used to examine the similarity between plasmids pFA7, pFA3, pJB1, and pHD131. This enzyme was used because its four-base-pair recognition sequence would result in considerably more cut sites in the plasmids and because it has been used to produce a map of pFA3 and pFA7 (29). The map published by Sox et al. (29) shows that the two largest *Alu*I fragments of pFA3 lie in an area which surrounds the position which we have found to contain the *Hin*dIII site. These fragments and a smaller intervening *Alu*I fragment, are partially deleted and fused to form a single fragment in pFA7 (29). Figure 2 shows a comparison of *Alu*I digests of pFA3, pFA7, pJB1, the 5.7-Mdal plasmid from strain HD115, and the 7.0-Mdal plasmids from the strains V1159, HD131, and 9265. It is evident that the two largest fragments cut from the 7.0-Mdal plasmids comigrate with the two largest fragments of pFA3. Similarly, the largest fragment cut from pJB1 and the 5.7-Mdal plasmid of strain HD115 comigrate with that of pFA7. Digests of the 7-Mdal plasmid of 9468 and the 5.7-Mdal plasmid of 109 showed identical *Alu*I restriction patterns to the other 7- and 5.7-Mdal plasmids, respectively (data not shown). Other than the difference in migration of the largest fragments, all other fragments of the 5.7- and 7.0-Mdal plasmids comigrate in acrylamide gels. A number of fragments found in the *H. ducreyi* plasmid digests which are absent from the gonococcal plasmid digests were presumed to be cut from the Tn*A* sequences present in the former and absent from the latter. This was confirmed by hybridization of nick-translated RSF1030 to *Alu*I-digested pJB1 and pHD131 DNA which had been transferred electrophoretically from acrylamide gels to diazobenzyloxymethyl paper (data not shown).

Tn*A* sequence of pHD131 is functional. Since pHD131 contained at least 2.2 Mdal of Tn*A* sequences, we wondered whether the Tn*A* sequence was functional. pHD131 and pUB307 were grown in the *recA* host JC6310 at 30°C for 50 generations. JC6310 containing these plasmids was mated with SF800, and transconjugants showing stable inheritance of linked resistance to tetracycline, kanamycin, and carbenicillin were isolated. Analysis of the plasmid complement of these strains showed a single plasmid with a molecular mass approximately 3 Mdal greater than that of pUB307. Molecular analysis of these recombinant plasmids showed that they contained three *Pst*I sites distributed as in Tn*A* which were not present in pUB307. Five recombinant plasmids were analyzed by restriction endonuclease digestion with *Bam*HI and *Eco*RI as previously described (6, 16). The Tn*A* insertion was shown to occur in at least four distinct sites in the five clones examined.

Electron microscope analysis of heteroduplexes was used to confirm the strong suggestion derived from the above experiments that the *H. ducreyi* plasmids contained complete Tn*A* sequences and that 7.0-Mdal *H. ducreyi* plasmids differ from 5.7-Mdal plasmids only in a 1.3-Mdal sequence containing a *Hin*dIII site, which is identical to a sequence present in pFA3. Figure 3 shows a series of heteroduplexes. It is evident that pFA3 is entirely homologous with pHD131, but that the latter contains 2.35 Mdal of additional sequences most of which are presumably Tn*A* sequences. Similarly pFA7 is entirely homologous to pJB1, except that the latter contains 2.35 Mdal of additional sequences which are also presumably Tn*A* sequences. pJB1 is entirely homologous to pHD131, except that the latter contains an insertion loop of 1.3 Mdal which was found to contain a *Hin*dIII site in other experiments (data not shown). As expected, heteroduplexes between pHD131 and pFA7 show that all pFA7 sequences are present in pHD131. Two insertion loops—one of 1.3 Mdal and the other of 2.35 Mdal (presumably Tn*A* sequences)—are seen. Again, other experiments showed that the 1.3-Mdal sequence contained a *Hin*dIII cleavage site.

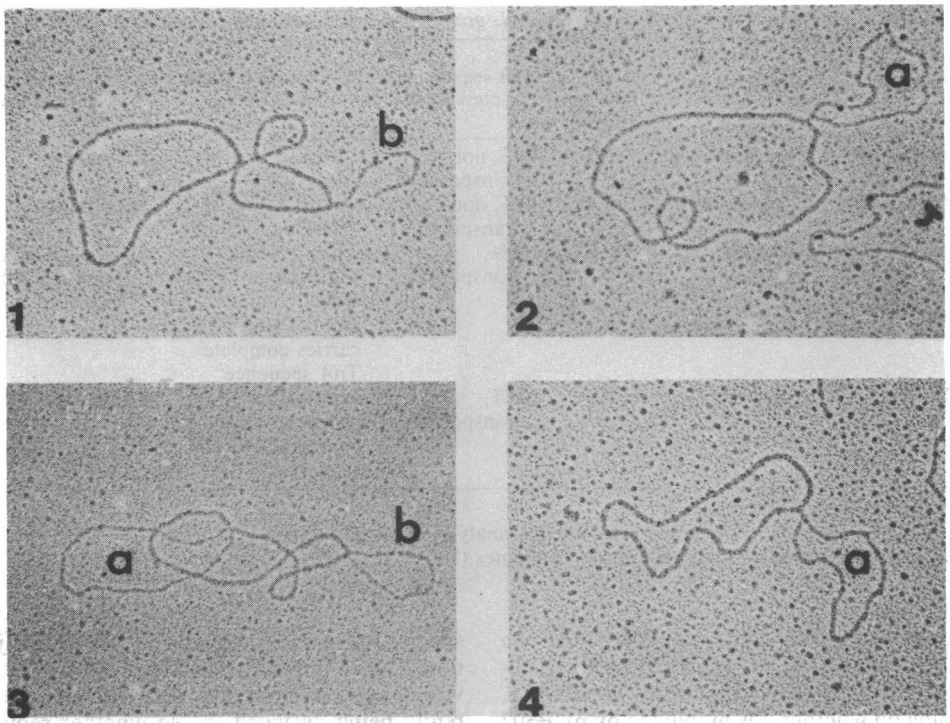

Fig. 3. Heteroduplexes of *H. ducreyi* and gonococcal beta-lactamase plasmids. Panels: 1, pHD131 and pJB1; 2, pHD131 and pFA3; 3, pHD131 and pFA7; 4, pJB1 and pFA7. Insertion-deletion loops labeled a have contour lengths corresponding to 2.35 Mdal and presumably contain Tn*A* sequences. Insertion-deletion loops labeled b are 1.3 Mdal in size and were found to contain the *Hin*dIII cut site in other experiments.

DISCUSSION

We have reported that beta-lactamase production in a strain of *H. ducreyi* isolated during an outbreak of chancroid in Winnipeg, Canada, was mediated by the 5.7-Mdal plasmid pJB1 (7). This plasmid was subsequently shown to contain 75% of pMRO360 (pFA3) sequences and to contain a physically complete and biologically functional Tn*A* sequence (6; unpublished data). The restriction enzyme map of a portion of pJB1 was shown to be identical to that of Tn*2* for the enzymes *Pst*I, *Bam*HI, *Bgl*I, and *Hin*cII (6). In this study, we have shown that beta-lactamase production in a variety of *H. ducreyi* strains isolated in Europe, North America, and Africa is mediated either by 5.7-Mdal plasmids which appear identical to pJB1 on the basis of restriction enzyme digestion or by 7.0-Mdal plasmids which are identical to pJB1, except that they possess an additional 1.3-Mdal sequence containing a *Hin*dIII site. Both types of plasmid contain a complete and functional Tn*A* sequence. It is of considerable interest that we have shown in the present study that the 7.0-Mdal plasmid pHD131 carries the entire sequences of pFA3, whereas pJB1 carries the entire sequence of pFA7. These findings are summarized in Table 3.

The finding that beta-lactamase specifying plasmids of *H. ducreyi* isolated from a variety of geographic locations share a common core of homologous sequences parallels the situation in *N. gonorrhoeae*, *H. influenzae*, and *Haemophilus parainfluenzae* (19, 24, 26, 30, 31). The widespread occurence of these highly homologous beta-lactamase specifying plasmids in *N. gonorrhoeae* and *Haemophilus* species is analogous to the widespread occurence of the 5.5-Mdal beta-lactamase specifying plasmid RSF1030 in enteric bacteria. Plasmids highly homologous to RSF1030 have been found in isolates of *Salmonella panama*, *Shigella dysenteriae* isolated in Bangladesh, Mexico, and Central America, and *Citrobacter freundii* (9). In *H. ducreyi* there does not appear to be a clear-cut geographical separation of the two molecular classes of plasmid as originally occurred in *N. gonorrhoeae* (24, 26).

The function of the 1.3-Mdal sequence containing the *Hin*dIII recognition site in pFA3 and pHD131 seems to be related to plasmid mobilization. Although Flett et al. found that the 24-Mdal gonococcal conjugative plasmid mobilized

TABLE 3. Comparison of *H. ducreyi* and *N. gonorrhoeae* beta-lactamase specifying plasmids

Plasmid	Host species	Molecular mass (Mdal)	TnA sequences present	Relationship to pFA3 (pMR0360)	Areas of isolation of similar plasmids
pFA3 (pMR0360)	*N. gonorrhoeae*	4.7	40%, not transposable		Far East
pFA7 (pMR0200)	*N. gonorrhoeae*	3.4	40%, not transposable	Result of 1.3-Mdal deletion of pFA3[a]	West Africa
pJB1	*H. ducreyi*	5.7	100%, transposable	Missing 1.3-Mdal sequence of pFA3 which contains *Hin*dIII site, carries complete TnA sequence[b]	Sweden, Kenya, Thailand, Atlanta, Winnipeg
pHD131	*H. ducreyi*	7.0	100%, transposable	Carries complete sequence of pFA3 and complete TnA sequence[b]	Kenya, Seattle[c]

[a] Assessed by restriction mapping (30).
[b] Assessed by electron microscopic heteroduplex analysis (6; this study).
[c] This strain was originally acquired in the Phillipines (18).

pJB1 and pFA3 with equal efficiency (15), we have found that pJB1 is mobilized 50 to 1,000-fold less efficiently than either pFA3 or pHD131, depending on whether it is mobilized by pUB307 or pHD147 (J. Brunton and W. Albritton, unpublished data).

Sox et al. were able to produce plasmids identical to the West African *N. gonorrhoeae* beta-lactamase specifying plasmid pFA7 by transformation of *N. gonorrhoeae* with purified pFA3 DNA (29). The implication of this study was that pFA7 might have been directly derived from pFA3 by deletion occurring during transformation of *N. gonorrhoeae* by plasmid DNA (29, 30). Two plasmid types (5.7-Mdal pJB1 and 7.0-Mdal pHD131) are found in *H. ducreyi* that are apparently identical to pFA7 and pFA3, respectively, except that they contain complete TnA sequences. This strongly suggests that the gonococcal plasmids could have been introduced to *N. gonorrhoeae* from *H. ducreyi* or another *Haemophilus* species. Deletion of the large portion of TnA sequences could have occurred either before or during transfer to *N. gonorrhoeae* since the deleted plasmids RSF0885 and pVE445 have been found in *H. parainfluenzae* and *H. influenzae*, respectively (14, 19).

How these plasmids could have been transferred from *Haemophilus* species to *N. gonorrhoeae* is not known, although Sparling et al. found that conjugal transfer to a 4.7-Mdal plasmid identical to pFA7 from a strain of *H. parainfluenzae* to *N. gonorrhoeae* did occur (30). However, using agarose gel electrophoresis of cleared lysates prepared by the Meyers method, they were able not to visualize the plasmid species presumably responsible for the conjugation. The conjugative plasmid pHD147 may provide the explanation (12). Experiments are currently being designed to see whether replicons homologous to pHD147 are capable of mobilizing pFA7 and pFA3 into *N. gonorrhoeae*.

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