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Plasmid-Coded Ampicillin Resistance in *Haemophilus ducreyi*

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Seven of the 96 ampicillin-resistant isolates of *Haemophilus ducreyi* reported in the preceding article (Bilgeri et al., Antimicrob. Agents Chemother. 22:686-688, 1982) were investigated and found to harbor plasmids of 3.95, 5.2, 5.8, and 6.4 megadaltons. All except the 5.8-megadalton plasmid have been shown to code for β-lactamase. The 6.4- and 5.2-megadalton plasmids of three isolates were conjugally transferable to a streptomycin-resistant mutant of *H. ducreyi* at high frequencies, perhaps due to the presence in these strains of a high-molecular-weight plasmid.

Hammond et al. (7) found that 3 of 19 strains of *Haemophilus ducreyi* isolated in an outbreak of chancroid in Winnipeg, Manitoba, Canada, produced β-lactamase, and Brunton et al. (3) found that all three harbored a 6.0×10^6-dalton plasmid which coded for β-lactamase but was not conjugally transferable to *Haemophilus influenzae*. In a later study, Handsfield et al. (8) found that seven ampicillin-resistant (Ap^r) *H. ducreyi* isolates from the United States and abroad carried plasmids of 3.6, 5.7, and 7.3 megadaltons (Mdal) coding for β-lactamase. Plasmid molecular weights were identical for isolates from epidemiologically linked cases and differed according to the geographic origin of the strains. Handsfield and co-workers did not investigate the transmissibility of these plasmids.

In a recent study, Bilgeri et al. (1) found that 96 of 103 strains of *H. ducreyi* isolated from patients suffering from chancroid in the Johannesburg area were Ap^r. We wondered, therefore, whether these resistant strains carried plasmids coding for β-lactamase and, if so, whether they were conjugally transferable, as this could possibly account for the extremely high percentage (93%) of Ap^r *H. ducreyi* strains isolated from patients suffering from chancroid. We report here the demonstration that all of the Ap^r *H. ducreyi* isolates studied carried plasmids coding for β-lactamase, ranging in size from 3.95 to 6.4 Mdal. Two of these plasmids are conjugally transferable at high frequencies to a streptomycin-resistant mutant of *H. ducreyi*, perhaps due to the presence in these donor stains of a high-molecular-weight plasmid.

H. ducreyi isolates were obtained from patients with clinical chancroid in the Johannesburg environs and were isolated at 35°C on MHIC medium (1) with 3 μg of vancomycin per ml to inhibit the growth of contaminating microorganisms. The Ap^r local isolates, GU 10, GU 36, GU 55, GU 68, GU 71, GU 75, and GU 80, were of low passage numbers to mitigate possible plasmid segregation. The *H. ducreyi* reference strain, ATCC 27722, was obtained from the American Type Culture Collection, Rockville, Md., and GU 74 was a local Ap^s isolate. *Escherichia coli* HB101(pBR322) was used as a control as it carries a 2.6-Mdal plasmid coding for ampicillin and tetracycline resistance (2). Minimum inhibitory concentrations of antibiotics were determined, and β-lactamase production was analyzed as described (1).

All of the Ap^r isolates were shown to produce TEM-type β-lactamase, and analysis of the minimum inhibitory concentrations of these strains showed that they were resistant to concentrations of ampicillin ranging from 16 to 128 μg/ml (1). To minimize the chance of spontaneous mutation to ampicillin resistance, 64 μg/ml was taken as the base line for ampicillin resistance, and all MHIC agar plates used for growth and selection of β-lactamase-producing strains contained 64 μg of ampicillin per ml. The Ap^r strains were stable and did not lose their antibiotic resistance even when subcultured 10 times onto MHIC agar without ampicillin.

To screen β-lactamase-producing strains for the presence of plasmids, the growth of *H. ducreyi* from two or three plates of MHIC agar was suspended and washed in TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris, pH 8.0). Cleared lysates were prepared and subjected to agarose gel electrophoresis as described by Meyers et al. (10), except that lysozyme treatment of the cells was followed by the addition of 1 mg of *N*-lauroyl sarcosine (Sigma) per ml for 30 min to effect complete cell lysis. As controls,

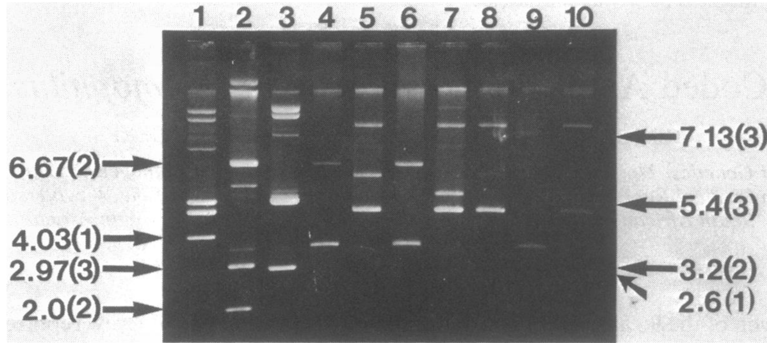

FIG. 1. Agarose gel electrophoretic patterns of plasmids isolated from *H. ducreyi*. The molecular weights of the control plasmids are indicated by arrows, with their lanes shown in parentheses. Lane 1; pHP3 (11.88 Mdal), pJW101 (4.03 Mdal), pBR322 (2.6 Mdal); lane 2, pDB248 (6.67 Mdal), pBD64 (3.2 Mdal), pC194 (2 Mdal); lane 3; pAW101 (7.13 Mdal), pBRZ1 (5.4 Mdal), pUB110 (2.97 Mdal). Lane 4; GU 10; lane 5; GU 36; lane 6; GU 55; lane 7; GU 68; lane 8; GU 71; lane 9; GU 75; lane 10; GU 80.

the Ap^s strains GU 74 and ATCC 27722 were used. All of the β-lactamase-producing strains carried plasmids (Fig. 1). The Ap^s strains did not (results not shown). It was of interest that the migration patterns of the plasmids from the different β-lactamase-producing strains in 0.7% agarose gels exhibited considerable heterogeneity.

Comparisons of the mobilities of the Ap^r plasmids with plasmids of known molecular weight (pC194, pBR322, pUB110, pBD64, pJW101, pBRZ1, pDB248, and pAW101, which range in size from 2 to 7.13 Mdal) showed that strains GU 10, GU 55, and GU 75 each carried a plasmid having a molecular mass of 3.95 Mdal, strains GU 36, GU 71, and GU 80 each carried two plasmids of 6.4 and 5.2 Mdal, and strain GU 68 carried a 5.2- and a 5.8-Mdal plasmid (Fig. 1).

To determine whether the plasmids coded for β-lactamase production and to ascertain whether the multiple plasmid bands found in some of the Ap^r strains represented different plasmids or merely different conformations of the same plasmid, *E. coli* C600 was transformed by the method of Cohen et al. (4), using either cleared lysates or DNA electroeluted at 90 mA for 2h out of individual plasmid bands into TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). The cleared lysate of strain HB101(pBR322), coding for resistance to ampicillin and tetracycline, and cleared lysates of two *H. ducreyi* Ap^s strains, GU 74 and ATCC 27722, were used as controls.

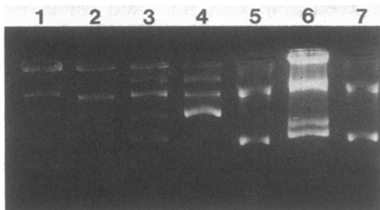

FIG. 2. Agarose gel electrophoretic patterns of plasmids isolated from *E. coli* transformed with *H. ducreyi* cleared lysates or with DNA electroeluted from *H. ducreyi* plasmid bands as follows: Lane 1; GU 10, slower migrating band; lane 2; GU 10, faster migrating band; lane 3; GU 36 cleared lysate; lane 4; GU 36, slower migrating band; lane 5; GU 36, faster migrating band; lane 6; GU 68, cleared lysate; lane 7; GU 68, faster migrating band.

All of the lysates of the β-lactamase-producing *H. ducreyi* isolates, as well as of strain HB101(pBR322), transformed *E. coli* C600 to ampicillin resistance, whereas the Ap^s strains did not. Efficiency of transformation by the *H. ducreyi* lysates ranged from 10^3 to 10^4 transformants per μg of DNA. Tetracycline resistance was only conferred by transformation with pBR322. The results of transformation of *E. coli* C600 by DNA electroeluted from individual plasmid bands (Fig. 2) indicated that strains GU 10, GU 55, and GU 75 each carried one Ap^r plasmid (3.95 Mdal; results shown for GU 10), and GU 36, GU 71, and GU 80 each carried two Ap^r plasmids (6.4 and 5.2 Mdal; results shown for GU 36); for GU 68, only the 5.2-Mdal plasmid could conclusively be shown to code for β-lactamase. Transformation efficiency with electroeluted DNA ranged from 1×10^2 to 5×10^2 transformants per μg of DNA.

To test for plasmid transfer, a spontaneous streptomycin-resistant mutant of the Ap^s strain ATCC 27722 was isolated for counterselection use in conjugation studies. This was done by subculturing onto increasing (log^2) levels of streptomycin. The mutant obtained grew well at 32 μg of streptomycin per ml, and the Ap^r isolates showed no spontaneous mutation to this level. Conjugation experiments were carried out

by spotting 0.1-ml samples of donor and recipient strains onto the surface of a chocolate agar plate, since the membrane filter technique of Thorne and Farrar (11) resulted in a marked loss of viability of all strains. All cultures were pretreated with DNase (25 μg/ml) to prevent the transformation of recipient bacteria by DNA released from donor cells. After overnight incubation at 37°C, the growth was scraped off the plate and suspended in 1 ml of Mueller-Hinton broth (1). Transconjugants were selected on chocolate agar or MHIC agar supplemented with 64 μg of ampicillin and 32 μg of streptomycin per ml. Donor and recipient strains were enumerated on chocolate agar or MHIC agar supplemented with 64 μg of ampicillin and 32 μg of streptomycin per ml. Spontaneous mutation of the donor strains to streptomycin resistance and of the recipient to ampicillin resistance was tested by plating donor and recipients on the counter-selecting antibiotic plate. Ampicillin transconjugants were checked for β-lactamase production.

Transfer of ampicillin resistance from *H. ducreyi* strains GU 36, GU 71, and GU 80 to the spontaneous streptomycin-resistant mutant of ATCC 27722 occurred at frequencies of 1×10^{-1} per donor and 2×10^{-3} per recipient. Transconjugants of GU 36, GU 71, and GU 80 carried both the 6.4- and the 5.2-Mdal plasmids (Fig. 3b; results shown for GU 36 and GU 80). It was surprising that Ap^r plasmids with such low molecular weights could code for their own transfer, so we screened GU 36, GU 71, and GU 80 for the presence of large plasmids by using a modification of the method of Kado and Liu (9) which specifically shows the presence of plasmids of high molecular weights (unpublished data). All three strains harbored large plasmids (Fig. 3a) whose mobilities on agarose were similar to that of the *Pseudomonas aeruginosa* plasmid RP4, which has a molecular size of 36 Mdal. These large plasmids were not found in the Ap^r *E. coli* transformants, nor in the *H. ducreyi* transconjugants. They could be mobilizing the 5.2- and 6.4-Mdal Ap^r plasmids of GU 36, GU 71, and GU 80. Deneer et al. (5) have also found in Ap^r isolates of *H. ducreyi* a large (23.5-Mdal) phenotypically cryptic plasmid which has mobilizing capabilities.

These results show that in the seven Ap^r isolates of *H. ducreyi* from the Johannesburg area tested, resistance is encoded by three plasmids with molecular masses of 3.95, 5.2, and 6.4 Mdal. The 6.4- and 5.2-Mdal plasmids of GU 36, GU 71, and GU 80 were transferable to a streptomycin-resistant mutant strain of *H. ducreyi*, possibly due to the fact that these strains harbor large-molecular-weight plasmids which could be capable of mobilizing the small Ap^r plasmids. These findings could explain the extraordinarily

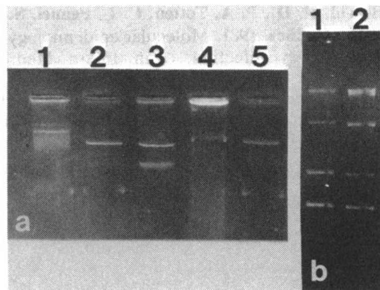

FIG. 3. Agarose gel electrophoretic patterns of (a) high-molecular-weight plasmids isolated according to Kado and Liu (9) and (b) plasmids isolated from transconjugants. (a) Lane 1; *Agrobacterium tumefaciens* C58 harboring a cryptic plasmid (300 Mdal) and the Ti plasmid (120 Mdal); lane 2; *E. coli* harboring the *P. aeruginosa* plasmid RP4 (36 Mdal); lane 3; GU 36; lane 4; GU 71; lane 5; GU 80. The diffuse bands in lanes 2 and 3 are chromosomal DNA. (b) Lane 1; GU 36 × ATCC 27722 transconjugant; lane 2; GU 80 × ATCC 27722 transconjugant.

high incidence of Ap^r *H. ducreyi* reported by Bilgeri et al. (1). Moreover, in contrast to the findings by Handsfield et al. (8), we find that geographically related strains carry very different plasmids. This would, therefore, indicate that the isolates with different plasmids are not epidemiologically related, and as this infection has been shown to be sexually transmitted (8) a variety of donor individuals must exist in the population.

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