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Characterization of Ampicillin Resistance Plasmids from *Haemophilus ducreyi*

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Seven strains of *Haemophilus ducreyi* from diverse geographic origins were analyzed for their plasmid content. All strains were multiply resistant, but only resistance to ampicillin was transferred to *Escherichia coli* by transformation. The *H. ducreyi* plasmids encoding for ampicillin resistance were 7.4, 5.7, and 3.6 megadaltons and encoded for part or all of Tn*A*, the ampicillin transposon. The relatedness of these plasmids was examined by restriction endonuclease digestion and DNA-DNA homology with isolated DNA fragments from Tn*A*.

Although chancroid is usually a disease of tropical and subtropical countries, recent outbreaks have occurred in Europe, Greenland, and North America (10, 17, 23). *Haemophilus ducreyi*, the organism associated with this disease, has infrequently been isolated from suspected cases, because it is a fastidious organism, difficult to isolate from other bacterial flora. Recent improvements in isolation media (9, 27) and procedures for identification (8) have allowed a more accurate assessment of the role of *H. ducreyi* in genital ulcers clinically resembling chancroid.

Strains of most pathogens can be differentiated on the basis of serotyping, bacteriocin or bacteriophage typing, biochemical differences, or auxotrophic markers; strain differentiation has not been possible with *H. ducreyi*, however. Markers used to identify strains could greatly enhance the understanding of the epidemiology of infection by this organism. A new technique which could be used to characterize and differentiate strains is restriction endonuclease fingerprinting of resident plasmids. This method has been used in other organisms both to document the spread of resistances from strain to strain and to evaluate the spread of resistant organisms within a patient population (20, 24, 30). We recently isolated six strains of antibiotic-resistant *H. ducreyi* from cases of chancroid in Seattle, Wash. Three of these patients had recently returned from the Philippines, Brazil, or Mexico, and three were epidemiologically linked to a patient who acquired the infection in the Philippines. We have reported that antibiotic resistance patterns and plasmid content differentiated these bacteria by the country of origin (11). In this study, we investigated further the nature of the antibiotic resistances in these strains and used restriction endonuclease fingerprinting and DNA-DNA homologies to further characterize the resident plasmids.

MATERIALS AND METHODS

Strains. The bacterial isolates used in this study are listed in Table 1. With the exception of strain 54198, which was kindly provided by W. L. Albritton and A. R. Ronald (University of Manitoba, Winnipeg, Manitoba), all strains were obtained from patients seen between May 1979 and January 1980 at the U.S. Public Health Service Hospital or at the Sexually Transmitted Disease Clinic, Harborview Medical Center, Seattle, Wash. Since all chancroid cases could be traced to persons returning from outside the United States or who had acquired their infection outside the United States, the country of origin is also listed in Table 1. The clinical evaluation of patients and isolation of organisms have been previously described (11). Briefly, specimens from patient lesions were inoculated onto agar plates containing GC agar base (BBL Microbiology Systems, Cockeysville, Md.) which was supplemented with 5% sheep blood, heated, and then further supplemented with 1% IsoVitaleX (BBL), 5 μg of vancomycin per ml, and 10% fetal calf serum. The inoculum was then streaked for isolation and incubated for up to 7 days in candle jars at 35°C. Wet gauze pads placed on the bottom of the jar provided the added humidity needed by these organisms (A. R. Ronald, personal communication). Colonies with typical morphology and Gram strains usually appeared in 2 to 4 days and were subcultured to chocolate agar plates without antibiotics. All strains were confirmed as *H. ducreyi* by their inability to synthesize porphyrins and the requirement for hemin but not NADH (8). The latter test was modified by adding glucose (0.1%), glutamine (0.01%), and cysteine (0.026%) to the GC agar base (Ronald, personal communication).

TABLE 1. *H. ducreyi* strains and their plasmids

Strain	Origin	Antimicrobial resistance phenotype[a]	Ap plasmid	Plasmid size (Mdal)
V-1157	Sexual contact of person infected in the Philippines	Ap Tc Cm	pUW113	7.4
V-1158	Sexual contact with above	Ap Tc Cm	pUW114	7.4
V-1159	Sexual contact with above	Ap Tc Cm	pUW115	7.4
V-1168	Brazil	Ap Tc	pUW116	3.6
V-1169	Philippines	Ap Tc Cm	pUW117	7.4
V-1171	Mexico	Ap Tc	pUW118	5.7
54198	Canada	Ap Tc	pJBI	5.7[b]

[a] All Ap strains produced beta-lactamase. Minimum inhibitory concentrations were: Pc, ≥32 μg/ml; Tc, ≥16 μg/ml; Cm, ≥16 μg/ml (11).
[b] Determined by Brunton et al. (2).

Purification of plasmid DNA. Cells were scraped from chocolate agar plates containing 1 μg of ampicillin per ml and lysed by a modification of the procedure of Elwell et al. (6). Briefly, the cells were suspended in a solution of 15% sucrose and 0.5 M Tris, pH 7.5, and then sequentially treated with 0.2 volume of lysozyme (10 mg/ml) and 0.4 volume of EDTA (0.25 M, pH 8). An equal volume of Triton lytic mix (0.1% Triton X-100, 0.05 M Tris, 0.0625 M EDTA, pH 8) was added, and lysis was accomplished by heating this mixture to 60°C after the addition of 0.02% Sarkosyl. Plasmids were purified by centrifugation in two successive cesium chloride-ethidium bromide density gradients, ethidium bromide was extracted with cesium chloride-saturated isopropanol, and the mixture was dialyzed against 10 mM Tris–1 mM EDTA, pH 7.5.

Electron microscopy. The technique used for electron microscopy of plasmid DNA has been described (14). Molecular weights were determined by comparing contour length measurements of the test plasmid to standard plasmids, ColE1 (4.2 megadaltons [Mdal]) or simian virus 40 (3.42 Mdal), contained in the same sample.

Transformation. Purified plasmid DNA from *H. ducreyi* was used to transform HB 101 (a plasmid-free strain of *Escherichia coli* [1]) by the methods of Cohen et al. (4). Plates used for selection contained 100 μg of ampicillin, 5 μg of tetracycline, or 50 or 100 μg of chloramphenicol. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.) except ampicillin, which was purchased from Bristol Laboratories (Syracuse, N.Y.).

Agarose gel electrophoresis. Purified plasmids were analyzed on 0.7% agarose gels as described by Meyers et al. (21). Restriction enzyme analysis of plasmids was accomplished by suspending purified plasmid DNA in a buffer composed of 5 mM $MgCl_2$–50 mM Tris, pH 7.5. Restriction enzymes *Pst*I and *Bam*HI (Bethesda Research Laboratories, Bethesda, Md.) were added, and the solution was incubated at 37°C. Fragments generated were resolved by electrophoresis on a 1% agarose gel.

Hybridization. *H. ducreyi* plasmid DNA was cleaved with *Pst*I and *Bam*HI restriction endonucleases and electrophoresed on agarose gels as described above. DNA in the gel was denatured in a solution of 0.2 M NaOH and 0.6 M NaCl for 45 min followed by neutralization in a solution of 0.6 M NaCl and 1 M Tris-hydrochloride, pH 7.0, for 45 min. DNA was then transferred to nitrocellulose filter paper by the Southern blotting technique (28). The position of DNA in the gel and thus on the blotted nitrocellulose paper was recorded by staining the gel in ethidium bromide and photographing it. DNA was fixed to the paper by heating at 80°C under vacuum for 2 h. Hybridization was performed by the procedure of Moseley et al. (22). By this method, filters were pretreated for 3 h in a solution of 50% formamide, 1× Denhart solution (5), 5× SSC (SSC = 0.015 M NaCl and 0.15 M sodium citrate), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. Hybridization was accomplished by incubating the filters overnight in the fresh solution described above and also containing 5×10^4 cpm of ^{32}P-labeled probe DNA (heat denatured) per ml and 100 μg of calf thymus DNA (heat denatured, sheared by sonication) per ml. Filters were washed in 5× SSC with 0.1% sodium dodecyl sulfate at 65°C for 45 min and then rinsed in 2× SSC at room temperature to remove excess probe DNA. These filters were air dried and exposed to X-Omat R X-ray film (Eastman Kodak Co., Rochester, N.Y.) with the appropriate intensifying screen. The positions of the hybridized DNA could be visualized after development of this film after exposure for 2 to 3 weeks at −70°C.

Preparation of ^{32}P probe DNA. The probe DNA used in this study was the ampicillin (Ap) transposon from RSF2124, a well-characterized plasmid composed of ColE1 and Tn*3*, a type of Ap transposon (26). Tn*A* has been subclassified into Tn*1*, Tn*2*, and Tn*3* based on fine genetic structure and the type of TEM β-lactamase encoded (13). However, since these transposons are highly homologous and were not distinguished by our techniques, we refer to them as Tn*A* here. The genes comprising Tn*3* have been previously located on the 1,929-, 919-, 659-base pair fragments after digestion with *Pst*I and *Bam*HI (12). By our calculations from agarose gel electrophoresis, the fragments were 1.2, 0.6, and 0.5 Mdal. To separate these genes from the rest of RSF 2124, the appropriate restriction fragments were cut out of a 1% agarose gel with a razor blade, combined, and recovered after electrophoresis through tubes containing 3% acrylamine and into dialysis bags attached to the bottoms of the tubes. These fragments were further purified through another agarose gel as above. This DNA was then phenol extracted, concentrated by ethanol precipitation, and resuspended in 10 mM Tris–1 mM EDTA, pH 7.5. The DNA was then labeled to a specific activity of 3×10^7

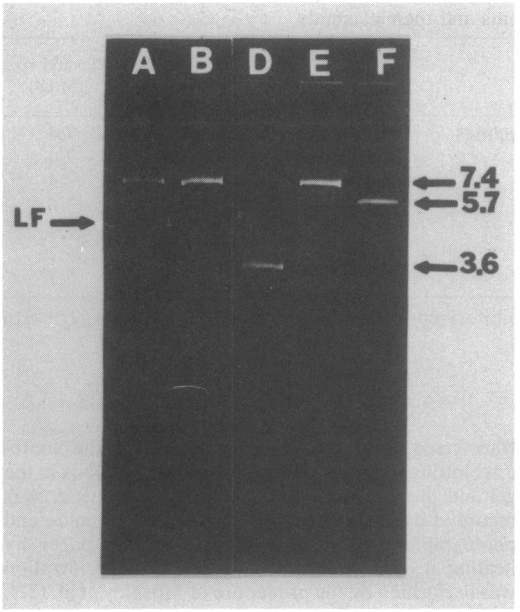

FIG. 1. Agarose gel electrophoresis of purified plasmid DNA. Plasmid DNA was obtained from *H. ducreyi* isolates (A) V-1157, (B) V-1158, (D) V-1168, (E) V-1169, and (F) V-1171. Corresponding molecular weights ($\times 10^6$) are indicated on the right. LF, Linear fragments of chromosomal DNA.

cpm per μg of DNA, using the nick translation technique of Maniatis et al. (19) and α-labeled dATP and dCTP (New England Nuclear Corp., Boston, Mass.).

RESULTS

Plasmid content. Single plasmids were found in all *H. ducreyi* strains tested (Fig. 1). Molecular weights as determined by electron microscopy are listed in Table 1 and correlated well with the sum of molecular weights of restriction fragments calculated from agarose gels. Plasmids in slots A, B, and E were isolated from the Philippine strains and have a similar size (7.4 Mdal). We had previously reported a molecular weight of 7.3×10^6 for the plasmids (11), but additional measurements showed that their sizes were closer to 7.4×10^6. The plasmid content of these strains thus could be distinguished from that of the isolates from Brazil (3.6 Mdal, slot D) and Mexico (5.7 Mdal, slot F).

Transformation. Ampicillin-resistant transformants were obtained after exposing HB 101 (a strain of *E. coli* with no plasmids) to plasmid DNA from each *H. ducreyi* strain. Agarose gel electrophoresis of these transformants revealed that they contained plasmids, although they were frequently smaller than the plasmids of the donor strain (data not shown). From these results, we concluded that Ap resistance was plasmid-borne in *H. ducreyi*. We used subsequent DNA hybridization to prove that these genes were present on the plasmids seen in Fig. 1. No transformants were obtained when the selection was for tetracycline or chloramphenicol, indicating that these genes were not present on the Ap plasmid. It is possible that these resistance determinants may be present on a plasmid not isolated by our technique, but further studies were not done.

Restriction enzyme analysis. We have previously shown that the *H. ducreyi* isolates could be differentiated on the basis of their antimicrobial susceptibilities and resident plasmid sizes (11). However, bacteria of the same species may have different plasmids of similar molecular mass. Consequently, restriction endonuclease fingerprinting is a more accurate method to establish plasmid identity. To determine whether the *H. ducreyi* plasmids harbored by these strains were similar, we digested them with the restriction enzymes *Bam*HI and *Pst*I and looked for similar digestion products in agarose gels.

The *H. ducreyi* plasmids could be distinguished by their restriction endonuclease digestion patterns (Fig. 2). Plasmids from strains originating in the Philippines (slots B, C, and E) had identical restriction digest patterns. In addition, strain V-1157, another isolate obtained from the Philippines, had patterns identical to those above (data not shown). These patterns could be distinguished from those of the Mexican (slot F) and Brazilian (slot D) strains. Thus, we have shown that V-1157, V-1158, and V-1159, strains obtained from sexual contacts, had similar if not identical plasmids.

Restriction enzyme analysis also revealed similarities in the *H. ducreyi* Ap plasmids. All Ap plasmid digests contained 0.6- and 0.9-Mdal fragments. In addition, all plasmids except one (strain V-1168, slot D) had 1.2- and 0.5-Mdal fragments. A strain of *H. ducreyi* isolated during an outbreak of chancroid in Canada was also analyzed by digestion with *Bam*HI and *Pst*I. The fragments produced (data not shown) were indistinguishable from those obtained from the plasmid in slot F in Fig. 2 (from strain V-1171, from a patient infected in Mexico).

DNA-DNA homology. To confirm that the Ap resistance genes were present on the *H. ducreyi* plasmids seen in Fig. 1 and to investigate the nature of these genes, we tested these plasmids for homology with Tn*A*. Figure 3 shows that this Ap transposon was located on the 0.5-, 0.6-, and 1.2-Mdal fragments of the plasmids from strains V-1157, V-1158, and V-1159 (slots A, B, and C). Similar fragments from the plasmids of strains V-1169, V-1171, and 54198 also showed homology with Tn*A* (data not shown). The remaining plasmid (strain V-1168, slot D) had only part of the Ap transposon, the 0.6-Mdal piece.

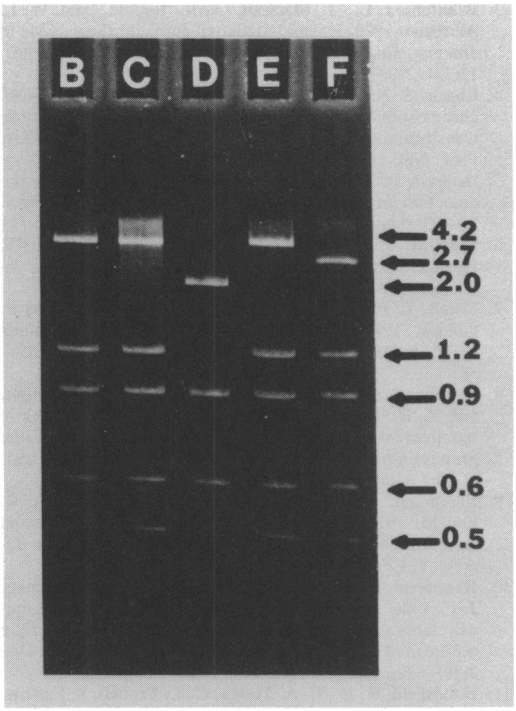

FIG. 2. Restriction endonuclease digestion of *H. ducreyi* Ap plasmids. DNA was digested with *Pst*I and *Bam*HI, and fragments were separated on a 1% agarose gel. Plasmid DNA in slots were from strains (B) V-1158, (C) V-1159, (D) V-1168, (E) V-1169, and (F) V-1171. Molecular weights listed on the right, ($\times 10^6$) were calculated from standards run on the same gel (lambda DNA cut with *Hind*III and lambda DNA cut with *Hind*III and *Eco*RI).

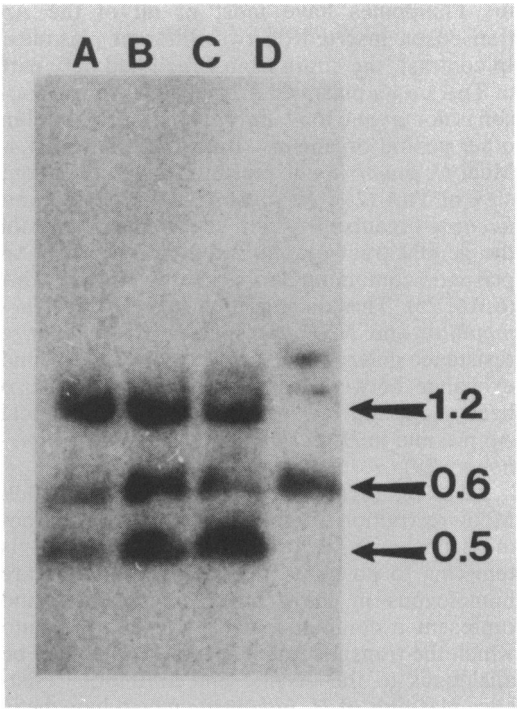

FIG. 3. Autoradiograph showing the hybridization of *H. ducreyi* Ap plasmids with the Ap transposon (Tn*A*) from RSF2124. Plasmids were digested with *Pst*I and *Bam*HI, electrophoresed on a 1% agarose gel, and then reacted with ^{32}P-labeled Ap transposon. Digested plasmids in slots A to D correspond to the same strains used to prepare plasmid DNA for slots A to F in Fig. 1 and 2: (A) V-1157, (B) V-1158, (C) V-1159, (D) V-1168. Positions of restriction enzyme-cut DNA were positioned from the gel used in this experiment and are indicated (in megadaltons) by arrows. The negative control, ColE1, does not encode for Ap resistance and showed no reaction with the probe (not shown).

DISCUSSION

In this study, we have shown that *H. ducreyi* strains possess at least three different types of Ap resistance plasmids. The plasmids differed with country of origin; strains from the Philippines, Brazil, and Mexico harbored plasmids of 7.4, 3.6, and 5.7 Mdal, respectively, and could also be differentiated by endonuclease fingerprinting. DNA homology studies revealed that one of these plasmids contained part, whereas the rest possessed most if not all, of Tn*A*, the Ap transposon encoding for TEM-type beta-lactamase. One Ap plasmid, harbored in a strain from Mexico, contained Tn*A* and was indistinguishable from the plasmid found in the isolate from Canada. Brunton et al. (2) demonstrated that this Canadian plasmid possessed an intact Tn*A* which could be transposed to coresident plasmids. This plasmid also was shown to encode for TEM-1-type beta-lactamase, the enzyme associated with Tn*2*, a subclass of Tn*A* (18).

Because of a lack of markers which can be used to distinguish *H. ducreyi* isolates, restriction enzyme fingerprinting may prove to be a valuable tool to study the epidemiology of these strains and their plasmids. In this paper we used this technique to document the sexual transmission of a particular *H. ducreyi* strain and to differentiate strains from different countries. This method has previously been used to analyze the spread of a variety of organisms, including penicillin-resistant *Neisseria gonorrhoeae* (31). Strains of *N. gonorrhoeae* carrying a 3.2-Mdal Ap plasmid predominate in Africa and caused an outbreak in England, whereas 4.4-Mdal Ap plasmids are found in isolates in the Philippines and the United States (25).

In addition to investigating the spread of *H. ducreyi* strains from person to person, we also studied the epidemiology of the Ap transposon from plasmid to plasmid. A comparison of Fig. 2 and 3 shows that the isolates from Mexico and

the Philippines have most or all of the Ap transposon inserted on two different plasmids. In contrast, the strain from Brazil had only part of Tn*A* on its plasmid. A truncated Ap transposon is not unique to *H. ducreyi*; it is also found in other genital organisms. Both the 3.2- and 4.4-Mdal *N. gonorrhoeae* plasmids have only about 40% of Tn*A* (25). *H. influenzae* and *H. parainfluenzae* organisms which we have isolated from the genital tract (unpublished data) harbor Ap plasmids containing approximately 40% of Tn*A* (6, 16, 25). These similarities suggest that *Haemophilus* and *Neisseria* species may exchange resistance determinants in vivo. In fact, plasmid exchange between these two genera has also been suggested by the isolation of an identical Ap plasmid in *H. parainfluenzae* and *N. gonorrhoeae* (29).

All *H. ducreyi* plasmids in our study had a 0.9-Mdal restriction fragment which was not homologous with Tn*A* (cf. Fig. 2 and 3). It is tempting to postulate that these fragments are homologous in the *H. ducreyi* plasmids and represent a common part of the plasmid onto which the transposon has inserted. This may be analogous to the cryptic and antibiotic resistance plasmids of *H. influenzae* which have been shown to be homologous (7), as have the different antibiotic-resistant plasmids of this species (15). Several investigators (7, 18) have suggested that the Ap transposon may insert on cryptic plasmids exchanged by *Haemophilus* and *Neisseria* species. This hypothesis is supported by our finding of similarities between the *H. ducreyi* Ap plasmids and by the presence of an abbreviated Tn*A* found on one *H. ducreyi* plasmid as on other *Haemophilus* and *Neisseria* plasmids. Further restriction enzyme analysis and DNA homology studies may reveal common structures shared by these plasmids and shed some light on the epidemiology of transposons and resistance plasmids in genital organisms.

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