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Plasmid-Mediated Tetracycline Resistance in *Haemophilus ducreyi*

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Clinical isolates of *Haemophilus ducreyi* were shown to be resistant to tetracycline. Resistance was associated in some strains with a 30-megadalton plasmid capable of transferring resistance in conjugative matings with other strains of *H. ducreyi* and other species of *Haemophilus*. Restriction endonuclease digestion patterns suggest a relationship between *H. ducreyi* plasmids and other tetracycline resistance plasmids in *Haemophilus*. The presence of plasmid-mediated resistance to the tetracyclines limits the use of these agents for the treatment of chancroid.

Tetracyclines have been reported as effective therapy for chancroid (4, 24). Recent studies, however, have reported clinical resistance and in vitro resistance of the causative organism, *Haemophilus ducreyi*, to the tetracyclines (10, 15, 16). In our laboratory, in vitro studies with a large collection of strains have shown over 90% of the isolates from selected geographic areas to be resistant to tetracyclines.

Since tetracycline resistance has been shown previously to be plasmid mediated in other species of *Haemophilus* (14), and since plasmid-mediated resistance to ampicillin (5–7) and sulfonamides (3) has been previously demonstrated in *H. ducreyi*, we looked for plasmid-mediated resistance to tetracyclines in this species.

We report here the demonstration and preliminary characterization of a conjugative plasmid encoding tetracycline resistance in clinical isolates of *H. ducreyi*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids named in this study are listed in Table 1. The remainder of the strains were clinical isolates of *H. ducreyi*.

Media. Strains of *H. ducreyi* were routinely grown on GC agar base (GIBCO Diagnostics, Madison, Wis.) supplemented with 1% hemoglobin and 1% CVA enrichment (GIBCO). MICs were determined by agar dilution as described previously (11).

Plasmid DNA. Strains were screened for the presence of plasmids as described by Meyers et al. (19) and previously reported (3, 6). Plasmid DNA for restriction digestion was prepared by a modification (8) of the method of Clewell and Helinski and isolated by cesium chloride-ethidium bromide equilibrium density gradient centrifugation as previously reported (3, 6).

Restriction endonuclease digestion. *Bgl*II, *Hin*dIII, and *Ava*I were purchased from New England Biolabs (Beverly, Mass.) or Boehringer Mannheim Corp. (Canada). Reactions were carried out as recommended by the suppliers. Electrophoresis and estimation of fragment molecular weight were as previously described (3, 6).

Conjugation procedure. Suspensions (10^8 CFU/ml) of the organisms were prepared from overnight cultures. Equal volumes of the donor and recipient were mixed. The organisms from 4 ml of these suspensions were filtered onto 0.45-μm polycarbonate filters. After overnight incubation on the surface of a chocolate agar plate, the organisms were resuspended and spread over the surface of chocolate agar containing tetracycline (4 μg/ml) and streptomycin (1,000 μg/ml). The clones isolated were screened for plasmid DNA, and their antibiotic resistance was confirmed by disk diffusion testing.

RESULTS

The distribution of MICs for 35 strains of *H. ducreyi* is shown in Fig. 1. Resistant strains were defined as those having an MIC ≥ 4 μg/ml. Tetracycline-resistant strains were found in every geographical area surveyed, with the percentage of resistant strains varying from 16 to 100 (Table 2).

Forty-one tetracycline-resistant strains were screened for the presence of plasmids. All but one strain contained plasmids similar in size to one of three previously described nonconjugative plasmids of molecular mass 4.9, 5.7, and 7.0 megadaltons (Mdal) (3, 6, 7). These plasmids are known not to carry the tetracycline resistance determinant. Ten of the strains, however, could be shown to have a 30-Mdal plasmid on initial screening which was associated with transfer of tetracycline resistance in conjugative matings with a laboratory-derived, streptomycin-resistant, tetracycline-susceptible strain of *H. ducreyi*. Three additional strains not showing a 30-Mdal plasmid on initial screening were able to transfer tetracycline resistance in conjugative matings with *H. ducreyi* 35000, and transconjugants from these matings had a 30-Mdal plasmid on screening. Similar observations have been made in conjugative matings with other species of *Haemophilus* (20, 22). The remainder of the strains had only the previously described nonconjugative plasmids and did not transfer tetracycline resistance in matings with *H. ducreyi* or other species of *Haemophilus*. There was no difference in the level of resistance between strains with or without demonstrable plasmids. These findings suggest that in these strains, the tetracycline resistance is chromosomally mediated.

The relationship between plasmids carrying the tetracycline resistance determinant and seen on initial plasmid screening and plasmids seen only in transconjugant strains was determined by restriction endonuclease digestion. Figure 2 shows the results of digestion with two restriction endonucleases. Strain HD131 did not demonstrate a 30-Mdal

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TABLE 1. Bacterial strains and plasmids

Strain	Plasmid complement (Mdal)	Phenotype	Source (reference)
H. ducreyi			
35000 Sm^r	None	Sm^r	Winnipeg (6)
HD131	7.0[a]	Ap^r Tc^r	Kenya (7)
HD9265	7.0, 30[b]	Ap^r Tc^r	Kenya (7)
V1157	7.0[c]	Ap^r Cm^r Tc^r	Seattle (23)
H. influenzae			
2265	34 (pHI2265)	Cm^r Tc^r	Winnipeg (1)
2289	30 (pHI2289)	Tc^r	Winnipeg

[a] In addition to the 7.0-Mdal plasmid (pHD131) previously shown to contain Tn*A* and not Tn*T* (7), this strain carried a 30-Mdal plasmid (pHD730)—not seen on initial plasmid screening—that contained the tetracycline resistance determinant.

[b] The 30-Mdal plasmid (pHD9265) carrying the tetracycline resistance determinant was shown not to carry the ampicillin resistance determinant. The ampicillin resistance determinant was presumably carried on the 7.0-Mdal plasmid in this strain as in strain HD131.

[c] As with strain HD131, this strain carried a 34-Mdal plasmid (pHD1157) containing the chloramphenicol and tetracycline resistance determinants which was not seen on initial plasmid screening. The 7.0-Mdal plasmid in this strain has been shown previously to carry the ampicillin resistance determinant (23).

plasmid on initial screening, but it did transfer tetracycline resistance, and the transconjugant contained a 30-Mdal plasmid. Strain HD9265 also transferred tetracycline resistance but demonstrated a 30-Mdal plasmid in the donor strain. Strain V1157 demonstrated only a 7.0-Mdal plasmid on initial screening but transferred both chloramphenicol and tetracycline resistance on conjugative matings, and a 34-Mdal plasmid was observed in the transconjugant. Similar restriction endonuclease patterns were observed with four other plasmids, two of which were not demonstrated on initial plasmid screening.

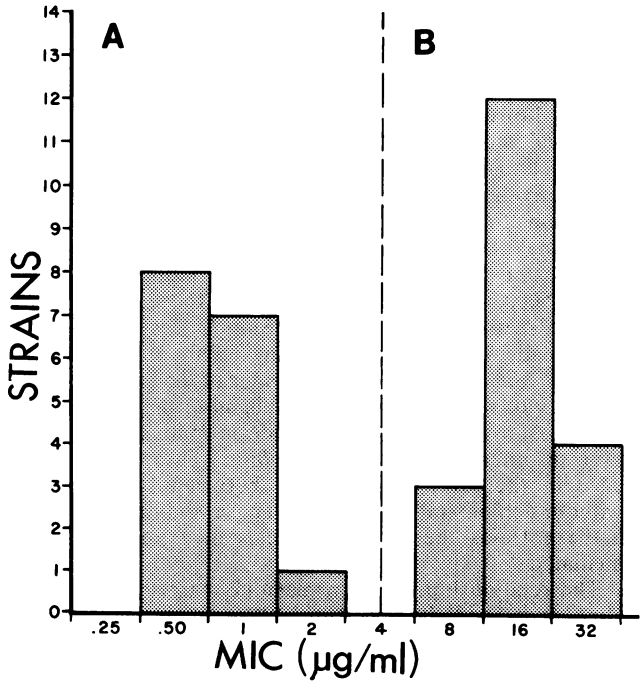

FIG. 1. Distribution of tetracycline MICs in susceptible (A) and resistant (B) clinical isolates of *H. ducreyi*.

TABLE 2. Tetracycline resistance in *H. ducreyi*

Geographic origin of strains	No. of strains	No. of tetracycline-resistant strains (%)	No. of strains transferring tetracycline resistance/no. tested
Canada	19	3 (16)	0/3
Atlanta	20	20 (100)	0/8
Kenya	209	199 (95)	13/30
Other[a]	8	5 (63)	Not done

[a] Includes strains from Amsterdam, Sweden, Thailand, and the United States other than Atlanta.

Ampicillin resistance was never cotransferred with tetracycline resistance in conjugative matings with these strains.

The relationship between *H. ducreyi* plasmids carrying tetracycline resistance and resistance plasmids of *Haemophilus influenzae* was determined by comparison of restriction endonuclease digestion patterns. The results with *Ava*I are shown in Fig. 3. A great deal of similarity was seen among the digestion patterns for all four plasmids from the two species. Similarities among the plasmids exceeded the reported size (9.3 kilobases) of tetracycline resistance transposons (13), suggesting relatedness in the plasmid core regions.

Incompatibility studies with ampicillin resistance plasmids of *H. influenzae* also suggested relatedness in the plasmid core regions, since all four plasmids were incompatible with an ampicillin resistance plasmid (pHI2316) originally isolated from a clinical strain of *H. influenzae* (L. Slaney and W. Albritton, unpublished data).

DISCUSSION

Plasmid-mediated antibiotic resistance in *H. ducreyi* has been previously described for ampicillin (5–7, 23) and sulfon-

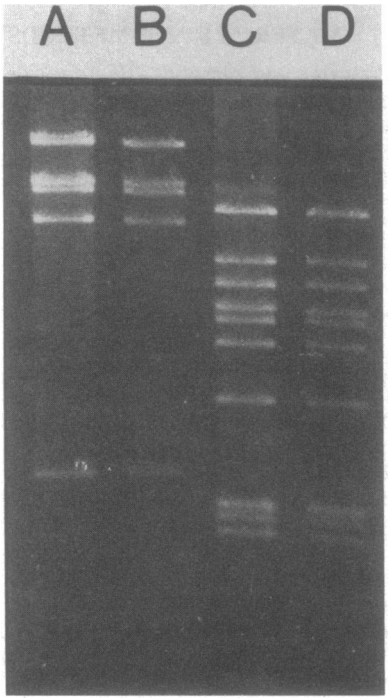

FIG. 2. Restriction endonuclease digestion patterns for tetracycline resistance plasmids of *H. ducreyi*. Lanes: A, pHD730 digested with *Bgl*II; B, pHD9265 digested with *Bgl*II; C, pHD730 digested with *Ava*I; D, pHD9265 digested with *Ava*I.

amides (3). Tetracycline- and chloramphenicol-resistant strains have also been described, but an association with plasmids was not demonstrated (10, 21). Plasmids mediating ampicillin resistance belong to a related group of nonconjugative plasmids described in *Neisseria gonorrhoeae* and other *Haemophilus* species (7, 17). The core region of this group of plasmids appears to be present in an indigenous plasmid found in *Haemophilus* spp. (2). However, the sulfonamide resistance plasmid, also a nonconjugative plasmid, appears to represent a direct extension of the enteric plasmid pool (3). Plasmids conferring tetracycline resistance appear to represent a group of related conjugative plasmids shared by several species of *Haemophilus*. Although tetracycline resistance plasmids found in *H. ducreyi* share restriction fragments with plasmids found in *H. influenzae*, they are not identical. Whether this can be taken to support the proposed multiclonal origin of these resistance plasmids (9, 14, 18), or whether it represents molecular modification of a common plasmid, is unclear. It is interesting, however, that ampicillin resistance was not associated with the tetracycline resistance plasmids of *H. ducreyi*, since a functional ampicillin resistance transposon has been demonstrated on nonconjugative plasmids in strains of *H. ducreyi* (7).

After recognition of plasmids conferring resistance to a single antibiotic in *H. influenzae* (8), plasmids conferring multiple resistance were described (12, 18). The recognition of linked tetracycline-chloramphenicol resistance in one strain of *H. ducreyi* in this study suggests that multiple-resistance plasmids may also become more prevalent.

Finally, standard therapy for chancroid has been sulfonamide or tetracycline. The demonstration of plasmid-mediated resistance to these antimicrobial agents and the reports of clinical failure with their use suggests that alternative therapies for chancroid are needed, based on in vitro resistance patterns and known mechanisms of resistance in other species of *Haemophilus*.

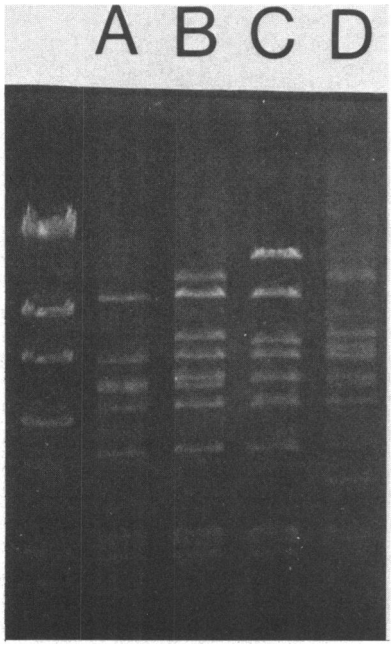

FIG. 3. *Ava*I digestion pattern for tetracycline resistance plasmids of *H. ducreyi* and *H. influenzae*. Lanes: A, pHI2289 from *H. influenzae*; B, pHD9265 from *H. ducreyi*; C, pHD1157 from *H. ducreyi*; D, pHI2265 from *H. influenzae*. A *Hin*dIII digest of λ DNA is shown in the left lane.

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