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Plasmid-Mediated Sulfonamide Resistance in *Haemophilus ducreyi*

WILLIAM L. ALBRITTON,[1]* JAMES L. BRUNTON,[2] LESLIE SLANEY,[1] AND IAN MACLEAN[1]

Departments of Medical Microbiology and Pediatrics, University of Manitoba, Winnipeg, Manitoba,[1] and Departments of Medical Microbiology and Medicine, University of Alberta, Edmonton, Alberta,[2] Canada

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Clinical isolates of *Haemophilus ducreyi* from patients with chancroid were shown to have one or more 4.9- to 7.0-megadalton non-self-transferable plasmids and to have in vitro resistance to sulfonamides. Transformation of *Escherichia coli* to sulfonamide resistance was associated with the acquisition of a 4.9-megadalton plasmid, which did not confer linked resistance to streptomycin. The guanine-plus-cytosine content of this plasmid was found to be 57%. Filter-blot hybridization and restriction endonuclease digestion studies suggested a relationship of this plasmid to RSF1010. Electron microscope heteroduplex analysis confirmed this relationship. The identification in *H. ducreyi* of a plasmid closely related to plasmids found in enteric species, rather than transposition of a resistance determinant to an indigenous plasmid, suggests that further dissemination of the enteric plasmid pool to this genus is possible since plasmid transfer between certain *Haemophilus* species is readily demonstrated.

Sulfonamides have been standard therapy for chancroid since the first successful report of their use in 1938 (11, 12, 25). Several recent reports, however, have described clinical resistance to sulfonamides, but in vitro studies of the causative species, *Haemophilus ducreyi*, were not included (10, 14, 17). Although clinical isolates of *H. ducreyi* recovered during an outbreak of chancroid in Winnipeg were susceptible to sulfisoxazole (9), up to 20% of the isolates recovered from a prospective study of genital ulcer disease in Nairobi, Kenya, have been resistant to sulfonamides (M. Fast, A. R. Ronald, and H. Nsanze, personal communication), and several recent isolates received from the Centers for Disease Control in Atlanta have been resistant to sulfonamides as well (F. Sottnek and R. Weaver, personal communication).

Since sulfonamide resistance has previously been reported to be plasmid mediated in the *Enterobacteriaceae* (1, 4, 20), and since we have demonstrated plasmid-mediated ampicillin resistance in *H. ducreyi* (2), we wondered whether sulfonamide resistance was also plasmid mediated and whether such *H. ducreyi* plasmids were related to those found in the *Enterobacteriaceae*.

We report here the demonstration and preliminary characterization of a 4.9-megadalton (Mdal) nonconjugative plasmid encoding only sulfonamide resistance in clinical isolates of *H. ducreyi*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The plasmids used as molecular weight standards in agarose gel electrophoresis were R1*drd-19*, RP4, RSF1010, and pMB8. These plasmids have been described previously (19).

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). Strains of *E. coli* were routinely grown on MacConkey or sheep blood agar. For sulfonamide disk susceptibility testing, strains of *H. ducreyi* were grown on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 50 μg of hemin per ml, 0.1% glucose, 0.01% glutamine, 0.025% cysteine, and 5% lysed horse blood. Minimal inhibitory concentrations for sulfonamides were determined for the *E. coli* transformants by agar dilution with Wellcotest agar (Burroughs-Wellcome Co., Research Triangle Park, N.C.).

Purification of plasmid DNA. Cleared lysates were prepared by a modification of the method of Clewell and Helinski in which Triton X-100 (0.1% final concentration) was substituted for Brij 58 (6). Covalently closed circular DNA was isolated by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

Determination of G+C content. Guanine plus cytosine (G+C) content was estimated as described by Mandel et al. (15) by determining the buoyant density of plasmid DNA in neutral CsCl gradients with a Beckman model E analytical centrifuge with *Clostridium perfringens* and *Micrococcus lysodeikticus* DNA as internal standards.

TABLE 1. Bacterial strains and their plasmids

Strain	Plasmid complement (Mdal)	Phenotype	Source
H. ducreyi			
HD148	4.9	Su^r, Tc^r	Kenya
HD109	4.9, 5.7	Su^r, Ap^r, Tc^r	Atlanta
HD131	7.0	Ap^r, Tc^r	Kenya
HD9468	4.9, 7.0	Su^r, Ap^r, Tc^r	Kenya
HD54198(pJB1)	5.7	Ap^r, Tc^r	Winnipeg
H. influenzae			
HI5257	4.9	—[a]	Winnipeg
HI437	2.0, 30	Ap^r	Winnipeg
E. coli			
C600 r^-m^+	—	r^-m^+, Thr^-, Leu^-, Thi^-	R. Gill (13)
C600(pHD148)	4.9	Su^r	Transformation of C600 with plasmid DNA from *H. ducreyi* HD148
C600(pHD131)	7.0	Ap^r	Transformation of C600 with plasmid DNA from *H. ducreyi* HD131
C600(pJB1)	5.7	Ap^r	Transformation of C600 with plasmid DNA from *H. ducreyi* HD54198(pJB1)
C600(RSF1010)	5.5	Su^r, Sm^r	S. Falkow (1)
C600(RSF1010::Tn*1*) Ap101[b]	8.7	Ap^r	R. Gill (13)
C600(RSF1010::Tn*1*) Ap111[b]	8.7	Ap^r, Su^r	R. Gill (13)
J53(pJB1)	5.7	Ap^r	Transformation of J53 with plasmid DNA from *H. ducreyi* HD54198

[a] —, None.

[b] RSF1010::Tn*1* plasmids Ap101 and Ap111 have the Tn*1* transposon inserted at 92.5 and 8.5%, respectively, of the distance from the left-hand end generated by *Eco*RI cleavage of RSF1010. Insertion of TN*A* in Ap101 resulted in inactivation of the sulfonamide resistance gene and increased susceptibility to streptomycin, whereas the insertion in Ap111 resulted in inactivation of the streptomycin resistance gene (13).

Filter-blot hybridization. Restriction endonuclease digestion fragments were separated in 1.5% agarose gels before being transferred to nitrocellulose sheets as described by Southern (24). Alternatively, covalently closed circular plasmid DNA in agarose gels was first depurinated with 0.25 M HCl before being denatured and transferred to nitrocellulose. Approximately 1×10^6 cpm of nick-translated probe DNA (16) was incubated with the filter-bound cold DNA in 2× SSC and 50% formamide at 37°C for 18 h. Therefore, assuming a G+C content of 55%, hybridization was carried out at 25°C below the melting temperature (18, 22). After hybridization the nitrocellulose was washed exhaustively in 2× SSC, dried, and subjected to autoradiography.

Electron microscope heteroduplex analysis. Approximately 0.1 μg of RSF1010::Tn*A* (Ap111) DNA or RSF1010::Tn*A* (Ap101) DNA cleaved with *Bam*HI was mixed with 0.1 μg of open circular pHD148 DNA. The molecules were denatured with 0.1 N NaOH and renatured with 0.1 M Tris-hydrochloride in 50% formamide (pH 8.5) for 5 h at 30°C. These conditions are about 25°C below the melting temperature (5, 18). The preparation was spread on a 20% formamide hypophase, picked up on Parlodion-coated grids, and shadowed with platinum-carbon (90:10). Shadowing was performed by D. Scraba and R. Bradley, Department of Biochemistry, University of Alberta, with a Balzers apparatus.

Restriction endonuclease digestion. *Eco*RI, *Hin*cII, and *Pst*I were purchased from New England Nuclear Biolabs (Beverly, Mass.) or Boehringer Mannheim Corp. (Canada). Reactions were carried out as recommended by the supplier and were halted by adding a stop mix, consisting of 20% Ficoll, 0.2 M EDTA, and 0.07% bromophenol blue. Digests were electrophoresed in 5% polyacrylamide, or 0.7 or 1.5% agarose gels. The molecular weight of fragments was estimated, with *Hin*cII-digested ϕX174 RF11 DNA or *Hin*dIII-digested λ DNA as reference markers.

Transformation with plasmid DNA. Purified plasmid DNA from strains of *H. ducreyi* was transformed into *E. coli* C600 by the method of Cohen et al. (3). Transformants were selected either on MacConkey agar supplemented with 25 μg of ampicillin per ml or Wellcotest agar supplemented with 100 μg of sulfadiazine per ml.

RESULTS

Sulfonamide-resistant strains of *H. ducreyi* were found to contain one or more of three nonconjugative plasmids shown in Fig. 1. The 4.9-Mdal plasmid has been found to date in all sulfonamide-resistant strains, whether singly or in combination with the 5.7- or 7.0-Mdal plasmid. The 5.7-Mdal plasmid confers resistance to ampicillin and has been previously described (2). The 7.0-Mdal plasmid also confers resistance to ampicillin and does not occur in strains containing the 5.7-Mdal plasmid (manuscript in preparation).

Transformation of *E. coli* C600 with purified

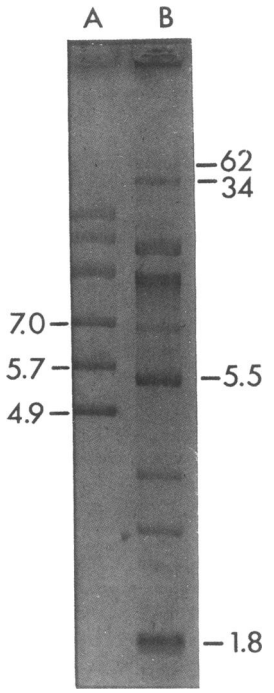

FIG. 1. Agarose gel electrophoresis of purified *H. ducreyi* plasmid DNA. (A) Pooled *H. ducreyi* plasmid DNA isolated by CsCl density gradient centrifugation from strains HD148, HD131, and HD54198. The calculated molecular weight ($\times 10^6$) is indicated in the margin. (B) Plasmid standards: R1*drd-19*, 62 Mdal; RP4, 34 Mdal; RSF1010, 5.5 Mdal; and pMB8, 1.8 Mdal. The molecular weight ($\times 10^6$) of the covalently closed circular form is indicated in the margin. Other bands in lanes A and B represent linear or open circular forms.

plasmid DNA from *H. ducreyi* clearly shows that sulfonamide resistance is associated only with the 4.9-Mdal plasmid and that this plasmid does not confer linked sulfonamide-streptomycin resistance as does the *E. coli* plasmid RSF1010 (Table 2).

The molecular weight of pHD148 calculated from its electrophoretic mobility in 0.7% agarose was 4.6 ± 0.24 Mdal. This is slightly below the molecular weight of 4.9 Mdal determined by contour length measurement using ϕX174 RF11 DNA as an internal standard. The G+C content was estimated as 57% compared to a value of 56% for RSF1010. This compares well with the previously reported value of 55% for RSF1010 (8).

Filter-blot hybridization was performed with nick-translated pHD148 as probe with depurinated covalently closed circular and open circular plasmid DNA bound to nitrocellulose filters after electrophoresis in a 0.7% agarose gel. Figure 2 shows that pHD148 is homologous only with covalently closed circular and open circular plasmid DNA of other sulfonamide resistance plasmids of *H. ducreyi* and the linked sulfonamide-streptomycin resistance plasmid RSF1010. HI437 (lane A) and HI5257 (lane G) are *H. influenzae* strains containing small "cryptic plasmids." HD9468 (lane D) and HD109 (lane E) are *H. ducreyi* strains containing the 7.0- and 5.7-Mdal ampicillin resistance plasmids, in addition to the 4.9-Mdal sulfonamide resistance plasmid. No homology is seen with the cryptic plasmids found in *H. influenzae* or the ampicillin resistance plasmids.

The restriction endonuclease patterns of RSF1010 were compared with that of pHD148 for the enzymes *Hin*cII, *Pst*I, the combination of both enzymes, and *Eco*RI (Table 3). *Eco*RI was found to cleave RSF1010 in a single site as previously reported (13) and to cleave pHD148 at two sites, generating fragments of approximately 4.3 and 0.6 Mdal. The patterns for *Hin*cII and *Pst*I are shown in Fig. 3. Identical patterns were observed for the sulfonamide resistance plasmids from HD9468 and HD109. Because the 0.5-Mdal fragments of pHD148 and RSF1010 were cleaved by *Pst*I to give a fragment of 0.42 Mdal, they were thought to be homologous. This was confirmed by filter-blot hybridization. Nick-

TABLE 2. Antimicrobial susceptibility of *E. coli* C600 and several plasmid transformants

Strain	Plasmid complement of transformant (Mdal)	Minimal inhibitory concn (μg/ml)[a] of:		
		Sulfadiazine	Streptomycin	β-lactamase
C600	—[b]	4	4	−
C600(pHD148)	4.9	>1,024	4	−
C600(pJB1)	5.7	4	4	+
C600(pHD131)	7.0	4	4	+
C600(RSF1010)	5.5	>1,024	256	−

[a] Differences between these values and the previously reported (13) minimal inhibitory concentrations of 0.5 μg/ml for streptomycin and 0.1 mg/ml for sulfadiazine (C600) and 60 μg/ml for streptomycin and 10 mg/ml for sulfadiazine (RSF1010) are probably due to differences in media and methods.

[b] —, None.

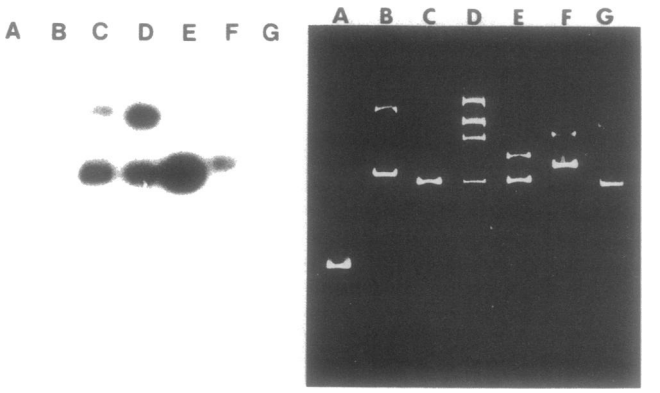

FIG. 2. Left, autoradiogram after hybridization; and right, agarose gel before transfer. Nick-translated pHD148 was used as a probe in a hybridization with filter-bound plasmids. Lane A, HI437; lane B, pJB1 (linear and open circular forms); lane C, HD148; lane D, HD9468; lane E, HD109; lane F, RSF1010; lane G, HI5257.

translated RSF1010 was shown to hybridize to the 0.5-Mdal *Hin*cII fragment, as well as the 0.42-Mdal *Pst*I-*Hin*cII fragment of pHD148. This was not surprising, since the 0.5-Mdal *Hin*cII fragment of RSF1010 is known to carry the sulfonamide resistance genes (2, 13). We were surprised, however, to find that there appeared to be extensive additional homology between RSF1010 and pHD148 (data not shown). Therefore, electron microscope heteroduplex analysis was used to further delineate the relationship between RSF1010 and pHD148. RSF1010::Tn*A* (Ap101) was used since the Tn*A* insertion occurs in the sulfonamide resistance gene and would allow this gene to be identified in a heteroduplex. Ap101 DNA was cleaved with *Bam*HI so that the Tn*A* insertion would appear as two single-stranded tails of 0.8 and 2.4 Mdal emanating from a very short duplex structure which represents the terminal inverted repeats. A typical heteroduplex is shown in Fig. 4. Seventy-nine percent of the pHD148 and 69% of the RSF1010 sequences are homologous and appear as duplex DNA. The remainder of the sequences are accounted for as a 0.8-Mdal insertion loop and a substitution loop having arms of 0.2 and 1.6 Mdal. Heteroduplexes between pHD148 and RSF1010::Tn*A* (Ap111) showed that the streptomycin-resistance-specifying sequences of RSF1010 are carried in the 1.6-Mdal limb of the substitution loop (Fig. 5), since the Tn*A* insertion has previously been shown to occur in the streptomycin resistance gene in this derivative (13).

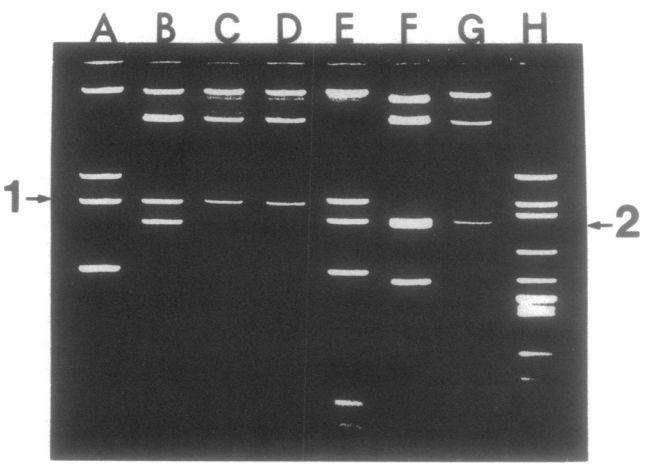

FIG. 3. Electrophoresis in 5% polyacrylamide gels of restriction endonuclease-generated fragments. Lane A, pHD148; lane B, a 3.7-Mdal Sm^r-Su^r plasmid from a clinical isolate of *Shigella sonnei*; lane C, RSF1010, respectively digested with *Hin*cII and *Pst*I; lane D, RSF1010 digested with *Hin*cII and *Eco*RI; lane E, pHD148; lane F, the 3.7-Mdal Sm^r-Su^r plasmid from *S. sonnei*; lane G, RSF1010, respectively digested with *Hin*cII and *Pst*I; and lane H, molecular weight reference: ϕX174 RF11 DNA digested with *Hin*cII.

TABLE 3. Size estimates for restriction endonuclease fragments generated by single and double digestion of pHD148 and RSF1010

Plasmid	Size estimate (Mdal) of fragments from digestion with:			
	*Hin*cII	*Pst*I	*Hin*cII-*Pst*I	*Eco*RI
pHD148	3.5, 0.6, 0.50,[a] 0.27	4.4, 0.52[a]	3.5, 0.52, 0.42[a], 0.27 (+ two fragments $<10^5$ daltons)	4.3, 0.6
RSF1010	3.7, 1.3, 0.50[a]	5.0, 0.52[a]	3.6, 1.3, 0.42[a] (+ two fragments $<10^5$ daltons)	Only one *Eco*RI site

[a] Fragments of RSF1010 known to carry the sulfonamide resistance determinant and thought to be identical to corresponding fragments cut from pHD148.

DISCUSSION

We have shown an association of a 4.9-Mdal plasmid in *H. ducreyi* with in vitro resistance to sulfonamides. It remains to be determined, however, whether such plasmid-mediated in vitro resistance can be associated with clinical failure in the treatment of chancroid and whether or not chromosomal or other plasmid-mediated sulfonamide resistance occurs in this species.

It is of interest that these sulfonamide resistance plasmids have a G+C content of 57% and are 79% related to the enteric streptomycin-sulfonamide resistance plasmid RSF1010, as determined by hybridization studies. The latter, or closely related plasmids, have been shown to occur in strains of *Salmonella, Proteus, Providencia*, and *Pseudomonas aeruginosa* (1, 8, 23). Previous studies have shown the 5.7-Mdal ampicillin resistance plasmid of *H. ducreyi* to have a G+C content of 41% and to be closely related to other small ampicillin resistance plasmids found in *H. influenzae, H. parainfluenzae*, and *N. gonorrhoeae* (2).

Other studies have demonstrated large conjugative plasmids with molecular weights of approximately 30 Mdal and G+C contents of 39% in *Haemophilus* species which carry transposable sequences specifying resistance to ampicil-

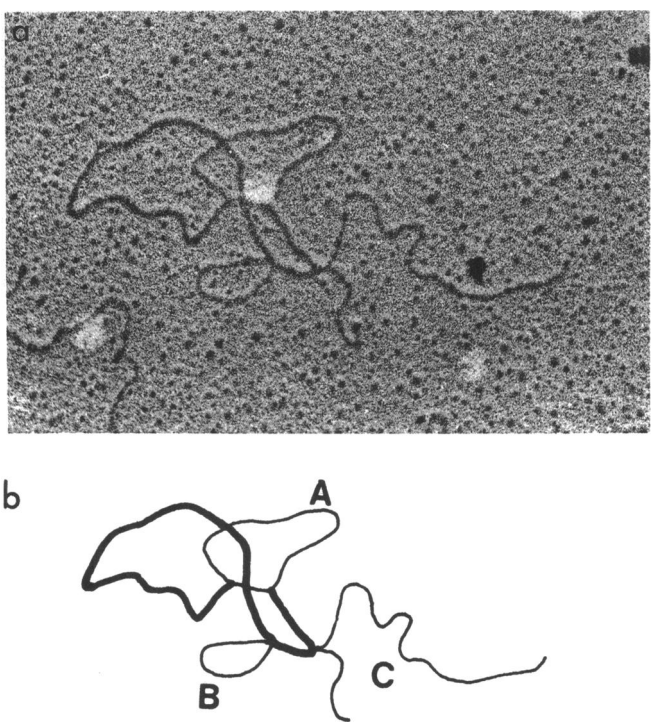

FIG. 4. (a) Heteroduplex between RSF1010::Tn*A* (Ap101) and pHD148. (b) Line drawing illustrating substitution loop (A), insertion loop (B), and insertion of two single-stranded tails of 0.8- and 2.4-Mdal at the sulfonamide resistance sequence (C). The tails are generated by cleavage of Ap101 at the *Bam*HI site within the Tn*A* sequences.

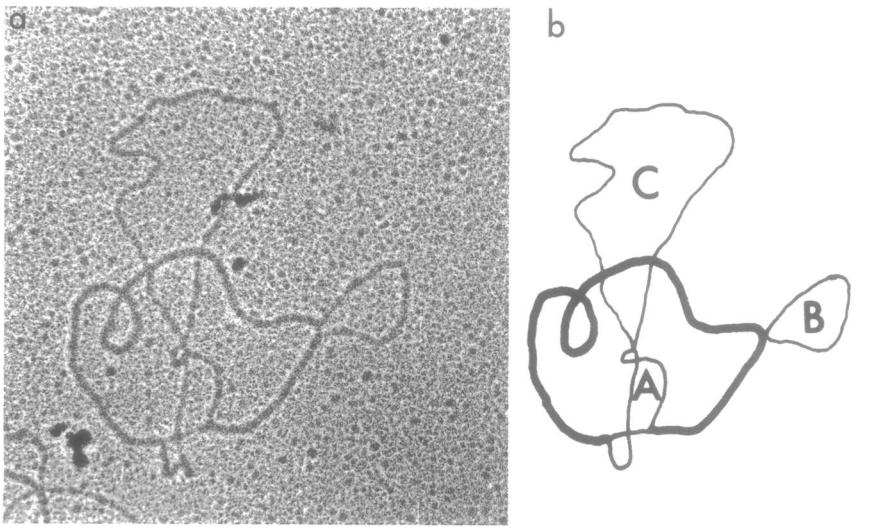

FIG. 5. (a) Heteroduplex between RSF1010::Tn*A* (Ap111) and pHD148 demonstrating insertion of Tn*A* into the 1.6-Mdal arm of the substitution loop. This identifies this sequence as carrying the streptomycin resistance genes. (b) Line drawing illustrating Tn*A* sequences (C), substitution loop (A), and insertion loop (B).

lin, tetracycline, chloramphenicol, and kanamycin (21). Extensive studies with these resistance plasmids have not shown any relationship other than that of their transposons with plasmids found in enteric bacteria, and it has been suggested that they arose by transposition of resistance determinants from enteric plasmids to cryptic plasmids resident in *Haemophilus* under conditions of mating in which the donor plasmid is lost after transfer (7). The presence of a 4.9-Mdal sulfonamide resistance plasmid in *H. ducreyi* that is highly homologous with RSF1010, a linked streptomycin-sulfonamide resistance plasmid of enteric origin, suggests that certain broad-host-range plasmids can be introduced into *H. ducreyi* and maintained. Whether such an event could result in sulfonamide resistance in *N. gonorrhoeae* or other *Haemophilus* species remains to be seen.

Finally, one strain of *H. ducreyi* isolated in Kenya has been found to contain a 24-Mdal cryptic plasmid capable of mobilizing the 4.9-Mdal sulfonamide resistance plasmid to *E. coli* by conjugative mating, and it appears to be stable in this host (unpublished data). Experiments are currently under way to determine whether mobilizing plasmids of enteric origin are capable of reintroducing this plasmid into *H. ducreyi*. In any event, the potential for further dissemination of the enteric antibiotic resistance pool to *H. ducreyi* and the impact such dissemination would have on the clinical management of chancroid are clear.

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