ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1985, p. 315–319
0066-4804/85/080315-05$02.00/0

Vol. 28, No. 2

Plasmid-Mediated Aminoglycoside Phosphotransferases in *Haemophilus ducreyi*

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Received 19 February 1985/Accepted 28 May 1985

Three clinical isolates of *Haemophilus ducreyi*, representing at least two subtypes, were shown to be resistant to streptomycin and kanamycin. They also produced a β-lactamase and chloramphenicol acetyltransferase and were resistant to tetracycline. In the three strains the resistance to both aminoglycoside antibiotics was encoded by a plasmid of ca. 4.7 kilobases which apparently did not carry ampicillin, chloramphenicol, or tetracycline resistance genes, as determined after transfer to *Escherichia coli* by transformation. Resistance to streptomycin and kanamycin was due to the presence of two aminoglycoside phosphotransferases (APH). The enzyme modifying kanamycin was a 3′,5″-APH of type I [APH(3′,5″)-I], as inferred from its substrate profile and immunological cross-reactivity with the APH(3′,5″)-I encoded by the transposable element Tn*903*. However, the APH(3′,5″)-I gene in *H. ducreyi* did not appear to be carried by Tn*903*.

Aminoglycosides have been reported as alternative therapy for chancroid, a sexually transmitted disease caused by *Haemophilus ducreyi*. This bacterium was found to be highly susceptible to streptomycin in vitro as early as 1946 (26), and its susceptibility to aminoglycosides was confirmed in vivo by the successful treatment of chancroid with streptomycin (4), kanamycin (25), and gentamicin (23).

Recently, however, treatment failures have been reported, accompanied by in vitro resistance in *H. ducreyi* to streptomycin and kanamycin in Singapore (31) and, additionally, to gentamicin in Amsterdam (36). A high frequency of kanamycin resistance has also been reported in strains of *H. ducreyi* from Thailand (37), but the mechanisms of resistance were not studied. During 1982 and 1983, we isolated, in Paris, three strains that were resistant to streptomycin and kanamycin from genital lesions of three male patients. These strains also produced a beta-lactamase and a chloramphenicol acetyltransferase, and they were resistant to tetracycline.

Aminoglycoside resistance has been shown previously to be plasmid mediated in other species of *Haemophilus* (9) and due to the presence of aminoglycoside-modifying enzymes (22). In *H. ducreyi*, plasmid-mediated resistance to ampicillin (3, 7), sulfonamides (1), and tetracycline (2) has been demonstrated. In light of these studies, we have searched for aminoglycoside-modifying enzymes in the resistant strains of *H. ducreyi* and tried to relate the resistance to plasmids present in these strains.

(This study was presented in part at the 24th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1984, Washington, D.C., abstr. no. 946).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains of *H. ducreyi* were routinely grown in PPLO broth or on PPLO agar (Difco Laboratories, Detroit, Mich.) containing 40% (vol/vol) Eagle minimal essential medium (Institut Pasteur Production, Marnes-la-Coquette, France) and 20% (vol/vol) fetal bovine serum (Industrie Biologique Francaise, Villeneuve-la-Garenne, France). All incubations were at 37°C in a 5% CO_2 humid atmosphere. The three *H. ducreyi* isolates were identified on the basis of colonial, morphological, and biochemical characteristics (8, 17).

Testing of antibiotic susceptibility and of antibiotic-inactivating enzymes. Antibiotic susceptibility was tested by an agar dilution method as previously described (33). Beta-lactamase production was detected by use of the rapid chromogenic cephalosporin test (28) on filter paper (BioMérieux, Charbonnières les Bains, France). Chloramphenicol acetyltransferase activity was assayed as previously described (32).

Characterization of outer membrane proteins. Total membranes from cells, after 24 h of growth and sonication (six bursts of 30 s, in ice), were prepared essentially as previously described (35). Sarkosyl-insoluble outer membranes (34) were prepared after incubation of ca. 100 μg of total membrane protein in 200 μl of 10 mM phosphate buffer (pH 7) containing 0.3% of Sarkosyl NL-97 for 30 min at room temperature and centrifugation at 40,000 × *g* for 45 min at 20°C. The pellet was suspended in sample buffer, boiled for 5 min, and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate as previously described (20).

Assay of aminoglycoside-modifying enzymes and gel filtration. *H. ducreyi* cells were grown for 24 h in liquid medium, 100,000 × *g* supernatants (S 100) were prepared after sonication (as above), and the enzymes were assayed with radioactive cofactors (see below) and binding of the modified antibiotics to phosphocellulose (Whatman P 81) as described by Haas and Dowding (14). S 100 from strain HD83011 was fractionated by filtration through a column of AcA54 (Réactifs IBF, Villeneuve-la-Garenne, France) equilibrated with buffer (50 mM Tris-hydrochloride, 10 mM MgCl, 200 mM NH_4Cl, 14 mM β-mercaptoethanol [pH 7.4]).

Electroblotting of S 100 proteins and reaction with anti-APH antisera. S 100 proteins were separated by electrophoresis (20) and electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell BA85) under the conditions described by Towbin et al. (38), except that the buffer contained 1% sodium dodecyl sulfate for 12 to 14 h, and no sodium dodecyl sulfate for 2 to 3 h, at 8 V/cm. Blocking of the nitrocellulose with bovine serum albumin

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TABLE 1. Bacterial strains used

Strain	Phenotype[b] of resistance	Country of infection	Reference
H. ducreyi[a]			
HD82006	Ap^r Cm^r Km^r Sm^r Tc^r	British Malaya (Singapore)	This paper
HD83004	Ap^r Cm^r Km^r Sm^r Tc^r	India	This paper
HD83011	Ap^r Cm^r Km^r Sm^r Tc^r	France	This paper
E. coli BM694	Nal^r		19

[a] Strains HD82006 and HD83011 were isolated at the Hôpital Saint-Louis (Paris); strain HD83004 was isolated at the Hôpital Labriboisière (Paris) and kindly provided by A. Felten.

[b] The nomenclature of phenotypic characters is that recommended by Novick et al. (27).

(5%; overnight), reaction with antisera against purified 3',5"-aminoglycoside phosphotransferase of type I [APH(3',5")-I] and APH(3')-II raised in rabbits (E. Collatz, unpublished data) and with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Nordic), and development with 4-chloro-1-naphthol (Merck) were carried out as described by Hawkes et al. (16).

Isolation of plasmid DNA, agarose gel electrophoresis, and bacterial transformation. The procedures described by Maniatis et al. (24) were followed. The alkaline lysis method was used for the rapid isolation of plasmid DNA, except that *H. ducreyi* cells were harvested from solid medium, agarose gel electrophoresis was performed in Tris-acetate buffer, and DNA was purified after electroelution into a trough cut into the agarose gel. The calcium chloride procedure was used for the transformation of *Escherichia coli* BM694 (19). Transformants were selected on LB agar containing streptomycin (30 μg/ml) or neomycin (50 μg/ml). The resistance phenotypes of the transformants were assayed by disk diffusion susceptibility testing on Mueller-Hinton agar.

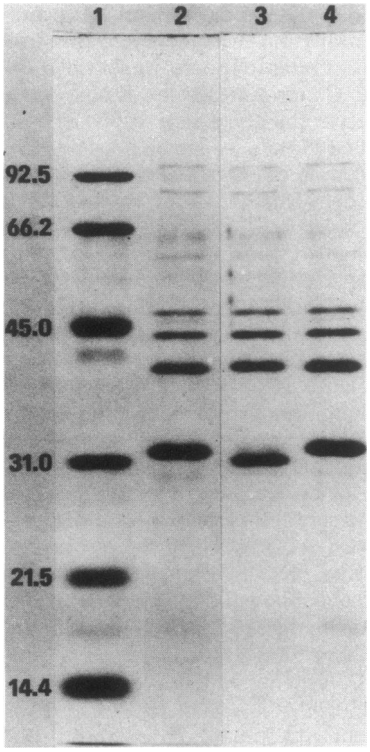

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Sarkosyl-insoluble outer membrane proteins of *H. ducreyi* strains. The concentrations of acrylamide and bis-acrylamide were 13 and 0.17% (wt/vol), respectively. Lane 1, molecular weight standards (Bio-Rad), from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soy bean trypsin inhibitor, and lysozyme. The numbers indicate the molecular size in kilodaltons. Lane 2, HD83004; lane 3, HD82006; and lane 4, HD83011.

TABLE 2. MICs of 10 antibiotics for the *H. ducreyi* strains[a]

Strain	MIC (μg/ml) of:									
	Str	Spc	Kan	Ami	Gen	Cmp	Tet	Min	Ery	Rif
HD82006	256	4	1,024	2	1	8	32	1	0.016	0.030
HD83004	128	4	1,024	2	1	8	32	2	0.008	0.008
HD83011	256	4	1,024	2	1	16	32	2	0.016	0.016

[a] All strains were beta-lactamase and chloramphenicol acetyltransferase producers (data not shown). Abbreviations: Str, streptomycin; Spc, spectinomycin; Kan, kanamycin; Ami, amikacin; Gen, gentamicin; Cmp, chloramphenicol; Tet, tetracycline; Min, minocycline; Ery, erythromycin; and Rif, rifampin.

Chemicals. [1-^{14}C]acetylcoenzyme A, [γ-^{32}P]ATP (triethylammonium salt), [γ-^{35}S]thio-ATP (triethylammonium salt), and [U-^{14}C]ATP (ammonium salt) were obtained from the Radiochemical Center, Amersham, England. Antibiotics were provided by the following laboratories: neomycin B, spectinomycin, and rifampin by Upjohn; lividomycin by Roger Bellon; butirosin by Warner Lambert; kanamycin and amikacin by Bristol; tobramycin, apramicin, and hygromycin B by Lilly; gentamicin complex, sisomicin, and netilmicin by Schering; fortimicin by Abbott; streptomycin and tetracycline by Specia; chloramphenicol and erythromycin by Roussel; and minocycline by Lederle.

RESULTS

Antibiotic susceptibility. The antibiotic susceptibility of the three strains of *H. ducreyi* is listed in Table 2. They had similar susceptibility patterns to the 10 different antibiotics tested. They were resistant to streptomycin, kanamycin, chloramphenicol, and tetracycline and susceptible to spectinomycin, amikacin, gentamicin, erythromycin, minocycline, and rifampin. All three strains produced a beta-lactamase and a chloramphenicol acetyltransferase (data not shown).

Outer membrane protein profiles. Odumeru et al. (29) recently suggested the possible value of subtyping on the basis of the outer membrane protein profiles for epidemiological studies of *H. ducreyi*. The profiles of the three aminoglycoside-resistant strains are shown in Fig. 1. Those from strains HD83004 and HD83011 were indistinguishable. The mobility of one protein of ca. 31,000 daltons in HD82006 differed from that of a possibly corresponding, slightly larger protein in the two other strains. We concluded that the three strains represent at least two subtypes. Although the proteins (Fig. 1) fall into the molecular weight range of those described and although the techniques employed were somewhat similar, we were not able to assign our strains to any of the subtypes established by Odumeru et al. (29). However, HD82006 may well correspond to the type 1 described by Taylor et al. (37).

Aminoglycoside-modifying enzymes. S 100 preparations

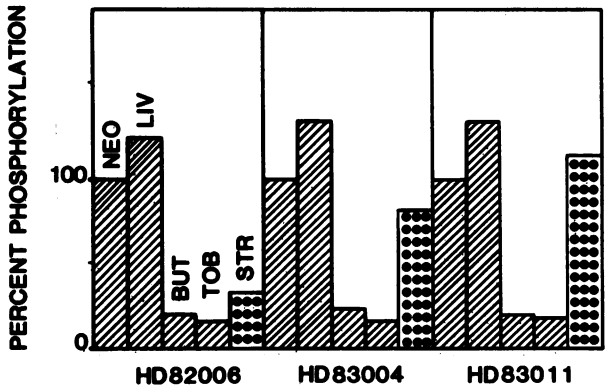

FIG. 2. Substrate profiles of the phosphotransferases in the aminoglycoside-resistant strains of *H. ducreyi*. Phosphorylation of 100% corresponds to ca. 10,000 cpm. Abbreviations: NEO, neomycin B; LIV, lividomycin; BUT, butirosin; TOB, tobramycin; and STR, streptomycin.

from the aminoglycoside-resistant strains were assayed for the presence of adenylyl-, acetyl-, and phosphotransferases with kanamycin and streptomycin as substrates. Phosphotransferases were mostly assayed with [γ-^{35}S]thio-ATP instead of [γ-^{32}P]ATP, because of the advantages of longer half-life of the radionucleotide, after it was found that this cofactor functions as a phosphate donor with a variety of APHs (E. L. Christian and E. Collatz, unpublished data). No adenylylating or acetylating activities were detected. Kanamycin and streptomycin were, however, phosphorylated. Since the substrate profile (Fig. 2) with the 2-deoxystreptamine derivatives was consistent with the presence of an APH(3′,5″)-I (11), we tried to separate it from the streptomycin-phosphorylating activity by gel filtration. Although

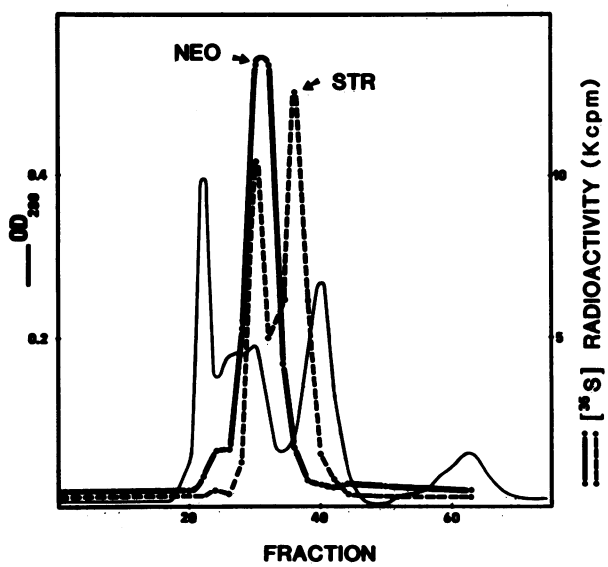

FIG. 3. Gel filtration of APHs from *H. ducreyi* 83011. An S 100 preparation (ca. 1 ml) was applied to a column (1.5 by 30 cm) of AcA54. The flow rate was 2 ml/h, and 0.8-ml fractions were collected. The enzyme activity was determined as previously described (14) with the substrates indicated (neomycin [NEO] and streptomycin [STR]) and [γ-^{35}S]thio-ATP as cofactor. OD_{280}, Optical density at 280 nm.

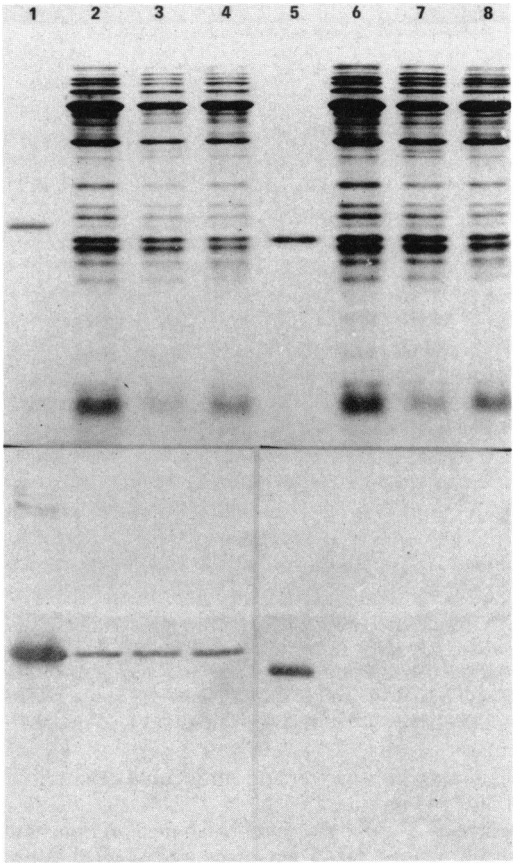

FIG. 4. Immunological characterization of the kanamycin phosphotransferase after electroblotting. Purified APHs and S 100 proteins were separated on polyacrylamide gels (15%) in the presence of sodium dodecyl sulfate and electrophoretically transferred to nitrocellulose sheets. Top, Stained with Coomassie blue; bottom, duplicate of the top, after transfer to nitrocellulose and reaction with anti-APH(3′,5″)-I (left) and anti-APH(3′)-II (right) raised in rabbits against the purified enzymes encoded by Tn*601*(*903*) and Tn*5*, respectively. Lane 1, APH(3′,5″)-I; lane 5, APH(3′)-II. Lanes 2 and 6, 3 and 7, and 4 and 8 contain HD82006, HD83004, and HD83011, respectively.

the enzyme activities were not completely separated (Fig. 3), it is apparent from the elution profile that two distinct phosphotransferases were present. The reason for the appearance of two peaks for the streptomycin-phosphorylating enzyme, and the site which it modifies, has not been studied. From an S 100 preparation of HB83011, a fraction devoid of streptomycin phosphotransferase activity was eluted from a column of immobilized kanamycin (data not shown). This preparation contained a phosphotransferase which modified neomycin and lividomycin to the extent shown in Fig. 2, but not butirosin, tobramycin, amikacin, gentamicin, sisomicin, netilmicin, apramicin, hygromycin, fortimicin, or spectinomycin, which is in agreement with the substrate profile of an APH(3′,5″)-I.

To characterize the kanamycin phosphotransferase further, we probed the S 100 proteins with antisera raised against the APH(3′,5″)-I encoded by Tn*601*(*903*) (10) and the APH(3′)-II encoded by Tn*5* (5) after electrophoretic separation and transfer to nitrocellulose sheets (Fig. 4). In the three *H. ducreyi* strains, one protein, which comigrated with the purified APH(3′,5″)-I, reacted with the corresponding anti-

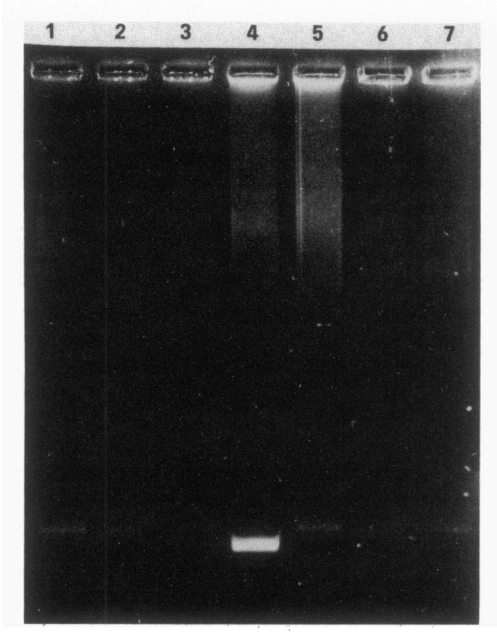

FIG. 5. Plasmid content of *E. coli* transformants. *E. coli* cells were transformed with DNA, extracted by alkaline lysis from *H. ducreyi* HD82006 (lanes 1 and 5), HD83004 (lanes 2 and 6), HD83011 (lanes 3 and 7), and *E. coli* (pBR322) (lane 4). Transformants were selected on kanamycin (lanes 1 to 3) or streptomycin (lanes 5 to 7).

body. There was, however, no noticeable reaction with the anti-APH(3')-II serum.

Association of aminoglycoside resistance and plasmid DNA. Extrachromosomal DNA was prepared from the three aminoglycoside-resistant strains by alkaline lysis and used to transform *E. coli*. Transformants selected in the presence of either streptomycin or kanamycin were resistant to both drugs but susceptible to gentamicin, ampicillin, chloramphenicol, and tetracycline. Each transformant had acquired a small plasmid (Fig. 5) of ca. 4.7 kilobases (data not shown). To ascertain that both resistance markers were carried by the 4.7-kilobase plasmid and not by DNA with lower mobility, which probably corresponded to different forms of that plasmid (Fig. 5), DNA from the fastest migrating band was eluted from the agarose gel and used for retransformation of *E. coli*. Resistance to streptomycin and kanamycin was again cotransferred.

DISCUSSION

Although aminoglycosides have not generally been considered drugs of choice for the treatment of chancroid, they have been used successfully (4, 23, 25). Treatment failures with streptomycin and kanamycin, accompanied by unexplored in vitro resistance, have occurred (31, 36) and may be explained by resistance mechanisms similar to those reported here.

In three multiply drug-resistant strains of *H. ducreyi* belonging to at least two subtypes (29, 37), as inferred from their outer membrane protein profiles, we found streptomycin and kanamycin resistance to be mediated by a 4.7-kilobase plasmid and due to the synthesis of two APHs. The streptomycin-phosphorylating activity was not characterized further. The kanamycin-modifying enzyme was an APH(3',5")-I, as inferred from its substrate profile (11) and immunological cross-reactivity with the phosphotransferase encoded by Tn*601*(*903*) (10). This is the second observation of a double resistance linked to one plasmid in *H. ducreyi*, after the recognition of a linked tetracycline-chloramphenicol resistance in one strain (2).

The 3',5"-phosphotransferases of type I are widespread among gram-negative bacteria (11) and frequently encoded on transposable elements (10, 18). We have not specifically searched for the presence of transposons, but it appears that the APH(3',5")-I is not encoded by Tn*903* since no *Pvu*II fragments were found after endonuclease digestion of the 4.7-kilobase plasmid (data not shown). There are, however, four *Pvu*II sites on Tn*903* (13). One typical and one variant APH(3',5")-I, both plasmid mediated, have been described in strains of *Haemophilus* species other than *H. ducreyi* (9, 22).

Since the original reports (31, 36), a high frequency of kanamycin resistance has been reported in strains of *H. ducreyi* from Thailand, but no information was provided about the resistance mechanism or the mode of transfer (37). We have carried out plasmid transfer only to *E. coli*, and by transformation, to identify the aminoglycoside resistance plasmid. It should be interesting to know whether there is resistance transfer by conjugation and whether such a transfer would be dependent on a mobilizing plasmid (12). Furthermore, a structural comparison with the small beta-lactamase-specifying plasmids in *H. ducreyi* and *Neisseria gonorrhoeae*, the probably related cryptic plasmids of *H. parainfluenzae* (6), as well as the small cryptic plasmid in *N. gonorrhoeae* should provide material for speculation on the origin of the aminoglycoside resistance plasmid in *H. ducreyi*.

The emergence and spread of antibiotic resistance plasmids among *H. ducreyi* have made ineffective several standard regimens for the treatment of chancroid, i.e., ampicillin (15), sulfonamides (25), tetracycline (25), chloramphenicol (21), and possibly trimethoprim (37). The alternative treatment with aminoglycosides should not be considered unless the susceptibility of isolates is carefully established.

No resistance to macrolides has been detected as yet in *H. ducreyi*. Therefore, third-generation cephalosporins, erythromycin and cotrimoxazole, which remain active on sulfonamide-resistant *H. ducreyi* (30), provide the most effective therapy of chancroid. In light of the decreasing number of useful antibiotics, it would seem reasonable to study the susceptibility of *H. ducreyi* to the newer quinolones.

ACKNOWLEDGMENTS

We thank Y. Perol for encouragement of and J. F. Acar for interest in this study. We acknowledge the expert technical assistance of M. C. Thebault. We are also grateful to E. L. Christian and G. Tran Van Nhieu for their assistance with the enzyme and plasmid analysis. We thank P. Lallemant for the art work and M. C. Daudon for secretarial assistance.

This work was supported in part by a grant from the Caisse Nationale d'Assurance Maladies des Travailleurs Salariés (to E.C.).

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