

## Characterization of Ampicillin Resistance Plasmids of *Haemophilus ducreyi* and *Neisseria gonorrhoeae* with Regard to Location of Origin of Transfer and Mobilization by a Conjugative Plasmid of *Haemophilus ducreyi*

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Restriction endonuclease maps of the ampicillin resistance plasmids of *Haemophilus ducreyi* and *Neisseria gonorrhoeae* show marked structural similarities. Transfer frequencies obtained by mobilization correlated with physical structure and were enhanced by increased homology with the conjugative plasmid. The origin of transfer of each plasmid was located within a specific restriction fragment.

We compared the ampicillin resistance plasmids of *Haemophilus ducreyi* and *Neisseria gonorrhoeae* with regard to structure, transfer frequency, and location of the origin of transfer site. Transfer studies were carried out in a background of *Haemophilus influenzae* Rd (1) and 1008 (5) in which all plasmids, including pHD147, were stably maintained. For other characterizations, plasmid DNA was isolated from a background of *H. influenzae* Rd or *Escherichia coli* C600 (4). Plasmids are described in Table 1.

We constructed more extensive restriction endonuclease maps of the four ampicillin resistance plasmids than those previously published (3, 20), as an aid to characterization. As illustrated in Fig. 1, a high degree of structural relatedness was evident, with two obvious exceptions. The plasmids of *H. ducreyi* contained complete ampicillin transposons (2, 3), whereas those of *N. gonorrhoeae* contained only partial transposons (7). Deletions due to transposition of the ampicillin transposon have been reported previously (15). The internal resolution site of the transposon shares sequence homology with bordering inverted repeat sequences (11) such that a recombination event between the central region and the left-hand inverted repeat could result in the partial transposon present in the gonococcal plasmids.

Plasmids pHD747 and p22209 contained a region of approximately 1.3 megadaltons (Mdal) that was absent from the other two plasmids. The presence of this region in a 4.4-Mdal gonococcal plasmid has been previously established and shown to contain a single site for restriction endonuclease *Hind*III (3). This region has also

been shown to be bounded by inverted repeat sequences (6). Insertion or excision of this element could account for the second structural difference in the four plasmids. Plasmids p88557, p22209, and pJB1 could all be derived from pHD747 by appropriate deletions; however, this conversion of structure has never been observed during *in vitro* manipulation.

We investigated the frequency of transfer of these four plasmids by *in vitro* conjugation, carried out essentially as previously described by Deneer et al. (5). Transfer was mediated by a phenotypically cryptic conjugative plasmid, pHD147, that was previously isolated from *H. ducreyi* (5). Mobilization frequencies were obtained (the mean of three replicates) for the four ampicillin resistance plasmids. All were efficiently mobilized to recipient cells by pHD147 and were stably maintained. The mobilization frequencies of the plasmids varied in a reproducible manner. Plasmid pHD747 was mobilized at the highest frequency ( $P < 0.01$ ),  $0.9 \times 10^{-4}$ . Plasmid p22209 was mobilized at the second highest frequency ( $P < 0.01$ ),  $0.6 \times 10^{-4}$ . The mobilization frequencies of plasmids pJB1 and p88557 were lowest and not significantly different, at  $0.7 \times 10^{-5}$  and  $0.8 \times 10^{-5}$ , respectively. A 1-ml portion of each parental strain was used in each mating. In triparental mating, *H. influenzae* Rd (pHD147), standardized to  $10^8$  CFUs/ml, acted as the initial donor. *H. influenzae* Rd (pHD747), Rd (pJB1), Rd (p22209), and Rd (p88557) ( $10^7$  CFUs/ml) acted as intermediate recipients. *H. influenzae* 1008 ( $10^6$  CFUs/ml) was the final recipient. Mobilization frequencies were calculated on the basis of the number of transconjugants per final recipient.

TABLE 1. Phenotype and source of plasmids

Plasmid	Size (10 <sup>6</sup> daltons)	Phenotype <sup>a</sup>	Species origin	Source
pHD147	23.5	Cryptic	<i>H. ducreyi</i>	Clinical isolate, Kenya
pHD747	7.0	Ap <sup>r</sup>	<i>H. ducreyi</i>	Clinical isolate, Kenya
pJB1	5.7	Ap <sup>r</sup>	<i>H. ducreyi</i>	Clinical isolate, Winnipeg
p22209	4.4	Ap <sup>r</sup>	<i>N. gonorrhoeae</i>	Clinical isolate, Winnipeg
p88557	3.2	Ap <sup>r</sup>	<i>N. gonorrhoeae</i>	Clinical isolate, Winnipeg

<sup>a</sup> Ap<sup>r</sup>, Ampicillin resistance.

Although the mobilization event can occur by two mechanisms, *in trans* or *in cis* (12), the mechanism by which plasmid pHD147 mediates transfer of the ampicillin resistance plasmids is unknown. Deneer et al. (5) noted consistent cotransfer of plasmid pHD147 during conjugation in all but one instance. The fact that a conjugative plasmid was not detected in this case may have been a result of the screening procedure (18) or loss of the plasmid after transfer. In our own experience, cotransfer of the conjugative plasmid always occurs. This would indicate that mobilization occurs *in cis*. Howev-

er, coinTEGRATION of the conjugative and nonconjugative plasmids is a necessary intermediate step of *in cis* mobilization (12), and coinTEGRATE molecules have never been detected in either *rec*<sup>+</sup> or *rec*<sup>-</sup> recipient strains.

It was interesting that plasmids pHD747 and p22209, mobilized at the highest frequencies, both contained the unique 1.3-Mdal transposon-like region. We investigated the possibility that homology might be present between this and other regions of the ampicillin resistance plasmids and the conjugative plasmid by the Southern blot procedure (19). Plasmid pHD147 was

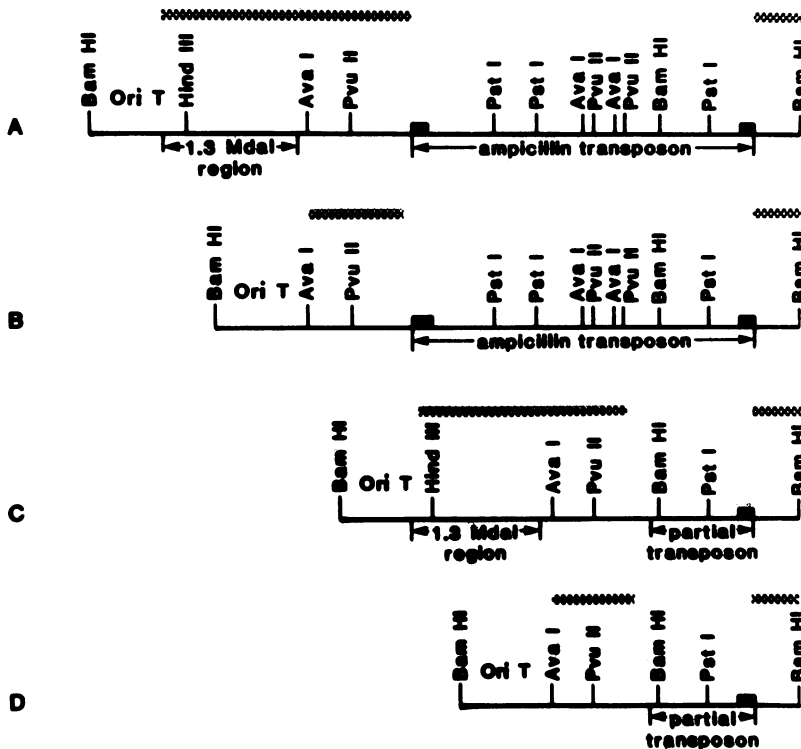


FIG. 1. Restriction endonuclease maps of the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae*. (A) The 7.0-Mdal plasmid pHD747 of *H. ducreyi*. (B) The 5.7-Mdal plasmid pJB1 of *H. ducreyi*. (C) The 4.4-Mdal plasmid p22209 of *N. gonorrhoeae*. (D) The 3.2-Mdal plasmid p88557 of *N. gonorrhoeae*. Regions homologous with the 23.5-Mdal plasmid pHD147 of *H. ducreyi* are indicated by cross-hatched lines above each map. The location of the OriT of each plasmid within the specific restriction fragment is indicated.

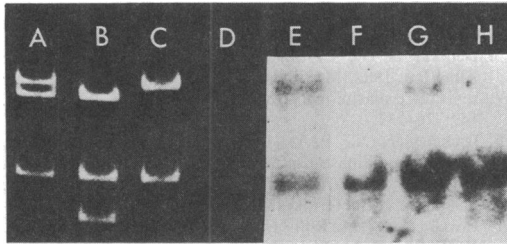


FIG. 2. Regions of the *H. ducreyi* and *N. gonorrhoeae* ampicillin resistance plasmids sharing homology with plasmid pHD147. Homology between the ampicillin resistance plasmids and plasmid pHD147 was determined by the Southern blot technique (19). Plasmid pHD147 was nicked translated by the method of Maniatis (14) and used to probe for homology. Hybridization was carried out at 68°C for 18 h with  $10^7$  cpm of [ $\alpha$ - $^{32}$ P]dCTP nick-translated probe per ml. Lanes A through D show restriction fragments resulting from a *Bam*HI-*Pvu*II digest of plasmids pHD747, p22209, pJB1, and p88557, respectively. Lanes E through H show the corresponding autoradiographs after blotting of the gel and hybridization with the plasmid pHD147 DNA probe.

nick translated by the technique of Maniatis (14) and was used as a probe for homology.

Figure 2 illustrates one restriction digest with the accompanying autoradiograph of the blotted fragments after hybridization with a pHD147 probe. The presence of homology is clearly visible. These regions and their known restriction sites are illustrated in Fig. 1. Although all four plasmids contain homologous regions, pHD747 and p22209 had more in common with pHD147. Included in the regions of homology was the 1.3-Mdal region previously described. Regions internal to the ampicillin transposon were not homologous with pHD147. Therefore, it was assumed that homology with fragments, including part of the transposon and the plasmid core, was due to the core region. This same reasoning was applied to fragments from plasmids pHD747 and p22209 containing the 1.3-Mdal region and a portion of the plasmid core. The 0.8-Mdal *Bam*HI-*Ava*I fragment from pJB1 and p88557 shared no homology with pHD147. This region has been shown by heteroduplex analysis to be homologous with much of the 1.1-Mdal *Bam*HI-*Hind*III fragment in pHD747 and p22209 (3). Therefore, it is assumed that homology with this region was due to the presence of a portion of the 1.3-Mdal region in this fragment.

Conjugal transfer of a plasmid is believed to be effected by the transfer of a specific single strand of plasmid DNA rendered linear by the introduction of a nick at a unique site (8, 9). The location of this nick site or origin of transfer (OriT) has been determined in vitro for several

plasmid species by treatment of plasmid DNA-protein relaxation complexes with protein-denaturing agents (9, 13, 16, 22). Recently, the DNA-protein complex of the 4.4-Mdal plasmid of *N. gonorrhoeae* was isolated (10). The structural similarity of this plasmid to plasmid p88557 of *N. gonorrhoeae*, as well as to plasmids pHD747 and pJB1 of *H. ducreyi* (Fig. 1), allowed us to attempt to isolate and relax these complexes in vitro for all four plasmids, as previously described (13). A limited nick-labeling procedure (16), followed by restriction digestion, allowed us to locate the unique nick site (OriT) within a specific restriction fragment of each plasmid species. The fragments resulting from the restriction endonuclease digestion of each plasmid as well as autoradiographs of those restriction digests are shown in Fig. 3. In each case, a unique fragment incorporated a label, indicating that the plasmids had been specifically nicked at a unique site by the in vitro treatment. The fragment containing the OriT of each plasmid is presented in the plasmid restriction maps shown in Fig. 1. The OriT's of both pHD747 and p22209 were located in the 1.1-Mdal *Bam*HI-*Hind*III fragments. The OriT's of pJB1 and

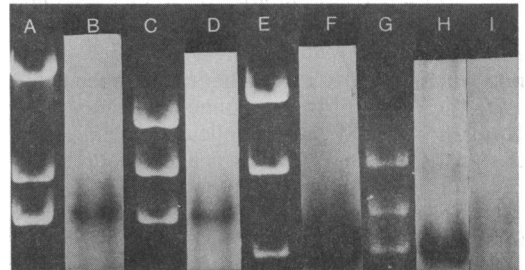


FIG. 3. Location of the OriT's of the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae*. Plasmid DNA-protein complexes recovered from cleared lysates of *H. influenzae* and *E. coli* were treated with sodium dodecyl sulfate and converted to the open circular form by introduction of a specific nick at OriT (13). The nick site was labeled by the translation procedure of Maniatis (14) at 4°C for 10 min. Labeled plasmids pHD747 and p22209 were treated with *Bam*HI and *Hind*III restriction endonucleases (lanes A and C, respectively). Corresponding autoradiographs (lanes B and D, respectively) show the  $^{32}$ P label specifically incorporated in the 1.1-Mdal *Bam*HI-*Hind*III fragment. Labeled plasmids pJB1 and p88557 were treated with *Bam*HI and *Ava*I restriction endonucleases (lanes E and G, respectively). Corresponding autoradiographs (lanes F and H, respectively) show  $^{32}$ P label specifically incorporated in the 0.8-Mdal *Bam*HI-*Ava*I fragment. As a control for nonspecific nicking, DNA-protein complexes were heat treated (13) to prevent specific nicking before translation. In this case, the label was not specifically incorporated, as shown in lane I.

p88557 were located in the 0.8 Mdal *Bam*HI-*Ava*I fragments.

Since the plasmids are structurally very similar, the presence of the OriT's in common fragments is not surprising. Since we were able to isolate DNA-protein complexes and relax those complexes *in vitro*, we assume that all four plasmids encode specific nicking protein(s) and so participate in their own mobilization. Other required functions, however, must be supplied by conjugative plasmid pHD147.

Our studies have shown a positive correlation between plasmid structure, homology with the conjugative plasmid, and mobilization frequency. However, we cannot make a definitive statement as to the exact mechanism of the mobilization process. The presence of an OriT site is a necessary feature of *in trans* mobilization, whereas homology with the conjugative plasmid is a necessary feature of *in cis* mobilization (12). All four ampicillin resistance plasmids possess both features. However, the enhanced transfer frequencies of plasmids pHD747 and p22209 could be related to the presence of an additional region of homology to pHD147. With regard to these two plasmids, mobilization by an *in cis* mechanism could account for the higher transfer frequencies.

The presence of homology between a conjugative plasmid isolated from *H. ducreyi* and resistance plasmids isolated from *N. gonorrhoeae* is noteworthy. Many researchers have speculated as to the origin of the resistance plasmids in *N. gonorrhoeae* and have implicated members of the genus *Haemophilus* as a likely source (17, 21). Our data support this hypothesis. This work was presented in part at the American Society for Microbiology meeting, New Orleans, March 1983.

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