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Origin and Direction of In Vitro Replication of *Haemophilus ducreyi* and *Neisseria gonorrhoeae* Ampicillin Resistance Plasmids

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The origin of replication of *Haemophilus ducreyi* and *Neisseria gonorrhoeae* ampicillin resistance plasmids was located by cloning *Bam*HI restriction fragments into vector plasmid pAT153 and a derivative plasmid, pAT2. Selection was made for plasmid maintenance in a *polA* mutant. Direction of replication was determined by in vitro replication of plasmid DNA in the presence of radiolabeled deoxynucleotide.

The ampicillin resistance plasmids of *Haemophilus ducreyi* and *Neisseria gonorrhoeae* (Table 1) are closely related in structure (4, 9). As part of an ongoing characterization of these plasmids, we have located a region, common to the four plasmids, that is essential for maintenance. In addition, we have determined that in vitro replication of the plasmids proceeds unidirectionally about the plasmid molecule.

Two plasmid vectors were used in this study. Plasmid pAT153 was a derivative of plasmid pBR322 (3), formed by deletion of the *Hae*II B and G fragments (13). This deletion eliminated the origin of the transfer site of plasmid pBR322, thereby limiting the ability of the derivative plasmid pAT153 to be mobilized.

The second vector, plasmid pAT2, was derived from plasmid pAT153 by spontaneous deletion in vitro of the β-lactamase gene. In an attempt to form chimeric molecules, plasmids p22209 and pAT153 were ligated at the unique *Pst*I site within the β-lactamase genes. *Escherichia coli* C600 (1) was transformed with the ligation mixture, and selection was made for tetracycline resistance. Transformants contained plasmid pAT2, a 2.7-kilobase (kb) plasmid that was smaller than either plasmid p22209 or pAT153. Restriction digest patterns of plasmid pAT2 were identical to those of plasmid pAT153 (data not shown) with two exceptions. Plasmid pAT2 did not have a *Pst*I restriction site, and the large *Hin*dIII-*Ava*I restriction fragment had lost 0.9 kb relative to the same fragment from plasmid pAT153. The β-lactamase gene had been deleted from plasmid pAT153, giving rise to plasmid pAT2.

Plasmid pAT153 and pAT2 required DNA polymerase I for replication as did parent plasmid pBR322 (8). Transformants were never observed after transformation of *E. coli* W3110 *polA1* (1, 7) with plasmid pAT153 or pAT2 DNA. Conversely, the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae* were maintained in this mutant. Plasmids pHD747 and pJB1 of *H. ducreyi* and plasmids p22209 and p88557 of *N. gonorrhoeae* each have two recognition sites for *Bam*HI restriction endonuclease (Fig. 1). Digestion with this enzyme resulted in the generation of two fragments. The smaller 2.2-kb fragment was homologous in all four plasmids (4) and contained the gene encoding β-lactamase production. We cloned this specific fragment into the unique *Bam*HI site of vector plasmid pAT2. *E. coli* C600 (1) was transformed (5) with ligated recombinant plasmid mixture, and selection was made for resistance of transformants to 20 μg of ampicillin per ml of medium. Plasmid DNA, isolated from transformants (2), was mapped with several restriction endonucleases to confirm that recombinant plasmid pAT2/SB was present (Fig. 1).

By using plasmid pAT153 as a vector, we similarly cloned the larger *Bam*HI fragment of the four ampicillin resistance plasmids. Selection of transformants was again made with ampicillin, but transformants were also checked for sensitivity to tetracycline. Transformants meeting both criteria were screened for the presence of recombinant molecules. Recombinant plasmids were mapped by restriction endonuclease digest to confirm that the large *Bam*HI fragment of each ampicillin resistance plasmid had been cloned.

We transformed *E. coli* W3110 *polA1* with recombinant plasmid DNA and selected for ampicillin resistance of transformants. Transformants were screened for plasmid content (5) and were found to contain recombinant plasmid pAT2/SB only. Neither plasmids pAT2, pAT153, nor any of the recombinant plasmids containing the larger *Bam*HI fragments of the ampicillin resistance plasmids could be maintained in the *polA* mutant. Therefore, the 2.2-kb *Bam*HI fragment contained the region of the ampicillin resistance plasmids that was required for maintenance.

The 2.2-kb *Bam*HI fragment contained a 1.4-kb portion of Tn*A* (4). Therefore, the origin of vegetative replication (OriV) was located within 0.8 kb of the remaining plasmid core (Fig. 2C).

Having located the OriV, we were interested in determining whether replication of the ampicillin resistance plasmids proceeded bi- or unidirectionally. Several workers have carried out in vitro replication studies of plasmid DNA in cell extracts to determine direction of replication and to confirm location of the replicative origin (6, 10–12). Since the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae* were stably maintained in *E. coli* C600, the in vitro replication studies employed cell extracts of this strain.

Covalently closed plasmid pHD747 acted as template for in vitro replication. Preparation of cell extracts and contents of the replication mixtures were essentially as described previously (10). However, the volume of the mixture was reduced to 75 μl, with 25 μl of cell extract. Although enzymes necessary for replication of DNA were supplied by the extract, the reaction mixture was supplemented with ribonucleotides and deoxyribonucleotides including [α-^{32}P]dCTP. After an incubation period of 10 min at 30°C, plasmid DNA was recovered as excluded radioactivity from

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TABLE 1. *H. ducreyi* and *N. gonorrhoeae* ampicillin resistance plasmids

Plasmid	Size (kb)	Phenotype	Species origin	Source
pHD747	10.6	Amp^{r}[a]	*H. ducreyi*	Clinical isolate, Kenya
pJB1	8.8	Amp^{r}	*H. ducreyi*	Clinical isolate, Winnipeg
p22209	6.8	Amp^{r}	*N. gonorrhoeae*	Clinical isolate, Winnipeg
p88557	5.0	Amp^{r}	*N. gonorrhoeae*	Clinical isolate, Winnipeg

[a] Amp^{r}, Ampicillin resistant.

a column of Sephadex G-100. The plasmid DNA was digested with *Bam*HI and *Hin*dIII restriction endonucleases, and fragments were resolved in 0.7 or 1.0% agarose gels. After drying the gel, autoradiography was carried out. Although all fragments contained label, the majority of the label was incorporated into the 1.7-kb *Bam*HI-*Hin*dIII fragment, immediately adjacent to the replicative origin (Fig. 2A). When replicated in the presence of an equal molar quantity of ddTTP and dTTP, label was again predominately incorporated into this fragment (Fig. 2B). A small amount of label was incorporated into the 2.2-kb *Bam*HI fragment under these conditions, whereas virtually no label was present in the 7.0-kb *Bam*HI-*Hin*dIII fragment. This was an indication that replication of the plasmid was proceeding unidirectionally, clockwise around the plasmid map (Fig. 2C). The OriV was situated very close to the *Bam*HI site, as label was rapidly incorporated into the 1.7-kb *Bam*HI-*Hin*dIII fragment. For significant label to be incorporated into the 2.2-kb *Bam*HI fragment, replication of the DNA molecules would have to go to completion.

Finally, we attempted to initiate in vitro replication at the OriV site by using prerestricted plasmid pHD747 DNA as a template. In this instance, the majority of label should be incorporated into the 2.2-kb *Bam*HI fragment. It was expected that some label might become incorporated into the

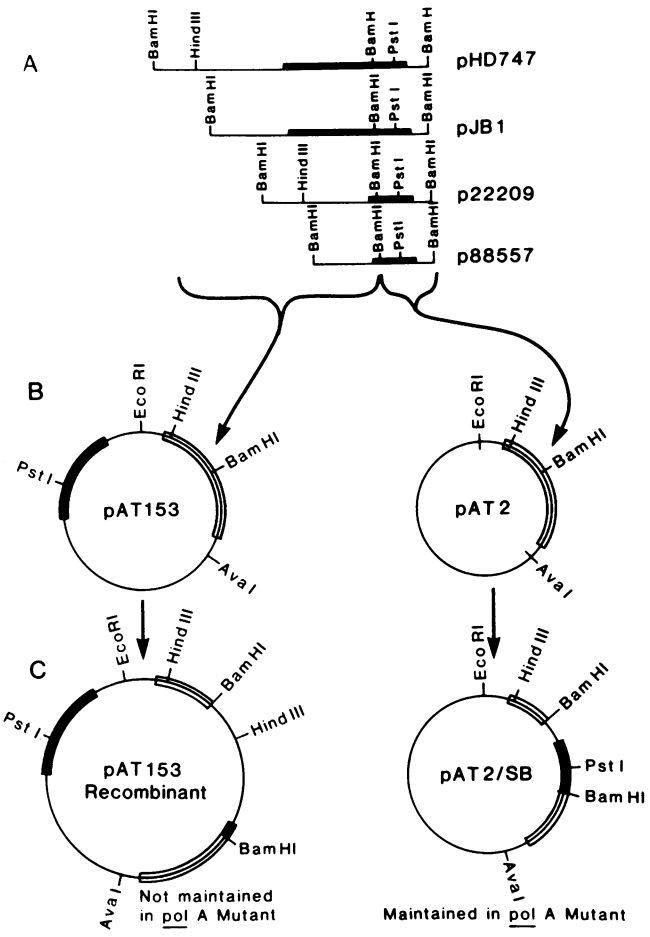

FIG. 1. Cloning strategy for determining location of the OriV. (A) Restriction maps of the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae*. These plasmids were restricted with *Bam*HI restriction endonuclease, and the larger *Bam*HI fragments were cloned into plasmid pAT153. (B) The smaller fragment was cloned into pAT2. Solid enhanced regions represent the ampicillin transposon, whereas open enhanced regions represent the tetracycline transposon. Ligation mixtures contained 0.5 pmol of DNA with respect to 5′ end in the ratio of 1:4, cloning vector to insert. The final volume of the mixtures was 20 μl with 1 U of T4 ligase (Bethesda Research Laboratories, Bethesda, Md.). *E. coli* C600 was transformed with 5 μl of the mixture. Recombinant plasmids were isolated from transformants and transformed into *E. coli* W3110 *thyA36 polA1* (1, 7). Plasmid pAT2/SB was maintained, whereas pAT153 recombinant plasmids (C) were not.

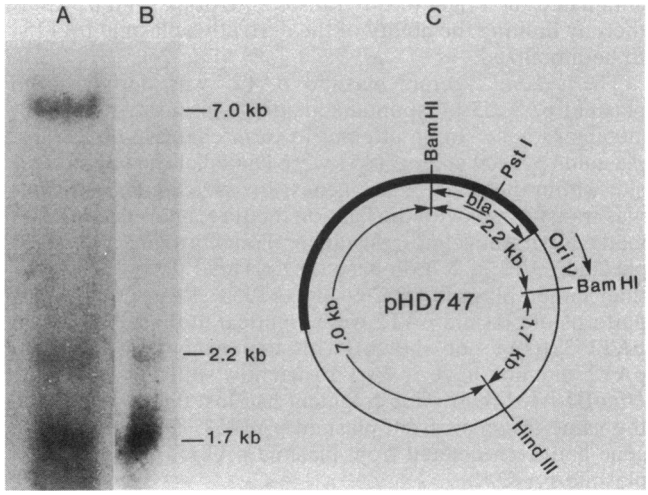

FIG. 2. In vitro replication of plasmid DNA. Cell extract of *E. coli* C600 was used for in vitro replication studies with plasmid pHD747. A 1-μl quantity of plasmid pHD747 DNA was replicated at 37°C for 10 min in cell extract supplemented with ribonucleotides and deoxyribonucleotides, including [α-^{32}P]dCTP (3,184 Ci/mM), at a final concentration of 1.5 μM. Plasmid DNA was recovered from the reaction mixture and digested with *Bam*HI and *Hin*dIII restriction enzymes. Fragments were resolved by electrophoresis through a 1.0% agarose gel. After drying, autoradiography was carried out. The autoradiograph is presented in (A). Plasmid pHD747 DNA was again replicated as described above, but with the addition of ddTTP at a final concentration of 25 μM. After recovery of plasmid DNA and digestion with *Bam*HI and *Hin*dIII restriction enzymes, fragments were resolved by electrophoresis through a 1.0% agarose gel. The autoradiograph of this gel is presented in (B). Plasmid pHD747 is illustrated in (C). The dark enhanced line shows the position of the ampicillin transposon. The β-lactamase gene is indicated by *bla*. The direction of replication proceeding clockwise about the plasmid from OriV is represented by the arrow situated above OriV.

remaining fragments due to repair of nicks and the filling in of cohesive ends of the fragments. The procedure was carried out as previously described, but after drying, restriction bands were cut from the gel. Label incorporated in each band was determined by counting duplicate samples in a scintillation counter and calculating radioactivity on the basis of counts per minute per base pair. The 2.2-kb *Bam*HI fragment containing the OriV site incorporated, on average, 30 cpm per base pair. The 1.7- and 7.0-kb *Bam*HI-*Hin*dIII fragments incorporated 5 and 6 cpm per base pair, respectively. These findings support the location of the OriV within the 2.2-kb *Bam*HI fragment.

By a combination of cloning studies and by in vitro replication of plasmid DNA, we have located the OriV of the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae* within a specific region of the plasmid core. In addition, by in vitro replication techniques, we have determined that replication of these plasmids proceeds unidirectionally about the plasmid molecules.

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