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IS*1*-Dependent Generation of High-Copy-Number Replicons from Bacteriophage P1 Ap Cm as a Mechanism of Gene Amplification

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Mutant P1 Ap Cm lysogens were isolated in which the drug resistance genes resident on the plasmid prophage P1 Ap Cm are amplified by a novel mechanism. The first step required for amplification is IS*1*-mediated rearrangement of the P1 Ap Cm prophage. The drug resistance genes are amplified from the rearranged P1 Ap Cm prophage by the formation of a plasmid (P1dR) which contains the two resistance genes. The P1dR plasmid is an independent replicon about one-half the size of P1 Ap Cm that can be maintained at a copy number eightfold higher than that at which P1 Ap Cm can be maintained. It contains no previously identified replication origin and is dependent on the Rec$^+$ function of the host.

Gene amplification in bacteria, phage, and plasmids occurs spontaneously at a frequency ranging from 10^{-1} to 10^{-5} (3). Sequences which flank a gene determine the size of the amplified region and its frequency of amplification. For most bacterial genes, amplification is dependent on homologous recombination. Usually after amplification the gene is present as a tandem multimer. Although such tandem multimers are unstable, strains in which a gene has been amplified can be isolated if the amplification results in a selectable phenotype.

For most drug resistance determinants, the level of resistance increases with increased gene dosage (47, 60). Selection for high levels of resistance therefore may result in the isolation of strains in which the drug resistance determinants have been amplified. One of the best-studied examples of this is the incFII group of R plasmids, whose drug resistance genes (r-det) are separated from the rest of the plasmid by IS*1* elements in direct orientation. These IS*1* elements serve as recombination sites for amplification of the r-det region (9, 42, 51), resulting in tandem r-det multimers. Although the amplified r-det region is present as an independent, supercoiled molecule under some conditions, it does not appear to be a replicon (11, 12, 41, 44, 51). The supercoiled molecules are lost when high drug concentration selection is removed, leaving the parent plasmid as the predominant form (28). Transposon Tn*2350* from plasmid R1*drd-19*, which can also be found as a supercoiled circle under some conditions (14–16), differs from other IS*1*-flanked r-det regions because it is an independent replicon and therefore does not have to be generated continuously from the parent plasmid.

P1 prophage is stably maintained as a plasmid with a copy number of about one per host chromosome in rapidly growing cells (31, 43, 47). By cloning of fragments of P1 into lambda (57, 58) or generation of miniplasmids in vitro (8) and in vivo (23), two replicons have been identified so far in P1. Both of these replicons are adjacent in the P1 genome. For replication, they both require sequences contained within the *Eco*RI-4 fragment of P1 Ap Cm (see Fig. 1) and, in addition, sequences either to the left (replicon 5L) or the right (replicon 5R) of *Eco*RI-4. One of these replicons (5L) has a high copy number and is unstable in Rec$^+$ hosts (57), so it probably is not the region normally used for P1 plasmid replication. The other replicon (5R), which has been studied most extensively, is maintained at a low copy number. The 5R replicon is thought to contain the sites and encode the functions normally used for P1 plasmid replication, because it is regulated like the whole P1 plasmid prophage (4) and it codes for a gene product required for plasmid DNA replication (1) in which mutants affecting copy number are located (6, 47).

To investigate the control of replication of the P1 plasmid prophage, mutants were isolated from P1 Ap Cm, in which the two drug resistance markers are widely separated, by selection for high-level resistance to both antibiotics (47). Some contained point mutations that altered the regulation of P1 plasmid copy number control (6); the others are described below.

MATERIALS AND METHODS

Media. LBB, LBA, and tryptone agar have been previously described (18, 46). Media were supplemented with 40 μg of thymine (Sigma Chemical Co.), 20 μg to 3 mg of ampicillin (reference standard from Bristol Laboratories), and 40 μg to 1 mg of chloramphenicol (Sigma) per ml.

Bacterial strains. The bacterial strains used are listed in Table 1.

Phage strains. The P1 Ap Cm phage (47) used is a hybrid made by crossing P1 Cm (33) with P1 Ap (63). P1 Ap Cm contains the ampicillin (53) transposon (Tn*902*) from P7 and the chloramphenicol transposon Tn*9* at the Cm0 site (47). Preparation of phage lysates, lysogen construction, and phage assays was performed as described previously (45, 61).

Isolation of mutant P1 Ap Cm lysogens resistant to high levels of ampicillin and chloramphenicol. Spontaneous mutants resistant to high drug levels were isolated as described previously (47). Overnight cultures of the P1 Ap Cm lysogens were plated for single colonies on LBA containing ampicillin (up to 3 mg/ml) and chloramphenicol (up to 1 mg/ml). Plates were incubated at 37°C (30°C for *Escherichia coli* KL399 and SK119) for up to 72 h. The resulting colonies were picked and streaked on the same selective medium before being grown in LBB containing 1.5 mg of ampicillin and 40 μg of chloramphenicol per ml. Cultures were stored frozen at −70°C in 20% glycerol. To ensure maintainance of

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Reference
K140	*recA*$^+$	47
N99	*rec*$^+$	47
KL398	*recA*$^+$ *thyA54*	35
KL399	Same as KL398, except *recA200*(Ts)	35
AB1187	*recB*$^+$	34
SK119	Same as AB1187, except *recB270*(Ts)	34

the high-resistance phenotype, we always grew the strains in 2 mg of ampicillin–500 μg of chloramphenicol per ml unless otherwise stated.

Isolation of plasmid and chromosomal DNA. For restriction analysis and transformation, plasmid DNA was isolated by the alkaline extraction procedure of Birnboim and Doly (7). For Southern (54) transfer procedures, plasmid and chromosomal DNAs were isolated by a sheared-lysate procedure, followed by two equilibrium centrifugation steps in a CsCl-ethidium bromide gradient (29).

Copy number measurements. Plasmid copy number was measured in exponential cultures as described previously (23). Restriction nuclease-digested plasmid DNA, isolated from log-phase cells containing both the pBR322 and the rearranged P1 Ap Cm derivatives of unknown copy number, was separated by agarose gel electrophoresis. The intensity of the pBR322 band was compared with that of the P1 Ap Cm derivative bands by densitometric tracings of a photographic negative of the gel. To calculate the relative copy number of the rearranged P1 derivative, we compared the ratio of the band intensity of the P1 Ap Cm derivative to that of pBR322 with the ratio of the band intensity of P1 Ap Cm to that of pBR322 in a control containing pBR322 and P1 Ap Cm. P1 Ap Cm is maintained at a copy number of about one (43, 47).

Transformation. The procedure used to transform bacteria has been described previously (17).

Restriction enzyme digestion and agarose gel electrophoresis. DNA was digested with restriction nucleases (Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals) under conditions recommended by Maniatis et al. (37). DNA fragments were separated by electrophoresis on 0.8% horizontal agarose gels in 40 mM Tris acetate (pH 8.1)–2 mM EDTA–0.5 μg of ethidium bromide per ml.

Purification of DNA fragments. Restriction nuclease-digested DNA was separated on a 0.8% low-melting-point agarose gel (SeaPlaque; FMC Corp., Marine Colloids Div.). The fragment(s) of interest was cut out of the gel and melted by incubation at 60°C for 5 min. We extracted the DNA from the agarose by mixing it with an equal volume of TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA)-saturated phenol. The dissolved phenol was extracted with water-saturated ether.

^{32}P labeling of DNA probes. *Eco*RI-digested P1 Ap Cm plasmid DNA, purified restriction fragments from P1 Ap Cm containing the dR-genic junction region, and an *Eco*RI fragment of plasmid pMob45 containing IS*1* (S. Hollingshead, Ph.D. thesis, University of Georgia, Athens, 1983) were used for making ^{32}P-labeled probes. We labeled the linear DNA with [α-^{32}P]dATP (Amersham Corp.; 3,000 Ci/mmol) by using the Klenow fragment of DNA polymerase (Boehringer Mannheim Biochemicals) as described by Feinberg and Vogelstein (21, 22). This labeled DNA was used without further purification for DNA-DNA hybridization.

DNA-DNA hybridization. Restriction nuclease-digested DNA samples, separated by gel electrophoresis, were transferred to nylon filters (Pall Biodyne A; 0.2-μm pore size) by a modification of the Southern technique (52, 54). Hybridizations were performed at 42°C in 50% formamide for at least 15 h in a sealed plastic bag containing 5 ml of fluid with denatured, ^{32}P-labeled probe (10^7 cpm) as described by Meinkoth and Wahl (38). The filters were washed at 65°C in 0.015 M NaCl plus 0.0015 M sodium citrate (pH 7) and autoradiographed with Kodak X-Omat AR film with a Dupont Hi-Plus intensifying screen. The films were exposed at −70°C for 16 h to 1 week.

RESULTS

Characterization of P1dR plasmids. When cells harboring the low-copy-number plasmid P1 Ap Cm were plated on high concentrations of both ampicillin and chloramphenicol, one class of isolates contained copy number mutants with point mutations in the replication region (6, 47). The phage from these strains transferred the high-copy-number phenotype when they lysogenized a new host. However, another class of isolates, not previously described, did not produce plaque-forming P1. This class of highly drug resistant strains, which was found at a frequency of about 5×10^{-8}, contained a plasmid prophage estimated from agarose gel electrophoresis to be about half the size of P1 Ap Cm (data not shown). Because these defective prophage contained the drug resistance determinants that result in high antibiotic resistance, we call them P1dR.

P1dR prophage DNA was digested with several restriction nucleases, and the fragments were compared with those of P1 Ap Cm. All of the P1dR prophage tested were missing a contiguous set of fragments and, in addition, had a single new junction fragment formed by joining the P1 DNA at the endpoints of the deletion (Fig. 1). The total size of 15 independent P1dR prophage was between 35 and 45.8 kilobases (kb) as determined by adding the sizes of their *Eco*RI fragments. The deletion endpoints were determined by measuring the size of the junction fragments produced after digestion of each of several different P1dR prophage with *Bam*HI, *Eco*RI, *Bgl*II, and *Pst*I-*Hin*dIII (data not shown). One deletion terminus (designated A on the left in Fig. 1) occurred at or near the same place in all 15 P1dR prophage tested (within a 2.7-kb region bounded by *Hin*dIII-*Pst*I-6 and *Bgl*II-9). The distance between this deletion terminus and the right-hand IS*1* of Tn*9* in P1 Ap Cm is less than 0.1 kb for the two P1dR prophage that have been most carefully mapped, so we presume that the deletion was generated by this IS*1* (see below). The deletion terminus at the right of the P1 map in Fig. 1 (called B) was different for each P1dR, although all of the termini lay within a region whose limits were defined by *Bam*HI-12 and *Eco*RI-5. If the right-hand IS*1* of Tn*9* formed the left deletion terminus, the right deletion terminus always occurred within a 10-kb region of the 104-kb P1 Ap Cm prophage (Fig. 1).

It seemed likely that the increased antibiotic resistance of the P1dR-containing strains resulted from an increase in copy number of the plasmid rather than from a regulatory mutation or gene duplication, since there were no restriction fragments present in disproportionate concentrations (data not shown). A representative plasmid, P1dR23, was found to be present as a 45-kb monomer with a copy number 8.6 times greater than that of P1 Ap Cm.

Instability of the high drug resistance phenotype. Since the P1dR prophage are deleted for all regions of P1 known to be involved in plasmid replication (1, 8, 57), they may not be replicons. To determine the stability of the P1dR forms, we

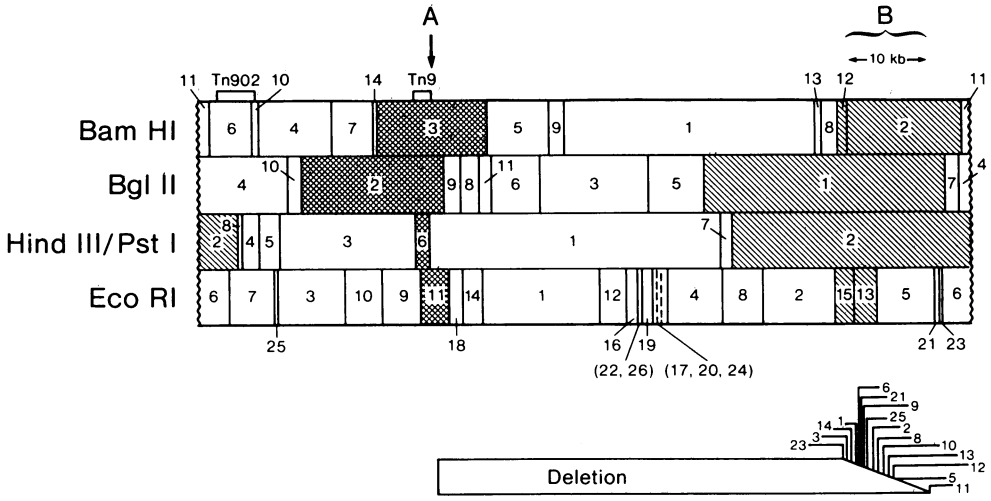

FIG. 1. Restriction map of P1 Ap Cm, showing the extent of the deletions in the P1dR prophage. The open bar shows the region deleted for 15 independent P1dR prophage. The A above the restriction map indicates the position of the deletion terminus, which is the same for all of the P1dR plasmids. The B above the restriction map indicates the region in which the variable P1dR deletion terminus is located. The shaded regions show which restriction fragments contain the deletion termini A and B. The restriction map of P1 Ap Cm is based on data from Bachi and Arber (5), Yun and Vapnek (63), Iida and Arber (30), and our unpublished data. The Tn*902* transposon is from the related phage P7 (53) and codes for ampicillin resistance. The Tn*9* transposon codes for chloramphenicol resistance. The positions of these two transposons are shown above the map. The P1 Ap Cm map is linearized at *loxP* for ease of display. *loxP* is the locus of site-specific recombination that generates the ends of the P1 genetic map which is linear, although the P1 prophage is circular (56).

grew the original highly drug-resistant strains without selection, plated the cells on nonselective medium, and then tested single cells for their levels of antibiotic resistance by replica plating. After 30 generations without selection, the fraction of highly drug-resistant cells was only 2×10^{-3}. Further growth without selection led to complete loss of high drug resistance. Eighteen independent "revertants" to low drug resistance had all lost the multicopy P1dR prophage and instead contained extra large P1 supercoiled DNA (ELPS) at a copy number of about one per *E. coli* chromosome (data not shown). Further growth of ELPS-containing cells in the absence of selection (40 generations) resulted in no loss of the ELPS plasmid.

DNA from two ELPS plasmids (descended, respectively, from a P1dR23 and a P1dR6 lysogen) was isolated and digested with restriction nucleases, and the fragments were separated on agarose gels. These ELPS plasmids contained almost all fragments of P1 Ap Cm intact, including the region with deletion terminus A of P1dR (*Eco*RI-11 and *Bam*HI-3; Fig. 1). The only fragments not present intact were those that contain the site of deletion terminus B of the P1dR prophage (*Bam*HI-12 and *Eco*RI-15 for the ELPS from the P1dR23-containing strain and *Bam*HI-2 and *Eco*RI-13 for the ELPS from the P1dR6-containing strain; Fig. 1). Densitometric tracings of the photographic negatives showed that all P1 Ap Cm fragments present in the P1dR plasmid were duplicated in the ELPS DNA. In addition, analysis of restriction nuclease digests revealed two fragments in the ELPS plasmids different in size from any present in either P1dR or P1 Ap Cm. These fragments, referred to as junction fragments, were isolated from ELPS DNA and mapped by both single and double digestion with *Eco*RI and *Bam*HI. *Pst*I digestion was used to determine whether Tn*9* or just IS*1* was present in each junction (data not shown). The structures determined for the two ELPS plasmids and their junction fragments are shown in Fig. 2. Some of the ELPS plasmids from low drug resistance derivatives of P1dR23 and P1dR6 lysogens have IS*1*-mediated deletions (13).

Structure of the DNA intermediate (dR-genic) in P1dR formation. Although P1dR plasmids are deleted for about half of the P1 Ap Cm genome, when high drug concentration selection was removed from P1dR lysogens, ELPS plasmids, which contain the entire P1 Ap Cm, were found (see above). Furthermore, marker rescue experiments and immunity tests (data not shown) demonstrated the presence in P1dR lysogens of P1 markers that should be absent judging from the restriction map of the P1dR prophage. These results suggest that a complete P1 Ap Cm genome is present in P1dR-containing strains. We suggest that this complete form is probably an intermediate in the generation of P1dR prophage (see below) and therefore refer to it as dR-genic.

The supercoiled and linear DNA fractions from a highly drug-resistant P1dR lysogen were tested by DNA hybridization (54) with a labeled P1 Ap Cm probe. The only P1-homologous fragments in the supercoiled DNA were those of the P1 prophage (data not shown). However, the expected P1 dR-genic DNA was present in the nonsupercoiled DNA fraction (Fig. 3). Trivial reasons for this result were ruled out by reconstruction experiments in which we were able to isolate and verify by gel electrophoresis the presence of large, supercoiled plasmids in mixtures of strains. P1 dR-genic DNA was not integrated at a unique site in the host chromosome, since no new junction fragments were found in Southern blots of DNA from two independent P1dR-containing strains (P1dR23 and P1dR6).

In P1dR-genic DNA, as in the ELPS plasmids (see above), all fragments of P1 Ap Cm were intact except for those containing the P1dR deletion terminus B. There were also two new junction fragments in the dR-genic DNA from strains containing either P1dR23 or P1dR6 which had the same sizes, respectively, as the junction fragments of the cognate ELPS plasmids. The detailed structures of these junction fragments were shown to be the same as the junctions in the ELPS plasmids by hybridization of dR-genic DNA with probes that hybridize to fragments spanning deletion termini A and B of each P1dR prophage (IS*1* and P1

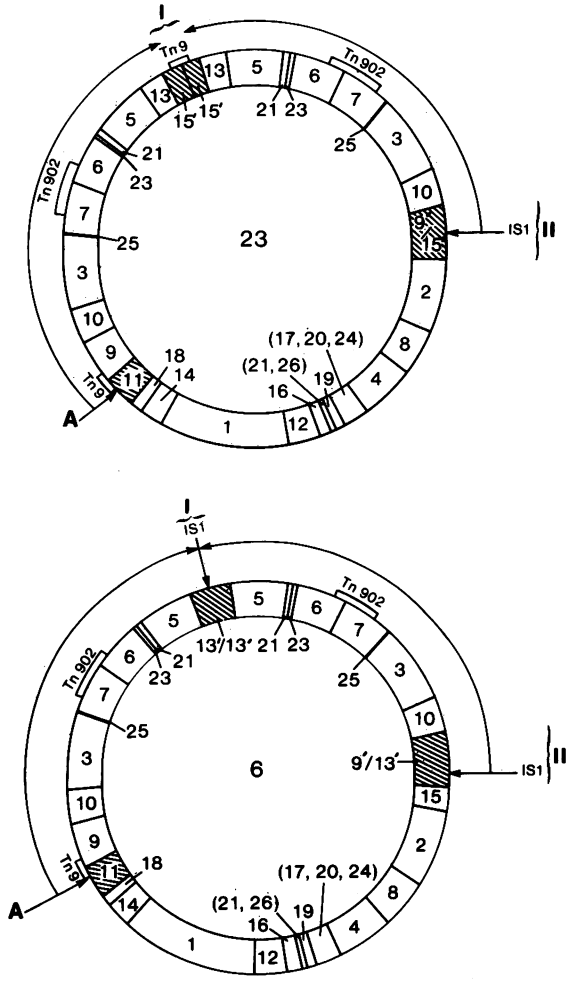

FIG. 2. *Eco*RI restriction maps of ELPS plasmids. The map labeled 23 is for an ELPS plasmid descended from a P1dR23-containing strain. The map labeled 6 is for an ELPS plasmid descended from a P1dR6-containing strain. The restriction fragments are numbered as in the unrearranged P1 Ap Cm map in Fig. 1. The locations of Tn*9*, Tn*902*, IS*1* elements, and the unrearranged copy of the P1dR deletion terminus A are indicated. The shaded regions show which *Eco*RI fragments contain the dR-genic junctions I and II.

Ap Cm *Eco*RI-10 for terminus A in both; P1 Ap Cm *Eco*RI-15 for P1dR23 terminus B and P1 Ap Cm *Eco*RI-13 for P1dR6 terminus B; Fig. 1 and Table 2).

In P1dR-genic or ELPS DNA from both strains, the entire P1dR plasmid was duplicated and inverted, forming junction fragments I and II. The basic structures of the dR-genic junctions were the same for both P1dR23- and P1dR6-containing strains (Fig. 2). It seems likely that ELPS DNA was derived from dR-genic DNA, since the only other P1 DNA in cells that give rise to ELPS is the defective P1dR prophage that lacks many P1 regions.

P1dR-genic DNA is present in ELPS-containing strains. When low drug resistance strains which contained ELPS DNA were plated on high-drug medium, the frequency of survival (10^{-2} to 10^{-5}) was at least 200-fold higher than that of the original P1 Ap Cm lysogen (5×10^{-8}). These highly resistant strains had lost the ELPS DNA and contained a P1dR plasmid. Since both ELPS and dR-genic DNAs contain at least one copy of the original P1dR region flanked by IS*1* elements, homologous recombination between these elements would generate this P1dR plasmid from either. Because P1dR lysogens are generated at a higher frequency from ELPS-containing strains than from the original P1 Ap Cm lysogen, it is likely that the ELPS-containing strain has already undergone the first step in generating a P1dR plasmid.

Although different P1dR plasmids contain different deletions (Fig. 1; see above), all highly resistant survivors of a single ELPS-containing strain had the same P1dR plasmid, and this plasmid was the same as that in the parental strain from which the low-resistance ELPS-containing strain was derived (data not shown). This suggests that the original P1 Ap Cm lysogen undergoes a change that dictates which P1dR it can generate and that this change persists in the presence of both the P1dR and ELPS plasmids. It seems likely that this change is the rearrangement of the P1 Ap Cm plasmid to produce the P1dR-genic form. In support of this, we found undeleted dR-genic DNA in strains containing ELPS plasmids which had undergone IS*1*-mediated deletions. For example, in one Southern blot experiment a strain with a deleted ELPS plasmid lacking junction I (Fig. 2) contained a fragment with the same structure as junction I in the nonsupercoiled DNA (data not shown).

Once formed, ELPS plasmids do not appear to require coresident dR-genic DNA as a template for replication. Although we have never observed a deletion in dR-genic DNA, deletions often occur in ELPS plasmids. Once a specific ELPS deletion plasmid is formed, no ELPS plasmids without deletions or with smaller deletions are found in descendents of this strain, as would be expected if the undeleted, coresident dR-genic form served as a template for their synthesis. This suggests that ELPS plasmids are replicons.

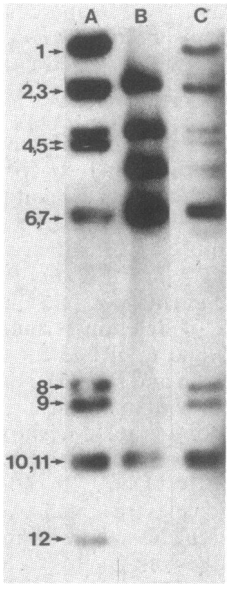

FIG. 3. Identification of P1 Ap Cm fragments missing from the P1dR prophage in the nonsupercoiled DNA fraction from a highly drug-resistant P1dR23-containing strain. Southern hybridization of *Bam*HI-digested DNA with a ^{32}P-labeled P1 Ap Cm probe (see Materials and Methods). The numbers and arrows at the left identify the *Bam*HI fragments of the P1 Ap Cm plasmid. Lanes: A, P1 Ap Cm supercoiled DNA; B, P1dR23 supercoiled DNA; C, nonsupercoiled DNA fraction from the P1dR23-containing strain.

Recombination dependence. We have been unable to isolate P1dR-containing derivatives of *recA*$^-$ strains. To determine whether the Rec$^+$ function is required only for generation of a P1dR plasmid or also for its maintenance, we isolated high drug resistance derivatives of P1 Ap Cm in temperature-sensitive *recA* and *recB* mutants at the permissive temperature. When these P1dR lysogens were grown at the nonpermissive temperature without drug selection, they lost the high-level drug resistance conferred by the P1dR plasmid much more rapidly than in the Rec$^+$ strain (Table 3). The dependence on *recB*$^+$ as well as *recA*$^+$ shows that maintenance of high drug resistance requires homologous recombination and not some other *recA*$^+$-dependent function like induction of the SOS pathway (62). Strains that lost the P1dR plasmid when grown under Rec$^-$ conditions all contained ELPS DNA (data not shown). Thus, although maintenance of the P1dR prophage is dependent on the Rec$^+$ pathway, maintenance of ELPS DNA does not require Rec$^+$.

TABLE 2. Southern blot analysis of dR-genic junction fragments[a]

P1dR strain	Enzyme[b]	dR-genic junction[c]	Fragment size (kb)	Probe[d]	Hybridization
P1dR23	*Bam*HI	I	3.6	IS*1*	+
				P1dR23/J[e]	+
	*Bgl*II	I	35	IS*1*	+
				*Eco*RI-13	+
				*Bam*HI-2	+
				*Eco*RI-15	+
P1dR6	*Eco*RI	I	7.9	IS*1*	+
				*Eco*RI-15	−
				*Eco*RI-13	+
				*Eco*RI-10	−
				*Bam*HI-2	+
P1dR23	*Bam*HI	II	5.7	IS*1*	+
				*Eco*RI-15	+
				*Eco*RI-13	−
	*Eco*RI	II	6.9	IS*1*	+
				*Eco*RI-15	+
				*Eco*RI-13	−
P1dR6	*Bam*HI	II	5.8	IS*1*	+
				*Eco*RI-15	+
				*Eco*RI-13	+
				*Eco*RI-10	+
				*Bam*HI-2	+
	*Eco*RI	II	5.1	P1dR6/J[e]	+

[a] The nonsupercoiled DNA fraction which contains dR-genic DNA was from the P1dR23- or P1dR6-containing, highly drug-resistant strain. Purified DNA was digested with restriction nucleases and hybridized as described in Materials and Methods. P1dR plasmid DNA and chromosomal DNA from the parental strain, which contains no P1 sequences, were always included on the Southern blots as controls.

[b] Restriction nucleases with which the nonsupercoiled DNA fraction was digested.

[c] See Fig. 2 for the structure of dR-genic DNA junctions I and II.

[d] DNA fragments were purified, and the probes were labeled as described in Materials and Methods. The IS*1* probe was isolated from pMob45 (Hollingshead, Ph.D. thesis) after digestion with *Eco*RI. The P1 Ap Cm fragments *Eco*RI-10, *Eco*RI-13, and *Bam*HI-2 were isolated from the P1dR23 plasmid, and *Eco*RI-15 was isolated from P1 Ap Cm (Fig. 1).

[e] Probe P1dR23/J is the *Eco*RI junction fragment isolated from the p1dR23 plasmid. It contains the portions of *Eco*RI-15 and *Eco*RI-11 that are in the P1dR23 plasmid (Fig. 1). Probe P1dR6/J is the *Bam*HI junction fragment isolated from the P1dR6 plasmid. It contains the portions of *Bam*HI-2 and *Bam*HI-3 that are in the P1dR6 plasmid (Fig. 1). Both probes contain Tn*9*.

TABLE 3. Recombination dependence of P1dR plasmids[a]

Plasmid	Host	Phenotype	Growth temp (°C)	Generations without drug selection	Fraction retaining P1dR[b,c]
P1dR23[d]	KL398	Rec$^+$	30	13	0.68
		Rec$^+$	42	13	0.66
P1dR23[d]	KL399	Rec$^+$	30	13.5	0.76
		RecA$^-$	42	13.5	1.6×10^{-3}
P1dR23[d]	AB1157	Rec$^+$	30	11.5	0.77
		Rec$^+$	42	11.5	0.58
P1dR23[d]	SK119	Rec$^+$	30	12.5	0.75
		RecB$^-$	42	12.5	1.2×10^{-4}
P1dR43[e]	KL399	RecA$^+$	30	23.5	0.74
		RecA$^-$	42	24	4.2×10^{-3}
P1dR43[e]	SK119	RecB$^+$	30	89	1
		RecB$^-$	42	85	$<5 \times 10^{-4}$
P1dR7[e]	KL399	RecA$^+$	30	51	0.73
		RecA$^-$	42	44	$<5 \times 10^{-3}$
P1dR7[e]	SK119	RecB$^+$	30	47	1
		RecB$^-$	42	47	7×10^{-3}

[a] Overnight cultures grown in LBB containing 2 mg of ampicillin and 500 μg of chloramphenicol per ml at the temperatures indicated were plated at appropriate dilutions on selective and nonselective media, and the plates were incubated at 30 or 37°C. The cultures were also diluted into drug-free LBB to about 100 cells per ml (determined by microscopic count) and grown overnight at the same temperature. This procedure was repeated either until high drug resistance was lost or the cells had been grown without selection for 150 generations.

[b] The fraction retaining P1dR was determined by dividing the cell titer on tryptone containing 2 mg of ampicillin and 500 μg of chloramphenicol per ml by the cell titer on tryptone. Since the presence of the P1dR form is required for high drug resistance (P1dR-genic DNA confers only low drug resistance), this fraction measures loss of the P1dR form. At least 90% of the cells retain low drug resistance.

[c] For P1dR43, the fraction that retained P1dR was the cell titer on tryptone containing 20 μg of ampicillin per ml divided by the cell titer on tryptone. For P1dR7, the fraction that retained P1dR was the cell titer on tryptone containing 40 μg of chloramphenicol per ml divided by the cell titer on tryptone.

[d] The recombination-dependent P1dR23 was measured in the presence of the dR-genic DNA.

[e] P1dR43 and P1dR7 were derived from P1dR23. They were present in strains lacking any other P1 DNA.

The P1dR plasmid is a replicon. The Rec$^+$ requirement for maintenance of the P1dR prophage suggests that P1dR might be unable to replicate in the absence of coresident P1dR-genic DNA. Instead, P1dR might be generated continuously from a replicating P1dR-genic copy by reciprocal recombination between IS*1* elements. However, the rate of recombination required to maintain a copy number of 8 to 9 P1dR plasmids per chromosome (see above) would be very high.

To test whether a P1dR plasmid replicates in the absence of other P1 DNA, we used P1dR23 plasmid DNA to transform a strain containing no P1 prophage. Double-drug-resistant transformants were obtained, and some contained the entire P1dR23. Hybridization of DNA isolated from the transformants to a labeled P1 DNA probe showed that none of the transformants contained P1dR-genic DNA (data not shown).

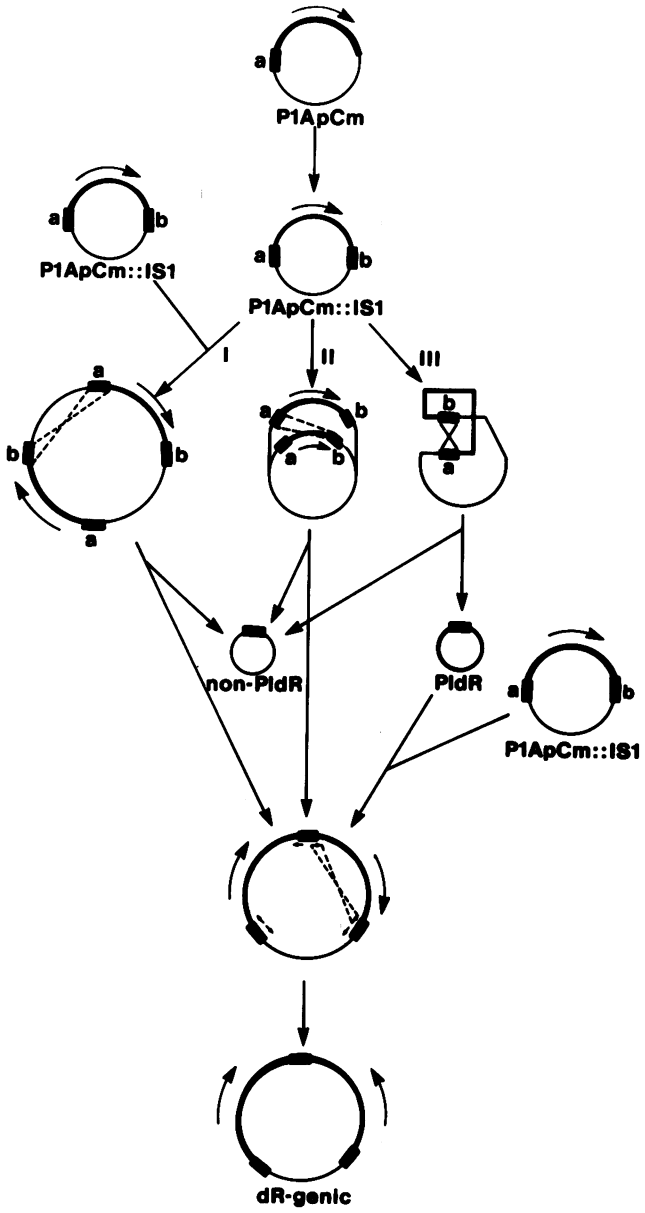

FIG. 4. Model(s) for the formation of dR-genic DNA (42). The boxes labeled a and b represent Tn*9* or IS*1*. Box a represents the Tn*9* resident in P1 Ap Cm, and b represents either IS*1* or Tn*9* (both have been found in independent mutants). The region of the DNA drawn with heavier lines represents the P1 Ap Cm region found in the PldR prophage. The arrows outside the circles show the orientation of the PldR region. The small arrows inside the circle represent inverted repeats found at the ends of IS*1* elements.

Most of the plasmids present in the transformants were derived from PldR23 by IS*1*-mediated deletions. It is probable that these deleted plasmids were present in the transforming DNA at a level undetectable by Southern blot analysis and had a selective advantage in transformation because of their small size (27). In agreement with this, the frequency of transformation of the 45-kb PldR23 plasmid was very low: 10^{-9} transformants per DNA molecule compared with 1.6×10^{-6} for the 2.7-kb pUC9 control. A 2.6-kb derivative of PldR23 transformed as efficiently as the control plasmid.

The Rec$^+$ dependence of PldR maintenance was observed in the original PldR-containing strains, which also contained PldR-genic DNA. To determine whether PldR replication requires Rec$^+$ function or whether the initial observations were caused by the presence of PldR-genic DNA, we tested the stability of PldR23 deletion derivatives at high temperature in a *recA*(Ts) and a *recB*(Ts) strain. Both plasmids were stable at low temperature in these strains but required both RecA$^+$ and RecB$^+$ (Table 3).

DISCUSSION

High-copy-number PldR plasmids probably arise from the large rearranged derivative of P1 Ap Cm, PldR-genic, which is found in the nonsupercoiled DNA fraction of PldR-containing strains. Because both of the dR-genic DNAs studied in detail contain a duplication of the PldR region (flanked by IS*1* elements), it seems likely that such duplications are required for either the formation of the PldR plasmid or maintenance of the PldR plasmid at a copy number sufficiently high for growth under selective conditions. When high antibiotic concentration selection is removed, these strains segregate derivatives that contain the PldR-genic form and a new ELPS form that has the same genetic organization as nonsupercoiled, PldR-genic DNA. It is not clear why the ELPS plasmid is lost when high drug concentration selects for PldR. We suggest that this might be due to competition among these P1 plasmids for a limited concentration of a factor required continuously for replication from any P1 origin (23).

The fact that IS*1* (or Tn*9*) is found at the fusion sites of both of the new dR-genic junctions (I and II in Fig. 2) suggests that the rearrangements are IS*1* (or Tn*9*) mediated. Tn*9* is a composite transposon which consists of a chloramphenicol resistance gene flanked by directly oriented IS*1* elements. Since the IS*1* elements contain everything necessary for Tn*9* transposition, the types of rearrangements mediated by Tn*9* are the same as those mediated by IS*1* alone (32).

At least three separate events are needed to generate dR-genic DNA from P1 Ap Cm (Fig. 4). The first intermediate in dR-genic formation, called P1 Ap Cm::IS*1*, is probably formed by the transposition of Tn*9* or IS*1* into a second site in the P1 Ap Cm prophage (the PldR deletion terminus B region; Fig. 1), providing the homology needed to generate gene duplication. This second Tn*9* or IS*1*, along with resident Tn*9*, defines the limits of the region that forms the PldR plasmid. The second intermediate is probably formed by duplication of the IS*1*-flanked PldR region in P1 Ap Cm::IS*1* DNA, which could proceed by one of three pathways (I, II, or III in Fig. 4) that have been proposed for the generation of tandem amplification (duplication) of other IS*1*-flanked resistance determinants (Tn*9* in P1 Cm [39] and the r-det regions in plasmids R100, R1, and NR1 [9, 42]). All three pathways involve recombination between IS*1* elements flanking the drug resistance genes.

The first step in pathway I (Fig. 4) is the formation by homologous recombination of a cointegrate between two identical P1 Ap Cm::IS*1* molecules. The second step is resolution of the cointegrate by recombination between flanking IS*1* elements into two products, a plasmid containing one copy of the DNA not found in PldR, non-PldR, and a DNA molecule that differs from the dR-genic form only in that the two copies of the PldR region are directly repeated. We have never seen a plasmid corresponding to non-PldR DNA. Although it should be able to replicate independently since it includes all the DNA making up a known P1

minireplicon (1, 8, 57), this non-P1dR plasmid would also contain the P1 incompatibility functions. Because antibiotics select for the other P1 forms, the non-P1dR DNA may be lost from the strain if it is formed.

Pathway II (Fig. 4) involves reciprocal intramolecular recombination between IS*1* elements at opposite ends of the P1dR region in the two daughters of a partially replicated P1 Ap Cm::IS*1* DNA. After the recombination event, completion of the replication cycle results in one daughter with a direct duplication of the P1dR region and one composed only of non-P1dR DNA. These are the same products as those obtained in pathway I.

The first step in pathway III (Fig. 4) is the dissociation of P1 Ap Cm::IS*1* DNA into two circles, P1dR and the non-P1dR, by homologous recombination between IS*1* elements. Recombination of the autonomous P1dR plasmid with a second P1 Ap Cm::IS*1* molecule generates the product with the directly repeated P1dR region. Because the dR-genic form is found in every strain producing a P1dR, it seems unlikely that it is a side product, as would be the case if pathway III were used.

All three pathways generate a product that contains a directly oriented duplication of the DNA making up the P1dR plasmid. However, in dR-genic DNA, the repeated regions are in inverted orientation. The small, imperfectly homologous inverted repeats (40) at the ends of the IS*1* elements bordering the duplication could presumably serve as sites for a homologous recombination event that inverts the region between them. Recombination between such small homologies has been shown to occur (2, 19, 20).

The direct orientation of the duplication of the P1dR region is probably not found in either of the dR-genic DNAs studied, because it would make the duplication unstable; one copy of the duplicated region would be lost by recombination between homologous sequences anywhere within the 35 to 46 kb of the duplication. On the other hand, recombination between homologous regions in an inverted orientation would merely result in inversion of the intervening DNA. The only effect this would have on the structure of the dR-genic DNA would be to change the orientation of the Tn*9* or IS*1* between the two copies of the duplication. It seems likely that both orientations of this Tn*9* exist within the population.

The P1dR plasmid could then be formed from the P1dR-genic DNA by intramolecular recombination between directly oriented IS*1* elements which flank the two copies of the P1dR region. In some cells in the dR-genic DNA-containing population, there should be a copy of the P1dR DNA region flanked by directly repeated IS*1* elements, since this region can be inverted. We were unable to determine whether formation of the P1dR plasmid is dependent on Rec^+ function, since P1dR maintenance requires this function.

Formation of the P1dR plasmid by intramolecular recombination within a nonreplicated molecule would lead to loss of one copy of the duplicated P1dR region from dR-genic DNA. We have never observed deletions occurring in P1dR-genic DNA, so we believe that the intramolecular recombination necessary to form the P1dR plasmid involves a replicative intermediate of the dR-genic DNA in which the P1dR region has already been duplicated.

The site of P1dR deletion terminus A (one IS*1* of the resident Tn*9*) appears to be the same for all of the mutants, presumably because this terminus is created by an IS*1*-promoted event. It is possible that we only found insertion of the IS*1* which marks deletion terminus B within the 10-kb region defined by *Bam*HI-12 and *Eco*RI-5 because too few P1dR plasmids were examined to identify one in the 6-kb region of P1 Ap Cm to the right of *Eco*RI-5 (Fig. 1; *Eco*RI-21, 23 and 6 up to the ampicillin resistance gene). However, high-resistance strains resulting from IS*1* insertions to the left of *Bam*HI-12 (Fig. 1) should have been found because this is a large region of DNA. Since the copy number of some plasmids is inversely proportional to size (23, 26, 48, 50, 59; our unpublished observations for P1dR), it seems possible that the large P1dR plasmids that would result from such insertions would have too low a copy number to be found in our selection.

Highly drug-resistant mutants were found at a frequency of about 5×10^{-8}, which indicates that the three steps (transposition of IS*1*, duplication of the IS*1*-flanked region, and inversion of the IS*1*-flanked region) that lead to their formation must occur at fairly high frequencies.

No frequencies have been reported for intramolecular transposition of both IS*1* and Tn*9*, but frequencies between 10^{-5} and 10^{-7} have been reported for their intermolecular transposition (10, 25, 55). Frequencies of at least 10^{-3} have been measured for spontaneous duplication of IS*1*-flanked drug resistance regions (28). There are no good estimates of the probable frequency for recombination between small homologies like the inverted repeats at the ends of IS*1*. However, if the IS*1* transposition occurred at a frequency of 10^{-5}, and the duplication of the IS*1*-flanked region occurred at 10^{-3}, the homologous recombination event leading to inversion of the IS*1*-flanked region would have to occur at a frequency greater than one. It is therefore likely that, for formation of P1dR either transposition or duplication occurs at a higher frequency than has been measured previously.

The P1dR plasmid is an independent replicon, although the P1dR prophage does not contain any of the P1 genome previously identified as required for replication. Replication of P1dR is, however, unusual in its dependence on the $RecA^+$ function of the host. Because lytic growth of many large phages, including P1, requires recombination for the late phase of replication (24, 36, 49, 64), it seems possible that, instead of using the normal plasmid replication origin and functions, P1dR utilizes the vegetative replication mode of the phage. We are currently testing this hypothesis and investigating the requirements for Rec^+-dependent replication of P1dR.

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