

Construction and Characterization of Amplifiable Multicopy DNA Cloning Vehicles Derived from the P15A Cryptic Miniplasmid

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Construction and characterization of a class of multicopy plasmid cloning vehicles containing the replication system of miniplasmid P15A are described. The constructed plasmids have cleavage sites within antibiotic resistance genes for a variety of commonly employed site-specific endonucleases, permitting convenient use of the insertional inactivation procedure for the selection of clones that contain hybrid DNA molecules. Although the constructed plasmids showed DNA sequence homology with the ColE1 plasmid within the replication region, were amplifiable by chloramphenicol or spectinomycin, required DNA polymerase I for replication, and shared other replication properties with ColE1, they were nevertheless compatible with ColE1. P15A-derived plasmids were not self-transmissible and were mobilized poorly by Hfr strains; however, mobilization was complemented by the presence of a ColE1 plasmid within the same cell.

The recent development of DNA cloning methods has made possible the introduction into *Escherichia coli* of genetic material from a wide variety of sources. Such experiments have involved principally use of cloning vehicles or vectors derived from plasmid ColE1 or pSC101 or from bacteriophage λ . Genetic manipulation of these replicons in vivo or in vitro has yielded a series of specialized cloning vehicles having individual properties suited to particular experimental needs (1, 5, 42, 47, 51, 53, 58). However, since all of the commonly used amplifiable multicopy plasmid cloning vehicles have been derived from the ColE1 replicon, experiments requiring the use of different amplifiable and compatible recombinant plasmids within the same bacterial cell have not been practical.

The cryptic plasmid derived from *E. coli* 15 is one of the smallest known naturally occurring replicons, consisting of approximately 2,300 base pairs. Although the size of the P15A plasmid allows for only two or three genes, earlier studies have shown that this plasmid contains all of the functions necessary for it to exist as an autonomous replicon (39). Because of its small size and its multicopy status (22), P15A seems to be an especially suitable source of a replication system for the construction of a series of special-purpose plasmid cloning vehicles. Although P15A does not code for detectable phenotypic properties, an indirect selection procedure (39) allows the plasmid to be separated from the other replicons

that are present concurrently in its natural host, *E. coli* 15; the P15A replicon can then be linked in vitro to a series of conveniently selectable antibiotic resistance determinants. The choice of resistance genes that contain cleavage sites for commonly available restriction endonucleases allows the simple isolation of hybrid clones by insertional inactivation (59).

The present communication describes the construction and characterization of a class of DNA cloning vehicles derived from miniplasmid P15A. The constructed plasmids carry antibiotic resistance genes that contain single cleavage sites for the *EcoRI*, *HindIII*, *SmaI*, *HincII*, *BamI*, *SaII*, *PstI*, and *XhoI* endonucleases and also contain cleavage sites for a number of other commonly employed site-specific endonucleases. Although the plasmids are amplifiable by either chloramphenicol or spectinomycin and share a number of other properties with ColE1, they are compatible with ColE1-derived replicons and can be propagated concurrently with such replicons in the same bacterial cell. Construction of the P15A-derived cloning vehicles described here employed a series of experimental techniques that appear to be generally applicable for the construction of other special-purpose plasmids.

MATERIALS AND METHODS

The plasmids and bacterial strains used in these experiments are listed in Tables 1 and 2.

TABLE 1. *Bacterial strains and previously described plasmids*

Strain/plasmid	Relevant properties	Reference/source
<i>E. coli</i> strains		
C600		2
CR34N	<i>nalA thy</i>	20
JC1569	<i>recA</i>	12
D7001	Hfr	10
SC293	<i>polA</i> (Ts)	59
Previously described plasmids		
pSC101	Tc ^r	18
pSC204	Tc ^r Km ^r	Km ^r fragment from R6-5 inserted into <i>EcoRI</i> site of pSC101 (19)
pSC105	Tc ^r Ap ^r	Temperature-sensitive replication mutant of pSC101 carrying Tn3 (40)
pSC212	Km ^r Ap ^r	Deletion mutant of pSC105::Tn3 (48)
pCS84	Km ^r	From S. Chang; Km ^r fragment from R6-5 (19) inserted into <i>EcoRI</i> site of pACYC184
P15A	Cryptic plasmid	22
ColE1-K30	ColE1 immunity and production	29, 34
pKT002	Cm ^r	Cm ^r fragment from R6-5 inserted into <i>EcoRI</i> site of ColE1 (Timmis et al., in press)
pFC012	(Sm ^r -Sp ^r)	Sm ^r -Sp ^r fragment of R6-5 inserted into <i>EcoRI</i> site of ColE1 (Timmis et al., in press)

TABLE 2. *Plasmids constructed in current study*^a

Plasmid	Antibiotic resistance determinant(s)	Mol wt (×10 ⁶)	Length (kb pairs)
pACYC139	Km ^r	4.5	6.75
pACYC140	Km ^r	3.6	5.4
pACYC142	Ap ^r Km ^r	7.5	10.8
pACYC175	Cm ^r Tc ^r	8	12.0
pACYC177	Ap ^r Km ^r	2.45	3.45
pACYC184	Cm ^r Tc ^r	2.60	3.9

^a Molecular weight and contour length data for the plasmids constructed in the current study were calculated from the agarose gel electrophoresis patterns shown in Fig. 2A and B, using fragments of bacteriophage T5 DNA as length standards (see Materials and Methods). The standard error in such estimations is ±10%.

Reverse transcriptase (specific activity, 70 U/ml) was a gift from M. Bishop, University of California, San Francisco. The various restriction endonucleases and the bacteriophage T4 DNA ligase used in these experiments were obtained from New England Biolabs, with the exceptions of *EcoRI* and *BamI* which were obtained from Miles Laboratories.

The conditions used for growth of bacterial cells and for the isolation of covalently closed circular plasmid DNA have been described (21), as have the procedures used for the transformation of *E. coli* (20).

Antibiotic concentrations used for routine selection were ampicillin (Ap, 25 µg/ml), kanamycin (Km, 25 µg/ml), chloramphenicol (Cm, 25 µg/ml), tetracycline (Tc, 10 µg/ml) and spectinomycin (Sp, 25 µg/ml). Amplification of plasmid DNA in the presence of Cm was carried out at a Cm concentration of 100 µg/ml as

described previously (13); amplification by Sp (J. Davies, personal communication) was carried out at an Sp concentration of 300 µg/ml.

Digestion of DNA by restriction endonucleases was accomplished by published procedures and standard assay conditions. The conditions used for reverse transcriptase reactions have been described (33). DNA ligase reactions were carried out in 0.025-ml volumes containing 50 mM tris(hydroxymethyl)aminomethane (Tris; pH 7.5), 1.0 mM ATP, 10 mM MgCl₂, 10 mM dithiothreitol, 0.01% gelatin, and DNA at a final concentration of 250 µg/ml (62). Reaction mixtures were incubated overnight at 14°C, and the enzyme was then inactivated at 65°C for 5 min.

Electrophoresis of DNA was carried out either in simple agarose gels in Tris-borate-ethylenediaminetetraacetate buffer as described by Sharp et al. (56) or in a composite gel system consisting of 0.2% acrylamide plus 0.5% agarose in Tris-borate-ethylenediaminetetraacetate buffer as described by Landy et al. (41). *HindIII*-generated fragments of bacteriophage T5 DNA (28) were used as molecular length standards in gels and were a gift from H. Bujard.

The methods used for electron microscope heteroduplex analysis of plasmid DNA sequence relationships have been described (7, 25, 55).

Elution of DNA fragments from agarose gels. After electrophoresis, agarose gels were stained by soaking in ethidium bromide (5 µg/ml) for 5 min and were then viewed under long-wavelength (300-nm) UV light to preclude excessive DNA nicking. To remove DNA bands from gels, the gels were frozen with dry ice, sliced, and macerated between two layers of Parafilm. The gel was then soaked in 10× the volume of TE buffer (0.01 M Tris [pH 8.0], 0.001 M ethylenediaminetetraacetate) for 4 to 6 h at 4°C, and centrifuged.

gation of the mixture at 40,000 rpm was carried out for 2.5 h at 4°C. The supernatant was extracted with butanol to remove ethidium bromide (6), extracted sequentially with phenol and ether, and lyophilized. The lyophilized material was suspended in 0.1 volume of TE and precipitated by addition of 2.5 volumes of cold absolute ethanol; the precipitate was suspended in TE at a final DNA concentration of 30 µg/ml.

RESULTS

Construction of the pACYC177 plasmid. Although P15A does not contain cleavage sites for any of the commonly employed restriction endonucleases that utilize a recognition site consisting of six or more nucleotides, the plasmid is cleaved once with the *HpaI* enzyme and is divided into two fragments of 1.06 and 0.99 kilobase (kb) when cleaved by the *HincII* endonu-

lease. The *HpaI* site on P15A DNA, which has a recognition sequence consisting of GTT AAC (29), is cleaved also by *HincII*, which has a recognition sequence consisting of CTPy PuAC (52). In the first step of construction of the pACYC177 plasmid (Fig. 1A), a DNA segment containing a Km resistance gene derived originally from the R6-5 plasmid, and inserted previously into the pSC101 plasmid to form pSC105 (19), was introduced into the *HpaI* site of P15A.

The pSC105 gene specifying Km resistance is located between an *EcoRI* site and a *SalI* site (our unpublished data). The pSC105 plasmid was digested with both of these enzymes, and a DNA fragment of the size calculated to be appropriate (4.6 kb) was isolated from agarose gels as indicated in Materials and Methods. The

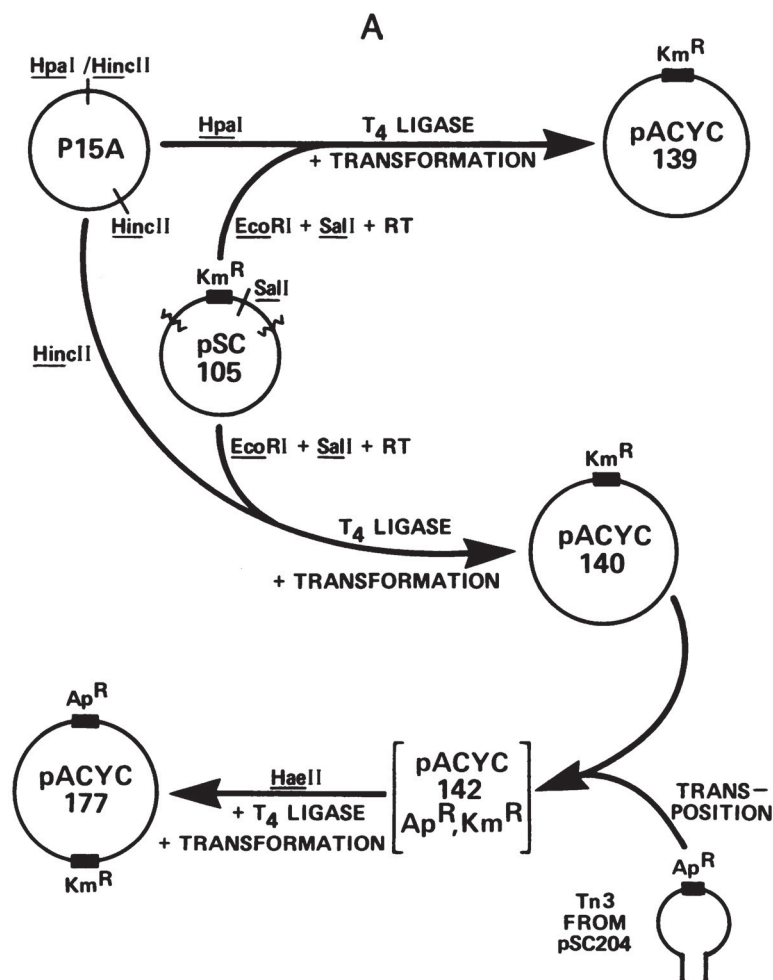


FIG. 1. Schematic diagrams showing the genealogy of cloning vehicles constructed in the current study. (A) Construction of the pACYC177 plasmid. (B) Construction of the pACYC184 plasmid. RT, RNA-dependent DNA polymerase (reverse transcriptase).

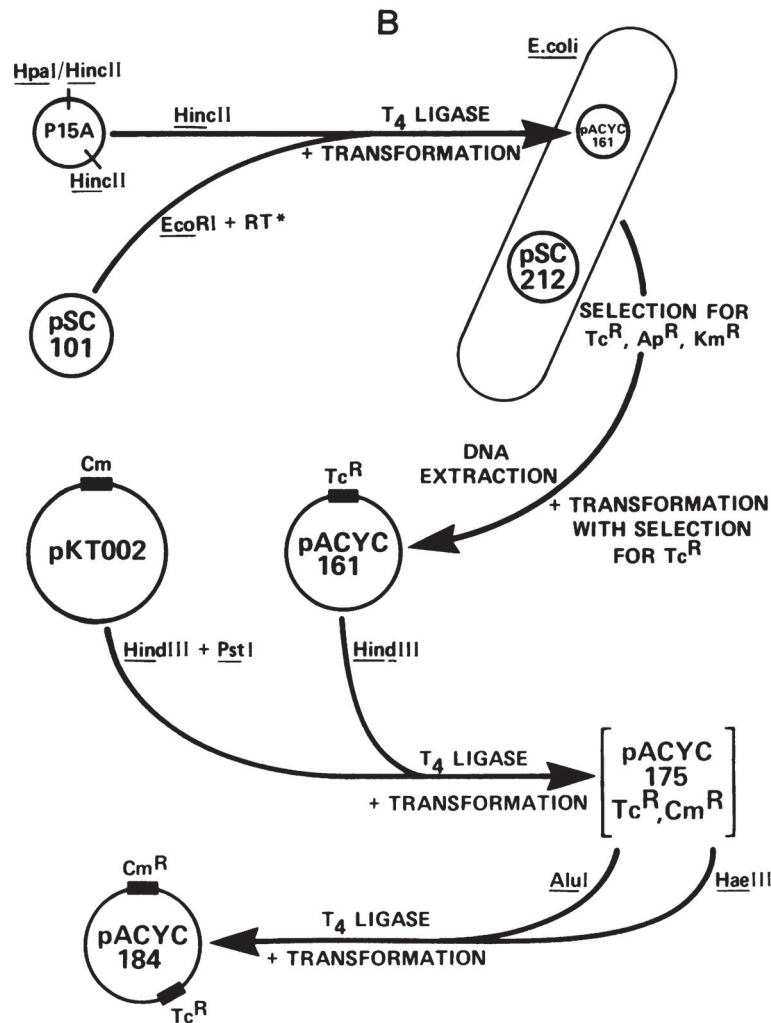


FIG. 1B.

single-strand 5' protruding ends generated by the *EcoRI* and *SalI* endonucleases were filled in by using reverse transcriptase (33), and the resulting blunt-ended DNA fragment was ligated to *HpaI*- or *HincII*-cleaved blunt-ended fragments of P15A DNA by using bacteriophage T₄ ligase. After transformation of the ligation mixture into *E. coli* C600, bacterial colonies resistant to Km were selected (frequency, ~10 Km^r colonies per μg of ligated DNA mixture). The plasmid isolated from a Km-resistant clone derived from the ligation mixture containing *HpaI*-digested P15A DNA was designated pACYC139, and the plasmid obtained from a similarly resistant clone derived from *HincII*-treated P15A DNA was designated pACYC140 (Fig. 1). The molecular weights of these plasmids, calculated

by agarose gel electrophoresis (Fig. 2) using *HindIII*-generated fragments of bacteriophage T5 DNA (28), are shown in Table 2.

pACYC140 was introduced by transformation into a C600 clone carrying the pSC204 plasmid. Clones that were concurrently resistant to both Ap and Km were selected at 45°C; since host bacteria are cured of the pSC204 plasmid at 45°C (39), and since translocation of Tn3 between plasmids occurs at a much higher frequency than between a plasmid and chromosome (40), the doubly resistant clones were expected to contain pACYC140 derivatives that have acquired the Ap resistance element Tn3. A plasmid (10.8 kb in length) isolated from one such clone was designated pACYC142.

Since translocation of Tn3 from pACYC142

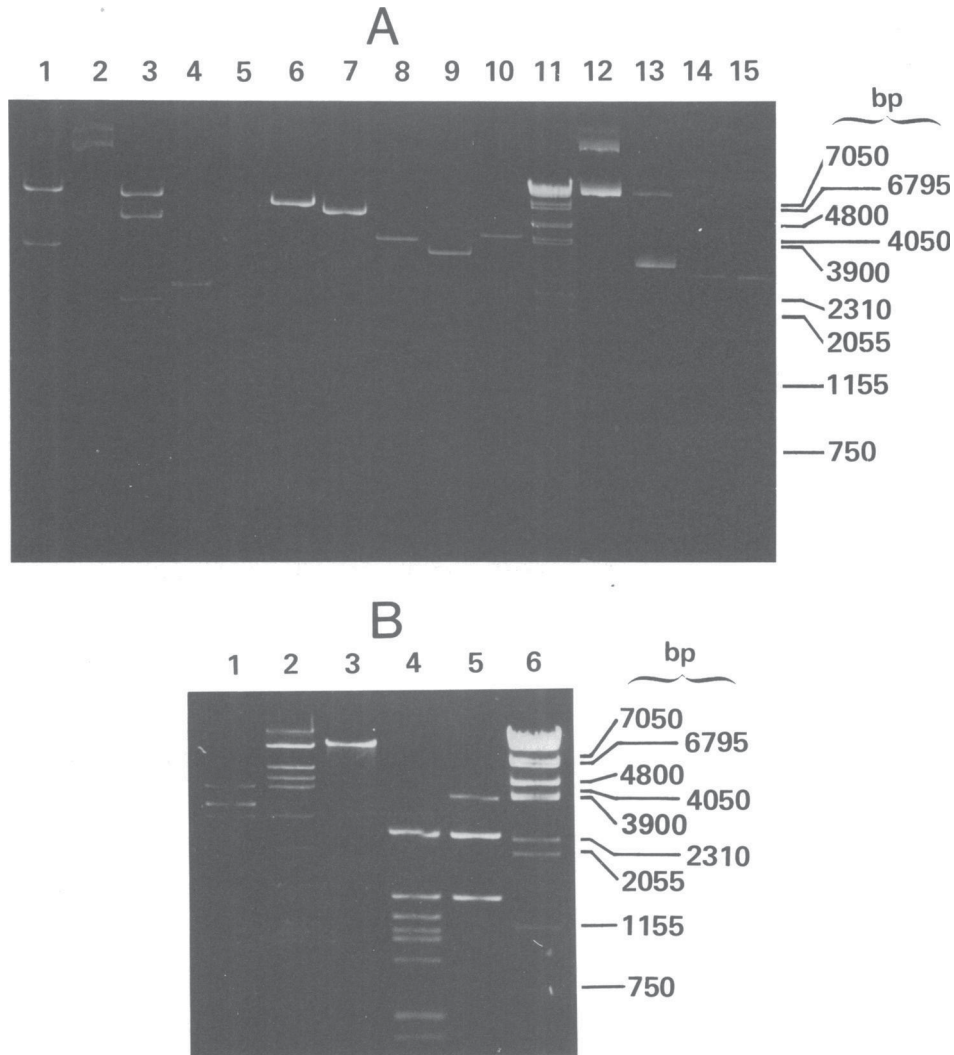


FIG. 2. Molecular length determinations of constructed plasmid DNA: agarose gel electrophoresis patterns. Molecular lengths were calculated from *Hind*III-generated fragments of bacteriophage T5 DNA (see text and below) and are accurate to $\pm 10\%$. (A) Lanes: (1) *Eco*RI-cleaved pACYC175 DNA, showing two fragments (10 and 3 kb); (2) pACYC175 plasmid, showing uncleaved covalently closed circular and open circular DNA bands (12 kb); (3) pSC105 DNA cleaved with *Eco*RI and *Sal*I endonucleases, showing three fragments (8.5, 5.4, and 1.8 kb) (a fourth fragment [0.637 kb] is not visible in the gel); (4) P15A plasmid cleaved with *Hpa*I endonuclease to give single linear fragment (2.17 kb); (5) P15A plasmid cleaved with *Hind*II and the nuclease, giving two fragments (1.06 and 0.99 kb); (6) pACYC139 plasmid cleaved to a single linear fragment (6.75 kb) by *Hind*III and endonuclease; (7) pACYC140 cleaved with *Hind*III endonuclease to yield a single linear fragment (5.4 kb); (8) pACYC177 plasmid cleaved with *Hind*III to yield a linear fragment (3.45 kb); (9) pACYC161 plasmid cleaved into a linear fragment by *Hind*III (3 kb); (10) pACYC184 plasmid cleaved into a linear fragment (3.9 kb) with *Hind*III endonuclease; (11) *Hind*III-generated fragments of bacteriophage T5 used as molecular length standards (29). The lengths of the fragments shown are (from top to bottom) 16.8, 15.45, 13.92, 13.05, 11.85, 10.74, 7.05, 6.80, 5.07, 4.88, 4.05, 3.9, 2.31, 2.05, 1.16, and 0.75 kb. Lane 12 shows multimers of uncleaved pACYC184 DNA extracted from *E. coli* C600. Lane 13 contains undigested DNA of the same plasmid isolated from JC1569 (*recA*), showing a molecular size characteristic of the monomeric plasmid. Lanes 14 and 15 show pACYC177 (monomeric) plasmid DNA isolated from strains C600 and JC1569, respectively. (B) Lanes: (1) pACYC175 DNA treated concurrently with *Hind*III and *Pst*I endonucleases; (2) pKT002 DNA digested with both *Hind*III and *Pst*I; (3) pACYC142 plasmid DNA cleaved with *Bam*I endonuclease; (4) pACYC142 plasmid DNA cleaved with *Hae*II endonuclease; (5) pACYC177 plasmid DNA treated with *Hae*II; (6) *Hind*III-generated fragments of bacteriophage T5 DNA, used as molecular size standard.

potentially could complicate the use of this plasmid as a cloning vehicle, and since the plasmid appeared to contain a substantial amount of extraneous DNA in addition to the genes coding for replication functions and for resistance to Km and Ap, the inverted repeat termini of Tn3 (38) were removed, and the plasmid was simultaneously reduced in size by a simple "scrambling" procedure. pACYC142 was treated with *HaeII* restriction endonuclease, which cleaves the plasmid into 10 fragments (Fig. 2B, lane 4) having 3' protruding ends. The DNA fragments present in the *HaeII* digest were ligated, and a culture of strain C600 was transformed with the ligated mix. Ten bacterial clones that were resistant to both Km and Ap were selected, and covalently closed circular plasmid DNA extracted from these clones was examined by electrophoresis in agarose gels. One of the plasmid DNA species isolated from a Km^r Ap^r transformant had a molecular weight of 3.45 kb (2.45×10^6) and was shown by retransformation to carry genes for resistance to both Ap and Km; this plasmid was designated pACYC177.

Construction of the pACYC184 plasmid. To introduce a gene for Tc resistance into the P15A plasmid, pSC101 was treated with *EcoRI* endonuclease at low ionic strength and pH 8.5; these experimental conditions are known to favor the *EcoRI** activity of the endonuclease which utilizes a four-base-pair recognition site (50). The resulting cohesive-ended fragments were treated with reverse transcriptase to create blunt ends and then were ligated to blunt-ended DNA fragments generated as a result of *HincII* digestion of P15A DNA (Fig. 1B).

The ligation mixture was used for transformation of a C600 clone carrying the pSC212 plasmid (48). This plasmid contains the pSC101 replication system and a gene that expresses resistance to Ap; however, a spontaneous deletion in pSC212 has eliminated the Tc resistance gene of this plasmid. Clones resistant to Ap and Tc were selected; since pSC212 and pSC101 utilize the same replication system and are incompatible, selection for the Ap resistance gene of pSC212 was expected to prevent establishment of any pSC101-derived recombinant replicons containing the Tc gene. Thus, the procedure selects for new plasmids that result from linkage of an *EcoRI**-generated Tc resistance fragment of pSC101 to the P15A plasmid replication system, which is compatible with pSC212.

To ensure segregation of any derivatives of the pSC101 replicon that might have been taken up by pSC212-containing cells, transformed clones were grown in medium containing Ap for 20 generations and were screened again for Tc resistance. Clones that expressed resistance to

Km, Ap, and Tc after 20 generations of growth in the absence of Tc selection were isolated and found to contain two plasmids: pSC212 and a P15A-derived replicon containing the Tc resistance gene from pSC101. DNA isolated from six separate clones containing the two plasmids was examined by electrophoresis on an agarose slab gel. The clone that contained the smallest plasmid was identified, and the two plasmids present in this clone were separated by transformation into *E. coli* C600. The Tc resistance plasmid thus obtained, which has a size of 3.0 kb (2.0×10^6 daltons), was designated pACYC161.

A gene that expresses resistance to Cm was added to pACYC161 as follows. pKT002 is a plasmid which consists of the pML21 (35) and a *HindIII* Cm^r plasmid derived from R6-5 (K. Timmis, F. Cabello, and S. N. Cohen, *Mol. Gen. Genet.*, in press). pKT002 was treated with both the *HindIII* and *PstI* restriction endonucleases (Fig. 2B, lane 2). Because the 3' protruding ends of DNA fragments generated by *PstI* join easily to each other, the resulting DNA fragments contain an internal *PstI* site and *HindIII* ends and can be introduced into the *HindIII* site of pACYC161. After these steps were carried out, the ligation mixture was transformed into strain C600, and Cm^r Tc^r clones were selected. One such clone contained a plasmid (pACYC175, 12 kb in length) in which a Cm resistance gene had been introduced onto pACYC161.

To reduce the size of the pACYC175 plasmid, three separate preparations of plasmid DNA were treated respectively with the *HaeIII*, *AluI*, and *HincII* endonucleases, mixed, and subjected to ligation. After transformation of strain C600 with the ligated DNA, clones resistant to both Tc and Cm were pooled, and covalently closed circular plasmid DNA extracted from the pooled clones was examined by electrophoresis in agarose gels. The mixture contained a series of plasmids ranging in size from 2.3×10^6 to 8.0×10^6 daltons. The most rapidly migrating (smallest) covalently closed circular DNA species was isolated from the gel and used for transformation of strain C600; individual Tc^r Cm^r clones were isolated after transformation. The plasmid from one of these clones, which was selected for further study, had a size of 3.75 kb (2.6×10^6 daltons) and was designated pACYC184 (Fig. 1B).

Endonuclease cleavage sites on the pACYC177 plasmid. Figures 3A and B show the agarose gel electrophoresis patterns of pACYC177 DNA that has been treated with combinations of restriction endonucleases. The electrophoresis pattern of *HaeII*- or *HaeIII*-digested ColE1 DNA (49) was used as a standard for calculation of fragment size. A map of restric-

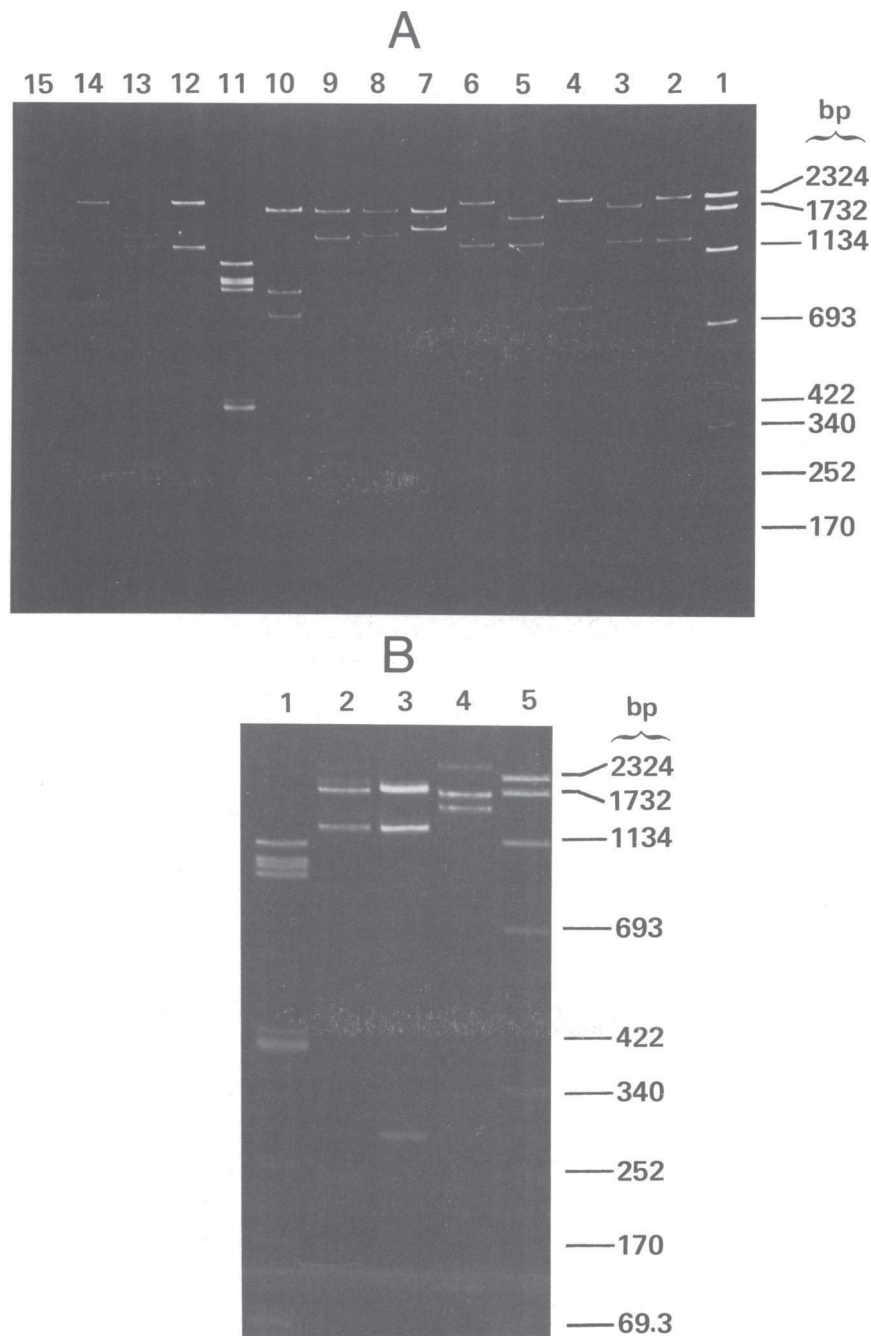


FIG. 3. Mapping of endonuclease cleavage sites on the pACYC177 plasmid by agarose-acrylamide gel electrophoresis. *ColE1* DNA digested with either *HaeII* (A, lane 1; B, lane 5) or *HaeIII* (A, lane 11; B, lane 1) endonuclease was used as the molecular size standard. (A) 2% acrylamide-0.5% agarose gel. The enzymes used for cleavage of pACYC177 were: lanes (2) *HincII* plus *HindIII*; (3) *HincII*, *HindIII*, and *PstI*; (4) *HincII*, *HindIII*, and *BamI*; (5) *HindII*, *HindIII*, and *XhoI*; (6) *HincII* plus *HindIII*; (7) *PvuI*; (8) *PvuI* plus *HindIII*; (9) *PvuI* plus *HincII*; (10) *PvuI* plus *BamI*; (12) *HaeII*; (13) *HaeII* plus *HincII*; (14) *HaeII* plus *HindIII*; (15) *HaeII* plus *BamI*. (B) 2.5% acrylamide-0.5% agarose gel. Lanes: (1) *ColE1* treated with *HaeIII* as a standard; (2) *HincII*, *HindIII*, and *SmaI*; (3) *HaeII* plus *BamI*; (4) *PvuI* plus *PstI*.

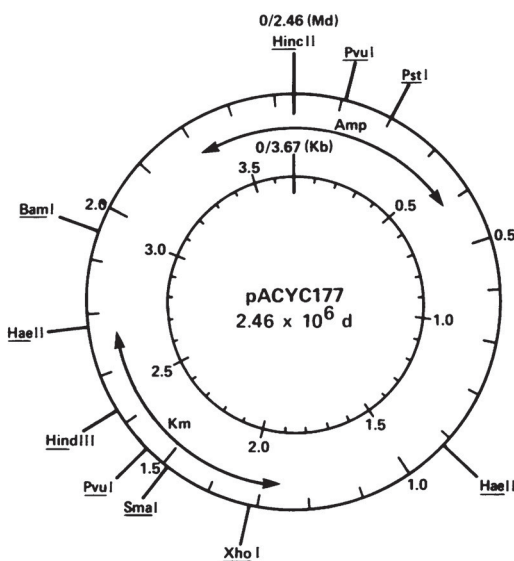


FIG. 4. Map of the pACYC177 plasmid, showing cleavage sites for the restriction endonucleases studied.

tion endonuclease-generated fragments of pACYC177 DNA (Fig. 4) was constructed after analysis of overlapping fragment data obtained from such gels.

Both the *HincII* and *PstI* endonucleases cleave the Ap gene; insertion of a DNA fragment at either of these cleavage sites interferes with expression of Ap resistance (53). The Km resistance gene contains cleavage sites for *HindIII* (Timmis et al., in press), *SmaI* (*XmaI*), and *XhoI* (A. C. Y. Chang, J. Sninsky, and S. N. Cohen, unpublished data), and introduction of a DNA fragment into any of these sites leads to inactivation of Km resistance. DNA fragments having blunt ends generated by any of a large number of different restriction endonucleases can be inserted at either the *HincII* or the *SmaI* site of pACYC177, and clones containing such hybrid plasmids can be identified by their sensitivity to either Ap or Km. DNA fragments having 5' protruding ends generated by other endonucleases can also be treated with reverse transcriptase to yield blunt ends, and the resulting fragments can be cloned at either the *HincII* or *SmaI* site of pACYC177. Alternatively, DNA fragments having cohesive ends generated by the *PstI*, *HindIII*, *XmaI*, or *XhoI* endonuclease can be cloned directly into sites present on the pACYC177 plasmid, and the hybrid plasmids can be detected by insertional inactivation. For both *PstI* and *SmaI* sites, the recognition sequence for the enzymes can be regenerated by addition of polydeoxyguanosine residues to the

vector, using the terminal transferase reaction (43).

The *BamI* cleavage site on pACYC177 does not inactivate a detectable gene; however, the site can be used for the cloning of DNA fragments that carry genes that are phenotypically expressed and directly detectable (e.g., *BamI*-generated *lac* and *trp* fragments of *E. coli*). The cycloserine selection procedure described by Curtiss et al. (23) and employed recently by Rodriguez et al. (53) to enrich bacterial cultures for an insertional inactivated Tc resistance gene can also be used for this purpose with pACYC177.

Endonuclease cleavage sites on the pACYC184 plasmid. Previous evidence (Timmis et al., in press) has indicated that an *EcoRI* cleavage site is contained within the Cm resistance gene of the R6-5 plasmid. Similarly, it has been shown that the Tc resistance gene of pSC101 contains cleavage sites for the *BamI*, *SaII*, and *HincII* endonucleases (18, 32, 49). Introduction of a foreign DNA fragment at the *HindIII* cleavage site also inactivates Tc resistance, except in instances where the introduced fragment provides a function to substitute for the apparently disrupted promoter region of the Tc operon (9, 57). Thus, the pACYC184 plasmid is suitable as a cloning vehicle for fragments generated by a variety of restriction endonucleases; insertional inactivation of either Cm or Tc resistance can be employed, and cultures can be enriched for chimeric plasmids by penicillin (24) or cycloserine (23) selection methods. The plasmid is especially useful for cloning *EcoRI*-generated fragments by insertional inactivation of Cm resistance, and recently it has been employed to demonstrate that the *EcoRI* endonuclease functions in vivo to accomplish site-specific genetic recombination (11).

A pACYC184 map showing the *EcoRI*, *BamI*, *SaII*, *HincII*, and *HaeII* cleavage sites is shown in Fig. 5. The acrylamide-agarose gel electrophoresis data used for construction of this map are shown in Fig. 6.

General properties of constructed plasmids. (i) Requirement for DNA polymerase I. It has previously been shown that, like ColE1, the P15A miniplasmid is present in multiple copies per bacterial chromosome (22) and that replication of P15A is inhibited by rifampin (45). To determine directly whether replicons derived from P15A are dependent upon DNA polymerase I for replication, pACYC139, pACYC177, and pACYC184 were separately introduced into *E. coli* SC291 *polA*(Ts). A ColE1-derived replicon, pFC012, which contains an Sm-Sp resistance gene fragment cloned at the *EcoRI* site of

ColE1, was employed as a positive control, and the pSC101 plasmid was used as a negative control. All of the P15A-derived constructed

plasmids and pFC012 were lost from bacterial cells grown at 42°C, whereas pSC101 replication was independent of the *polA* function (Table 3).

(ii) **Ability to undergo amplification.** The constructed plasmids pACYC139, pACYC177, and pACYC184 exist in either *E. coli* C600 or in a *recA* host (strain JC1569) at about 20 copies per chromosome during normal bacterial growth (Table 4). Whereas pACYC139 and pACYC177 were found to undergo only limited amplification in the presence of either Cm or Sp in *E. coli* C600 or JC1569, greater than 10-fold amplification of pACYC184 occurred in strain C600 in the presence of Sp and 5-fold amplification was observed in strain JC1569. Because pACYC184 carries a gene for Cm resistance, amplification of this plasmid in the presence of Cm could not be tested. For this reason, a derivative of pACYC184 (i.e., pCS84) that contains an *E. coli* DNA fragment inserted into the *EcoRI* site within the Cm gene was used in the Cm amplification studies carried out with this plasmid (Table 4). It is of some interest that the pACYC177 and pACYC184 plasmids, which are approximately the same size and were derived from the same replicon, show different abilities to undergo amplification of plasmid copy number in the presence of Sp.

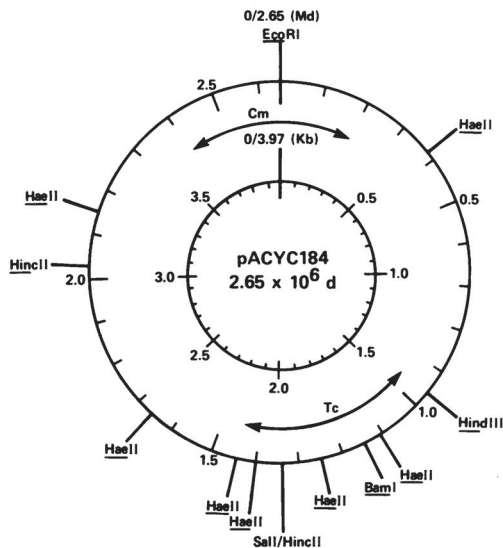


FIG. 5. Map of the pACYC184 plasmid, showing cleavage sites for the resistance endonucleases studied.

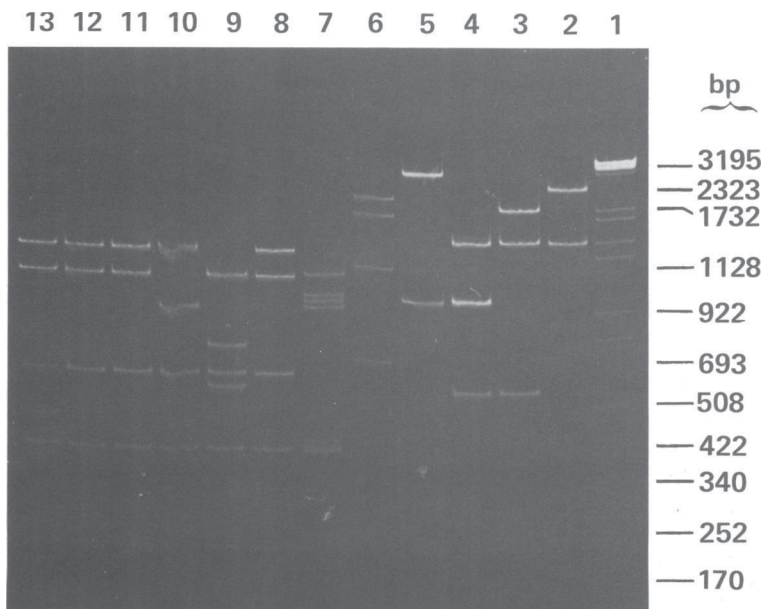


FIG. 6. Mapping of endonuclease cleavage sites on the pACYC184 plasmid. Electrophoresis was carried out on composite 2.0% acrylamide-0.5% agarose gel in Tris-borate-ethylenediaminetetraacetate buffer. Lane: (1) Bacteriophage λ DNA treated with *EcoRI* and *HindIII* endonucleases was used as a molecular length standard DNA fragment size. Lanes: (2) pACYC184 treated with *HindIII* and *EcoRI*; (3) *HindIII*, *EcoRI*, and *Sall*; (4) *HindIII*, *EcoRI*, and *HincII*. Lane: (5) pACYC184 treated with *HincII*. Lane: (6) *ColE1* treated with *HaeII*, used as a standard. Lane: (7) *ColE1* treated with *HaeIII* used as a standard. Lanes: (8) pACYC184 treated with *HaeII*; (9) *HaeII* and *EcoRI*; (10) *HaeII* and *HindIII*; (11) *HaeII* and *BamI*; (12) *HaeII* and *Sall*; (13) *HaeII* and *HincII*.

TABLE 3. Requirement of constructed plasmids for DNA polymerase I^a

Bacterial strain and plasmid	Antibiotic used	Colony no. ($\times 10^2$) with antibiotic at:		Colony no. ($\times 10^2$) without antibiotic at:	
		42°C	32°C	42°C	32°C
SC291	None			8.5	9
SC291(pFC012)	Sp	0	8.4	8.5	8.6
SC291(pACYC139)	Km	0	9	8.5	8.7
SC291(pACYC177)	Km	0	8	8.3	8.5
SC291(pACYC184)	Cm	0	8	8.5	8.7
SC291(pACYC101)	Tc	5	7	7.5	7.3

^a Plasmids were introduced into bacterial cells by transformation (20). Cultures of bacteria that contained the plasmid listed were grown exponentially at 32°C in Penassay broth (PAB) containing appropriate antibiotics. Diluted portions, each including about 1,000 cells of the liquid culture, were placed onto PAB agar plates containing or lacking antibiotics and were incubated at either 42 or 32°C overnight. Cultures of the *polA*(Ts) strain (SC291) containing either the pFC012 or pSC101 plasmid were used as positive and negative controls, respectively. Before the experiment, the *polA*(Ts) status of the host bacteria was confirmed by sensitivity to 0.03% methyl methene sulfonate (46). Cultures of *E. coli* C600 (*polA*⁺) containing each of the plasmids tested yielded similar numbers of colonies in the presence of antibiotics at 42 or 32°C.

TABLE 4. Ability of different P15A derivative plasmids to undergo amplification

Bacterial strain and plasmid	Plasmid copy no./chromosome ^a	
	During exponential growth	After amplification
C600(pACYC139)	20	44
JC1569(pACYC139)	21	33
C600(pACYC177)	22	43
JC1569(pACYC177)	22	45
C600(pACYC184)	18	184
JC1569(pACYC184)	18	104
C600(pSC84)	16	200

^a The plasmid copy number per *E. coli* chromosome equivalent during normal exponential growth or after addition of either Cm (180 μ g/ml) or Sp (300 μ g/ml) is shown. Plasmid copy number was determined by cesium chloride-ethidium bromide equilibrium centrifugation of radioactively labeled total DNA as described by Cabello et al. (8). The procedure yields a minimal copy number that is estimated to be accurate to $\pm 10\%$. JC1569 is a *recA* strain of *E. coli* K-12.

Recently it has been reported that certain plasmids derived from the ColE1 replicon form multimers in a *recA*⁺ host (4). As shown in lanes 12 through 15 of Fig. 2A, agarose gel electrophoresis of uncleaved DNA of the pACYC184 plasmid indicated that this plasmid also underwent multimer formation in the *E. coli recA*⁺ strain, C600, but not in the *recA* host, JC1569. In contrast, pACYC139 and pACYC177 did not form multimers in either *recA*⁺ or *recA* bacteria, even though these plasmids were derived from the same replicon as pACYC184. Thus, in this limited group of plasmids there appears to be a correlation between the ability of a plasmid to undergo amplification in the presence of a pro-

tein synthesis inhibitor and its capacity to form multimers.

Incompatibility relationships to P15A-derived plasmids. It has been reported that about 60% of P15A plasmid DNA is homologous with the ColE1 plasmid (31). The DNA sequence homology between the two plasmids, the multi-copy status of both ColE1 and P15A, and the requirement of these replicons for DNA polymerase I suggested that both plasmids might share a common replication system. Since replication properties of plasmids appear to be closely associated with incompatibility functions (60), we studied the incompatibility relationships of P15A derivatives with each other and with a ColE1-derived replicon, pFC012 (Timmis et al., in press).

pFC012 and pACYC139 were introduced sequentially by transformation into the *E. coli recA* strain, JC1569. During isolation of transformants, selection for both the Sp resistance determinant of pFC012 and the Km resistance of pACYC139 was maintained. Cultures were diluted into media lacking both antibiotics and, after 60, 120, or 180 generations of growth in such media, bacteria were checked for the presence of the antibiotic resistance genes carried by either plasmid. The results of this experiment (Table 5) indicate that both plasmids are stably and compatibly maintained for at least 180 generations of growth in the absence of selection. An analogous experiment involving pML21 (which consists of a mini-ColE1 plasmid linked to a Km resistance gene) and the Ap-resistant pACYC184 plasmid provided additional evidence that ColE1-derived and P15A-derived replicons are compatible.

When pACYC139 and pACYC184 were introduced by transformation under the selective

pressure of antibiotics (Km and Ap) into a single bacterial clone, incompatibility between the two plasmids was readily demonstrable. After 180 generations of growth in the absence of antibiotics, 95% of the bacterial cells had lost pACYC184, whereas 5% had lost pACYC139. When pACYC177 and pACYC184 were tested for incompatibility in the same cell by the same procedure, and with the same two antibiotics (i.e., Cm and Km), loss of pACYC177 occurred from more than 95% of cells after 180 generations of nonselected growth. Although the absence of distinguishing phenotypic markers on pACYC139 and pACYC177 prevented us from testing these two plasmids directly in combination, the data obtained suggest that there is a hierarchy that determines which of the three constructed plasmids is most likely to be lost as a consequence of expression of incompatibility. The basis for this apparent hierarchy is unknown.

Identification of regions of replication activity on constructed plasmids. Analysis of pACYC139-pACYC184 heteroduplex (Fig. 7A) shows a single area of homology between the two plasmids, calculated from heteroduplex measurements to be approximately 860 base pairs in length. Since both pACYC139 and pACYC184 are derived from the P15A replicon, and since these plasmids carry different antibiotic resistance determinants, we infer that this single region of homology contains the replication regions of the plasmids.

A heteroduplex between linear pACYC139

DNA cleaved at the *Hind*III site and *Eco*RI-cleaved linear ColE1 DNA (Fig. 7B) shows a single 960-base pair duplex region of homology that includes the replication region of pACYC139 identified in Fig. 7A. This homology extends over a segment located between 9 and 25% of the ColE1 length from the *Eco*RI cleavage site of ColE1; in earlier investigations (36, 44, 62) the ColE1 origin of replication was localized at a point 18% of the plasmid length from the *Eco*RI cleavage site. Taken together, those findings suggest that the replication regions of pACYC139 and pACYC184 (and, by inference, of pACYC177 and other P15A-derived plasmids) include the DNA segment that contains the replication origin of ColE1. However, it is not known whether the same nucleotide sequence within this region functions as the replication origin for both P15A and ColE1. These heteroduplex results also suggest that pACYC184 lacks some of the DNA sequences that pACYC139 and ColE1 have in common.

Studies of ColE1 and its derivatives have indicated the presence of three cleavage sites for the *Hae*II restriction endonuclease in close proximity to the origin of plasmid replication (49). However, agarose gel electrophoresis analysis of *Hae*II digestive patterns of pACYC177 and ColE1 (Fig. 3) indicates no common band despite the apparent homology found within this region by heteroduplex analysis.

Ability of constructed plasmids to be mobilized by Hfr. Like ColE1 and most other small plasmids, derivatives of the P15A replicon

TABLE 5. Incompatibility of pairs of P15A-derived and ColE1-derived plasmids^a

Plasmids and phenotypic markers tested	No. of dilutions	No. of colonies tested	No. of colonies resistant to determinant(s) expressed by first plasmid	No. of colonies resistant to determinant expressed by second plasmid	No. of colonies resistant to determinants expressed by both plasmids
pACYC139 (Km) × pFC012 (Sp)	2 4	154 175	154 175 (Km)	154 175 (Sp)	154 175 (Km Sp)
pACYC184 (Tc Cm) × pML21 (Km)	6	198	198 (Tc Cm)	198 (Km)	198 (Km Tc Cm)
pACYC184 (Tc Cm) × pACYC139 (Km)	3 6	154 160	13 0 (Tc Cm)	141 160 (Km)	1 0 (Km Tc Cm)
pACYC184 (Tc Cm) × pACYC177 (Km)	3 6	153 166	149 156 (Tc Cm)	4 10 (Km)	0 0 (Tc Cm Km)

^a Plasmid pairs were introduced concurrently into *E. coli* JC1569 (*recA*) by transformation, and bacteria were grown on PAB plates under the selective pressure of two or three antibiotics as shown in column 1. Three separate isolates were picked from each plate, grown overnight in L broth containing the antibiotics indicated, and simultaneously tested for the presence of both plasmids in the clone. Overnight cultures were diluted $\times 10^{-6}$ into fresh medium lacking antibiotics and grown to the late exponential phase. Dilutions were repeated (each $\times 10^{-6}$) the number of times indicated, and the final culture growth was plated onto PAB plates lacking antibiotics. Colonies were picked at random and tested in duplicate for expression of the antibiotic resistance determinants shown.

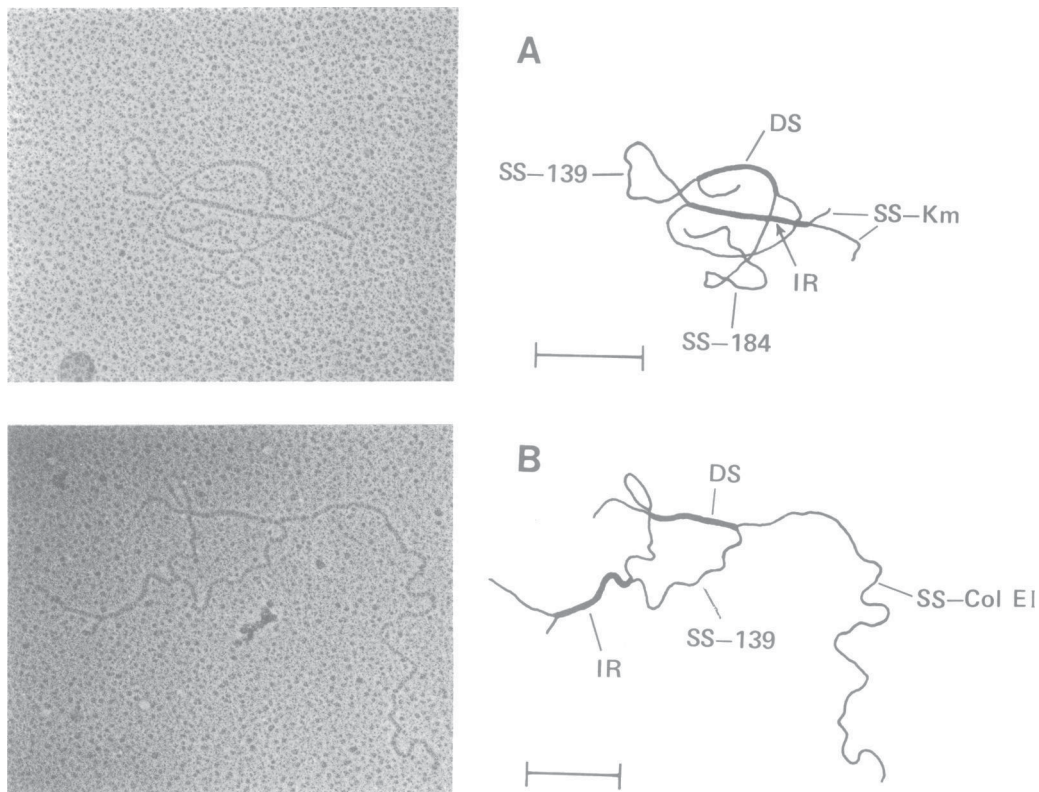


FIG. 7. (A) Heteroduplex between *Hind*III-cleaved pACYC139 plasmid DNA and *Eco*RI-cleaved pACYC184 plasmid. The inverted repeat (IR) stem of the *Hind*III-cleaved *Km* resistance gene loop (SS-*Km*) of pACYC139 serves as a marker for localizing the duplex region of homology (DS) on the pACYC139 plasmid. The remaining nonhomologous segments of pACYC139 (SS-139) and pACYC184 (SS-184) are indicated. (B) Heteroduplex between *Eco*RI-cleaved *ColE1* linear DNA and *Hind*III-cleaved pACYC139 DNA. The duplex region of homology (DS) is localized on the pACYC139 genome with respect to the inverted repeat (IR) stem of the *Km'* gene hairpin loop structure and is also localized in relation to the ends of *Eco*RI-treated linear *ColE1* DNA. The nonhomologous segments of the two plasmids are indicated by SS-139 and SS-*ColE1*. Double-stranded molecules of pACYC184 or *ColE1* served as molecular length standards. Single-stranded lengths were calculated by subtracting the length of double-strand segments from the length of the entire molecule and then assigning single-strand kilobase lengths in proportion to the measured lengths. Such electron microscopy measurements are accurate to $\pm 5\%$ (48).

do not carry functions for self-transfer between bacterial cells. However, at least some small plasmids can be mobilized from bacteria that contain fertility plasmids (37). Recent evidence (26, 37) has suggested that the relaxation complex shown earlier to be associated with *ColE1* (14) plays a primary role in mobilization of this plasmid.

Mobilization of the pACYA139, pACYC177, and pACYC184 plasmids, all of which were derived from the P15A replicon, was investigated by using a bacterial strain that has a chromosomally integrated fertility plasmid (Hfr Cavalli, D7001). The *ColE1*-derived pFC002 plasmid was mobilized at a high frequency in the Hfr strain, whereas mobilization of pSC101 was not detect-

able (Table 6). The pACYC139 plasmid, which contains most of the original P15A replicon, was mobilized at approximately the same frequency as mini-*ColE1* and the pBR series of constructed plasmids that use the *ColE1* replication system (54). pACYC177 and pACYC184, which lacks a 160-base pair segment of homology with *ColE1* that is present in pACYC139, were mobilized less efficiently than pACYC139.

Since plasmids derived from the P15A replicon are compatible with *ColE1*, it was possible to test directly whether *ColE1* can complement the inability of P15A derivatives to be mobilized by an Hfr strain (Table 5). A function provided by *ColE1* in *trans* accomplished mobilization of the pACYC139 plasmid at the same frequency

as ColE1 itself. The frequency of mobilization of pACYC177 and pACYC184 increased by two to three orders of magnitude in the presence of ColE1. Moreover, mobilization of the apparently unrelated pSC101 plasmid by the Hfr strain increased from undetectable levels to $\sim 10^{-6}$ transconjugants per recipient cell in the presence of ColE1. Analysis of transconjugant clones by agarose gel electrophoresis (A. C. Y. Chang and S. N. Cohen, unpublished data) indicated that ColE1- and P15A-derived plasmids exist as separate replicons after mobilization, whereas ColE1 accomplishes mobilization of pSC101 by recombining with the latter plasmid. These studies will be reported elsewhere.

The pML21 plasmid, which is itself mobilized poorly by the Hfr strain (Table 6), had no detectable effect on mobilization of either pACYC184 or pSC101. These findings suggest that functions provided by ColE1 but not by pML21 can interact with sites located on P15A derivatives and on pSC101 to promote plasmid mobilization.

DISCUSSION

Earlier studies have indicated that the P15A cryptic plasmid possesses some of the same replication characteristics as the ColE1 plasmid: both are present in multiple copies (3, 22) and the replication systems of both are sensitive to rifampin (30, 45). The investigations reported here were undertaken initially with the intent of attaching easily detectable and selectable "marker" genes to P15A to facilitate molecular and genetic studies of the replication region. A second goal was the construction of a series of P15A-derived small plasmid cloning vehicles that contain restriction endonuclease cleavage sites located in readily assayable genes. The plasmids we constructed constitute a class of amplifiable multicopy DNA cloning vehicles that can exist compatibly with ColE1-derived plasmids within bacterial cells and which also have a number of other properties that make them useful as general and special-purpose cloning vehicles.

Initial separation of the P15A cryptic plasmid

TABLE 6. Mobilization of plasmids by Hfr strain^a

Plasmid(s)	No. of recipient cells ($\times 10^6$)	Selective markers	No. of transconjugants	Frequency of mobilization (no. of transconjugants per recipient cell)
None	3.8	<i>nal lac</i>	3×10^7	1×10^{-1}
pFC012	5.1	<i>nal Sp</i>	6.5×10^7	1×10^{-1}
pML21	4	<i>nal Km</i>	6.5×10^2	1.5×10^{-6}
pSC101	4	<i>nal Tc</i>	0	$< 2.5 \times 10^{-9}$
pACYC139	4	<i>nal Km</i>	7.5×10^2	1.75×10^{-6}
pACYC140	4	<i>nal Km</i>	5×10^2	1.25×10^{-6}
pACYC177	4.4	<i>nal Km</i>	5×10^1	1.1×10^{-7}
pACYC184	4.5	<i>nal Tc</i>	1.5×10^1	3.3×10^{-8}
pFC012 and pSC101	6	<i>nal Sp</i>	8×10^7 (pFC012)	1.3×10^{-1} (pFC012)
	6	<i>nal Tc</i>	8.5×10^2 (pSC101)	1.4×10^{-6} (pSC101)
pFC012 and pACYC139	6	<i>nal Sp</i>	1.0×10^8 (pFC012)	1.6×10^{-1} (pFC012)
		<i>nal Km</i>	8.3×10^7 (pACYC139)	1.4×10^{-1} (pACYC139)
pFC012 and pACYC140	7	<i>nal Sp</i>	8×10^7	1.1×10^{-1} (pFC012)
		<i>nal Km</i>	7×10^7 (pACYC140)	1×10^{-1} (pACYC140)
pFC012 and pACYC177	6	<i>nal Sp</i>	6.2×10^7 (pFC012)	1×10^{-1} (pFC012)
		<i>nal Km</i>	2.6×10^4 (pACYC177)	4×10^{-5} (pACYC177)
pFC012 and pACYC184	5.5	<i>nal Sp</i>	6.2×10^7 (pFC012)	1.2×10^{-1} (pFC012)
		<i>nal Cm</i>	1.0×10^4 (pACYC184)	2×10^{-5} (pACYC184)
pML21 and pACYC184	6	<i>nal Km</i>	1×10^3 (pML21)	1.4×10^{-6} (pML21)
		<i>nal Cm</i>	1×10^1 (pACYC184)	1.4×10^{-8} (pACYC184)

^a Plasmids were introduced either singly or concurrently into the Hfr strain D7001 (Cavalli) by transformation. For assay of mobilization frequencies, cell cultures inoculated from single colonies were grown overnight in Penassay broth (PAB) containing the appropriate antibiotics. The next morning, cultures were diluted 1:100 into fresh PAB containing no antibiotics and were grown until the optical density at 650 nm was 0.05; the donor and recipient strains were then mixed 1:1 and incubated at 37°C for 4 h without shaking. Cultures were then plated onto PAB agar containing appropriate antibiotics or onto McConkey agar containing lactose as a carbon source for identification of *lac*⁺ colonies.

from the other plasmids present concurrently in the original host, *E. coli* 15T⁻, was made possible by an indirect selection procedure that used an indicator plasmid to identify bacterial cells that had been cotransformed with the cryptic plasmid (39). Once isolated, the P15A replicon was employed as a basic building block for the construction of other plasmids by addition of restriction endonuclease-generated DNA fragments containing genes cleaved from other plasmid sources. In these constructions, the "scrambling" of blunt-ended DNA fragments by several different endonucleases proved to be an effective method for eliminating segments of DNA that were not essential for either replication or selection of the plasmid. The presence of a cleavage site for one of the endonucleases in an essential gene does not preclude use of this method, since the selection for antibiotic resistance and replication ability is powerful enough to yield molecules that contain reformed and functional genes.

Our results indicate that plasmids derived from P15A are homologous with ColE1, as determined by electron microscope heteroduplex analysis, in an area of ColE1 shown previously to contain the plasmid replication origin. Nevertheless, the P15A and ColE1 plasmids appear to differ in specific nucleotide bases within the replication region, as shown by their different *Hae*II restriction endonuclease patterns. Analogous nucleotide base pair changes (i.e., microevolution) leading to altered restriction endonuclease cleavage patterns in regions of DNA that appear to be identical within the limits of resolution of heteroduplex analysis have been observed previously in plasmid DNA (16; K. N. Timmis, F. Cabello, and S. N. Cohen, submitted for publication).

The replication properties of P15A derivatives also suggest that the P15A plasmid may be ancestrally related to ColE1: the ColE1 and P15A replicons require DNA polymerase I for replication; they exist normally in multiple copies per chromosome; and, like ColE1, the P15A-derived plasmids continue to replicate when bacterial cells carrying the plasmids are grown in the presence of the protein synthesis inhibitor Cm or Sp (Table 4). However, the P15A replicons are compatible with the ColE1 plasmid and can coexist stably with ColE1 within the same bacterial cell while at the same time being incompatible with each other, indicating that incompatibility functions are expressed by P15A.

Although the pACYC139, pACYC177, and pACYC184 plasmids presumably utilize the same P15A-derived replication system, an apparent hierarchy of segregation as a result of

incompatibility was observed among the three plasmids. pACYC139, which contains more of the original P15A genome than the other two plasmids, was most stable; loss of the pACYC184 plasmid occurred from 99% of the bacterial cells carrying both the pACYC139 and pACYC184 replicons. However, when present in cells carrying pACYC177, pACYC184 was retained by more than 95% of the bacteria. It is not clear at present whether the hierarchy of incompatibility is determined by genetic information derived from P15A or whether it results from differences in the location or nature of the DNA segments added to the basic replicon.

The three P15A-derived plasmids studied here were all amplified in copy number in the presence of either Cm or Sp. However, the extent of amplification differed substantially from plasmid to plasmid and in a *recA*⁺ versus *recA*⁻ host. The pACYC184 plasmid was observed to undergo the greatest degree of amplification; interestingly this was the only one of the three P15A-derived replicons studied that was found to form multimers. Such multimer formation occurred in *recA*⁺ but not in *recA*⁻ bacteria—as was the case with previously studied plasmids derived from the ColE1 replicon (4). pACYC139 and pACYC177, which showed only limited amplification in the presence of Cm or Sp, showed no multimer formation in either *recA*⁺ or *recA*⁻ bacteria. Although the amplification of plasmid copy number is not necessary for the production of multimers in ColE1-derived plasmids (4), the apparent correlation between amplifiability and multimer formation observed in our studies with P15A-derived plasmids suggests the possibility of a relationship between these properties.

It has long been recognized that certain nonconjugative plasmids such as ColE1 can be mobilized to other bacteria at high frequency by F⁺ or Hfr strains (15). Other nonconjugative plasmids, such as pSC101, are mobilized poorly by the same fertility systems. Recently, evidence has accumulated to suggest a strong correlation between the ability of ColE1 plasmid DNA to be relaxed and the ability of that plasmid to be transferred by conjugation (26, 37).

Mobilization of the three P15A-derived plasmids studied in these investigations occurred at a frequency six or seven orders of magnitude lower than the frequency observed for ColE1. Two of the three plasmids (pACYC177 and pACYC184) were mobilized approximately 10- to 40-fold less efficiently than pACYC139, which contains the largest amount of the original P15A plasmid DNA as noted above. The ability of all three plasmids to be transferred as autonomous replicons was complemented by a concurrently

present ColE1 replicon, indicating that a *trans*-acting function provided by ColE1 is involved in such plasmid mobilization. Similar complementation of a transfer-deficient ColE1 by a concurrently present ColE1 or ColK plasmid has recently been reported (63). As a result of complementation, mobilization of pACYC139 occurred at the same frequency as mobilization of ColE1 itself; the pACYC177 and pACYC184 plasmids, which were mobilized less well than pACYC139 in the absence of ColE1, failed to show the same extent of mobilization as pACYC139 when complemented. These findings suggest that several different genetic functions may be involved in plasmid mobilization. Study of the relaxation complexes of these various constructed plasmids should help define further the role of the relaxation complex in this process.

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