Plasmid ColE1 as a Molecular Vehicle for Cloning and Amplification of DNA

(*Escherichia coli*/trp operon/*in vitro* recombination/DNA replication/amplified enzyme levels)

VICKERS HERSHFIELD*, HERBERT W. BOYER†, CHARLES YANOFSKY‡,
MICHAEL A. LOVETT*, AND DONALD R. HELINSKI*

* Department of Biology, University of California, San Diego, La Jolla, Calif. 92037; † Department of Microbiology, University of California, San Francisco Medical Center, San Francisco, Calif. 94133; and ‡ Department of Biology, Stanford University, Stanford, Calif. 94305

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ABSTRACT DNA fragments obtained from *EcoRI* endonuclease digestion of bacteriophage ϕ 80pt190 (*trp*⁺) and the plasmid ColE1 were covalently joined with polynucleotide ligase. Transformation of *Escherichia coli trp*⁻ strains to tryptophan independence with the recombinant DNA selected for reconstituted ColE1 plasmids containing the tryptophan operon and the ϕ 80 immunity region. Similarly, an *EcoRI* endonuclease generated fragment of plasmid pSC105 DNA containing the genetic determinant of kanamycin resistance was inserted into the ColE1 plasmid and recovered in *E. coli*. The plasmids containing the *trp* operon (ColE1-*trp*) and the kanamycin resistance gene were maintained under logarithmic growth conditions at a level of 25-30 copies per cell and accumulate to the extent of several hundred copies per cell in the presence of chloramphenicol. Cells carrying the ColE1-*trp* plasmid determined the production of highly elevated levels of *trp* operon-specific mRNA and tryptophan biosynthetic enzymes.

Two recent reports described the molecular cloning of prokaryotic and eukaryotic DNA in *Escherichia coli* after recombination of DNA molecules *in vitro* (1, 2). Molecular cloning of DNA can be defined as the isolation in a bacterial cell of a fragment of DNA in covalent linkage with an independently replicating plasmid serving as a molecular vehicle. Thus, a homogeneous population (or clone) of DNA molecules obtained from the bacterial progeny of such a cell can be considered to be derived from one parental molecule.

There are two essential steps involved in the cloning procedure. First, the covalent insertion of DNA into the plasmid vehicle is accomplished through recombination *in vitro*. Second, the recombinant DNA is transformed into *E. coli* and recovered either by selection of a function controlled by the plasmid, the integrated DNA, or both. The key enzymatic step in the recombination procedure is provided by a restriction endonuclease that cleaves a unique nucleotide sequence to produce complementary cohesive termini that can be covalently rejoined at random with polynucleotide ligase (3-5). In the previous reports of cloning (1, 2) the restriction endonuclease *EcoRI* was used to insert DNA fragments into the bacterial plasmid pSC101 (6), which served as the molecular vehicle.

The pSC101 plasmid belongs to a class of extrachromosomal elements (F, R1, ColIb) that replicate under stringent control and exist as 1-2 copies per chromosome (7). Another class of extrachromosomal elements, including the plasmids R6K and colicinogenic factor E1 (ColE1), are under relaxed con-

trol and exist in multiple copies per cell in *E. coli* (7, 8). There are about 24 copies of plasmid ColE1 per cell under normal growth conditions (9). Furthermore, after the addition of chloramphenicol the chromosome stops replicating after a short period of time, whereas ColE1 DNA synthesis continues for 12-16 hr, and eventually 1000-3000 copies of the plasmid are accumulated per cell (10, 11). In addition to its relaxed mode of replication, the size of the ColE1 plasmid [4.2×10^6 daltons (12)] and the presence of a single *EcoRI* substrate site in the molecule (M. Lovett and D. R. Helinski, *Proc. Nat. Acad. Sci. USA*, submitted) suggested that this element offers particular advantages as a vehicle for the molecular cloning and amplification of DNA. In this paper we report the use of the ColE1 plasmid as a vehicle for the molecular cloning of the tryptophan operon of *E. coli* and a fragment of an R-plasmid.

MATERIALS AND METHODS

All strains of bacteria used in this study were derivatives of *E. coli* K12. Covalently closed circular plasmid DNA from JC411Thy⁻/ColE1 (11), C600/pSC105 (1), and strains containing the recombinant plasmids constructed in this study were purified as described (13). Bacteriophage ϕ 80pt190 (14) DNA was prepared as described elsewhere (15).

Purification and conditions for use of the *EcoRI* restriction endonuclease (16) and *E. coli* polynucleotide ligase (17) have been described. The ColE1-*trp* plasmid was constructed as follows. In a volume of 200 μ l [100 mM Tris·HCl (pH 7.5), 5 mM MgCl₂, 50 mM NaCl], 5.7 μ g of ColE1 and 6.0 μ g of ϕ 80pt190 were digested to completion with homogeneously purified *EcoRI* endonuclease. Complete digestion was monitored by electrophoresis of the fragments in an agarose gel (see Fig. 2). The ϕ 80pt190 DNA is cleaved 11 times by the *EcoRI* endonuclease to produce 12 fragments (R. Helling, H. Goodman, and H. Boyer, unpublished observation). The endonuclease was inactivated by heating at 65° for 5 min, the digest was dialyzed overnight against 5 mM Tris·HCl (pH 7.5), and the sample was concentrated to 50 μ l. The *EcoRI*-generated fragments were ligated as described at a concentration of 75 pmoles of fragments or 150 pmoles/ml of *EcoRI* termini (5).

The procedure used for transformation was that of Cohen, Chang, and Hsu (18), except that the cells were grown to $A_{590} = 0.600$ and after exposure to DNA were incubated in L-broth for 90 min. The cells were collected and resuspended in 10 mM NaCl before they were plated. C600 *trpR*⁻ Δ *trpE5* (MV1), C600 *trpR*⁻ *trpE* 10220 *recA* (MV2), C600 Δ *trpE5* (MV10), and C600 Δ *trpE5* *recA* (MV12) were used as re-

Abbreviations: TEN buffer, 20 mM Tris·HCl, 1 mM EDTA, 20 mM NaCl (pH 7.5); Cam, chloramphenicol.

FIG. 1. Structural properties of plasmids pVH5 and pML2. (A and B). Tritium-labeled plasmid DNA was prepared by growth of the bacteria MV1/pVH5 (A) and MV2/pML2 (B) in M9-casamino acids-glucose B1 medium (10 ml) containing [^3H]-thymine (10 $\mu\text{Ci/ml}$) and deoxyadenosine (250 $\mu\text{g/ml}$) as described (13). Bacterial cultures were harvested, lysed as described (13), and centrifuged for 40 hr at 38,000 rpm at 20° in a cesium chloride gradient in a Spinco 50 Ti rotor. Collection of the gradients and assay of fractions for radioactivity have been described (9). (C and D). After isopropanol extraction of the ethidium bromide (20) from fractions 5–9 of peak A in gradient A (C) and fractions 7–10 of peak A in gradient B (D), the DNA was diluted with 2 volumes of TEN buffer and concentrated by precipitation with 6 volumes of absolute alcohol and 0.3 volume of 3 M sodium acetate (pH 6.0). The precipitated DNA was resuspended in a small volume of TEN buffer and centrifuged in the presence of [^{14}C]thymine-labeled ColE1 marker DNA (23S) (12) on a 5–20% sucrose gradient. The gradients were centrifuged for 100 min at 45,000 rpm in a Spinco SW 50.1 rotor. Fractions (0.12 ml) were collected onto Whatman no. 1 filter paper, and the radioisotope was counted as described (11).

cipients in the transformations. *trpR*[−] is the structural gene for the *trp* repressor and ΔtrpE5 is a deletion entirely within *trpE* and removing most of this gene. Cultures transformed for nutritional markers were plated on Vogel-Bonner agar supplemented with 50 $\mu\text{g/ml}$ of the nonselective amino acids, 0.2% (w/v) glucose, and 5 $\mu\text{g/ml}$ of required vitamins. L-broth and L-broth agar were used for nonnutritional selections. Drug concentrations used were 25 $\mu\text{g/ml}$.

Transformants to colicin immunity were initially selected on a lawn of a culture of a mutant strain carrying ColE1 (isolated by J. Collins) that lyses and massively overproduces colicin at 43°. Clones that grew up in this background were picked and tested for colicin production by the overlay procedure of Fredericq (19) or for immunity to colicin E1 by testing growth of the clone in a background of purified colicins E1 and E2. Clones containing ColE1 are immune to colicin E1 but remain sensitive to colicin E2.

RESULTS

Construction of ColE1-*trp* Plasmids. About 2 μg of the $\phi 80\text{pt190}$ DNA and ColE1 DNA that had been treated with *EcoRI* endonuclease and ligated was used to transform C600 *trpR*[−] ΔtrpE5 (MV1). Clones were selected for their ability to grow in the absence of tryptophan. Two size classes of transformants were obtained. The least numerous were Trp⁺ clones that appeared to represent recombinants between

FIG. 2. Agarose slab gel electrophoresis of DNA digested by *EcoRI* endonuclease. The digested DNA (0.2–0.6 μg) was applied to the sample slots in 25- μl volumes containing 9% glycerol (w/v). Electrophoresis was carried out at a constant 100 volts for 2.75 hr in 0.8% agarose gel (Tris-borate buffer). The slab gel apparatus and buffer system were described (16). The gel was stained in electrophoresis buffer with 0.5 μg of ethidium bromide/ml (24) for 10 min and photographed on a short-wave UV transilluminator. The fragments of the phage λ generated by *EcoRI* endonuclease were used as standard markers (ref. 25; R. Davis, unpublished observation; R. Helling, H. Goodman, and H. Boyer, unpublished observation) for estimation of molecular weights. The λ and $\phi 80\text{pt190}$ fragments were heated at 65° for 5 min before application of the sample to dissociate the hydrogen-bonded cohesive ends of the genomes. (A and G) λ fragments. The molecular weight estimates are: 13.70, 4.68, (3.70 and 3.56 fragments appear as one band here), 3.03, and 2.09×10^6 , respectively. (B) pSC105 fragments; molecular weights 5.8 and 4.5×10^6 . The origin of the faint band is unknown, but may represent a population of deleted pSC105. (C) pML2 fragments; molecular weights 4.5 and 4.2×10^6 . (D) ColE1 linear molecule; molecular weight 4.2×10^6 . (E) pVH5 fragments; molecular weights 8.5, 4.2, and 1.6×10^6 . (F) $\phi 80\text{pt190}$ fragments; molecular weights 8.5, 4.4, 4.0, 3.9, 3.4, and 1.6×10^6 . Six smaller fragments are not visible because either they have migrated off the gel or too little DNA was present to be visible.

the *trp*⁺ DNA and the chromosome, since these clones had no extrachromosomal DNA and were sensitive to colicin. The most numerous class of transformants contained *trp* genes on the ColE1 plasmid. Clones containing ColE1 plasmids with the *trp* genes inserted at their *EcoRI* site were unable to produce colicin E1; however, the immunity property (Colⁱ^{imm}) of the plasmid was retained. All ColE1 plasmids containing *trp* were designated with the prefix pVH. *trp*⁺ clones containing these recombinant plasmids were resistant to $\phi 80$, indicating the presence of the $\phi 80$ repressor gene. Strains carrying these plasmids are still sensitive to phage T1, indicating that neither *tonA* nor *tonB* had mutated.

Construction of ColE1-kan Plasmids. The plasmid pSC105 DNA can be cleaved with the *EcoRI* endonuclease to produce two DNA fragments having molecular weights corresponding to 5.8×10^6 and 4.5×10^6 (1). The smaller fragment, containing the *kan* resistance determinant, was purified by preparative sucrose gradient centrifugation of pSC105 DNA that had been treated with *EcoRI* endonuclease and pre-

precipitated with ColE1 DNA that had been treated with *EcoRI* endonuclease. The precipitated DNA mixture was resuspended in TEN [20 mM Tris·HCl, 1 mM EDTA, 20 mM NaCl (pH 7.5)] buffer and used to transform C600 *trpR*⁻ *trpE10220 recA*(MV2). A clone resistant to kanamycin, designated MV2/pML2, was selected for further analysis. It was found to be immune to colicin E1 and sensitive to tetracycline and ϕ 80, but it did not produce colicin E1.

Properties of Plasmid DNA Purified from the Transformants. DNA was purified from pure clones of transformed cells by dye-cesium chloride gradient centrifugation. Covalently closed circular DNA was found in the preparations from the MV1/pVH5 and MV2/pML2 strains, as indicated by the band of DNA with the higher buoyant density (peak A, Fig. 1A and B). No supercoiled DNA peak was found in similarly prepared lysates of the recipient strain. The DNA in the supercoiled peak in each case sedimented faster than normal ColE1 DNA (Fig. 1C and D). The sedimentation coefficients of the faster sedimenting peak of DNA correspond to molecular weights of about 15×10^6 and 9×10^6 for plasmids pVH5 and pML2, respectively. The slower sedimenting peaks correspond to the open circular DNA form of these plasmid DNAs. The plasmid DNA isolated from these transformants could in turn transform other *E. coli* strains at a high efficiency (about $10^4/\mu\text{g}$ in each case) for either Trp⁺ or Kan^r. In both cases, immunity to colicin accompanied the selected marker. In triple-cross matings (21) the Trp⁺ and Col^{imm} characteristics of the pVH plasmids cotransferred when mobilized by the plasmid R64-11.

Agarose Gel Electrophoresis of *EcoRI* Endonuclease Digests. Purified ColE1-*trp* plasmid DNA from five independent Trp⁺ clones yielded three fragments on *EcoRI* endonuclease treatment, while the pML2 plasmid yielded two DNA fragments (Fig. 2). All of the plasmids tested yielded a fragment corresponding to linear ColE1 DNA (molecular weight 4.2×10^6). The other fragments had molecular weights of 8.5×10^6 and 1.6×10^6 for the pVH plasmids and 4.5×10^6 for plasmid pML2. The 4.5×10^6 -dalton fragment, derived from pML2, corresponds to the molecular weight previously determined for the *kan* fragment present in pSC105 (1). The 8.5×10^6 - and 1.6×10^6 -dalton fragments correspond to fragments identifiable in *EcoRI*-treated ϕ 80pt190. The *trp* operon genes represents about one-third of the mass of the larger fragment, while the smaller piece is exclusively ϕ 80-DNA since it is present in digests of wild-type ϕ 80 (R. Helling, H. Goodman, and H. Boyer, unpublished observation). The sum of the molecular weights of the fragments obtained from each pVH plasmid totals 14.3×10^6 , which is in close agreement with the molecular weight as determined by sucrose gradient analysis and electron microscopy analysis of isolated plasmid pVH5.

Transformation with *EcoRI* Endonuclease Cleavage Fragments of pVH5. Plasmid DNA from the strain C600 Δ *trpE5 recA*/pVH5 was purified and digested to completion with *EcoRI* endonuclease. The three fragments were separated by centrifugation through a neutral sucrose gradient, and all combinations of the purified fragments and each fragment alone was used to transform C600 Δ *trpE5* for Trp⁺. Trp⁺ transformants that were immune to colicin E1 were obtained at a low frequency only when all three pieces were recombined. When C600 Δ *trpE5* was transformed with the linear

FIG. 3. The extent of replication of plasmids ColE1, pVH5, and pML2 in the presence of Cam. Cultures (MV12/pVH5 and MV2/pML2) were grown in 100 ml of M9-casamino acids-glucose medium containing 250 $\mu\text{g}/\text{ml}$ of deoxyadenosine, and [^3H]thymine (20 $\mu\text{Ci}/\text{ml}$). Tryptophan (50 $\mu\text{g}/\text{ml}$) was added to the MV2/pML2 culture. Samples of 20 ml were removed at 0, 2, 4, 6, and 22 hr after the addition of Cam (250 $\mu\text{g}/\text{ml}$) and placed on ice. KCN was added at a final concentration of 1 mM. Sarkosyl lysates (10) were prepared and portions were centrifuged in cesium chloride-ethidium bromide gradients. Fractions were collected and assayed as described in Fig. 1. The amount of plasmid DNA was estimated on the basis of the number of counts in the supercoiled peak as a percent of the total counts enclosed in both the supercoiled and open peaks. Previously obtained data (10) on the replication of ColE1 in the presence of Cam are included in this figure for comparison. ●, ColE1; ○ and □, pVH5 (separate experiments); Δ, pML2.

4.2×10^6 -dalton fragment, colicin-immune clones were obtained that produced colicin E1.

Replication in the Presence of Chloramphenicol. One of the characteristics of the ColE1 plasmid is its ability to continue replication in the presence of chloramphenicol (Cam) under conditions where protein synthesis and chromosomal DNA synthesis have ceased (10, 11). Replication of the constructed plasmids was followed by removal of samples of cultures over a 22-hr period after the addition of Cam. Sarkosyl lysates of each sample were prepared and centrifuged to equilibrium in dye-cesium chloride gradients. When the gradients were observed with UV light, the concentration of the DNA band representing covalently closed, circular DNA increased as a function of incubation in the presence of Cam. Fig. 3 represents a summary of the data obtained on the extent of DNA replication in the presence of Cam of strains containing ColE1, pVH5, and pML2. The level of plasmid DNA is expressed as a percent of the total DNA isolated. At the time of addition of Cam, the C600 *trpR*⁺ Δ *trpE5 recA*(MV12)/pVH5 strain possessed 6.4% of its DNA as plasmid, which is equivalent to 11 copies/chromosome or 22 copies/cell, assuming about two genome equivalents per cell (22, 23). In the case of C600 *trpR*⁻ *trpE10220 recA*(MV2)/pML2, about 5.2% of the total DNA was plasmid, equivalent to 14 copies/chromosome or 28 copies/cell. Both pVH5 and pML2 follow the kinetics reported earlier (10) for the replication of ColE1 in the presence of Cam. After 12 hr of incubation, cells containing these plasmids accumulated in each case an amount of plasmid DNA equivalent to 45% of the total DNA comple-

TABLE 1. *trp* Enzyme levels in plasmid-containing strains

Strain	Growth supplement	Specific activity		Stability (% <i>trp</i> ⁻ colonies)
		ASase	TSase α	
W3110 <i>trpR</i> ⁺	Trp	0.02	0.08	
W3110 <i>trpR</i> ⁻	Trp	1.4	15	
C600 <i>trpR</i> ⁺				
Δ <i>trpE5 recA</i> /pVH5	0	0.77	12.3	0
	Trp	0.77	14.7	0
	IA	70	308	0
C600 <i>trpR</i> ⁺				
Δ <i>trpE5 recA</i> /pVH15	0	0.71	12.2	0
	Trp	0.85	14.9	0
	IA	59	289	0
C600 <i>trpR</i> ⁻				
<i>trpE10220 recA</i> /pVH15	0	9.4	134	~50
	Trp	0.17	19	~95

Cultures were grown to stationary phase at 37° in glucose-M9 minimal medium supplemented where indicated with L-tryptophan (Trp, 50 μ g/ml) or 3-indolylacrylic acid (IA, 10 μ g/ml). Cells were harvested, washed, and disrupted by sonic oscillation. The debris was removed by centrifugation, and the supernatant solutions were assayed for anthranilate synthetase (ASase) and for tryptophan synthetase α (TSase α), as described elsewhere (26). Stability of the plasmid was determined by streaking a loopful from each culture on tryptophan-supplemented agar and replicating the colonies that appeared to minimal agar.

ment of the cell. This corresponds to 359 copies of pVH5, 564 copies of pML2, and 1200 copies of ColE1 per cell.

trp Operon Enzyme and Messenger RNA Levels. Several strains carrying the ColE1-*trp* plasmids were assayed for *trp* operon enzymes and *trp* mRNA. If operon expression were strictly a function of the number of copies of the *trp* operon per cell, we would expect *trp* enzyme and *trp* mRNA levels to be about 11-fold higher than those of appropriate plasmid-free strains. Table 1 shows that in cultures grown with excess tryptophan, the anthranilate synthetase and tryptophan synthetase α levels of the *trpR*⁺/pVH strains are 40-fold and 18-fold higher, respectively, than those of the W3110 *trpR*⁺ control strain. The estimate of the increase in the tryptophan synthetase α level is the more reliable of the two because the anthranilate synthetase level of repressed W3110 *trpR*⁺ is too low to be measured accurately. Thus, the *trp* operon enzyme levels are in reasonable agreement with estimates of the number of operon copies per cell. Enzyme levels are identical in plasmid-containing strains grown with and without exogenous tryptophan (Table 1). This is due to the excretion of tryptophan by these strains and, therefore, excess tryptophan is present under both growth conditions. The data in Table 1 also indicate that there is a sufficient level of *trp* repressor in the *trpR*⁺ plasmid-containing strains to effect repression of all *trp* operons. Where repression is relieved by addition of the tryptophan analog, 3-indolylacrylic acid, to *trpR*⁺ plasmid-containing strains, the *trp* enzyme levels are elevated and account for at least 20–25% of the cellular protein. Stability tests performed on samples of the *trpR*⁺/pVH cultures failed to detect segregation of plasmid-free bacteria.

Enzyme levels were also determined in C600 *trpR*⁻ *rec*⁻ and *rec*⁺ plasmid-containing strains, but plasmid loss from these strains precludes meaningful interpretation of the values obtained (Table 1). The anthranilate synthetase level

TABLE 2. *trp* mRNA formation in plasmid-containing strains

Strain	Pretreatment	% of counts in mRNA hybridized to	
		ϕ 80 DNA	ϕ 80pt190 DNA — ϕ 80 DNA
W3110 <i>trpR</i> ⁻	+ Trp, 10 min	0	0.33, 0.41
	— Cam		
	+ Trp, 10 min	0.22, 0.24	0.14
C600 <i>trpR</i> ⁺	+ IA, 10 min	0.30, 0.20	4.8
Δ <i>trpE5</i>	+ Cam, 15 hr	9.8	16
<i>recA</i> /pVH15	minimal, 10 min	9.8	16
	+ Trp, 10 min	9.5	18

Cultures were grown in M9 minimal medium plus 0.1% acid-hydrolyzed casein, thiamine (1 μ g/ml), and 0.2% glucose to a cell density of 3.4×10^8 /ml. Samples (10 ml each) were added to flasks at 37° containing L-tryptophan (Trp, 20 μ g/ml) or 3-indolylacrylic acid (IA, 20 μ g/ml). After 10 min of vigorous shaking, 0.2 ml of [³H]uridine (1 mCi/ml; 26 Ci/mmol) was added to each flask and the cells were killed after 1 min. A second culture was supplemented with Cam (150 μ g/ml) and shaken at 37° for 15 hr. The culture was centrifuged, and the cells were washed and resuspended in the supplemented minimal medium mentioned above. Samples (10 ml each) were added to flasks with or without L-tryptophan and, after a 10-min incubation period, labeling was performed as described above. RNA was extracted and annealed to denatured DNA (10 μ g per filter) immobilized on nitrocellulose filters as described elsewhere (27). Hybridization to denatured DNA of phage lambda on control filters was about 0.01%.

(+ tryptophan) in a culture of *trpR*⁻ *recA*/pVH15 growing in the presence or absence of tryptophan indicates that there is less than one functioning ColE1-*trp* plasmid per cell (compare with the value for W3110 *trpR*⁻). On minimal medium lacking tryptophan, plasmid-containing cells are selectively favored and, therefore, enzyme levels are elevated.

RNA-DNA hybridization experiments were performed with RNA extracted from cultures pulse-labeled for 1 min with [³H]uridine. Label was detected by hybridization to ϕ 80 DNA and to *trp* operon DNA (Table 2). ϕ 80-specific mRNA was expected since segments of the ϕ 80 genome are present in the pVH plasmids and since ϕ 80 immunity is expressed by pVH-containing strains. Comparisons of the *trp* mRNA level of the culture labeled in the presence of excess tryptophan with that of the *trpR*⁻ control strain indicates that about one-third as much *trp* mRNA is being synthesized. Since the W3110 *trpR*⁻ mRNA level is expected to be 30- to 50-fold higher than that of repressed W3110 *trpR*⁺, these findings suggest that there is 10- to 15-fold greater *trp* mRNA production in the plasmid-containing *trpR*⁺ strain than in a haploid *trpR*⁺ strain. The lower *trp* mRNA level of the *trpR*⁺ plasmid-containing strain (+ tryptophan) than that of the *trpR*⁻ control culture demonstrates, in agreement with enzyme measurements, that there is sufficient repressor in the plasmid-containing cells to repress all *trp* operons. After depression of the *trp* operons of a *trpR*⁺/pVH culture by addition of indolylacrylic acid, the *trp* mRNA level was about 13-fold higher than that of the *trpR*⁻ control culture. After incubation with Cam for 15 hr, the relative rate of synthesis of ϕ 80 mRNA increased 50-fold over that of the

starting culture, while *trp* mRNA synthesis also increased appreciably.

DISCUSSION

In this paper ColE1 has been shown to serve as an effective molecular vehicle for cloning and amplifying specific regions of unrelated DNA. On the basis of several criteria it was demonstrated that the plasmids contained cloned DNA. (a) The sizes of the plasmids obtained were larger than the normal ColE1 plasmid. (b) Purified plasmid DNA from the transformed clones could in turn transform cells for the phenotype of the inserted DNA fragment and colicin immunity. (c) Treatment of the plasmids from the transformed cells with the *Eco*RI endonuclease generates fragments of sizes corresponding to those expected for the ColE1 DNA and the inserted unrelated DNA. (d) The ColE1-*trp* plasmid strains produce tryptophan biosynthetic enzymes and *trp* mRNA at elevated levels consistent with the estimate of 11 copies of the *trp* operon per chromosome.

The construction of two ColE1 hybrid plasmids with different fragments of DNA resulted in the loss of colicin production, but did not alter the ability of these plasmids to determine immunity to colicin E1. This result indicates that the *Eco*RI site may be in the structural gene for colicin or in a gene that regulates colicin production. The recovery of clones that were both colicin-immune and colicin-producing by transformation with the 4.2×10^6 -dalton fragment demonstrates that the colicin gene was not irreversibly inactivated by the recombination event. This finding suggests that immunity to and production of colicin are determined by separate genes.

Cells containing the ColE1-*trp* plasmid are unstable when they are also *trp* repressor deficient. Consistent with this is the observation that on media containing tryptophan the colony size of *trpR*⁻ plasmid-free segregants is larger than that of corresponding plasmid-containing strains. Presumably, the excessive amounts of *trp* operon products produced by *trpR*⁻ plasmid-containing strains result in growth retardation.

The cloning of the 1.6×10^6 -dalton fragment in each of the ColE1-*trp* plasmids obtained from several independent isolates was not anticipated. The observation that these plasmids confer to the cell immunity to $\phi 80$ can be explained by the presence of the 1.6×10^6 -dalton fragment, which contains the $\phi 80$ immunity region (R. Helling, H. Goodman, and H. Boyer, unpublished observation). The cocloning of this fragment is most likely required for repression of the *N* gene found to be located near the *trp* operon in the 8.5×10^6 -dalton fragment. Presumably, large amounts of the *N* gene product would be lethal or detrimental to the cell.

ColE1 replication does not appear to be affected by the insertion of DNA at the *Eco*RI site. About 22–28 copies of each recombinant plasmid per cell were found, and plasmid replication continues in the presence of chloramphenicol to the extent that the final yield of plasmid DNA is approximately that observed for normal ColE1 DNA. *trp* mRNA production also increases appreciably after an extended period in the presence of chloramphenicol. Thus, it should be possible to obtain large amounts of any DNA cloned in the ColE1 plasmid. In addition, the demonstration of a gene dosage effect for the *trp* operon should provide a useful approach for obtaining large quantities of the gene products of the *trp* operon as well as the products of other genes that can be cloned in this plasmid.

During these studies, a *trp*⁻ segregant on one of the ColE1-*trp*⁺ clones was isolated that remained immune to colicin E1. This cell contained a plasmid that has a molecular weight of 2.2×10^6 , of which about 2.0×10^6 dalton was shown to be homologous to ColE1 by heteroduplex analysis (V. Hershfield, M. Lovett, L. Chow, H. Boyer, and D. Helinski, unpublished observation). In logarithmically growing cells this plasmid is present to the extent of 100–200 copies per cell. The reason for this derepressed DNA synthesis is unclear, but the finding that the mini-ColE1 plasmid has retained the *Eco*RI restriction site makes it potentially an even more effective vehicle for amplification of specific genetic information.

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