Proc. Nat. Acad. Sci. USA
Vol. 72, No. 11, pp. 4361–4365, November 1975
Biochemistry

Biochemical construction and selection of hybrid plasmids containing specific segments of the *Escherichia coli* genome

(molecular hybrids/E1 colicinogenic factor/arabinose operon/tryptophan operon/leucine operon)

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Communicated by Thomas C. Bruice, August 29, 1975

ABSTRACT Using a poly(dA·dT) "connector" method, a population of annealed hybrid circular DNAs was constructed *in vitro;* each hybrid DNA circle containing one full-length molecule of poly(dT)-tailed DNA from E1 colicinogenic factor (Col E1) fragmented by *Eco*RI endonuclease annealed to any one of a collection of poly(dA)-tailed linear DNA fragments of the entire *E. coli* genome. This annealed, but unligated, hybrid DNA was used to transform several different auxotrophic mutants of *E. coli,* and by direct selection, bacterial clones were isolated which contained specific hybrid plasmids. In this manner, bacterial strains containing Col E1 hybrid plasmids carrying the entire tryptophan operon or the arabinose and leucine operons were isolated. The methods described should allow the molecular cloning of any portion of the *E. coli* genome by selection from a pool of DNA molecules containing at least several hundred different hybrids representing the entire bacterial genome.

Several recent reports have described molecular cloning of bacterial and eukaryotic DNAs on various plasmids in *Escherichia coli* (1–4). In no case, however, has a specific piece of *E. coli* DNA, which codes for known RNA or protein, been cloned without first enriching for that specific DNA or "precloning" the DNA using a phage vector. We have been seeking efficient ways to clone specific genes and operons of *E. coli* by direct selection from a pool of hybrid plasmids which are constructed *in vitro* and which contain pieces of DNA representing the entire *E. coli* genome.

Hershfield *et al.* (2) have described the distinct advantages of the E1 colicinogenic factor (Col E1) when used as a vehicle for cloning and amplification of *E. coli* DNA. Using this plasmid vector, we report the cloning of regions of the *E. coli* chromosome, specifically those portions containing the arabinose, leucine, and tryptophan operons, by direct selection from a pool of hybrid plasmid molecules derived from the *Eco*RI endonuclease digestion products of *E. coli* DNA. The hybrid plasmids were constructed *in vitro* using poly(dA·dT) "connectors", by the method described by Lobban and Kaiser (5) and Jackson *et al.* (6). The procedures described in this paper should permit the molecular cloning of any portion of the *E. coli* genome for the study of specific gene systems and their products.

METHODS AND MATERIALS

Bacterial Strains. The following strains, all derivatives of *E. coli* K12, were used as recipients for transformation: NL20-028 (*ΔaraC766 Δlac514*), NL20-127 (*ΔaraC766 Δlac514 recA*), NL20-008 (*ΔaraCOIBA*), NL20-047 (*ΔaraCOIBAD*), NL20-000 (*leu*), MV10 (C600 *ΔtrpE5 thr leu thi*), MV12 (C600 *ΔtrpE5 recA thr leu thi*), DM8 (*ΔtrpEDCBA-tonB*), SB2 (C600 *thr leu thi thy str Δtrp-tonB hsm hsr*).

DNA. Covalently closed circular plasmid DNA from *E. coli* strain JC411/Col E1 (7) and hybrid plasmid DNA from transformants isolated in this study were purified as described (8, 9). High-molecular-weight *E. coli* DNA (90 to 200 × 10^6 daltons) from strain CS520 (*HfrC trpA58 metB glyVsu58*) was purified according to the phenol extraction procedure described by Saito and Miura (10), omitting RNase treatments.

Enzymes. *Eco*RI restriction endonuclease was purified and used according to the methods of P. J. Greene *et al.* (11), with certain modifications (M.-T. Hsu, T. Landers and P. Berg, personal communication). Phage λ-exonuclease (contributed by Peter Lobban and Dale Kaiser) was prepared according to Little *et al.* (12). Calf thymus deoxynucleotidyl terminal transferase, prepared according to Kato *et al.* (13), was given to us by I. R. Lehman and R. Ratliff.

Construction of Hybrid Col E1–*E. coli* DNA Annealed Circles. Col E1 covalently closed circular DNA or *E. coli* DNA was digested to completion with *Eco*RI restriction endonuclease in a reaction mixture containing 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 100 μg/ml of gelatin, and 100 μg/ml of DNA. The reaction was allowed to proceed for 30 min at 37°, then immediately chilled to 0° in an ice bath. One-half volume of 200 mM K-glycine (pH 9.4), 12 mM $MgCl_2$ was added to the reaction mixture with 400 units/ml of λ-exonuclease and the mixture was allowed to incubate on ice for 30 min. NaEDTA (10 mM) was added to stop the reaction and the DNA (L_{RI} exo) was immediately phenol extracted and precipitated in 67% ethanol. It was previously determined that the λ-exonuclease treatment removes approximately 25 nucleotides from the 5′-phosphoryl termini of the DNA (L_{RI}). This treatment facilitates the subsequent terminal transferase reaction (5, 6).

Extensions of poly(dA) or poly(dT) were added to the 3′-hydroxyl termini of the DNAs as described by Lobban and Kaiser (5). Poly(dT) tails were added to Col E1 DNA (L_{RI} exo) in a reaction containing the following: 100 mM K-cacodylate (pH 7.0), 8 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 7.5 mM KH_2PO_4, 0.5 mM $CoCl_2$, 150 μg/ml of bovine serum albumin, 0.1 mM [^{3}H]dTTP, 100 μg/ml of DNA, and 55 μg/ml of terminal transferase. The reaction was incubated for 25 min at 37° and terminated by the addition of NaEDTA (10 mM). Polythymidylylated Col E1 DNA [(L_{RI} exo)-$(dT)_n$] was phenol extracted and ethanol precipitated. Under the above conditions approximately 300 residues of dT were added per molecule DNA, or 150 residues per end.

Abbreviations: Col E1, plasmid directing production of colicin E1; L_{RI}, a linear DNA molecule generated by digestion with *Eco*RI restriction endonuclease; exo, refers to DNA partially digested by γ-exonuclease; $E1^r$, resistant to colicin E1; Ara^+, able to utilize L-arabinose; Leu^+, Trp^+, able to grow without exogenous leucine or tryptophan, respectively.

Poly(dA) extensions were added to *E. coli* DNA (L_{RI} exo) in a reaction mixture similar to that described above, omitting the $CoCl_2$, and using 0.1 mM [^{3}H]dATP, 100 μg/ml of *E. coli* DNA (L_{RI} exo) and 180 μg/ml of terminal transferase. The reaction was incubated at 37° for 45 min, terminated by addition of NaEDTA (10 mM), and the polydeoxyadenylylated *E. coli* DNA [(L_{RI} exo)-$(dA)_n$] was phenol extracted and ethanol precipitated. Approximately 300 residues of dA were added per molecule of DNA, or 150 residues per end, assuming a genome equivalent of *E. coli* DNA (2.7×10^9 daltons) is cut into approximately 400 pieces by the *Eco*RI restriction endonuclease. The average size of *E. coli* DNA (L_{RI} exo) is approximately 6.3×10^6 daltons, as determined by zone sedimentation in neutral sucrose.

Equal amounts (by weight) of Col E1 (L_{RI} exo)-$(dT)_{150}$ DNA and *E. coli* (L_{RI} exo)-$(dA)_{150}$ DNA were mixed at 5 μg/ml in 10 mM Tris·HCl (pH 8.0), 100 mM NaCl, 1 mM NaEDTA, and allowed to anneal for 1 hr at 46°, 1 hr at 37°, and 1 hr at 23°, or for 16 hr at 37°. After annealing, DNA samples were prepared for electron microscopy by the aqueous method of Davis *et al.* (14) and molecules were scored according to structure. Annealed DNA preparations were concentrated by ethanol precipitation and stored at 4° in 10 mM Tris·HCl (pH 8.0), 10 mM NaCl, 1 mM NaEDTA.

Transformation and Selections. The procedure used for transformation [a modification of the method of Mandel and Higa (15)] was that described by Wensink *et al.* (1), except that after exposure to DNA the cells were grown for 2 hr in L-broth. The cells were then harvested by centrifugation and resuspended in minimal salts solution before plating on selective media using standard techniques.

Transformants to colicin immunity were selected or tested by cross-streaking on L-broth agar plates which were spread or streaked with crude preparations of colicins E1 or E2. Clones containing Col E1 or Col E1–*E. coli* hybrid plasmids are immune to colicin E1 and sensitive to colicin E2.

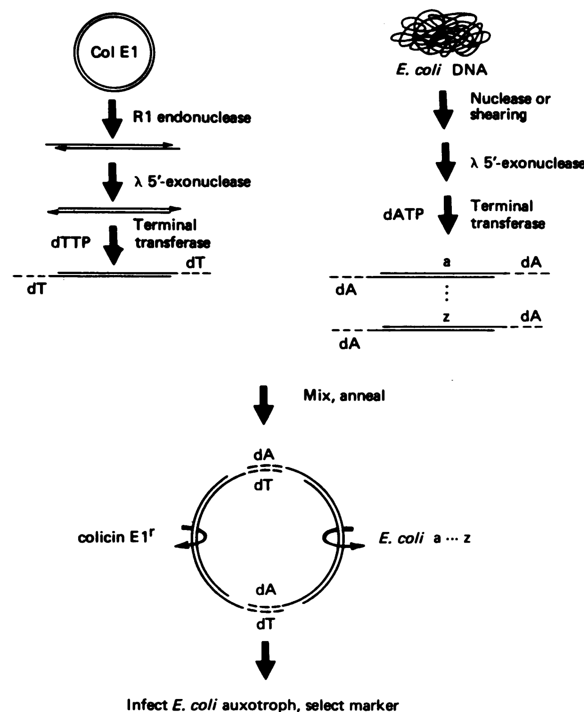

FIG. 1. Method for construction and selection of hybrid Col E1–*E. coli* DNA plasmids.

RESULTS

Construction of hybrid Col E1–*E. coli* annealed circular DNA

The method used to construct hybrid Col E1–*E. coli* DNA circles is a modification of procedures already described (1, 5, 6) (Fig. 1). Both Col E1 and *E. coli* DNAs are digested to completion with *Eco*RI endonuclease, treated with λ-exonuclease to remove approximately 25 bases from their 5'-phosphoryl termini, and tailed with poly$(dT)_{150}$ and poly$(dA)_{150}$, respectively, at their 3'-hydroxyl termini using the calf thymus deoxynucleotidyl terminal transferase. Alternatively, the *E. coli* DNA can be fragmented by hydrodynamic shearing before attachment of poly(dA). The two DNAs are then mixed and annealed at low concentration (5 μg/ml). The hybrid DNA preparation is concentrated and used directly, without ligation, for transformation of an *E. coli* auxotroph and the selection of a specific marker.

Annealed hybrid DNA was spread for electron microscopy (14), examined in the electron microscope, and individual molecules were scored according to structure (Table 1). Percentage yields of the various structures varied somewhat depending on conditions of annealing. If the hybrid DNA preparation was annealed for 16 hr at 37°, 10% of the molecules examined were circles and an additional 17% were

Table 1. Structures of annealed hybrid DNA molecules

DNA	Annealing conditions	Structures seen, %				
		Linear	Branched linear	Circular	Branched circular	Tangled
1. Col E1 (L_{RI} exo)-$(dA)_{150}$	46°, 1 hr; 37°, 1 hr; 23°, 1 hr	98	0	1	0	1
2. *E. coli* (L_{RI} exo)-$(dT)_{150}$	Same	91	0	0	0	9
3. Mixture, 1 & 2 (5 μg/ml)	Same	48	4	6	20	21
4. Mixture, 1 & 2 (5 μg/ml)	37°, 16 hr	58	5	10	17	11

DNA samples were spread for electron microscopy as described in *Methods and Materials.* At least 100 molecules of each DNA preparation were scored.

Table 2. Transformation efficiency of annealed circular hybrid DNA

DNA	Recipient	Selection	Transformants/μg of DNA
Col E1 (I) (circular)	SB2(C600)	Colicin E1^r	6×10^4
Col E1 (L_{RI} exo)-$(dA)_{150}$	SB2(C600)	Colicin E1^r	<1
Col E1 (L_{RI} exo)-$(dT)_{150}$ + *E. coli* (L_{RI} exo)-$(dA)_{150}$	SB2(C600)	Colicin E1^r	$\sim 10^3$
Col E1 (L_{RI} exo)-$(dT)_{150}$ + *E. coli* (L_{RI} exo)-$(dT)_{150}$	NL20-127 (K12 Δ*araC766 recA*)	Ara$^+$	0.2 (2/2 colicin E1^r)
Col E1 (L_{RI} exo)-$(dA)_{150}$ + *E. coli* (L_{RI} exo)-$(dT)_{150}$	MV10 (C600 Δ*trpE5 rec*$^+$)	Trp$^+$	4 (12/45 colicin E1^r)

E1^r, resistant to colicin E1; Ara$^+$, able to utilize L-arabinose; Trp$^+$, able to grow without exogenous tryptophan.

branched circles. The remainder of the molecules were mostly linear structures.

Transformation efficiency of annealed hybrid DNA

Annealed hybrid DNA was used in several transformations of *E. coli* auxotrophs (Table 2). Col E1 circular plasmid DNA alone, when used to transform strain SB2(C600) to colicin E1 immunity, routinely yielded approximately 6×10^4 transformants per μg of DNA on L-broth–colicin E1 plates. The transformants were resistant to colicin E1 but sensitive to colicin E2 (see *Methods and Materials*). Annealed hybrid DNA, when used in similar transformations, gave approximately 10^3 transformants per μg of DNA. Again, these transformants were resistant to colicin E1, but sensitive to colicin E2. The Col E1 (L_{RI} exo)-$(dT)_{150}$ DNA gave less than one colicin E1-resistant transformant per μg when used alone.

Hybrid DNA was then used to transform strain MV10 (Δ*trpE5 rec*$^+$) and colonies were selected for the ability to grow in the absence of tryptophan. (Although the donor DNA was *trpA58*, transformants were Trp$^+$ since the MV10 recipient has a wild-type *trpA* gene.) Forty-five isolates were obtained (4 transformants/μg of DNA), twelve of which were resistant to colicin E1 and sensitive to colicin E2. The remaining 33 colonies were Trp$^+$ but were sensitive to both colicins, and presumably were recombinants between *trp*$^+$ DNA and the chromosome. They were either lacking plasmid or the ability to express colicin E1 resistance. We have not seen this type of recombinant transformant if the *E. coli* recipient is *recA*, however.

The preparation of annealed hybrid DNA was also used to transform strain NL20-127 (Δ*araC recA*) and colonies were

Table 3. Transformation efficiencies of various hybrid plasmid DNAs

Plasmid DNA	Recipient	Selection	Transformants/μg DNA
pLC1 (Col E1–*ara*)	NL20-0127 (K12 Δ*araC766 recA*)	Ara$^+$	2.2×10^3 (100% colicin E1^r)
pLC1	NL20-008 (K12 Δ*araCOIBA*)	Ara$^+$	3.2×10^3 (91% colicin E1^r)
pLC1	NL20-047 (K12 Δ*araCOIBAD*)	Ara$^+$	3.8×10^3 (100% colicin E1^r)
pLC1	NL20-000 (K12 *leu*)	Leu$^+$	8.8×10^3 (100% colicin E1^r)
pLC3 (Col E1–*ara*)	NL20-028 (K12 Δ*araC766*)	Ara$^+$	7.4×10^3* (100% colicin E1^r)
pLC3	NL20-008 (K12 Δ*araCOIBA*)	Ara$^+$	<1
pLC3	NL20-047 (K12 Δ*araCOIBAD*)	Ara$^+$	<1
pLC3	NL20-000 (K12 *leu*)	Leu$^+$	7.8×10^3 (100% colicin E1^r)
pLC19 (Col E1–*trp*)	MV12 (C600 Δ*trpE5 recA*)	Trp$^+$	1.8×10^3 (100% colicin E1^r)
pLC19	DM8 (W3110 Δ*trpEDCBA-tonB*)	Indole$^+$†	1.9×10^3 (74% colicin E1^r)

Leu$^+$, able to grow without exogenous leucine.
* Data supplied by N. Lee.
† The selection was for indole$^+$ (indole utilization), not Trp$^+$, since the *E. coli* DNA from which the hybrid plasmid was constructed was isolated from strain CS520 (*trpA58*).

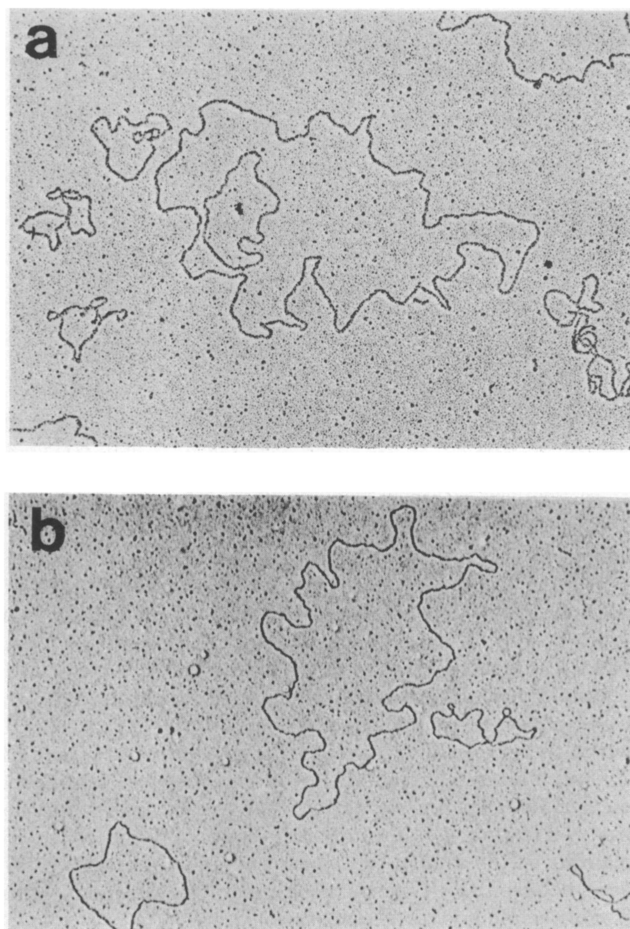

FIG. 2. Electron micrographs of (a) pLC3 (Col E1–*ara*) DNA and (b) pLC5 (Col E1–*trp*) DNA spread with Col E1 DNA as described in *Materials and Methods*. The pLC3 DNA is 4.3 times the length of Col E1 DNA or approximately 18×10^6 daltons. The pLC5 DNA is 3.6 times the length of Col E1 DNA or approximately 15×10^6 daltons.

selected on minimal arabinose plates. Two candidates which could utilize L-arabinose were obtained at an efficiency of 0.2 transformants/μg of DNA, and both were colicin E1 resistant and colicin E2 sensitive.

Attempts to obtain a hybrid plasmid carrying the lactose operon were unsuccessful. There is some evidence, however, for an *Eco*RI restriction endonuclease site in the *lacZ* gene (Clarke and Carbon, unpublished work; see *Note Added in Proof*). Thus, annealed hybrid DNA composed of plasmid and sheared *E. coli* DNA would need to be used to select for hybrid plasmids of this type. Such plasmids, which carry an *Eco*RI restriction endonuclease site within a well-characterized *E. coli* operon, may be useful for cloning other types of DNA.

Characterization of hybrid plasmids

Covalently closed circular plasmid DNA was isolated from both of the Ara$^+$ isolates and two of the Trp$^+$ isolates by preparing cleared lysates of cells carrying the hybrid plasmids and banding plasmid DNA in CsCl–ethidium bromide gradients (8, 9). The Col E1–*ara* plasmids were designated pLC1 and pLC3, and the Col E1–*trp* plasmids, pLC5 and pLC19.

Relaxed circular plasmid DNA from each of the four isolates was spread for electron microscopy using relaxed circular Col E1 DNA as a standard (Fig. 2). Projections of the molecules were traced on paper and measured with a map measurer. Both the pLC1 and pLC3 DNAs were approximately 4.3 times the length of Col E1 DNA or about 18×10^6 daltons, while pLC5 and pLC19 DNAs were 3.6 times the length of Col E1 DNA or 15×10^6 daltons.

Transformations were carried out using pLC1, pLC3, and pLC19 DNAs and various auxotrophic recipients (Table 3). As anticipated, pLC1 (Col E1–*ara*) DNA and pLC3 (Col E1–*ara*) DNA transformed strains NL20-0127 (Δ*araC recA*) and NL20-028 (*araC rec*$^+$) to Ara$^+$ with high efficiency. Likewise pLC19 (Col E1–*trp*) DNA transformed strain MV12 (Δ*trpE5 recA*) to Trp$^+$ with high efficiency.

Further transformations were carried out with pLC1 and pLC3 DNAs, selecting for Ara$^+$, using two strains carrying longer deletions, NL20-008 (Δ*araCOIBA*) and NL20-047 (Δ*araCOIBAD*). As shown in Table 3, pLC1 DNA transformed both strains to Ara$^+$ with high efficiency, whereas pLC3 DNA transformed neither. Apparently pLC1 carries the entire arabinose operon, but pLC3 does not. Since the hybrid plasmids were made from *Eco*RI endonuclease products of *E. coli* DNA, pLC1 and pLC3 should be identical. Indeed, no difference in the length of their DNAs was detected in electron microscopic measurements. Both plasmid DNAs are resistant to digestion with *Eco*RI endonuclease, so pLC1 was not originally constructed from a partial enzyme digestion product. Several reasons may be offered to explain the differences between the two plasmids. The *E. coli* DNA fragment from which pLC3 was constructed may have been sheared at a region close to the *araC* gene prior to *Eco*RI digestion. On the other hand, the pLC3 plasmid DNA could have undergone a small deletion, excising a portion of the arabinose operon. Finally, the *E. coli* DNA fragment from which pLC3 was originally constructed may have been excessively digested with λ-exonuclease, resulting in the loss of several genes. We surmise that the *ara* region must be close to that portion of the DNA that was treated with λ-exonuclease and joined to poly(dA). Both pLC1 and pLC3 DNAs transform strain NL20-000 (*leu*) to Leu$^+$ with high efficiency (Table 3). Since the two plasmids carry pieces of *E. coli* DNA of approximately 14×10^6 daltons and since *ara* and *leu* are 50% cotransducible with phage P1 (16) and map very close together on the chromosome (17), the portions of the arabinose and leucine operons carried by the plasmids probably define the extreme ends of the *E. coli* DNA contained in pLC1 and pLC3.

Hershfield *et al.* (2) have shown previously that there are no *Eco*RI endonuclease sites within the tryptophan operon. The hybrid plasmid pLC19 (Col E1–*trp*) carries *trpE*, *trpD*, *trpC*, *trpB*, and presumably *trpA58* (Table 3). It was necessary to select for indole utilization when strain DM8 (Δ*trp EDCBA–tonB*) was transformed with pLC19 DNA, because the DNA from which the hybrid plasmid was constructed was isolated from strain CS520 (*trpA58*), which grows in the absence of tryptophan only when supplied with indole. We have not yet established the presence of the *trpA* region on pLC19, however.

The usefulness of the plasmid Col E1 as a vehicle for amplification of *E. coli* DNA, mRNA, and proteins has been previously demonstrated by Hershfield *et al.*, in that strains carrying the Col E1–*trp* plasmid pVH15 show greatly elevated *trp* operon message and enzyme levels (2). We have found similarly elevated levels of *trp* mRNA and *ara* mRNA and proteins in strains carrying pLC5, pLC19, and pLC3. For example, after treatment of the cells with chloramphen-

icol (8), up to 38% of pulse-labeled messenger RNA in cells harboring pLC3 is specific for arabinose operon genes (Nancy Lee, personal communication).

DISCUSSION

This paper reports a general procedure by which specific regions of the *E. coli* genome can be obtained on plasmids and cloned. Using this procedure, hybrid plasmids such as Col E1-*ara* or Col E1-*trp* can be selected directly from a pool of hybrid molecules derived from the *Eco*RI endonuclease digestion products of *E. coli* DNA. The plasmids were constructed from *Eco*RI endonuclease digests of Col E1 DNA and *E. coli* DNA using the poly(dA-dT) "connector" method (1, 5, 6). An important feature of this method is that, in our case, for example, a molecule of linear vector Col E1 (L_{RI} exo)-$(dT)_n$ DNA can only be annealed with a tailed fragment of *E. coli* (L_{RI} exo)-$(dA)_n$ DNA, and other intermolecular or intramolecular annealings are prevented; thus the yield of hybrid product is greatly increased. This aspect is particularly important when attempting to select a specific molecule from a pool of several hundred different hybrids. In contrast, DNA joining methods that depend on the annealing and ligation of cohesive ends generated by restriction endonuclease are subject to the disadvantage that the yield of hybrid circular DNA is small, and it is thus difficult or impossible to select a particular DNA segment from a large population of fragmented DNA molecules. An additional advantage of the poly(dA-dT) "connector" method is that enzymatic ligation of the annealed hybrid circular DNA is unnecessary; unsealed DNA preparations are infectious and are converted to covalently closed circular DNA molecules *in vivo*.

In principle, it should be possible to obtain any portion of the *E. coli* chromosome on a plasmid by the methods described here, particularly if random *E. coli* DNA segments of a chosen size produced by hydrodynamic shearing are employed. For example, we are presently using the plasmid vector pCR1 and sheared *E. coli* DNA to obtain a population of covalently closed circular hybrid plasmids representing the entire *E. coli* genome. Plasmid pCR1 is a derivative of the Col E1-*kan* plasmid pML2 (2) in which one of the two *Eco*R1 endonuclease sites has been deleted (C. Covey, D. Richardson, and J. Carbon, unpublished data). By transfecting with hybrid pCR1-*E. coli* DNA and selecting for kanamycin resistance, a population of cells can be isolated which contains hybrid plasmids representing the entire *E. coli* chromosome. A preparation of covalently closed circular plasmid DNA can be isolated from these cells and this DNA will transform appropriate recipients with high efficiency to any phenotype for which there is a selection. Alternatively, if the cells that are originally transformed with hybrid pCR1-*E. coli* DNA carry the F factor, them a specific plasmid may be selected directly by conjugal cotransfer of the hybrid plasmid and F factor from the kanamycin resistant transformants into an appropriate recipient.

There are many obvious advantages in having particular gene systems isolated and cloned on plasmids carried by bacteria, particularly if the plasmids are under relaxed control and are present in many copies per cell, such that amplification of gene products can occur (2). Specific isolation of groups of contiguous genes facilitates the study of chromosome structure and yields mapping information. For example, knowing the size of pLC1, the Col E1 hybrid plasmid which carries the arabinose operon and at least a portion of the leucine operon, we can estimate that there are no more than 15 kilobases between *ara* and *leu*.

Finally, hybrid plasmids containing specific *E. coli* genes may be easily manipulated or made smaller by shearing or endonuclease digestion. New vectors can then be isolated which bear known *E. coli* gene systems that may be useful in the isolation and cloning of genes from other organisms. Thus, the insertion of foreign DNA into an *E. coli* operon, bringing the expression of that DNA under control of bacterial regulatory sequences, should be readily achieved.

Note Added in Proof. When applied to *E. coli* DNA fragmented randomly by hydrodynamic shearing, this method has enabled us to isolate several hybrid col E1 plasmids, including col E1-*lac*.

We are grateful to Denise Richardson for valuable technical assistance, and to Nancy Lee for permission to quote unpublished work and for the gift of several bacterial strains. This work was supported by Research Grants CA-11034 and CA-15941 from the National Cancer Institute, U.S. Public Health Service.

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