percent gave low levels. Figure 3 summarizes this analysis and shows the behavior of both classes of λ H2 phage in transductional tests. We assume that these two types represented the behavior of phage carrying phase 2–on, or phase 2–off. The process of phase transition was asymmetric, that is, the rate of transition from on to off appeared to be at least tenfold higher relative to the rate in the reverse direction (off to on). The picture is similar to that initially observed by Stocker (*3*). He found that the transition from phase 2–on to phase 2–off occurred at about ten times the frequency of the reverse transition. On the basis of these experiments, we can define two populations of λ phage, one that carries H2–off in almost all of the population and another which has mostly H2–on.

To determine whether the "inversion bubble" is correlated with expression of the different states of H2, DNA's from the different λ populations were prepared, denatured, and reannealed; and the frequency of inversions was measured. Three hundred molecules of λ H2–off DNA were scored, and 2.5 percent had the "inversion bubble." One hundred and fifty molecules of DNA carrying λ H2–on were scored, and 12 percent were found to carry the inversion. When equal amounts of these preparations were mixed, denatured, and reannealed, 34 percent of 150 molecules were found to have the inversion. The large increase in frequency obtained when the two populations were mixed indicated that it was the heteroduplex between phase 2–on and phase 2–off regions that formed the "inversion bubble." On the basis of these experiments we suggest that phase variation is determined by a specific region adjacent to the H2 gene which can undergo an inversion. In one configuration it allows the H2 gene and perhaps adjacent genes (*13*) to be transcribed. In the opposite configuration it does not allow H2 transcription. This hypothesis is shown schematically in Fig. 4. We cannot formally rule out the possibility that a specific transposition of heterologous DNA into the phase-controlling site is responsible for the "inversion bubble." However, in recent experiments DNA molecules associated by a duplex region which corresponded to the "inversion bubble" with nonhomologous single-stranded ends, have been observed. These results suggest that with respect to the "bubble" the notion that it is generated by an inversion is the simplest model that explains all of our observations. It raises questions about the specific mechanisms involved in regulating the transition. These can be approached experimentally with the use of the cloned DNA.

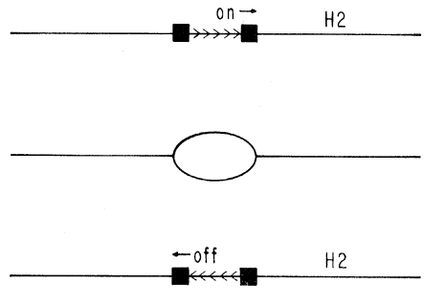

Fig. 4. A model for phase variation. An inversion of the sequence between the dark blocks could result in a change in the orientation of a promoter contained within the region or it could allow read through from a promoter outside the region.

Evidence in a variety of different systems has accumulated suggesting that a recombinational event, an inversion (*14*), or a transposition (*15*) can be involved in regulating gene expression. These findings taken together with our present view of phase variation suggest that it may be worthwhile to reexamine the notion that directed recombinational events play a role in cell differentiation and development.

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9 February 1977

Gene Cloning and Containment Properties of Plasmid Col E1 and Its Derivatives

Abstract. *Colicinogenic plasmid E1 (Col E1) and Col E1 derivatives offer advantages as plasmid cloning vehicles with regard to both utility and biological containment. The Col E1 derivative pCR1 does not alter those essential characteristics of the enfeebled* Escherichia coli *strain χ1776 that make this strain particularly useful as a host-vehicle system for recombinant DNA research.*

In this report we discuss the properties of colicinogenic plasmid E1 (Col E1) and certain Col E1 derivatives obtained in our laboratory that make these plasmids particularly suited as cloning vehicles for recombinant DNA research.

Col E1 is a relatively small, covalently closed, circular DNA molecule (4.2 megadaltons in size) (*1*) that is present in multiple copies in *Escherichia coli*—about 20 to 30 molecules per cell (*2*). When grown in the presence of chloramphenicol, Col E1 is amplified; that is, bacterial chromosomal replication ceases, but Col E1 continues to replicate until approximately 45 percent of the cellular DNA is Col E1 DNA (*2, 3*). With the use of this amplification step, high yields of both Col E1 DNA and also any DNA sequence that is inserted into the plasmid are readily obtained (*4*).

Since Col E1 contains a nonessential region, a single site sensitive to the restriction endonuclease Eco RI, Eco RI–digested DNA can be inserted into the plasmid after cleavage with this restriction enzyme. Recombinant plasmids constructed by insertion at the Eco RI site are readily detected since they no longer produce the antibiotic protein colicin E1 (*4*). Col E1 is a nonconjugative plasmid; that is, it is unable to promote its own transfer from one bacterial cell to another (*5*). However, the conjugal transfer of Col E1 is promoted by self-transmissible (conjugative) plasmids (*6*). Conjugative plasmids vary greatly in their ability to promote the transfer of Col E1.

There are several unique derivatives of Col E1 that have been developed for use

as cloning vehicles. Mini-Col E1 (pVH51) is a spontaneously-isolated plasmid (2.1 megadaltons) in which approximately one-half of Col E1 DNA is deleted (*7*). pVH51 has an even higher copy number than does Col E1 (approximately 114 copies per cell), amplifies in chloramphenicol to the same extent as Col E1, and retains the single Eco RI site. With regard to containment of the plasmid, it is of importance that pVH51 is mobilized by a conjugative plasmid at a much lower frequency than Col E1 (*7*). Similar low molecular weight derivatives of Col E1–like plasmids that carry antibiotic resistance genes and are extensively used as plasmid cloning vehicles have been constructed (*8*).

Kanamycin resistant (Km^r) derivatives of plasmids Col E1 and mini-Col E1 have been constructed in vitro by insertion of a 4.5-megadalton kanamycin-resistance fragment into the Eco RI site of Col E1 (*4*) and mini-Col E1 (*7*). One of the two Eco RI sites of Col E1-*kan* (designated pML2) has been removed by exonuclease treatment of pML2 after partial digestion with Eco RI (*9*). This plasmid, designated pCR1 (*9*), has two properties that make it advantageous for gene cloning. First, although Col E1 and pVH51 lack easily detectable phenotypes that facilitate detection, pCR1 specifies kanamycin resistance, a very effective selective marker. Second, since one Eco RI site has been deleted, DNA can be inserted into the remaining Eco RI site of pCR1 without loss of the Km^r marker. A restriction map of pCR1 is shown in Fig. 1.

In view of the advantages of pCR1 as a cloning vehicle, the effect of this plasmid on the survivability properties of the enfeebled *E. coli* strain χ1776 (*10*) were examined to determine the suitability of χ1776 (pCR1) as an EK2 host-vector system for gene cloning.

In the absence of exogenous diaminopimelic acid (DAP), a cell wall constituent, the viability of strain χ1776 decreases to a level of at most 10^{-8} of the starting cell concentration (Fig. 2A) as observed by R. Curtiss III and co-workers. Death of χ1776 resulting from lack of DAP also occurs in the presence of another strain, χ1780, that carries the conjugative plasmid R64 drd 11, indicating that at least the strain χ1780 does not rescue χ1776 by cross-feeding (Fig. 2B). The presence of pCR1 in χ1776 does not prevent or alter death due to lack of DAP (Fig. 2C), nor can χ1780 "rescue" pCR1 by mobilization of pCR1 out of χ1776 and into χ1780 (Fig. 2D).

Another important property of strain χ1776 is its sensitivity to bile salts, a characteristic that should reduce the survival of this organism in the digestive tract. Strain χ1776 carrying pCR1 shows no increased resistance to bile salts than χ1776 without pCR1 (Table 1). Indeed, the data suggest that χ1776 (pCR1) is even more sensitive to bile salts than χ1776. These results essentially confirm the containment properties of χ1776 observed by R. Curtiss III and co-workers and demonstrate that pCR1 does not significantly alter these properties.

Table 1. Average plating efficiencies (*P*) of χ1776 and χ1776 (pCR1) as a function of bile salts concentration. Exponential and early stationary cultures of χ1776 and χ1776 (pCR1) grown at 37°C in L2 broth were concentrated by centrifugation and resuspended in 1.0 ml of L2 broth. Dilutions were made in the same medium and plated onto L2 agar containing 0, 0.15, 0.37, or 0.75 percent Bacto bile salts No. 3. Survival on plates containing bile salts was determined by means of the titer on plates without bile salts. The plating efficiency (*P*) is log (viable count of χ1776) − log [viable count χ1776 (pCR1)] for each concentration of bile salts. The survival of χ1776 and χ1776 (pCR1) at the different bile salts concentrations ranged from 2.9×10^{-2} to 9×10^{-7} at 0.15 percent, 6.9×10^{-7} to 3.4×10^{-9} at 0.37 percent, and 7.1×10^{-8} to 1.5×10^{-9} at 0.75 percent.

Bile salts (%)	Average plating efficiency ($P \pm$ S.E.)	Experiments (No.)
0.15	1.7 ± 4.3	4
0.37	0.9 ± 0.56	5
0.75	0.8 ± 0.31	5

Finally, the ability of conjugative plasmids to promote the transfer of pCR1 out of χ1776 was tested in triparental matings using (i) donor strains χ1776 (pCR1) or C600 (pCR1) as a control, (ii) nine *E. coli* strains (*11*) carrying different R (antibiotic resistance) plasmids capable of mobilizing pCR1, and (iii) an *E. coli* recipient. The nine R plasmids tested for mobilization of pCR1 were R64 drd 11, R6K, R16, N-3, R27, R46, R471a, RIP175, and R71a. The R factors mobilized pCR1 from χ1776 at frequencies below 10^{-8} under conditions when the mobilization of pCR1 from the control strain C600 (pCR1) occurred at frequencies of 5×10^{-4} to 10^{-7} after 25 hours of mating. Other investigators have shown that a sex plasmid, the F1 plasmid, can mobilize Col E1 derivatives from strains analogous to C600 at frequencies approximately 100-fold higher than what was observed with these nine R plasmids (*12*). This difference could be explained if F and its derivatives are more efficient than R plasmids at mobilizing Col E1.

The recovery of pCR1 from χ1776 as covalently closed circular DNA is similar to that found for the C600 strain using sarkosyl (*13*) or Triton-X100 (*14*) lysis. However, amplification in the presence of chloramphenicol (*4*) yielded at best 5 percent of the total DNA as covalently closed circles.

Containment can also be facilitated through the use of special plasmid cloning vehicles as well as enfeebled hosts. For instance, since drug-resistant strains of *E. coli* have a selective advantage for establishment in the gut of animals being starved (*15*) or treated with antibiotics

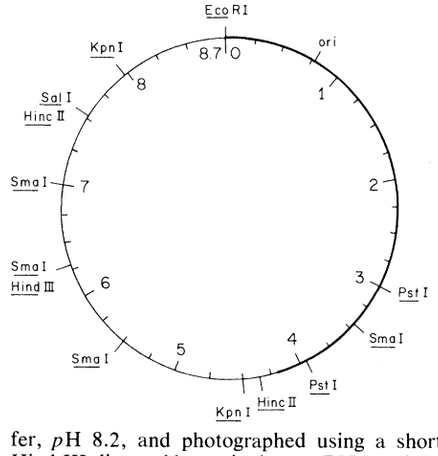

Fig. 1. Restriction map of the Col E1 derivative pCR1. Purified pCR1 DNA was digested at 37°C in buffer consisting of 7 m*M* tris, *p*H 7.4, 60 m*M* NaCl, 7 m*M* $MgCl_2$, and 7 m*M* mercaptoethanol for all enzymes except Hpa I [10 m*M* tris, *p*H 7.4, 10 m*M* $MgCl_2$, 6 m*M* KCl, 1 m*M* dithiothreitol (DTT)], and Eco RI (100 m*M* tris, *p*H 7.4, 50 m*M* NaCl, 6 m*M* $MgCl_2$, and 6 m*M* mercaptoethanol). Reactions were stopped by heating at 65°C for 10 minutes, and 20 μl of bromophenol blue dye containing 25 percent glycerol and 5 percent sodium dodecyl sulfate was added to each reaction mixture. Digests were subjected to electrophoresis in 1.0 percent, 1.2 percent, or 1.4 percent agarose gels at 120 to 140 volts at room temperature for approximately 2 hours. After electrophoresis, gels were stained for 30 minutes with ethidium bromide in tris-borate buffer, *p*H 8.2, and photographed using a shortwave ultraviolet transilluminator. Eco RI- or Hind III-digested bacteriophage λ DNA and Eco RI–digested pML2 (Col E1-*kan*) were used as molecular weight standards. The enzymes Bam HI, Hpa I, and Bgl II did not cleave pCR1. Hinc II also cleaves the smaller Hinc II fragment twice, producing three fragments of 0.5, 1.0, and 1.3 megadaltons. The relative order of these fragments has not been conclusively determined, but the largest (1.3 megadaltons) is cleaved by Hind III. Hae II and Hae III cleave Col E1 into 6 and 14 fragments, respectively (*19*). Insertion into the Hind III site inactivates Km^r (D. Figurski and R. Meyer, unpublished results), but insertion into the Sal I site does not (*20*). Loss of the small Pst I fragment does not inhibit Col E1 replication (A. Otsuka, unpublished results). The divisions represent megadaltons of DNA. The kanamycin determinant is denoted by the lighter section of the map.

(*16*), plasmid cloning vehicles carrying selective markers other than antibiotic resistance may be preferred in certain circumstances. For this purpose, two plasmids that carry genes for tryptophan biosynthesis (pVH151, Col E1-*trpE trpD*; and pVH153, mini-Col E1-*trpE trpD*) were constructed in collaboration with C. Yanofsky. The *trp* fragment (4.7 megadaltons) was obtained by Eco RI digestion of λ-*trp* bacteriophage λED10f(bot) and contains the poED region of the tryptophan operon (*17*). The *trp* fragment was inserted at the Eco RI site of Col E1 and mini-Col E1. At present, experiments are in progress to delete one of the two Eco RI sites in each plasmid to facilitate the insertion of exogenous DNA.

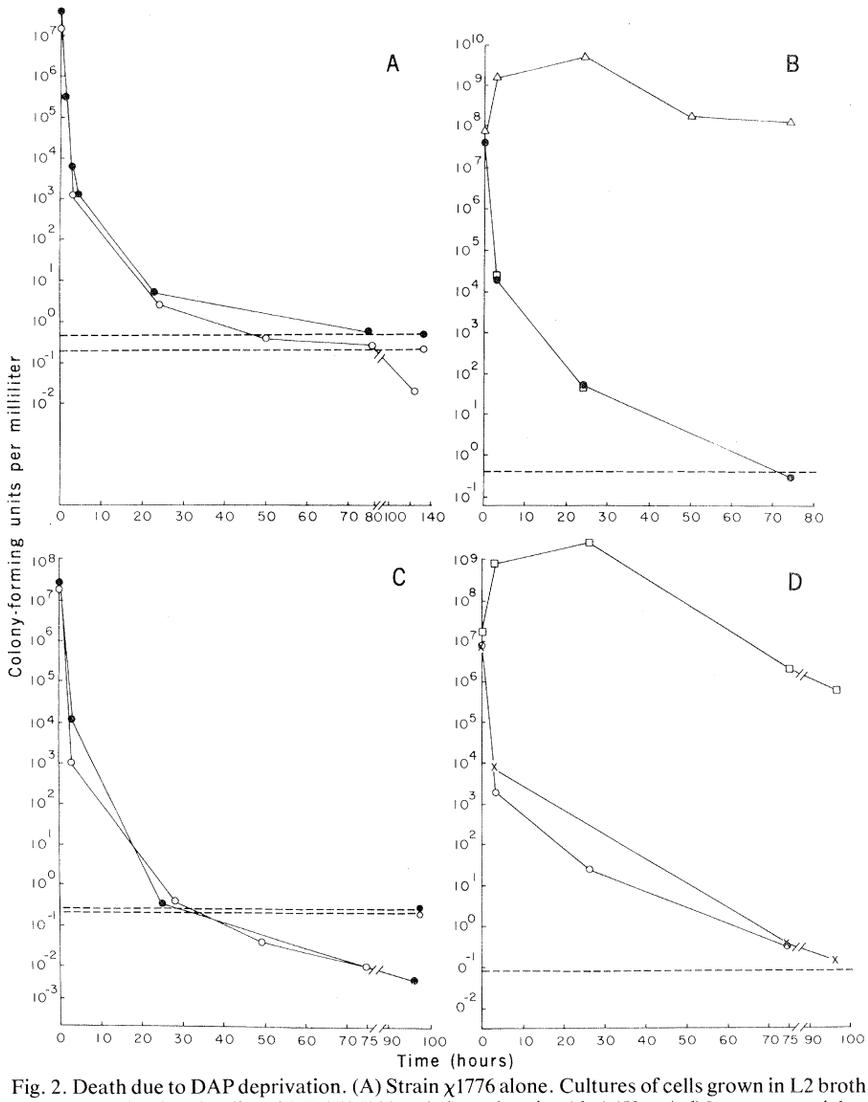

Fig. 2. Death due to DAP deprivation. (A) Strain χ1776 alone. Cultures of cells grown in L2 broth [L broth + diaminopimelic acid (DAP) (200 μg/ml) + thymine (thy) (50 μg/ml)] to exponential or early stationary phase were harvested by centrifugation at 15°C. The pellets were washed and resuspended in 10 ml of BSG buffer (8.5 g of NaCl, 0.3 g of KH_2PO_4, 0.6 g of $NaHPO_4$, and 100 mg of gelatin per liter) and were used to inoculate DAP^- medium (L broth containing thy and lacking NaCl), at a cell concentration of 5 ml/liter. Cultures were incubated with shaking at 37°C. Samples were removed at the times indicated and titered on L2 agar. At later times, cells were concentrated by filtration, and colonies were grown on sterile Millipore filters placed on L2 agar. Controls showed that viability was not affected by filtration. The dotted line indicates the level at which the number of colony-forming units have decreased by 10^8 from the original titer. The symbols (○) and (●) represent points from two different experiments. (B) Strain χ1776 in the presence of χ1780. Cultures of both strains were treated as described in (A). Titers were determined with the use of the following media: χ1776, L2 agar plus nalidixic acid (50 μg/ml) (●); χ1780, MacConkey lactose agar (Δ); and χ1776 (R64), L2 agar plus nalidixic acid plus tetracycline-HCl (25 μg/ml) (□). Strain χ1780 [*tsx-63 supE42* λ^- *his-53 lysA32 T3*^{r} *xyl-14 arg-65* (R64 drd 11 Sm^rTc^r)] was obtained from R. Curtiss III. (C) Strain χ1776 (pCR1) alone. Cultures were treated and the titers were determined as described in the legend to Fig. 2A. The symbols (○) and (●) represent data from two different experiments. (D) Strain χ1776 (pCR1) in the presence of χ1780. Titers were determined using the following media: χ1776, L2 agar + nalidixic acid (○); χ1780, MacConkey lactose agar (□); χ1776 (pCR1) and χ1780 (pCR1), L2 agar plus kanamycin sulfate (25 μg/ml) (×).

Other plasmid vehicles that may also facilitate containment are those that are disabled by a mutation in the plasmid. One such type, pJC307, a Col E1 plasmid that is unable to replicate at 37°C, has been isolated (*18*). The thermosensitive phenotype of this mutant suggests that Col E1 codes for at least one protein essential for its own replication and has led to our current efforts to isolate amber-suppressible Col E1 mutants that will be dependent upon a specific host genotype for replication and maintenance. The use of such mutant plasmid vectors should increase significantly the degree of biological containment afforded by an enfeebled host strain.

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10. The *E. coli* strain χ1776 *F⁻tonA53 dapD8 minA1 supE42 Δ40 (gal-uvrB) λ⁻ minB2 rfb-2 nalA25 thyA57 metC65 T3*^{r} *Δ29 (bioH-asd) cycB2 cycA1 hsdR2* was developed by R. Curtiss III and his colleagues as an enfeebled host for recombinant DNA research.
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