TEMPERATURE-SENSITIVE MUTANTS FOR THE REPLICATION OF PLASMIDS IN *ESCHERICHIA COLI* I. ISOLATION AND SPECIFICITY OF HOST AND PLASMID MUTATIONS[1]

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ABSTRACT

Temperature-sensitive mutants of *Escherichia coli* defective in the replication of the plasmid colicinogenic factor E1 (*Col*E_1) were isolated following mutagenesis of *E. coli* K12 strain carrying the *Col*E_1 factor. Following the mutagenic treatment an enrichment procedure utilizing the replacement of thymine with bromouracil in the *Col*E_1 DNA duplicated at the restrictive temperature was used. The mutants isolated following this enrichment step were the result of a mutation event either in the host chromosome or in the *Col*E_1 plasmid. The host mutants fell into three phenotypic classes based on the effect each mutation had on the maintenance of a variety of other extrachromosomal DNA elements. Phenotypic class I mutations affected all *E. coli* plasmids, both the I and F sex factor types as well as the *Col*E_1 factor. Phenotypic class II mutations affected the maintenance of the *Col*E_1 and the F sex factor type plasmids and not the I type, while phenotypic class III mutations affected only *Col*E_1 replication. None of these mutations was found to have a significant effect on the replication of the *E. coli* chromosome. The plasmid-linked mutations fell into two phenotypic classes on the basis of the ability of the *Flac* episome to complement the mutation in the *Col*E_1 plasmid.

A property common to all the extrachromosomal elements (plasmids) of *Escherichia coli* is the capability of autonomous replication followed by the distribution of plasmid molecules to the daughter cells during cellular division. The number of copies in a cell population is carefully controlled and the rate of segregation of cells without the plasmid DNA is very low for the sex factors and rare for most non-sex factor plasmids.

The mechanism of this replication and segregation of plasmid DNA has been a subject which has received much attention in the past few years. JACOB, BRENNER and CUZIN (1963) postulated that every autonomous unit of replication should be considered a replicon, a replication unit carrying one or more genes

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that control its own replication. In support of the replicon hypothesis properties of a series of temperature-sensitive replication mutants of an *Flac* factor were described (CUZIN and JACOB 1967). Certain of these mutations were located on the *Flac* factor and could be complemented by the presence of a normal *Fgal* factor. In the same study (CUZIN and JACOB 1967) the authors reported the isolation of plasmid replication mutants that map on the *E. coli* chromosome. These mutants have been shown to specifically affect the replication of *Flac* or *Fgal* factors but not an F-type *R* factor (HIROTA *et al.* 1968). The gene products involved in these mutations have not been identified, but it is clear from these studies that the genetic control of these extrachromosomal replicons lies both on the bacterial chromosome and on the plasmid DNA.

The colicinogenic factor E_1 (*Col*E_1) is a plasmid element lacking sex-factor properties. The *Col*E_1 DNA molecule has a molecular weight of 4.2×10^6 daltons and in contrast to the *E. coli* sex factors, exists in the cells in multiple copies (BAZARAL and HELINSKI 1968). The *Col*E_1 factor can be isolated as either a supercoiled circular DNA molecule or as a relaxation complex of supercoiled DNA and protein (CLEWELL and HELINSKI 1969, 1970). Unlike the *E. coli* sex factors, these plasmid molecules are unable to promote either their own conjugal transfer from cell to cell or the transfer of chromosomal DNA. The transfer of the *Col*E_1 factor, however, can be promoted by a sex factor present in the same cell.

The role of chromosomal genes in controlling the replication of factors of the *Col*E_1 type is poorly understood. GOEBEL (1970) has shown that a *dnaA* (initiation) mutation, known to affect chromosomal DNA synthesis, causes a rapid cessation of *Col*E_1 synthesis when shifted to the non-permissive temperature. A *dna*B (chain elongation) mutant was found to synthesize *Col*E_1 DNA at a diminishing rate under conditions where chromosomal DNA synthesis is immediately shut off (GOEBEL 1970). In a study of plasmid replication in the *E. coli* *pol*A1 mutant (DNA polymerase I) of *E. coli*, it was shown that the *Col*E_1 and *Col*E_2 factors required DNA polymerase I for their normal maintenance (KINGSBURY and HELINSKI 1970). The maintenance of various sex-factor plasmids was unaffected in this mutant (KINGSBURY and HELINSKI 1970; D. KINGSBURY and D. HELINSKI, submitted for publication).

In the present study mutations were induced in an *E. coli* strain carrying the *Col*E_1 plasmid that resulted in defective replication of the *Col*E_1 plasmid at a non-permissive temperature. The mutations were located either on the host chromosome, or on the *Col*E_1 plasmid. The specificity of these mutations with respect to their effect on the maintenance of other plasmids is described. Various other properties of plasmids in these mutants are considered in an accompanying report (KINGSBURY, SIECKMANN and HELINSKI 1973).

MATERIALS AND METHODS

Bacterial strains: The bacterial strains used throughout this study are described in Table 1. C6000 *Flac* was obtained from M. COHEN, J5-3 (R1drd19) from E. MEYNELL via W. BELSER and J5-3 (R64drd11) from E. MEYNELL. The origin of the other strains was our own collection (HELINSKI and HERSCHMAN 1967).

The thymine-requiring mutant of JC411 (*Col*E_1), DK9, was made by the procedure of

TABLE 1

Bacterial strains and their characteristics

Strain	Characteristics*	Parent strain
JC411	His, argG, metB, Leu, malA, Xyl, lacY, strA	
JC411 (*Col*E_1)	same as JC411 except *Col*E_1+ (K30)	JC411
DK9	same as JC411 (*Col*E_1) except Thy⁻	JC411 (*Col*E_1)
DK100	same as DK9 except Mal+	DK9
CR34	Thr, Leu, Thi, Thy, Lac	
CR34 (*Col*E_1)	same as CR34 except *Col*E_1+ (K30)	CR34
CR34 (*Col*E_1) (*Flac*)	same as CR34 (*Col*E_1) except *Flac*+ (C6000)	CR34 (*Col*E_1)
CR34 (*Col*E_2)	same as CR34 except *Col*E_2+ (*Shigella* P9)	CR34
C6000 *Flac*	Thr, Leu, Thi, *Flac*+	
K30	*Col*E_1+, *Col*V+	
K94	*Col*V+	
CA53	*Col*Ia+	
C600/az	Thr, Leu, Thi, Lac, Az-r	
J5-3 (R1)	Pro⁻met⁻ *R1*+ (fi+, Km, Cm, Sm, Su, Cm)	
J5-3 (R1drd19)	same as J5-3 *R1* except derepressed (drd19)	
J5-3 (R64)	Pro⁻met⁻ *R64*+ (fi⁻, Tc, Sm)	
J5-3 (*R64*drd11)	same as J5-3 *R64* except derepressed (drd11)	
YS40	His, Pro, str-r	
YS40/E	same as YS40 except colicin E-r	YS40
YS40/V	same as YS40 except colicin V-r	YS40
YS40/azE	same as YS40 except colicin E-r, azide-r	YS40
YS40/azV	same as YS40 except colicin V-r, azide-r	YS40
Sal. LT2 (*Col*Ib)	*Salmonella typhimurium* LT2, cysD36 *Col*Ib+ (*Shigella* P9)	*S. typhimurium* LT2

* Unless otherwise indicated all-strains are *E. coli* derivatives. Gene symbols and usage are those suggested by TAYLOR and TROTTER (1967). Str-r and azide-r refer to resistance to streptomycin and sodium azide, respectively. Colicin E-r and V-r refer to resistance to colicin E_1 and colicin V, respectively. Colicinogenic factors determining the production of colicins E_1, E_2, V, Ia and Ib are designated *Col*E_1 and *Col*E_2, *Col*V, *Col*Ia, and *Col*Ib, respectively.

SMITH (1967), utilizing the selection of spontaneous mutants by growth of the bacteria in a minimal meduim containing 350 μg/ml aminopterin (kindly provided by Lederle Laboratories, Pearl River, N.Y.) and 200 μg/ml thymine. After plating and re-isolation, cells capable of growing on 2 μg/ml thymine were selected.

Strain DK100 was selected as a maltose positive revertant of DK9 which is unable to ferment maltose. DK100 simultaneously acquired sensitivity to bacteriophage λ.

Media and growth conditions: The defined media used throughout this study consisted of M9 phosphate buffered salts supplemented with the particular requirements for each strain. M9 salts contained the following per liter: 6.0g Na_2HPO_4, 3.0g KH_2PO_4 5.0g NaCl, 1.0g NH_4Cl, 10^{-3}M $MgSO_4$ and 10^{-4}M $CaCl_2$. When amino acids were required they were added at a level of 50 μg/ml; thiamine at 2 μg/ml; and thymine was added at a level of 20 μg/ml in solid media and 2 μg/ml in liquid. Difco Vitamin-Free Casamino acids were used at a concentration of 4 g/l.

The carbon source, either glucose or glycerol, was added to a final concentration of 0.4%. When appropriate to make agar plates, Bacto Agar (Difco) was added at 17.5 g/l.

Carbohydrate fermentation was tested on MacConkey agar (Difco) containing a final concentration of 1% of the sugar or sugars to be tested.

L-medium or AB3 medium was used for the detection of colicin production and the performance of genetic crosses. L-medium consisted of 10.0 g/l tryptone (Difco), 5.0 g/l yeast extract (Difco), 5.0 g/l NaCl and 1.0 g/l glucose. The pH was adjusted to 7.0 with 5N NaOH. AB3 medium consisted of 17.5 g/l Difco Antibiotic Medium 3. For solid media 17.5 g/l Difco agar were added to the above (hard agar).

When required, antibiotics were added to the various media at the following concentrations: streptomycin 50–100 μg/ml, chloramphenicol 10 μg/ml, tetracycline 10μg/ml and sulfanilamide 20 μg/ml. The streptomycin, tetracycline and sulfanilamide were purchased from Calbiochem; the chloramphenicol was a gift of the Parke Davis Company. Sodium azide (NaN_3) was added to a final concentration of 2×10^{-3} M when required.

Cell densities were estimated from the optical densities determined with a Klett-Summerson colorimeter using a number 540 filter. One hundred Klett units corresponds to approximately 4.5×10^8 bacteria per ml.

Colicin assays and detection of colicinogenic colonies: Colicin production by single colonies was assayed by stabbing each colony into a fresh AB3 plate and incubating at the appropriate temperature for 18–24 hours The surface of this plate was then exposed to chloroform vapors for a 3 min period to kill the bacterial colonies. The plate was then overlayed with 3 ml of soft agar containing 5×10^7 indicator bacteria, followed by an additional overnight incubation at 37°C.

The quantitative determination of transfer frequency of various *Col* factors was determined by the triple overlay techniques of FREDERICQ (1957). This procedure involves plating the colicinogenic cells in 2.5 ml of soft agar, followed by a second layer of 2.5 ml of soft agar containing no bacteria. After overnight incubation at 37°C, a third soft agar layer containing the indicator bacteria is applied and the plates are reincubated overnight. The individual colicinogenic colonies gave a clear zone in the turbid lawn of indicator cells.

Transfer of the colicinogenic factors: Transfer of the *Col*E_1 factor, which lacks sex factor activity, was promoted by either the *Col*V factor or an *Flac* factor. Log phase cultures were prepared by growth of the donor and recipient cultures in L-broth at 37°C with shaking for aeration. When the cell densities reached approximately 5×10^8/ml, the donor and recipient cultures were mixed in equal proportions and incubated in static culture for varying periods of time and temperature depending on the particular experiment. Generally the donor culture was either K30 or CR34 (*Col*E_1) (*Flac*) which are streptomycin-sensitive. Whenever possible streptomycin counterselection was used to eliminate the donor cells. If streptomycin could not be used, then the donor cells were counterselected by starving for one or more amino acids.

The *Col*E_2 factor, which also lacks sex factor activity, was transferred as described by SMITH *et al.* (1963) utilizing the *Col*Ib (*Shigella* P9) of *Salmonella typhimurium* LT2 (*Col*Ib) to promote the transfer. The mating periods for the *Col*E_2 transfer varied from 30 to 90 minutes.

The *Col*V, *Col*Ia, *Col*Ib, *R*1 and *R*64 factors were generally transferred by overnight growth of the donor and recipient culture together in static culture, followed by plating on selective agar.

Mutagenesis and purification of the mutants: All of the mutants isolated in this study were induced by treating the cells with N-methyl-N′-nitro-N-nitrosoguanidine (NTG). Various modifications of the method of ADELBERG, MANDEL and CHEN (1965) were used throughout.

Bacterial cultures of DK9, DK100 or CR34 (*Col*E_1) were grown to mid-log phase in either L-broth or AB3 at 37°C. The cultures were aerated by vigorous shaking and were usually 10 ml in volume. At the appropriate cell density (5×10^8/ml) the culture was placed in a sterile centrifuge tube and pelleted by centrifugation at 10,000 rpm for 10 minutes in the Sorvall RC 2-B.

In the case of *short-term mutagenesis*, the pelleted bacterial cells were resuspended in 1 ml of tris-malate buffer (TM buffer, 0.2 M tris HCl, 0.2 M maleic acid, pH 6.0) and added to 9.0 ml of TM buffer containing 1 mg of NTG. This suspension was placed in a 125 ml erlenmeyer flask and shaken at 37°C for 20 minutes. At the end of this 20 minute exposure to NTG the cells were again pelleted by centrifugation.

In the case of *long-term mutagenesis*, the pelleted bacterial cells were transferred to 30 ml of AB3 and shaken for 20 minutes at 33°C. Chloramphenicol was then added to give a final concentration of 33 μg/ml and the incubation continued for 90 minutes. After this period the bacterial chromosome had stopped replication, however, *Col*E_1 factor replication continued (CLEWELL and HELINSKI 1972). At this point NTG was added to a final concentration of 1 mg/ml and the culture was incubated for 12 additional hours. Following this incubation the cells were pelleted by centrifugation.

Following either mutagenesis regime the pelleted bacterial cells were resuspended in M9 salts in order to wash out any remaining NTG. Following this washing, the cells were again pelleted as above.

The segregation step employed at this stage also was dependent on the type of mutation desired and on the type of mutagenesis applied. Following short-term mutagenesis the bacterial pellet was resuspended in 30 ml of M9, Casamino acids, glucose broth containing 1 μg/ml thymine and shaken at 33°C for 2 generations to allow segregation of the chromosomal mutations. Following long-term mutagenesis the cells were resuspended in 30 ml of AB3 broth and grown at 33°C. The culture was transferred several times to allow approximately 10 to 20 generations after mutagenesis. Following this segregation period, the cells were centrifuged to remove the rich medium. One-third of a 30 ml culture was resuspended in M9, Casamino acids, glucose medium.

At this stage all of the cultures were handled similarly, regardless of the mutagenic regime used. The cultures were adjusted to approximately 50 to 70 Klett units and the level of thymine adjusted to 1 μg/ml. The cultures were shifted to a water bath shaker at 43°C and shaken for 90 minutes. Chloramphenicol was then added to a final concentration of 33 μg/ml and the cultures shaken for an additional 90 minutes. After this period in chloramphenicol, replication of the bacterial chromosome stopped. 5-Bromourail (BU) was then added at a final concentration of 300 μg/ml, which was 300 fold the concentration of thymine in the medium. At this stage the flasks were wrapped in aluminum foil to prevent any exposure to light.

The cultures, still at 43°C, were shaken for an additional 10–15 hours to allow maximum replication of the *Col*E_1 plasmid in BU. Following this incubation the 30 ml of culture was centrifuged at 10,000 rpm for 10 minutes to pellet the cells. The bacteria were then resuspended in 1 ml of M9 salts and placed as a drop in the center of a sterile plastic petri dish. On top of this petri dish containing the 1 ml of cell suspension was placed another petri dish containing 50 ml of a saturated solution of thymine in water. Finally, this plate usually was covered by a third glass petri dish of water. A high-intensity white light source, either a 250 watt heat lamp or an 8 watt fluorescent lamp, was placed 18 inches or 6 inches, respectively, from the surface of the petri dish of water. The light was used to irradiate the BU-labeled culture for 20 minutes. This 1 ml bacterial suspension was then placed in AB3 broth for 4–8 hours of growth at 33°C. Following this growth period the culture was diluted and plated on AB3 agar at 33°C. The dilutions plated were designed to give the maximum number of well-isolated colonies per plate. The resulting colonies were then tested for their temperature sensitivity for growth at 43°C and for colicin production at 43°C and 33°C. Those colonies which grew well and produced colicin at 33°C but not 43°C were tentatively identified as mutants and selected for further study.

Single colonies were checked for the presence of mutations and mutant clones were restreaked twice to insure purity. The mutant and parent stocks were routinely stored by stabbing into deeps of AB3 medium containing 0.9% Difco Agar.

RESULTS

Isolation and gross localization of mutants temperature sensitive for the replication of ColE_1*:* The large number of copies of *Col*E_1 DNA per chromosome (approximately 10–15) (CLEWELL and HELINSKI 1972) necessitated the development of a technique that was capable of enriching for a mutation that might occur in only one of the 15 copies. The procedure described in MATERIALS AND METHODS is basically an adaptation of the BU-incorporation procedure of BONHOEFFER and SCHALLER (1965). The starting cultures were either DK9, DK100

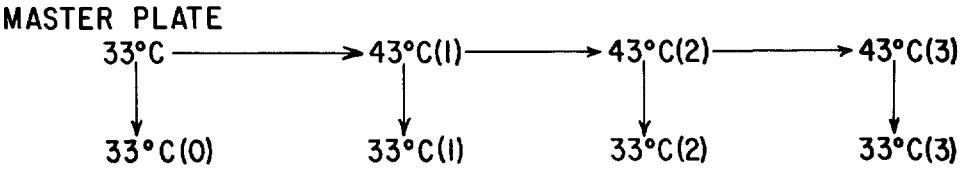

FIGURE 1.—Plate screening procedure for determining the loss of bacterial plasmids. The passage numbering system designates the number of exposures to 43°C. In every case the passages at 43°C were done under non-selective plating conditions to allow either free expression or loss of the plasmid markers.

or CR34 (*Col*E_1), all of which are *E. coli* K12 strains that contain the plasmid *Col*E_1 and require thymine for growth. DK100, a derivative of DK9, is sensitive to coliphage λ and is the parent of most of the host mutants isolated in this study.

Two mutagenic procedures were used, each designed to give a particular class of mutants. "Short-term" mutagenesis was designed to give predominantly mutations which lie on the bacterial chromosome, while "long-term" mutagenesis was designed to give almost entirely mutations on the *Col*E_1 factor itself.

The frequency of recovery of mutants varied widely between experiments. The highest rate in any experiment was one mutant colony in 10, while the lowest rate in any experiment was 1 in 300. To avoid the isolation of more than one descendent of a single mutant, no two mutants from the same mutagenic treatment and selection were kept unless each demonstrated unique phenotypic properties in one or more of the screening procedures.

Every prospective mutant was isolated because of its ability to produce a detectable zone of colicin at the non-permissive temperature. To establish that these isolates were in fact maintenance mutants rather than colicin induction mutants or colicin structural mutants the screening procedure outlined in Figure 1 was adopted. This test is based on the expected irreversible loss of the *Col*E_1 factor at high temperature in a plasmid replication mutant. Each mutant was stabbed into an AB3 plate at 33°C and 43°C and grown overnight. The colony from the 43°C plate was passed to a second 43°C plate and a 33°C plate by picking from the colony edge with a sterile toothpick and stabbing into the two new plates. The parent colony was chloroform-treated and overlayed with indicator bacteria. The procedure was continued for 3 passages at 43°C to allow dilution of the copies of the *Col*E_1 factor from the population following the termination of replication. Every mutant that lost the ability to produce colicin at 33°C following passage at 43°C was tentatively considered a replication mutant. Although this criterion does not distinguish between replication and segregation mutants, it was found that all of the mutants isolated when tested, subsequently, for *Col*E_1 DNA synthesis and the rate of formation of non-colicinogenic cells in cultures incubated at 43°C, behaved as replication mutants.

Temperature-sensitive mutants were initially characterized with respect to whether the mutational site was present on the host chromosome or on the *Col*E_1 plasmid. This was readily determined by examining the mutant strains for the co-transfer of the temperature-sensitive replication property and the *Col*E_1 plas-

mid. The *Col*E_1 factor lacks sex factor activity and, therefore, transfer of the factor requires the presence of a sex factor plasmid element in the donor cell. The system utilized in this study was to promote *Col*E_1 transfer with an *Flac* factor. To avoid complications due to the presence of a second plasmid, recipients were examined that received the *Col*E_1 plasmid but not the *Flac* factor. The advantage of this system is that when a *Lac*$^-$ female is used, those colonies which have received only *Col*E_1 can be easily differentiated from those receiving both *Col*E_1 and *Flac* by plating on MacConkey agar containing lactose. In addition, under the conditions employed, the transfer frequency of the *Flac* factor was low.

Each DK9 or DK100 mutant strain was crossed with C6000 *Flac*. *Lac*$^+$ and streptomycin-resistant colonies were selected from this cross on MacConkey agar containing lactose and streptomycin. The mutant strains carrying the *Flac* plasmid were then crossed with CR34 for 3–5 hours and the DK9 or DK100 donors were counter-selected nutritionally by the omission of histidine, methione and arginine from the medium. The colicinogenic CR34 isolates resulting from the cross were checked, subsequently, for the temperature sensitivity of *Col*E_1 replication and the presence of *Flac*. The results of these experiments are shown in Table 2. Those *Col*E_1 plasmids, which in the absence of *Flac* failed to show any temperature sensitivity, were considered normal and therefore it was concluded

TABLE 2

Gross localization of the mutational site

Isolate number	Parent strain	Method of mutagenesis*	Site of mutation
13, 14, 18, 19, 20, 22 23, 24, 25, 28, 30, 21	DK9	Short-term	Chromosome
29, 32, 34, 35, 36, 37 46, 49, 59, 61a, 62 63, 64, 65, 66, 67, 68, 70, 71, 72, 73a,	DK9	Short-term	*Col*E_1
75, 76, 77, 78a, 79, 80a, 81, 82, 83, 84, 86, 87, 88, 89, 91, 100 107a, 109a, 119a, 126a, 126b, 128, 129a, 131	DK9	Long-term	*Col*E_1
160, 164, 166, 171 191, 192, 201, 203, 203, 211, 213, 214, 215, 216, 218, 221,	CR34 (*Flac*) (*Col*E_1)	Short-term	Chromosome
223, 224, 225, 226, 227, 228, 231, 232, 233, 234, 235, 236, 237.	DK100	Short-term	Chromosome

* The short-term and long-term mutagenesis procedures are described in MATERIALS AND METHODS.

TABLE 3

Maintenance of extrachromosomal elements at 43°C in the chromosomal-located mutants

Mutant number	Extrachromosomal element	Expression of the plasmid element*						
		33(0)	43(1)	33(1)	43(2)	33(2)	43(3)	33(3)
	*Col*E$_1$	+	−	+	−	−	−	−
	*Col*E$_2$	+	−	+	−	±	−	−
	*Col*V	+	−	−	−	−	−	−
13, 18	*Flac*	+	−	−	−	−	−	−
	*Col*Ia	+	+	+	+	+	+	+
	*Col*Ib	+	+	+	+	+	+	+
	*R*1drd19	+	−	−	−	−	−	−
	*R*64	+	+	+	+	+	+	+
14, 19,	*Col*E$_1$	+	−	−	−	−	−	−
23, 24,	*Col*V	+	−	−	−	−	−	−
25, 28,	*Flac*	+	−	−	−	−	−	−
30, 31,	*Col*Ia	+	+	+	+	+	+	+
33, 192,	*Col*Ib	+	+	+	+	+	+	+
203, 223,	*R*1drd19	+	−	−	−	−	−	−
234	*R*64	+	+	+	+	+	+	+
	*Col*E$_1$	+	−	−	−	−	−	−
	*Col*V	+	−	−	−	−	−	−
	Flac	+	−	−	−	−	−	−
20, 191,	*Col*Ia	+	−	−	−	−	−	−
236, 237	*Col*Ib	+	−	−	−	−	−	−
	*R*1drd19	+	−	−	−	−	−	−
	*R*64	+	−	−	−	−	−	−
	*Col*E$_1$	+	−	±	−	−	−	−
	*Col*E$_2$	+	−	+	−	±	−	−
	*Col*V	+	−	−	−	−	−	−
22, 45	*Flac*	+	−	−	−	−	−	−
	*Col*Ia	+	−	−	−	−	−	−
	*Col*Ib	+	−	−	−	−	−	−
	*R*1drd19	+	−	−	−	−	−	−
	*R*64	+	−	−	−	−	−	−
164, 166, 171,	*Col*E$_1$	+	−	−	−	−	−	−
201, 205, 211,	*Col*V	+	+	+	+	+	+	+
215, 218, 221,	*Flac*	+	+	+	+	+	+	+
225, 226, 227,	*Col*Ib	+	+	+	+	+	+	+
231, 232, 233,	*R*1drd19	+	+	+	+	+	+	+
235	*R*64	+	+	+	+	+	+	+
	*Col*E$_1$	+	−	+	−	−	−	−
	*Col*V	+	+	+	+	+	+	+
213, 214,	*Flac*	+	unable to grow on MacConkey at 43°C					
228	*Col*Ib	+	+	+	+	+	+	+
	*R*1drd19	+	+	continued growth poor at 43°C				
	*R*64	+	+	continued growth poor at 43°C				

Mutant number	Extrachromosomal element	Expression of the plasmid element*						
		33(0)	43(1)	33(1)	43(2)	33(2)	43(3)	33(3)
	*Col*E$_1$	+	—	—	—	—	—	—
	*Col*E$_2$	+	+	+	+	+	not done	
	*Col*V	+	+	+	+	+	+	+
214, 216	*Flac*	+	+	+	+	+	+	+
	*Col*Ib	+	+	+	+	+	+	+
	*R*1drd19	+	+	+	+	+	+	+
	*R*64	+	+	+	+	+	+	+

* The designation of passages is described in Figure 1. (+) refers to the positive expression of detectable plasmid genes and (—) refers to no detectable plasmid functions.

that in those cases the temperature-sensitive mutation in the original colicinogenic strain was on the bacterial chromosome. The chromosome location of many, but not all, of these mutants was corroborated by temperature-curing the mutants for the *Col*E$_1$ plasmid and crossing a wild-type *Col*E$_1$ into the cells.

In every cross testing for the gross localization of the mutations 25 to 100 colonies were examined. There was considerable heterogeneity in the behavior of those colonies derived from parents resulting from the long-term mutagenesis. This lead to the conclusion that the long-term mutagenesis produced cells carrying a mixed population of plasmid molecules with respect to their temperature sensitivity and, therefore, before these mutants could be studied as a homogeneous population, the *Col*E$_1$ factors had to be transferred into a new host. The temperature sensitive CR34 (*Col*E$_1$) colonies which resulted from the crosses with the mutants behaved as homogeneous populations and are the plasmid mutant strains used in the remainder of this study. With only six exceptions (see Table 2) all of the plasmid-located mutations were derived as a result of the long-term mutagenesis procedure and none of the chromosomally located mutations were isolated by the long-term procedure.

Properties of the chromosome-located mutants: The separation of the mutants into two clearly defined groups indicated that it was possible to isolate a number of chromosomal mutations which, although resulting in defective *Col*E$_1$ replication, did not interfere with chromosome replication. Additional experiments were designed to examine the specificity of these mutations with respect to other plasmids and also the complementation of these mutations by other extrachromosomal elements.

A variety of extrachromosomal elements were introduced into the chromosomal-located mutants and the replication of these elements and their effect on *Col*E$_1$ replication was examined. Every mutant strain examined, as shown in Table 3, contained either *Col*E$_1$ alone, or *Col*E$_1$ plus the plasmid element to be tested. The only exception is *Col*E$_2$ which was introduced into mutants which had been cured of *Col*E$_1$ by growth at 43°C.

In no case did any of the plasmid elements tested complement the chromosome-located mutations and allow *Col*E$_1$ to replicate. The plasmids examined fell into two sex factor and four compatability classes. The F-type class included *Col*V,

Flac and *R*1drd19. *R*1drd19 is able to co-exist in the same cell as an F_1, or F' factor, whereas *ColV* and *Flac* are incompatible. The *Col*I-type plasmids included *Col*Ia, *Col*Ib and *R*64. *Col*Ia and *Col*Ib are incompatible, whereas *R*64 is able to co-exist with either of the *Col*I factors (NOVICK 1969).

As summarized in Table 4 the chromosome-located mutants fall into three well-defined specificity classes. The classes very in their ability to replicate the different sex-pilus-type factors. Group I mutants were defective in the replication of all of the extrachromosomal elements examined. Group II mutants were normal only with respect to the I-type plasmids. Mutants of group III were defective specifically for *Col*E_1 replication.

A select number of the mutants were tested to determine whether or not a single mutational event was responsible for the temperature-sensitive replication patterns observed. Cultures of various mutants were grown for several generations in AB3 broth at 43°C. This period of growth at the restrictive temperature results in the segregation of the *Col*E_1 plasmid and, correspondingly, loss of

TABLE 4

Classification of chromosomal mutants by phenotypic groups

Plasmid	Pilus type	Mutant class		
		I	II	III
ColE_1	None	ts*	ts	ts
ColE_2	None	ts	ts	wt
Flac, *ColV*,*R*1	F	ts	ts	wt
*Col*Ib, *R*64	I	ts	wt	wt
		Mutant numbers		
		TS20	TS13**	TS164
		22**	14	166
		45**	18**	171
		191	19	201
		236	23	205**
		237	25	211
			28	213
			30	214**
			31	215
			33	216**
			160	218
			192	221
			203	224
			223	225
			234	226
				227
				228
				231
				232
				233
				235

* ts and wt refer to temperature-sensitive and wild-type, respectively.
** Only these mutants were examined for their ability to replicate *Col*E_2.

TABLE 5

Maintenance of plasmids at 43°C in revertants of host mutants

Mutant number	Group	Number tested	Plasmid	Expression of the plasmid element*						
				33(0)	43(1)	33(1)	43(2)	33(2)	43(3)	33(3)
TS13	II	5	*Col*E$_1$	+	+	+	+	+	+	+
			*Col*V	+	+	+	+	+	+	+
			Flac	+	+	+	+	+	+	+
TS22	I	50	*Col*E$_1$	+	+	+	+	+	+	+
			*Col*V	+	+	+	+	+	+	+
			Flac	+	+	+	+	+	nt	nt
			*Col*Ia	+	+	+	+	+	nt	nt
			*R*1drd19	+	+	+	+	+	nt	nt
			*R*64	+	+	+	+	+	nt	nt
TS24	II	5	*Col*E$_1$	+	+	+	+	+	+	+
			*Col*V	+	+	+	+	+	+	+
TS30	II	25	*Col*E$_1$	+	+	+	+	+	+	+
			*Col*V	+	+	+	+	+	nt	nt
			Flac	+	+	+	+	+	nt	nt
			*R*1drd19	+	+	+	+	+	nt	nt

* The designation of passages is described in Figure 1. nt indicates not tested. (+) refers to the positive expression of plasmid functions.

immunity to colicin E$_1$. The loss of immunity results in death of the cell due to the colicin E$_1$ present in the culture and an enrichment of revertant cells in the population. After 10 to 15 generations the culture was plated for isolated colonies. These colonies were screened for revertants or cells that were now able to produce colicin E$_1$ at 43°C. Many of the resulting colonies, including representatives of all three phenotypic groups, were examined for the replication of other extrachromosomal elements. These data are given in Table 5. In every case examined, the maintenance of other plasmid elements was normal at 43°C. From the reversion data presented here it is likely that in most cases the pleiotropic effect of the mutation with respect to plasmid specificity is due to a single mutational event.

Properties of those mutants located on the Col*E$_1$ plasmid:* The transfer of the mutant *Col*E$_1$ plasmid molecules from DK9 to CR34 resulted in colicinogenic CR34 cells that exhibited a wide variety of temperature-sensitive phenotypes. A likely interpretation of this result was that the original DK9 mutant strain contained a mixture of mutant and wild-type *Col*E$_1$ plasmid molecules. The results of the transfer experiments indicated that the population of *Col*E$_1$ molecules in the CR34 cells derived from the cross with the DK9 (*Flac*) mutants was very homogeneous in any single colony isolate, suggesting that during transfer only a single copy of the *Col*E$_1$ molecule is transferred. The necessity for a homogeneous population of plasmid molecules for the proper analysis of the nature of the temperature-sensitive mutation required that further studies on these mutants employ the temperature-sensitive CR34 (*Col*E$_1$) mutant strains.

TABLE 6

Maintenance of ColE_1 *in plasmid-located mutants*

Mutant number	Presence of *Flac*	Maintenance of *Col*E_1* 33(0)	43(1)	33(1)	43(2)	33(2)	43(3)	33(3)
29, 35, 36, 37, 59, 65,	—	+	±	+	±	±	—	—
89, 126a, 126b, 128, 129a, 131	+	+	±	+	+	+	—	—
32	—	+	±	+	+	+	—	—
	+	not examined						
34, 49, 62, 63, 64, 66, 68, 70, 71, 73a, 76, 77, 80a, 81, 83, 86, 87, 91, 107a, 109a, 119a	—	+	—	—	—	—	—	—
	+	+	—	+	—	—	—	—
45, 46, 61, 79	—	+	—	—	—	—	—	—
	+	not examined						
67, 72	—	not examined						
	+	+	—	—	—	—	—	—
75, 78a, 82, 84, 88	—	+	—	—	—	—	—	—
	+	+	+	+	+	+	+	+

* Passage designations are described in Figure 1. (+) and (—) refer to the positive and negative expression of plasmid functions, respectively.

The rate of loss of the *Col*E_1 plasmid and the effect of the presence of the *Flac* sex factor genes on the loss of the plasmid were examined in the CR34 derivatives. Table 6 gives the results of these experiments. The rate of loss of *Col*E_1 by the plasmid mutants is generally somewhat slower than from the chromosomal mutants under the conditions employed. The presence of the *Flac* factor had little or no effect on the replication of the *Col*E_1 factor in the case of all of the plasmid mutants except for TS75, TS78A, TS82, TS84 and TS88, where the *Flac* appeared to complement the *Col*E_1 mutation and permit replication at the non-permissive temperature.

In the course of these plate screening experiments the effect of inhibition of plasmid replication on the growth of a cell in certain cases was apparent. Some of the plasmid mutants grew very poorly on the plates at the nonpermissive temperature for one or two plate passages. When cured (*Col*E_1^-) cells began to appear, the growth rate began to approach that of the wild-type cells.

DISCUSSION

The isolation of mutants of *E. coli* defective in *Col*E_1 DNA synthesis was made possible by the ability of the cell to replace thymine with BU in the DNA and to replicate *Col*E_1 in the absence of protein synthesis. The use of BU to isolate DNA synthesis mutants was first exploited by BONHOEFFER and SCHALLER

(1965) who showed that the incorporation of BU into chromosomal DNA was a lethal event when the cells were exposed, subsequently, to long-wave-length ultraviolet light. The ability of *Col*E$_1$ to continue replication for at least several hours following the cessation of protein synthesis was critical to the success of the isolation procedure. Unfortunately, this aspect of the procedure does not make it broadly applicable to all bacterial plasmids since synthesis of at least certain other *E. coli* plasmids stops more quickly following chloramphenicol treatment. The F_1 factor probably initiates, on the average, less than one additional round of replication (BAZARAL and HELINSKI 1970), and the *Col*I factors appear to be very similar in this respect after the addition of chloramphenicol. The possible use of chromosomal DNA synthesis mutants which allow F factor replication (e.g. CRT46) (HIROTA *et al.* 1968) has not been fully explored but is potentially useful for the isolation of temperature-sensitive mutants of other *E. coli* plasmids by a procedure similar to that employed in this study.

Not unexpectedly, the *Col*E$_1$ replication mutants fell into two groups with respect to the location of the mutational site—chromosomal and plasmid. Those mutants obtained from long-term mutagenesis appeared to contain not one type of mutant *Col*E$_1$ plasmid molecule per cell, but a mixture of wild-type and mutant plasmid molecules. This was observed in nearly every strain isolated from the long-term procedure and several isolates showed as many as six phenotypes following transfer to CR34. Often a CR34 recipient cell received a *Col*E$_1$ plasmid that did not demonstrate a temperature-sensitive phenotype, whereas the parent strain had. This suggests that there may have been wild-type plasmid molecules in the original mutant cells which were unable to complement the temperature-sensitive mutants. The failure to complement may have been due to their small number or due to the nature of the temperature-sensitive mutation itself. The demonstration of the multiple phenotypes resulting from long-term mutagenesis and the homogeneous behavior of the CR34 (*Col*E$_1$) strains resulting from the transfer experiments suggest that only a single type of *Col*E$_1$ molecule was transferred to the female cell during mating.

On the basis of the ability or inability of *Flac* to provide the necessary replication function, the plasmid mutants fell into two categories: those mutations which are unique to *Col*E$_1$ and cannot be complemented by *Flac* and those mutations which involve functions that are common to both *Flac* and *Col*E$_1$ and can be supplied by either. This type of study yields only a minimum number of classes and a more careful study with other factors may be necessary to obtain a further subclassification of the mutants.

The host mutants can be divided functionally into three classes, based on the replication of plasmids of various sex factor types. Mutants of the first group are defective in the replication of all the bacterial plasmids tested. Preliminary studies have shown that at least two group I mutants (TS22 and TS45) will not allow replication of temperate bacteriophages λ and P1 at 43°C while they will allow growth of bacteriophage T4 at the restrictive temperature.

The group II mutants allow the normal replication of the plasmids of the *Col*I type but the replication of other plasmids is defective. The restriction of repli-

cation of the F-type plasmids does not follow the patterns of compatibility but does follow the sex pilus type. The group III mutants appear to affect only *Col*E_1. Although not all of this group have been tested for *Col*E_2 replication, those that have were found to replicate *Col*E_2 normally. The group III mutants have been further subdivided into two functionally distinct groups based on the thermal lability of their DNA polymerase I. It has been demonstrated that the *Col*E_1 factor is not maintained in a mutant lacking DNA polymerase I activity (polA1), whereas all other bacterial plasmids tested are maintained normally in this mutant (KINGSBURY and HELINSKI 1970). When the group III mutants were examined for their DNA polymerase I activity some (group IIIa) exhibited a temperature-sensitive DNA polymerase I while others were normal for this property (group IIIb) (KINGSBURY and HELINSKI 1973). Other properties of the various mutants in the three groups are described in an accompanying communication (KINGSBURY, SIECKMANN and HELINSKI 1973).

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