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

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ABSTRACT

Host mutations in *Escherichia coli* K12 selected for the temperature-sensitive replication of the bacterial plasmid colicinogenic factor E_1 (*Col*E_1) exhibit a pleiotropic effect with respect to the effect of the mutation on other extrachromosomal elements. The mutations also vary with respect to the time of incubation of the cells at 43°C required for complete cessation of *Col*E_1 DNA synthesis. While the synthesis of the bacterial chromosome appears unaffected, supercoiled *Col*E_1 DNA replication stops immediately in some mutants and gradually decreases during several generations of cell growth before stopping in others. Mutations isolated in the *Col*E_1 plasmid resulted in only a gradual cessation of *Col*E_1 DNA synthesis over several generations of cell growth at 43°C. Conjugal transfer of the *Col*E_1 and *Col*V factors occurs normally in the host mutants when the transfer is carried out at the permissive temperature; however, the presence of a group I mutation in the donor cell prohibited conjugal transfer of either plasmid DNA at 43°C to a normal recipient cell. Similarly, the presence of this mutation in the recipient prevented the establishment of *Col*E_1 or *Col*V in the mutant recipient cell upon conjugation with a normal donor at 43°C. Various host *Col*E_1 replication mutants carrying either *Col*E_1 or *Col*E_2 were also defective in the mitomycin C-induced production of colicin E_1 or colicin E_2 at 43°C. The majority of the host mutations examined exhibited a temperature sensitivity to growth in deoxycholate in addition to the inhibition of plasmid DNA replication, suggesting a membrane alteration in these mutants when grown at the restrictive temperature.

THE accompanying paper (KINGSBURY and HELINSKI 1973a) reported the isolation and characterization of a series of *Escherichia coli* mutants temperature-sensitive for the maintenance of the plasmid, colicinogenic factor E_1 (*Col*E_1). The mutations were located either on the bacterial chromosome or on the *Col*E_1 plasmid. The plasmid mutants were separated into two classes on the basis of the ability or inability of the sex-factor plasmid, *Flac* to complement the plasmid-located mutations. The host mutants fell into three phenotypic classes based on

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the specificity of the mutations towards a variety of different plasmid elements. Group I mutants are defective in the maintenance of F-type and I-type plasmids in addition to the *Col*E$_1$ plasmid. Group II mutants are normal with respect to I-type plasmid maintenance, but defective for the F-type and *Col*E$_1$ plasmids. Finally, the group III mutants are defective specifically for the replication of the *Col*E$_1$ plasmid.

In this report some of the physiological and biochemical properties of these mutants are explored. The kinetics of the loss of various plasmids at the non-permissive temperature in certain of the mutants is presented as well as the demonstrations of the effect of both chromosomal and plasmid mutations on the replication of *Col*E$_1$ supercoiled DNA. In addition, this study describes the effects of these mutations on the conjugal transfer of the *Col*E$_1$ plasmid, the induction of colicin E$_1$ production, and the sensitivity of the cells to deoxycholate.

MATERIALS AND METHODS

Bacterial strains and media: The bacterial strains and growth media used throughout this study are described in the accompanying paper (KINGSBURY and HELINSKI 1973).

Detection of plasmid-containing cells: The quantitative determination of transfer frequency of various *Col* factors was determined by the triple overlay technique of FREDERICQ (1957). R-factor-containing cells were detected by plating on agar containing all of the antibiotics to which the R-factor is known to carry resistance. Where multiple platings for more than a single R-factor were involved standard replica plating techniques were used.

Sucrose gradient velocity centrifugation: Sucrose gradient centrifugation was performed in a Spinco Model L2 or L4 ultracentrifuge in either an SW65 or SW50.1 rotor at 50,000 rpm and 45,000 rpm, respectively. Centrifugation was done at 15°C for 150 min. Linear 5% to 20% sucrose gradients (5 ml) in high salt-TES buffer (0.05 M Tris, 0.5 M NaCl, 0.005 M EDTA, pH 8.0) were used. Fraction of 0.15 ml were collected by drop from the bottom of each cellulose nitrate tube through a 20 gauge needle directly onto Whatman #1 filter paper squares and counted as previously described (BAZARAL and HELINSKI 1968).

Reagents: All radioisotopes were purchased from New England Nuclear Co. The specific activity of the [^{3}H]-thymidine was 25 ci/mMole. Sarkosyl N30 was obtained from the Geigy Chemical Co., Brij 58 from McKesson Chemicals, Triton X100 from Atlas Chemicals and lysozyme from Calbiochem.

Measurement of colicin activity: The quantitation of colicin in liquid culture was carried out by treating one ml of colicinogenic culture with 0.1 ml of chloroform, serially diluting the chloroformed culture in L-broth, and spotting a drop of each dilution on an AB3 agar plate freshly overlayed with approximately 10^7 indicator bacteria in 3 ml of soft agar. The plate was then incubated for 15 hours at 37°C prior to inspection for colicin zones. The number of colicin units per ml was defined as the highest dilution which gave a clear zone of inhibition of growth of the indicator bacteria.

RESULTS

Growth properties and rate of segregation of R plasmids: The difference between chromosome-located mutants of groups I and II with respect to their ability to maintain the I-type plasmids (*Col*Ia, *Col*Ib and *R*64) after growth of the cells on solid medium at 43°C was tested further by determining the rate of segregation in liquid medium at 43°C of the F-type plasmid *R*1 and the I-type plasmid *R*64 from the mutant strains TS22 (Group I) and TS30 (Group II) carrying these two R factors in addition to the *Col*E$_1$ plasmid. As shown in Figure 1 the

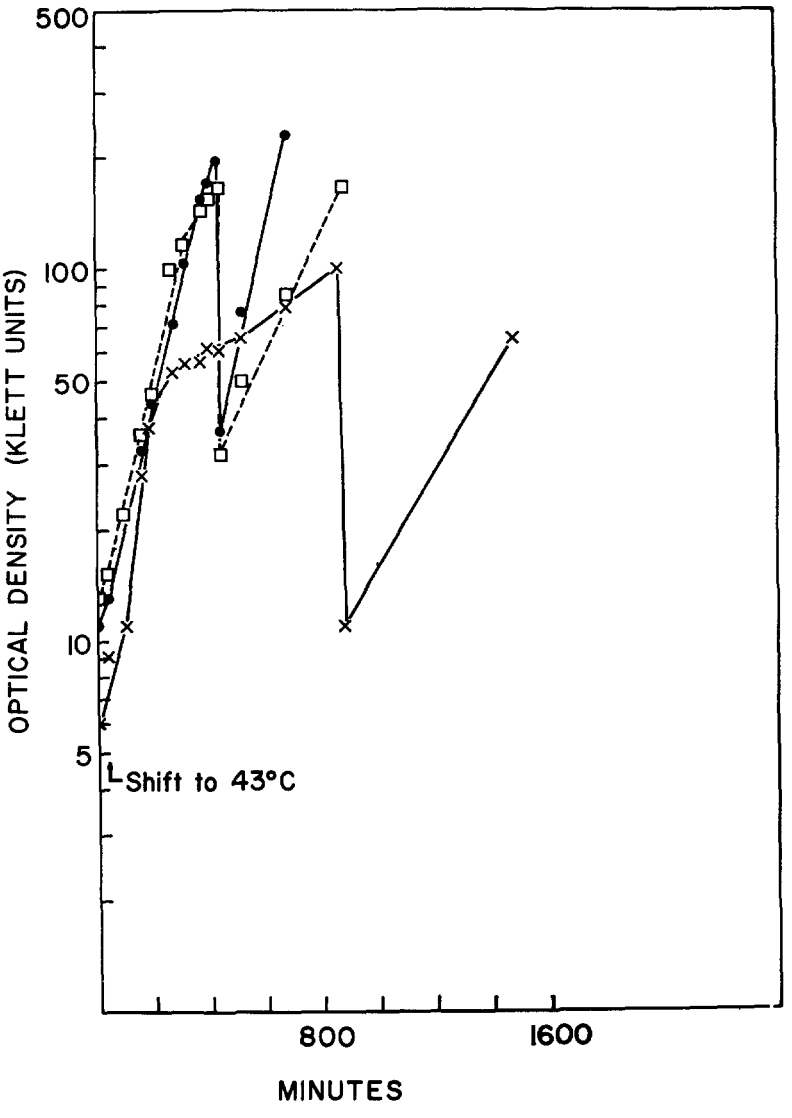

FIGURE 1.—Growth of R-factor containing wild-type and mutant strains of bacteria at the non-permissive temperature. The cells, each containing *ColE*$_1$, *R1*, and *R64*, were grown in M 9 salts, Casamino acids, glucose medium at 33°C and shifted to 43°C: DK9 (*R1*)(*R64*) (●—●); TS22 (*R1*)(*R64*) (×—×); TS30 (*R1*)(*R64*) (□—□).

two mutant strains and the wild-type control strain grow at a similar rate at 43°C for a time period equivalent to two to three generations. At this point there is a striking decrease in the growth rate of the Group I (TS22 (*R1*)(*R64*)) mutant and a diminution in the rate of growth of the Group II mutant (TS30 (*R1*)(*R64*)). The growth of TS22 (*R1*)(*R64*) virtually stops for approximately 45 minutes before resuming at a much lower rate. The onset of this change in growth

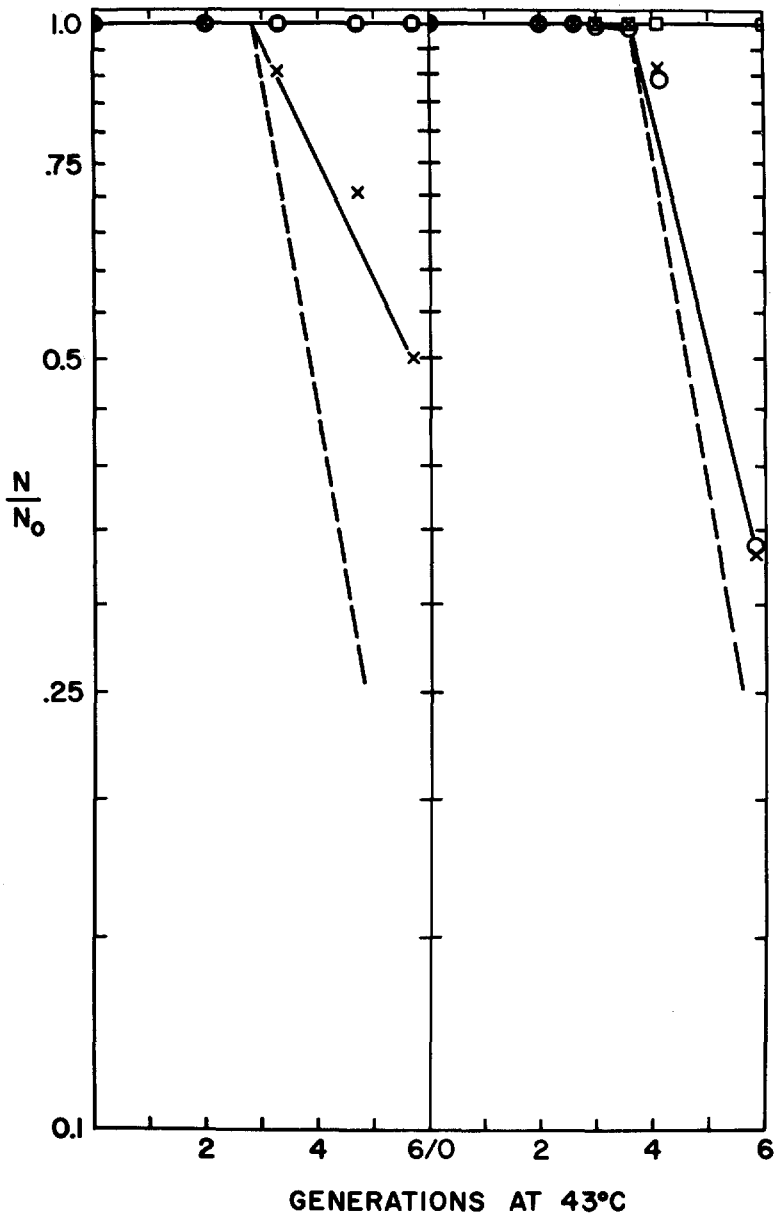

FIGURE 2.—Rate of segregation of R-factors from temperature-sensitive mutant bacteria grown at the non-permissive temperature. N is the number of R-factor containing bacteria and N_0 is the total bacterial count.

Left panel: TS30 (*R*1)(*R*64) and the wild-type strain DK9 (*R*1)(*R*64) growing at 43°C in M9 plus glucose plus Casamino acids medium: TS30 containing *R*1 (×—×); TS30 containing *R*64; (●—●); DK9 containing both *R*1 and *R*64 (□—□).

Right panel: TS22 (*R*1)(*R*64) and the wild-type strain DK9 (*R*1)(*R*64) growing as described for TS30 (*R*1)(*R*64): TS22 containing *R*1 (×—×); TS22 containing *R*64 (○—○); DK9 containing both *R*1 and *R*64 (□—□).

The dashed line in each panel is the theoretical rate of dilution of the R-factor molecules if there

rate corresponds to the time when *R1*⁻ and *R64*⁻ segregants of TS22 (*R1*) (*R64*) and *R1*⁻ segregants of TS30 (*R1*) (*R64*) appear (Figure 2). The differential loss of *R64* in the two mutant strains is consistent with the reported F-type and I-type plasmid specificity of the Group I mutants and F-type specificity of the Group II mutants. Both of these mutants were selected on the basis of their temperature-sensitive defect in *Col*E$_1$ maintenance. It was observed consistently that the segregant daughter cells in the case of the TS22 mutant simultaneously lose both *R1* and *R64*. The decreased growth rate of the mutant strains requires the presence of the plasmid elements. The TS22 (*R1*) (*R64*) mutant strain, temperature-cured for all plasmids, grows normally at 43°C.

*Col*E$_1$ *DNA synthesis in mutant strains:* To directly test the effect of the temperature-sensitive mutations on plasmid synthesis, the rate of incorporation of ^{3}H-thymidine into *Col*E$_1$ DNA after shifting the cells to 43°C was examined for several of the mutant strains. Cultures of various temperature-sensitive mutants were grown in M9 glucose medium supplemented with Casamino acids and 2 μg/ml thymidine at 33°C to a Klett$_{540}$ reading of 80 (2×10^8 cells/ml) and then shifted to 43°C. A 10 ml portion was removed and subjected to a 20 min pulse of ^{3}H-thymidine (100 μc/ml; 0 generation pulse), followed by placing the culture in ice water to stop any further synthesis. The remainder of the culture was grown to a Klett$_{540}$ reading of 160 and diluted one-fold with prewarmed (47°C) broth to maintain the temperature at 43°C. A 10 ml portion was removed and pulse-labeled for 20 min (1 generation pulse). The remaining culture was grown to a Klett$_{540}$ reading of 160 and diluted to a Klett$_{540}$ of 40. This culture was grown to a Klett$_{540}$ reading of 80 and pulse-labeled for 20 min (3 generation pulse). Brij-DOC lysates were prepared from the various cell samples as previously described (CLEWELL and HELINSKI 1969). After removing a portion of the crude lysate for determination of total incorporation of ^{3}H-thymidine into DNA, a clearing spin was performed at 46,000 × g for 25 minutes. The amount of labeled supercoiled *Col*E$_1$ DNA in each cleared lysate was estimated by sucrose density gradient analysis. The centrifugation conditions permit the detection of open circular DNA as well as unusual *Col*E$_1$ DNA forms (for example, multiple length circles) that may accumulate under these conditions. Figure 3 shows the sucrose density gradient profiles of the wild-type strain DK9 and the mutant strains TS22 and TS214 labeled at zero, one and three generations after the temperature shift. It is clear from these gradient profiles that supercoiled *Col*E$_1$ DNA synthesis is shut off in TS214 immediately after the temperature shift. This result is consistent with the finding of a temperature-sensitive DNA polymerase I in TS214 which is required for *Col*E$_1$ DNA synthesis (KINGSBURY and HELINSKI 1973b). In the case of the TS22 mutant, however, supercoiled *Col*E$_1$ DNA shuts off gradually, with residual synthesis detected even after three generations of growth at 43°C. In no case was there an unusual *Col*E$_1$ DNA species detected in these gradients.

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were complete cessation of replication of the R-factor after approximately three generations of growth in the case of TS30 (*R1*) (*R64*) and three and one-half generations of growth in the case of TS22 (*R1*) (*R64*).

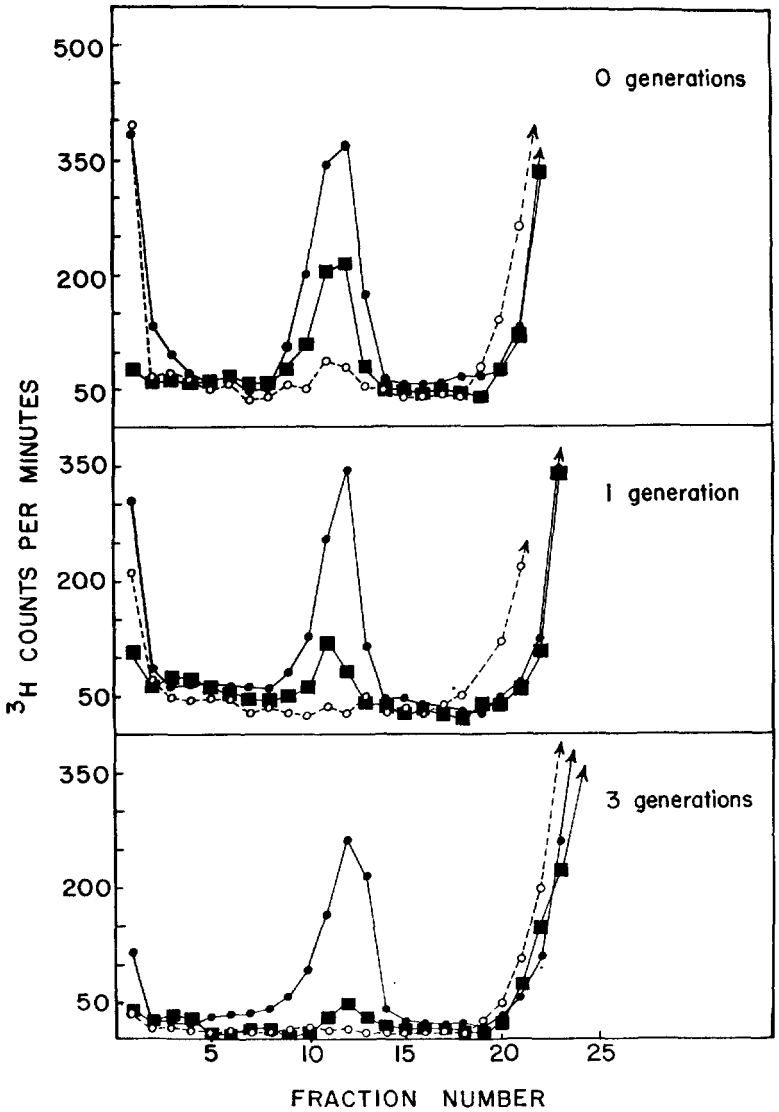

FIGURE 3.—Sucrose gradient analysis of *Col*E$_1$ DNA pulse-labeled for 20 min at 43°C following various periods of preincubation at 43°C. The details of labeling and preparation of the sample are given in the text. In each case 0.2 ml of a cleared lysate identically prepared for each strain was placed on the top of the gradient. The major peak in each gradient is at the position expected for supercoiled *Col*E$_1$ DNA. The peaks of open circular *Col*E$_1$ DNA or a supercoiled dimer of *Col*E$_1$ DNA would be approximately five fractions to the right, or left, respectively, of the monomer supercoiled *Col*E$_1$ DNA peak. Sedimentation is from right to left in these profiles. (A), (B) and (C) represent composite gradients of *Col*E$_1$ DNA from zero, one and three generations, respectively, of growth of DK9 (●—●), TS22 (■—■) and TS214 (○—○) at 43°C.

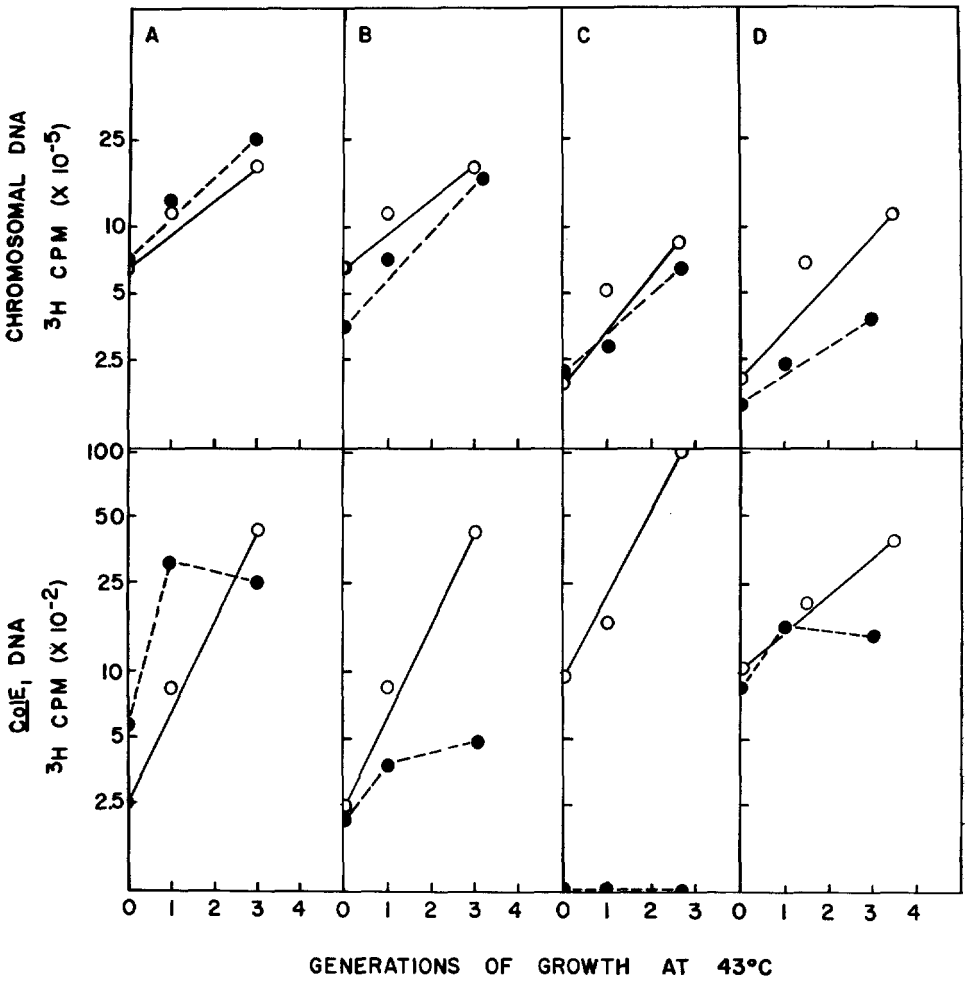

FIGURE 4.—Rate of *Col*E$_1$ DNA and chromosomal DNA synthesis at 43°C during 20 min pulses at various periods following the temperature shift. Chromosomal DNA synthesis was determined by measuring the incorporation of ^{3}H-thymidine into DNA in crude lysates prepared from the various samples. Plasmid DNA synthesis was determined on cleared lysates by sucrose gradient analysis as described in Figure 3. Each set of graphs compare the rate of chromosome and plasmid synthesis in the wild-type parent strain (○—○) with the rate in the mutant (●—●). Each mutant tested is shown together with its parent strain, labeled at the same time in the same lot of medium. (A) TS22 and DK9; (B) TS30 and DK9; (C) TS214 and DK100; (D) TS31 and DK9.

Figure 4 shows the rate of growth and labeling of both chromosomal DNA and *Col*E$_1$ DNA in four chromosome-located mutants. The rate of DNA synthesis was computed by multiplying the incorporation for a twenty-minute labeling period by the number of generations at 43°C. Clearly, the results from this procedure are dependent on the efficiency of recovery of the *Col*E$_1$ DNA in the cleared lysate of samples taken at various times at 43°C. Repeated experiments

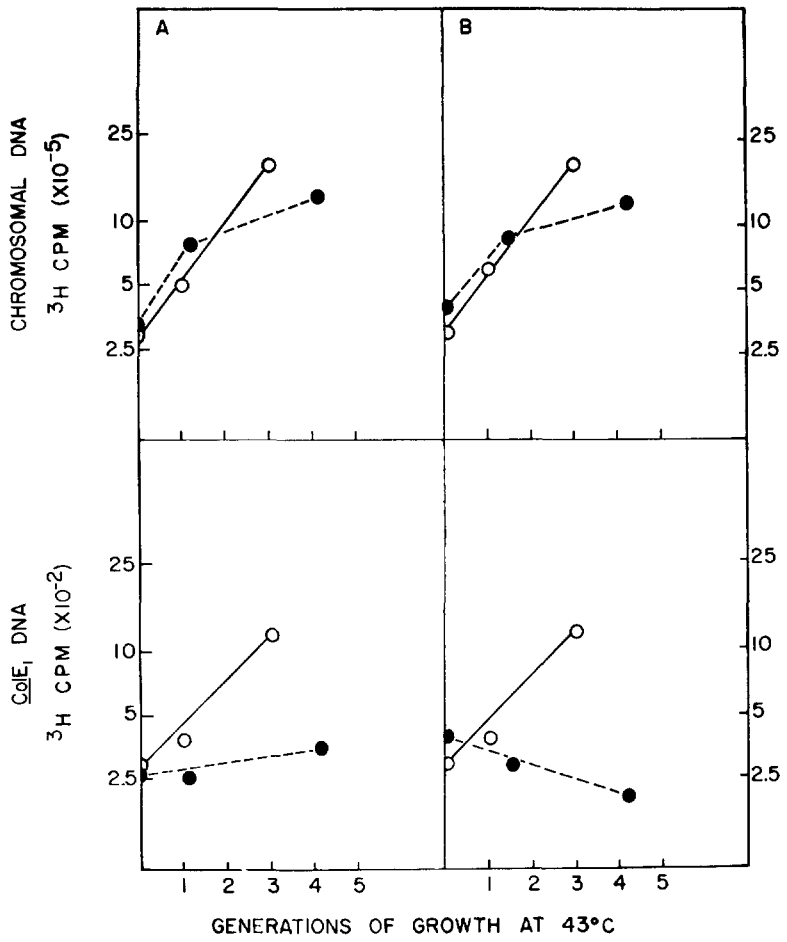

FIGURE 5.—Rate of *Col*E$_1$ DNA and chromosomal DNA synthesis at 43°C during 20 min pulses at various periods following the temperature shift. The isogenic wild-type control was always labeled at the same time in the same lot of medium as the mutant. Procedures used are described in Figure 4. (A) Rate of chromosomal and plasmid DNA synthesis in TS88 (●—●) and CR34 (*Col*E$_1$) (○—○); (B) rate of chromosomal and plasmid DNA synthesis in TS75 (●—●) and CR34 (*Col*E$_1$) (○—○).

with DK9 indicated that, generally, the recovery of *Col*E$_1$ DNA is reproducible; however, on occasion the recovery was unexplainably low. Thus, it was necessary to carry out this procedure several times in each instance to minimize the possibility that the data were in error due to spurious recovery problems. All of the mutants examined in pulse-labeling experiments showed normal or nearly normal rates of chromosomal DNA synthesis. The rate of *Col*E$_1$ DNA synthesis varied between mutants, but generally showed a relatively slow shut down occurring between one and three generations of growth at 43°C. The only

mutants that demonstrated an immediate shut-off of *Col*E_1 DNA synthesis were the Group III mutants that possess a temperature-sensitive DNA polymerase I (KINGSBURY and HELINSKI 1973b). In the case of each mutant examined by this procedure, significant levels of open circular *Col*E_1 DNA or multiple circular *Col*E_1 DNA forms were not detected.

Pulse-labeling experiments indentical to those described for the chromosome-located mutants were performed on ten plasmid-located mutants that were temperature-sensitive for *Col*E_1 maintenance. In general *Col*E_1 DNA synthesis at 43°C was more variable between mutants and did not stop as quickly in the plasmid mutants when compared with chromosome-located mutants. Figure 5 shows the rate of chromosomal and plasmid DNA synthesis in two plasmid mutants which showed a significant decrease in the rate of *Col*E_1 DNA synthesis before three generations of growth at 43°C. Several mutants showed virtually no effect in supercoiled *Col*E_1 DNA synthesis after three generations of growth at 43°C. In the case of the mutants TS88 and TS75 the rate of chromosomal DNA synthesis decreased significantly between one and three generations of growth; however, the rate of chromosomal DNA synthesis at 43°C remained significantly above the rate of *Col*E_1 DNA synthesis.

The requirement for plasmid DNA synthesis in conjugal transfer: The role of plasmid DNA replication in plasmid transfer was studied utilizing the various temperature-sensitive mutants as either the donor or recipient cells. To examine the possible requirement for replication in the donor cell the *Col*E_1 containing DK9 and TS22 strains were made colicinogenic for *Col*V by mating with the *E. coli* strain K94. These doubly-colicinogenic strains were then examined for donor ability. In addition, TS22 was cured of *Col*E_1 by growing the colicinogenic culture at 43°C until non-colicinogenic segregants could be isolated. The resulting non-colicinogenic strain (TS22C) was used as a recipient in the crosses.

The donor and recipient (C600/Az) cultures were grown at 33°C in L-broth until the culture reached a $Klett_{540}$ reading of 80. At this point, portions were removed and donor and recipient cells were mixed (1:1); incubation followed for 90 min at 43°C. The remainder of the culture was shifted to 43°C, grown to a $Klett_{540}$ reading of 160 and then diluted one-fold. After removal of a portion for mating (43°C for 90 min) the culture was grown to a $Klett_{540}$ of 160, diluted two-fold and then grown to a $Klett_{540}$ of 80, at which time the third cross (43°C, 90 min) was performed. Controls were carried out in a similar manner except all incubations were at 33°C. The results of the experiments are shown in Table 1.

To examine the role of DNA replication in the recipient the cured strain of TS22 (TS22C) was crossed with K30 to examine both *Col*E_1 and *Col*V transfer. The experiments were carried out as described above in the case of the temperature-sensitive donor. The results of these crosses also are shown in Table 1.

In both sets of crosses using the mutant cells as either donor or recipient it is clear that the inability of the cells to replicate the plasmid DNA interfered with transfer in the case of the mutant donor, and, at least, establishment in the case of the mutant recipient. In TS22 (*Col*V) there was a more immediate effect on *Col*E_1 transfer than on *Col*V transfer which shut down gradually over three

TABLE 1

Ability to TS22 to act as a donor or a recipient of ColE_1 *and* Col*V in conjugal transfer**

Donor strain	Recipient strain	Plasmid	Transfer temperature (°C)	Zero generation		One generation		Three generations	
				Number cells/total cells	*Col*+	Number cells/total cells	*Col*+	Number cells/total cells	*Col*+
TS22(*Col*V)	C600/AZ	*Col*E_1 (K30)	33	190/417	45.5	210/350	60	——	—
		ColE_1 (K30)	43	2/138	1.4	0/150	0	0/196	0
		*Col*V (K94)	33	186/420	44.2	——	—	——	—
		*Col*V (K94)	43	57/114	50.0	31/148	20.9	6/203	2.9
DK9(*Col*V)	C600/AZ	*Col*E_1 (K30)	33	262/500	52.4	238/350	68	——	—
		ColE_1 (K30)	43	29/140	20.7	80/189	42.1	79/226	35.4
		*Col*V (K94)	33	260/490	53.0	260/447	58	——	—
		*Col*V (K94)	43	38/156	24.4	104/202	51.4	128/262	49.2
K30	TS22C	*Col*E_1 (K30)	33	102/196	51	120/252	48	——	—
		ColE_1 (K30)	43	0/116	0	0/76	0	0/190	0
		*Col*V (K94)	33	298/624	47	157/449	29	118/433	27
		*Col*V (K94)	43	248/509	48	221/563	39	0/362	0
K30	JC411	*Col*E_1 (K30)	33	90/130	69	182/206	88	——	—
		ColE_1 (K30)	43	123/164	75.0	98/128	76.5	78/120	65.0
		*Col*V (K94)	33	48/98	49	42/119	36	126/394	31
		*Col*V (K94)	43	73/161	46	42/137	33	213/516	42

* Cross conditions are indicated in the text.

TABLE 2

Induction of colicin in temperature-sensitive mutants at 43°C and 33°C

Strain and mutant group	Colicin titer units/ml			
	E_1		E_2	
	33°C	43°C*	33°C	43°C*
DK9 (wild-type)	8	64	400	8,000
TS13 (II)	16	< 2	800	200
TS18 (II)	4	< 2	400	4,000
TS22 (I)	4	< 2	800	100
TS45 (I)	16	< 2	400	1,000
TS214 (III)	8	< 2	400	8,000

* Each strain was grown at 43°C for two generations and induced by adding 0.3 μg/ml mitomycin C.

generations. The effect of the mutation on *Col*V transfer is similar to the rate of shut-down of *Col*E_1 DNA synthesis observed in the pulse-labeling experiments. The immediate inability of *Col*E_1 to be transferred either into or out of TS22 differs from the delayed shut-off of *Col*E_1 DNA synthesis observed in the pulse labeling experiments.

The requirements for plasmid DNA replication for induced colicin E_1 *and colicin* E_2 *production:* To examine the requirement of *Col* factor synthesis for colicin-induction, various temperature-sensitive mutants containing *Col*E_1 or *Col*E_2 were induced under permissive and non-permissive conditions for *Col* factor replication. The cells were grown in L-broth at 33°C to a $Klett_{540}$ reading of 25 at which time one-half of the culture was shifted to 43°C and the other one-half left at 33°C. When the culture reached a $Klett_{540}$ of 100 (two generations of growth at 43°C), mitomycin C was added to a final concentration of 0.3 μg/ml. The cultures were shaken for an additional 90 min and then treated with chloroform. The colicin titer was then determined. The results of the induction experiments are shown in Table 2. For both colicin E_1 and colicin E_2 the induced levels of colicin production were decreased in the mutant strains. The experiments with the *Col*E_1 plasmid indicate that virtually no colicin is produced in the absence of *Col*E_1 DNA synthesis. A low level of colicin E_2 was produced at the restrictive temperature in several of the mutants and in the case of TS18 the level reached approximately the level of the control strain. TS214, which maintains, to a partial extent, the *Col*E_2 plasmid but not *Col*E_1, shows a control level of colicin-induction for colicin E_2 but no detectable colicin E_1 after induction with mitomycin C. These results generally are consistent with a requirement for *Col*E_1 and *Col*E_2 DNA synthesis for the induced production of colicin E_1 and colicin E_2, respectively, (DeWitt and Helinski 1965) and with the observed effect of a temperature-sensitive lesion in chromosomal DNA replication on *Col*E_1 replication and colicin E_1 induction (Goebel 1970).

Effect of deoxycholate on growth of the mutant strains: In an effort to test the possibility of a membrane defect as a result of the temperature-sensitive mutations the growth of various mutant strains was examined in the presence of

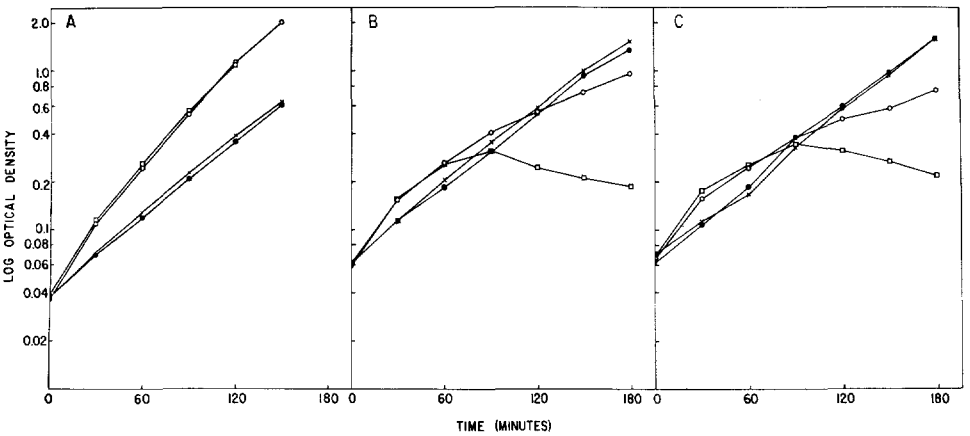

FIGURE 6.—The growth of plasmid replication mutants in the presence of 0.1% deoxycholate. Log phase cultures grown in L-broth at 33°C were diluted into fresh L-broth with or without 0.1% DOC and incubated with shaking at either 33°C or 43°C. At periodic intervals optical density (O.D.540) measurements were made. Each panel compares four growth flasks for each organism. (A) Parental strain DK9; (B) group I mutant TS22; and (C) group II mutant TS30. Growth at 33°C without DOC (●—●) and with DOC (×—×) is compared with growth at 43°C without (○—○) and with DOC (□—□).

deoxycholate at both the permissive and non-permissive temperatures. Figure 6 shows the growth of the wild-type strain and the mutant strains TS22 and TS30 after transferring early log-phase cells at a concentration of 5×10^7/ml in L-broth medium at 33°C to L-broth medium with or without 0.1% deoxycholate at either 33°C or 43°C. Samples were taken from the shaking culture at various intervals to determine the optical density of the culture. Following 90 min of growth at 43°C in the presence of deoxycholate, lysis ensued in the case of the two mutant strains. Under these conditions the wild-type strain continued to grow normally. The lag period of approximately two generations before lysis of the cells is approximately of the duration observed for the arrest of plasmid DNA synthesis.

To determine the deoxycholate sensitivity of many of the mutant strains a more rapid plate-assay procedure was developed. Various concentrations of deoxycholate were added to agar plates of AB3 medium and the growth of the wild-type and mutant strains at 33°C and 43°C was determined by spotting a standard concentration of cells on the plate (0.01 ml of 5×10^6/ml) followed by overnight incubation at the appropriate temperature. Following this incubation period the plates were examined for development of the bacterial colonies. Each plate had a control parental strain and several mutants on it. Table 3 shows the results of these experiments carried out over a range of deoxycholate concentration from 0.05% to 0.6%. At the higher concentrations the deoxycholate forms a flocculent precipitate in the agar.

Deoxycholate inhibited the growth of almost all of the mutant strains at the higher temperature. The only exceptions were TS18, TS205, TS216 and TS232.

TABLE 3

*Sensitivity of mutant strains to DOC**

Temperature of incubation 33°C	Percent DOC 0	0.05	0.1	0.2	0.4	0.6
DK9	++	++	++	++	++	++
TS18	++	++	++	++	++	++
TS20	++	++	++	++	++	++
TS22	++	++	++	++	++	++
TS28	++	++	±	±	±	±
TS30	++	++	++	++	++	++
TS31	++	++	++	++	++	++
TS45	++	++	++	++	++	++
TS201	++	++	++	++	++	++
TS203	++	++	++	++	++	++
TS205	++	++	++	++	not spotted	++
TS214	++	++	++	++	++	++
TS216	++	++	++	++	++	++
TS232	++	++	—	±	—	—
43°C						
DK9	++	++	++	++	++	++
TS18	++	++	++	++	++	++
TS20	+	±	—	—	—	—
TS22	++	+	—	—	—	—
TS28	++	±	—	—	—	—
TS30	++	++	+	±	—	—
TS31	+	±	—	—	—	—
TS45	++	++	++	+	+	+
TS201	++	++	±	±	—	—
TS203	+	—	—	—	—	—
TS205	++	++	++	++	++	++
TS214	++	++	—	—	—	—
TS216	++	++	++	++	+	+
TS232	++	++	++	++	+	±
TS235	+	±	—	—	—	—

* (++) and (+) refer to normal and subnormal growth, respectively, at the temperature indicated. (±) and (—) refer to marginal and no growth, respectively, at the temperature indicated.

Two of these mutants, TS216 and TS232, have been shown to be defective in DNA polymerase I (KINGSBURY and HELINSKI 1973b). An unexpected result was the sensitivity to deoxycholate of TS214, also a temperature-sensitive DNA polymerase I mutant.

DISCUSSION

Although the chromosome- and plasmid- located mutations affecting *Col*E$_1$ replication show considerable variability in their plasmid specificity and other phenotypic properties, several general conclusions can be derived from the studies to date with these mutants. It is clear that genes located both on the host chromosome and the *Col*E$_1$ plasmid directly, or indirectly, are involved in the determina-

tion of *Col*E$_1$ replication. This expected finding is not unlike the situation with the *Flac* factor in *E. coli* (JACOB, BRENNER and CUZIN 1963) and penicillinase plasmids in *Staphylococcus aureus* (NOVICK 1969). In both of these systems host chromosome and plasmid mutations were shown to affect the maintenance of plasmid elements.

It is also clear that a chromosome-located mutation can exhibit a pleiotropic effect with respect to the specificity of the mutation towards different plasmid elements. Thus, a single and revertible mutational event can result in the temperature-sensitive replication of *Col*E$_1$ and several F-type and I-type plasmids. Similarly, chromosome-located mutations in *S. aureus* have been shown to result in the defective replication of two distinct compatibility classes of penicillinase plasmids (NOVICK 1969). In addition, there are many reported cases of chromosome-located mutants in *E. coli* that affect chromosomal DNA synthesis and the maintenance of other replicons present in the same cell (GROSS 1971). Unexpecedly, an additional relationship between the replication of F-type plasmids and *Col*E$_1$ was indicated by the group II mutants which are defective in *Col*E$_1$ and F-type plasmid replication but normal in the maintenance of I-type plasmids. A corresponding class of mutants specific for I-type and *Col*E$_1$ plasmid replication was not detected. This may, however, reflect some bias in the mutagenesis and selection procedures employed rather than any special functional relationship between the F-type and *Col*E$_1$ plasmid replication systems.

Group III mutations which exhibit a defect specifically in *Col*E$_1$ DNA replication have been shown to be of at least two types on the basis of the temperature-sensitive DNA polymerase I found in certain but not all of the mutants in this group (KINGSBURY and HELINSKI 1973b). Mutants in this group that exhibit a normal DNA polymerase I are more sensitive than the wild-type strain to deoxycholate at the non-permissive temperature.

The role of plasmid DNA duplication in the conjugal transfer of plasmids was examined in a group I mutant (TS22) in this study and a DNA polymerase I (group III) mutant described in another report (KINGSBURY and HELINSKI 1973b). The results with each type of mutant clearly indicated that plasmid replication is required in both the donor and recipient cells if conjugal transfer and establishment of the plasmid is to occur. Consistent with the plasmid replication data, TS22 was unable to transfer or serve as an effective recipient of *Col*V or *Col*E$_1$ at 43°C. On the other hand, the *Col*E$_1$ specific group III mutant showed normal behavior to *Col*V but was ineffective as a donor or recipient of *Col*E$_1$ at 43°C. These results are consistent with a model of plasmid DNA transfer that involves replication of the plasmid DNA both in the donor for effective transfer and in the recipient for either reception or establishment of the plasmid element. The finding of MARINUS and ADELBERG (1970) that eight different chromosomal DNA replication mutants had no effect on the transfer of the F-type plasmid, *Flac*, and the results of this study suggest that the conjugal transfer of plasmid DNA is largely, if not entirely, under the control of the plasmid DNA replication system.

The various temperature-sensitive plasmid replication mutants, although gen-

erally normal in host DNA synthesis, do not necessarily represent mutations in genes that are concerned only with plasmid DNA synthesis. The selection procedure for the mutants demanded substantial or normal cell growth at 43°C. Therefore, certain of the mutations may be in genes that control both chromosomal DNA and plasmid DNA synthesis, but the mutations may have resulted in altered proteins that are defective only in plasmid DNA synthesis. In fact, several of the mutants carrying *Col*E$_1$ demonstrated an altered growth rate at 43°C and as shown in the case of TS22 (*R*1) (*R*64) and TS30 (*R*1) (*R*64) (Figure 1) the growth of the cells was seriously disrupted at 43°C at the time that plasmid DNA synthesis was inhibited. This observation of the effect of inhibition of plasmid DNA synthesis on cell growth has also been reported by HOHN and KORN (1970) in the case of the *Flac* factor. These observations suggest an interrelationship between plasmid DNA replication and vital processes in the cell as possibly chromosomal DNA replication. Clearly, many of the plasmid DNA replication mutants exhibit a temperature-dependent sensitivity to deoxycholate, suggesting changes in the membrane structure in the cell. The possible additional effect of the inhibition of plasmid DNA synthesis, conceivably on the cell membrane, may have severe consequences for cell growth.

The mutants examined exhibited variable lag periods before the rate of plasmid DNA synthesis decreased. Except for the immediate shut-off, group III mutants that were defective in DNA polymerase I, the other mutants generally showed delayed shut-down of plasmid DNA synthesis. The lag period before plasmid DNA synthesis inhibition may reflect a rapid temperature-dependent inactivation of newly-synthesized protein determined by the mutant gene, but delayed thermal inactivation of protein synthesized under the permissive conditions. Until the nature of the biochemical defect in these delayed shut-off mutants is determined, however, a number of possibilities remain open to account for the lag in inhibition of plasmid DNA replication. Given the suggested membrane alteration in many of these mutants and the biochemical complexity of membrane-mediated events, it is perhaps not unexpected that the plasmid DNA inhibition kinetics are not readily interpretable with respect to the nature of the replication defect.

It will be of interest to determine the number of distinguishable genes mutated in the various temperature-sensitive replication mutants. It is also relevant to our understanding of plasmid DNA replication to determine the potential of a single gene on the chromosome to mutate to any one of the three different phenotypic groups that the various mutants comprise. The mutants described in this study presently are being mapped to attempt to answer these questions.

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