PHENETHYL ALCOHOL RESISTANCE IN *ESCHERICHIA COLI*. III. A TEMPERATURE-SENSITIVE MUTATION (*dnaP*) AFFECTING DNA REPLICATION

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ABSTRACT

A temperature-sensitive DNA replication mutant of *E. coli* K-12 was isolated among the mutants selected for phenethyl alcohol resistance at low temperatures. This mutation, designated as *dnaP18*, affects sensitivity of the cell to phenethyl alcohol, sodium deoxycholate and rifampicin, presumably due to an alteration in the membrane structure. At high temperatures (e.g., 42°), synthesis of DNA, but not RNA or protein, is arrested, leading to the formation of "filaments" in which no septum formation is apparent. Nucleoids observed under electron microscope seem to become dispersed and DNA fibrils less condensed, which may explain the loss of viability under these conditions. Genetic analyses, including reversion studies, indicate that a recessive *dnaP* mutation located between *cya* and *metE* on the chromosome is responsible for both alterations of the membrane properties and temperature sensitivity. The *dnaP18* mutation does not affect growth of phage T4 or lambda under conditions where host DNA replication is completely inhibited. Kinetic studies of DNA replication and cell division in this mutant after the temperature shift from 30 to 42°, and during the subsequent recovery at 30°, accumulated evidence suggesting that DNA replication comes to a halt at 42° upon completion of a cycle already initiated before the temperature shift. Since the recovery of DNA synthesis after exposure to 42° does not depend on protein or RNA synthesis or other energy-requiring processes, the product of the mutant *dnaP* gene appears to be reversibly inactivated at 42°. Taken together with the recessive nature of the present mutation, it was suggested that one of the membrane proteins involved in initiation of DNA replication is affected in this mutant.

A number of temperature-sensitive mutants primarily affecting DNA replication in *Escherichia coli* have been isolated and characterized in several laboratories. These mutants were obtained either without any particular selection (KOHIYAMA *et al.* 1966; WECHSLER and GROSS 1971) or after selection for inability to replicate the chromosomal DNA at high temperatures (BONHOEFFER and SCHALLER 1965; FANGMAN and NOVICK 1968; KUEMPEL 1969). When cultures of these mutants are transferred to high temperatures, DNA synthesis stops either immediately or after a lag which permits completion of the replication cycle already initiated before the temperature shift. At least two genes (*dnaA* and *C*) have been reported to be involved in the initiation of DNA replication in *E. coli*, whereas five genes (*dnaB*, *D*, *E*, *F* and *G*) are known whose mutation

leads to an immediate cessation of DNA replication at high temperatures, implying that the chain elongation step is affected in these mutants (see reviews by GROSS 1972; SMITH 1973).

An involvement of the bacterial membrane in DNA replication has been experimentally substantiated, after it was originally proposed by JACOB, BRENNER and CUZIN (1963) (GANESAN and LEDERBERG 1965; SMITH and HANAWALT 1967; SUEOKA and QUINN 1968; FIELDING and FOX 1970; YAMAGUCHI, MURAKAMI and YOSHIKAWA 1971). Specific alterations of the membrane proteins have also been reported in some temperature-sensitive DNA replication mutants upon shift to high temperatures (INOUYE and GUTHRIE 1969; INOUYE and PARDEE 1970; SHAPIRO *et al.* 1970; SICCARDI *et al.* 1971, 1972; LAZDUNSKI and SHAPIRO 1973).

Our own approach to this problem has been through analysis of mutants of *E. coli* that are resistant to phenethyl alcohol (PEA) (YURA and WADA 1968; WADA and YURA 1971). In *E. coli*, PEA is thought to act at the cell membrane (SILVER and WENDT 1967) and to selectively inhibit the *initiation* of a new cycle of DNA replication without interfering with the completion of a cycle already initiated (TREICK and KONETZKA 1964; LARK and LARK 1966). Thus, we have identified and characterized a chromosomal mutation, *pea*, responsible for the resistance to PEA in the derivatives of strain C600 (YURA and WADA 1968). We have also studied a mutation at another locus (*maf*) affecting the maintenance of an autonomous F factor in the same strain. Replication of the F factor rather than F factor distribution during cell division appeared to be inhibited by PEA when a strain carried the *maf* mutant allele (WADA and YURA 1971).

In the course of these investigations, a number of temperature-sensitive PEA-resistant mutants were isolated from several PEA-sensitive strains (WADA and YURA 1968). These mutants were resistant to PEA at 30° and failed to grow at 42° even in the absence of PEA. DNA synthesis seemed to be specifically affected in some of these mutants, resulting in the formation of "filaments" after prolonged incubation at high temperatures. The results of detailed analysis of one such mutant (KY2750) are reported in this paper. It was revealed that a single mutation is responsible for both alterations of the membrane properties (sensitivities to PEA, deoxycholate, and to rifampicin) and of temperature sensitivity. The chromosomal replication at the stage of initiation appears to be inhibited when the mutant cells are exposed to high temperatures. In view of these as well as the genetic mapping data presented below, this mutation seems to define a novel gene involved in DNA replication and may tentatively be designated as *dnaP18* (P stands for phenethyl alcohol).

MATERIALS AND METHODS

Bacterial and phage strains: Bacterial strains used are all derivatives of *E. coli* K12, and their origins and genetic characters are listed in Table 1. Bacteriophages T4D and P1*vir* were obtained from DRS. T. MINAGAWA and J. TOMIZAWA, respectively.

Media: Peptone-glucose (PG) medium contained 20g polypeptone (Wako Drug Co.), 5 g

TABLE 1

Bacterial strains used and their known genetic characters

Strain	Sex	Genetic characters*	Origin†
PA678	F⁻	*thr, leu, thi, lac, gal, mal, xyl, mtl, ara, tonA, azi, str*	JACOB-WOLLMAN
KY2053	F⁻	*his, met, trp*; other markers same as in PA678	PA678
KY2750	F⁻	*dnaP18*; other markers same as in KY2053	KY2053
KY2900	F⁻	*mal*+; other markers same as in KY2750	KY2750
KY2901	F⁻	*thyA*; other markers same as in KY2750	KY2750
KY6007	F⁻	*thyA*; other markers same as in KY2053	KY2053
KY9115	F⁻	*met*+; other markers same as in KY2750	KY2750
CS101	Hfr	*met, tonA*	J. TOMIZAWA
BC73	F⁻	*trp, ilv, phoS, tna, str, lac*	N. OTSUJI
AB2277	F⁻	*pro, trp, his, ilv, metE, thi, lac, gal, mal, mtl, ara, str, tsx, tonB*	E. ADELBERG
GP1	Hfr	*ilv, metE, cya, thi*	T. YOKOTA
W3350	F⁻	*gal1, gal2, lac*	H. OZEKI

* Gene symbols are those employed by TAYLOR and TROTTER (1972).
† Origin as indicated here does not necessarily represent an immediate origin.

NaCl and 2 g glucose per liter (pH 7.4). Minimal medium E (VOGEL and BONNER 1956) was usually supplemented with 0.5% glucose, 0.2% Difco Casamino Acids, and 20 μg/ml of L-tryptophan and 2 μg/ml of thiamine (medium E-Casamino acids). Thymine (50 μg/ml) was further supplemented for experiments with the thymine-requiring strain.

Chemicals: Thymidine-6-^{3}H and uracil-2-^{14}C were obtained from Radiochemical Centre (Amersham), and thymine-6-^{3}H from Dai-ichi Pure Chemicals Co. (Tokyo). L-Arginine [3-^{3}H(N)] and L-arginine-^{14}C(U) were the products of New England Nuclear (Boston), whereas D-glucose-C-d7 (98 atom % as ^{2}H) and $^{15}NH_4Cl$ (95 atom % as ^{15}N) were purchased from Merck (Canada) and Hikari Kogyo (Tokyo), respectively. Phenethyl alcohol, deoxycholate and rifampicin were supplied by Nakarai Chemicals Co. (Kyoto), E. Merck (Darmstadt), and Lepetit spa. (Milano), respectively. Chloramphenicol was obtained from Sankyo Chemical Co. (Tokyo).

Determination of DNA synthesis: Incorporation of ^{3}H-thymidine or ^{3}H-thymine into acid-insoluble fraction was measured to determine DNA synthesis, unless otherwise stated. Thus, either ^{3}H-thymidine (0.4 μc/8 μg/ml) or ^{3}H-thymine (2 μc/50 μg/ml) plus 250 μg/ml of deoxyadenosine was added to PG medium or medium E-Casamino acids. An overnight culture grown in the radioactive medium at 30° was diluted 20-fold in the same medium, incubated further to the log phase and was used for various experiments. Aliquots (0.5 ml) were taken at intervals and were added to 3 ml of cold 5% trichloroacetic acid. After standing for at least 30 min in ice, cells were centrifuged, washed twice with cold trichloroacetic acid and were suspended in 0.5ml 2M NH_4OH. Contents were then transferred into a vial, 10 ml of Bray mixture added, and radioactivity determined in a liquid scintillation counter.

Isolation of the mutant: PEA-resistant mutants were isolated from a multiply marked strain of *E. coli* KY2053 that had been derived from strain PA678. Cells were treated with N-methyl-N′-nitro-N-nitrosoguanidine, washed and incubated in PG medium to allow for segregation, and plated on medium E-Casamino acids containing 0.22% re-distilled PEA and 1.5% agar. After incubation at 30° for 3 days, resistant colonies appeared at a frequency of about 10^{-7}. Among these resistant mutants, some were found to be unable to grow at 42° on PG medium even in the absence of PEA. One of these temperature-sensitive mutants *dnaP18* (strain KY2750) was chosen for further study, since this mutant seemed to be specifically affected in DNA synthesis and cell division at high temperatures, as will be shown below.

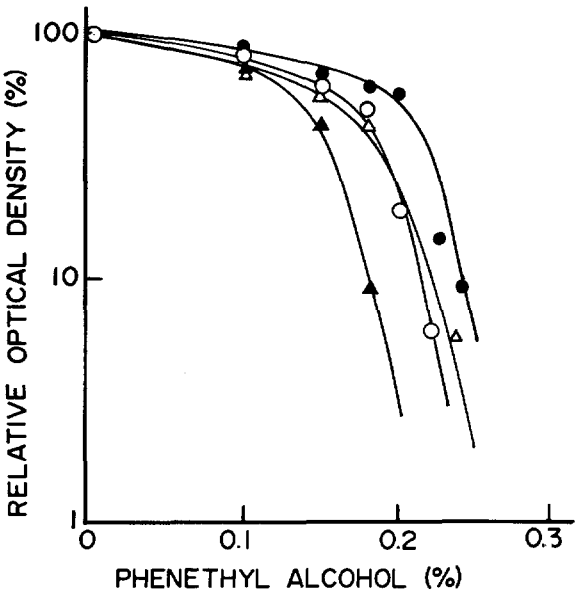


FIGURE 1.—Growth of bacteria in the presence of PEA. PG media containing various concentrations of PEA were inoculated with 10^6 cells/ml of each strain and were incubated by standing at 30° for 48 hr. Turbidity was measured in a Klett-Summerson colorimeter with a No. 54 filter. ○——○, Wild type (KY2053): ●——●, Mutant (KY2750); △——△, Ts+ revertant #1; ▲——▲, Ts+ revertant #2.

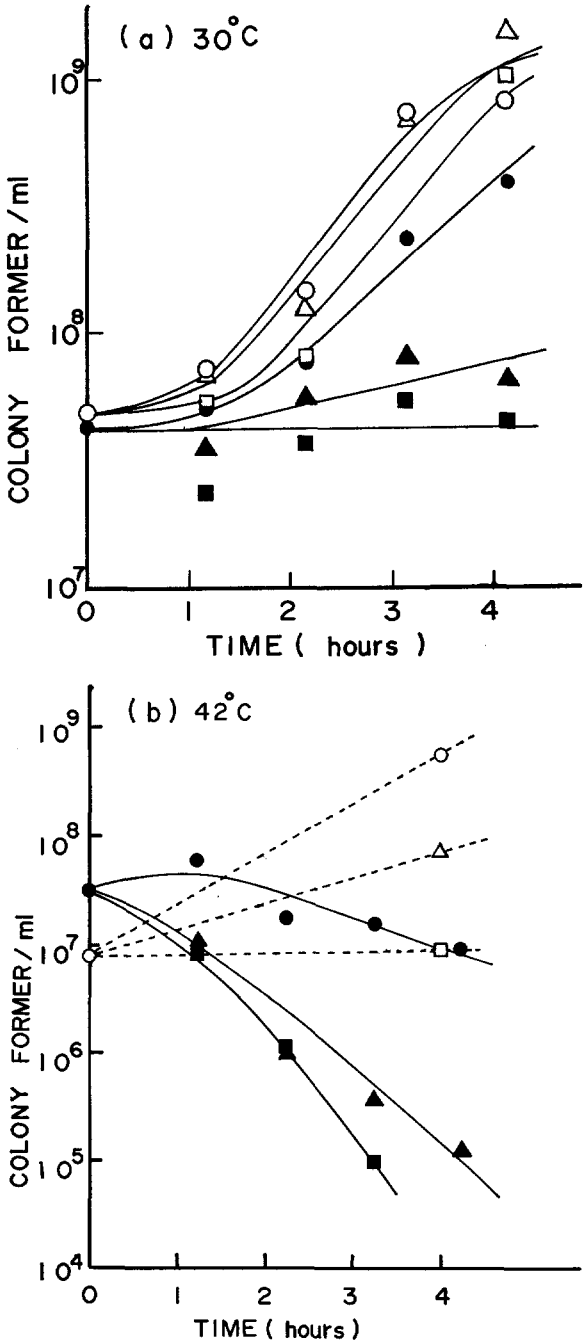
RESULTS

Alteration of the membrane properties in the mutant: As was expected from the selection employed, the mutant KY2750 was shown to be more resistant to PEA than the parental strain when grown in a liquid medium at 30° (Figure 1). The mutant was subsequently found to be altered in sensitivities to deoxycholate and to rifampicin. As shown in Figure 2, deoxycholate (DOC) at 0.25% strongly inhibited growth of the mutant, but not the wild type, at 30°. At 42°, the marked loss of colony formers was observed with the mutant in the presence of DOC. The mutant was also shown to be sensitive to low concentrations of rifampicin that had little effect on the wild-type bacteria (Figure 3). These results strongly suggest that the structure of some membrane component(s) has been altered in this mutant.

Specific cessation of DNA synthesis and cell division at high temperatures: When a mutant culture grown in PG medium at 30° was transferred to 42°, DNA

FIGURE 2.—Effect of DOC on the cell viability. Log-phase cultures (PG medium) of wild type (KY2053) and the mutant (KY2750) grown at 30° were diluted several-fold in prewarmed PG medium with or without DOC. Cultures were shaken at 30° (a) or 42° (b), and aliquots taken at intervals were diluted and plated on PG agar to score the number of colony formers at 30°. Open symbols are for the wild type, and closed symbols for the mutant. ○, ●, control; △, ▲, 0.25% DOC; □, ■, 0.5% DOC.

synthesis stopped after 60 to 90 min, resulting in about 50% increase in DNA as determined by ^{3}H-thymidine incorporation. In contrast, RNA synthesis as measured by ^{14}C-uracil incorporation proceeded faster at 42° than at 30°, as did



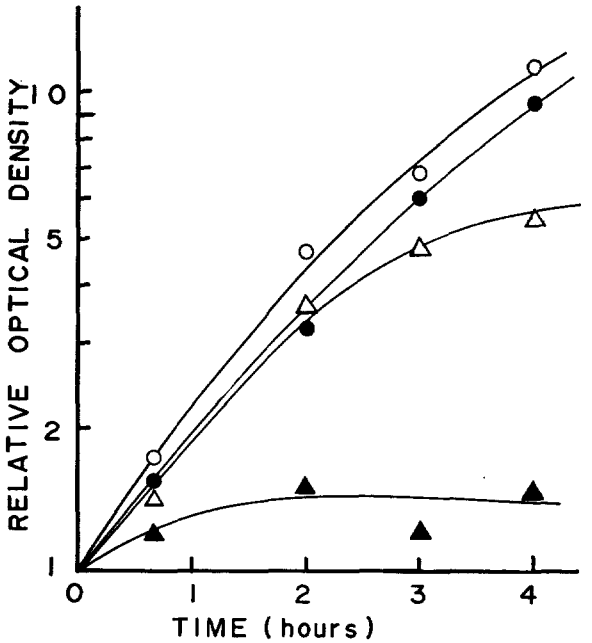


FIGURE 3.—Growth of bacteria in the presence of a low concentration of rifampicin. Log-phase cultures in PG medium of wild type (KY2053) and the mutant (KY2750) were used to inoculate the same medium with or without rifampicin (8 μg/ml) at about 5×10^7 cells/ml. Optical density was followed during shaking at 30°. O——O, wild type; △——△, wild type with rifampicin; ●——●, mutant; ▲——▲, mutant with rifampicin.

the parental strain. Similar results were obtained when the temperature was shifted to 40°, or when a thymine-requiring derivative (KY2901) of the mutant was examined using ^{3}H-thymine to label the DNA. Figure 4 presents the results of colorimetric determination of DNA, RNA and protein in this mutant grown at 30° or at 42° in medium E-Casamino acids. These results clearly indicate that DNA synthesis is specifically arrested at high temperatures in the mutant KY2750. In a separate experiment, cells previously labeled with ^{3}H-thymidine at 30° were washed and incubated in the absence of thymidine at 42°. No difference in acid-soluble radioactivity was found between the mutant and wild-type cultures for at least 1 hr, suggesting that DNA degradation is not accelerated in the mutant.

Cell division also stopped shortly after the cessation of DNA synthesis, leading to about a threefold increase in cell number during 3-hr incubation at 42° (Figure 5a). On the other hand, relatively little increase in number of viable cells (colony formers) was observed after the temperature shift, followed by the gradual decrease upon prolonged incubation (Figure 5b). The later phenomenon was particularly striking in a PG medium, where about 90% of colony formers were lost in 5 hr at 42°. Such a loss of viability was partially prevented by higher concentrations of salt (1.5% NaCl) added to the medium. The higher salt concen-

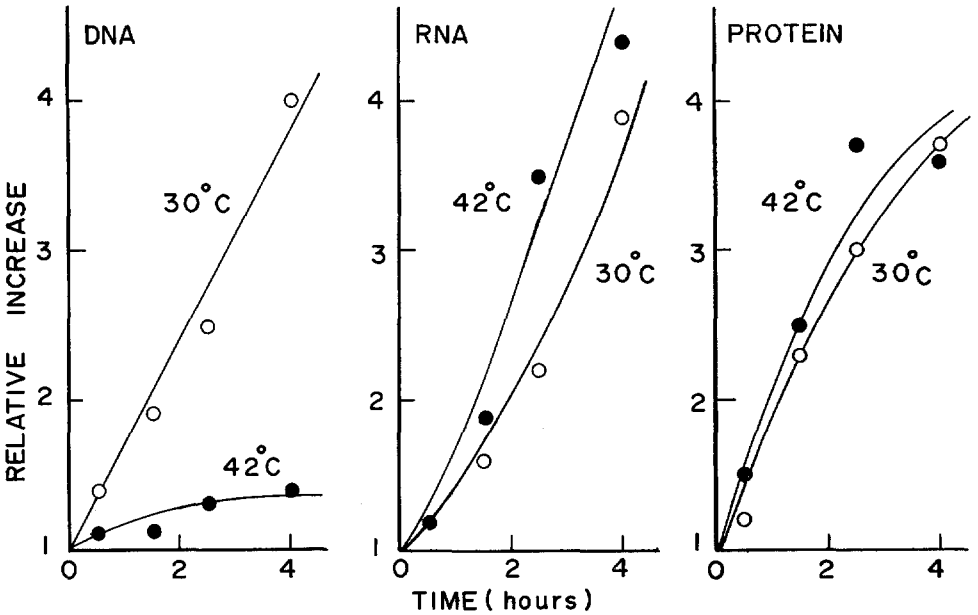


FIGURE 4.—Effect of temperature on DNA, RNA and protein synthesis in the mutant. A log-phase culture of the mutant (KY2901) in medium E-Casamino acids supplemented with tryptophan and thymine was grown to 1.5×10^8/ml, divided into two, and shaken at 30° or 42°, respectively. Aliquots were taken at the times indicated for determination of DNA, RNA and protein by colorimetric methods, essentially as described by BERRAH and KONETZKA (1962). Relative increases over the initial values are plotted.

tration failed to relieve the effect of high temperatures on DNA synthesis, however.

When mutant cells that had been aerated at 42° for 90 min were infected by phage T4D or lambda, they could still support the growth of these phages just as well as those grown at 30°, in spite of their complete inability to replicate the chromosomal DNA (Table 2). Thus the adverse effect of high temperatures on DNA synthesis in this mutant seems to be restricted to the chromosomal DNA. In this connection, the exposure of the mutant cells harboring an F*gal* or ColVB*trp* episome to 42° for 2 to 4 hr did not lead to the segregation of clones that had lost the episome at higher frequencies. DNA synthesis in these cells gradually stopped at 42° as in the mutant F^- bacteria.

Resumption of DNA synthesis upon return to low temperatures: When a mutant culture that had been kept at 42° for 1 to 4 hr was shifted back to 30°, DNA synthesis resumed with little lag. Addition of chloramphenicol (100 or 150 μg/ml) to the culture 15 min before or at the time of temperature shift, or of rifampicin (50 μg/ml) at the time of the shift, did not affect the initial doubling of the DNA, indicating that neither protein nor RNA synthesis is required for this resumption of DNA replication. Figure 6 presents a typical result obtained with strain KY2901. Similar results were obtained with strain KY2750 using ^{3}H-thymidine to label the DNA. In these experiments, the amounts of residual

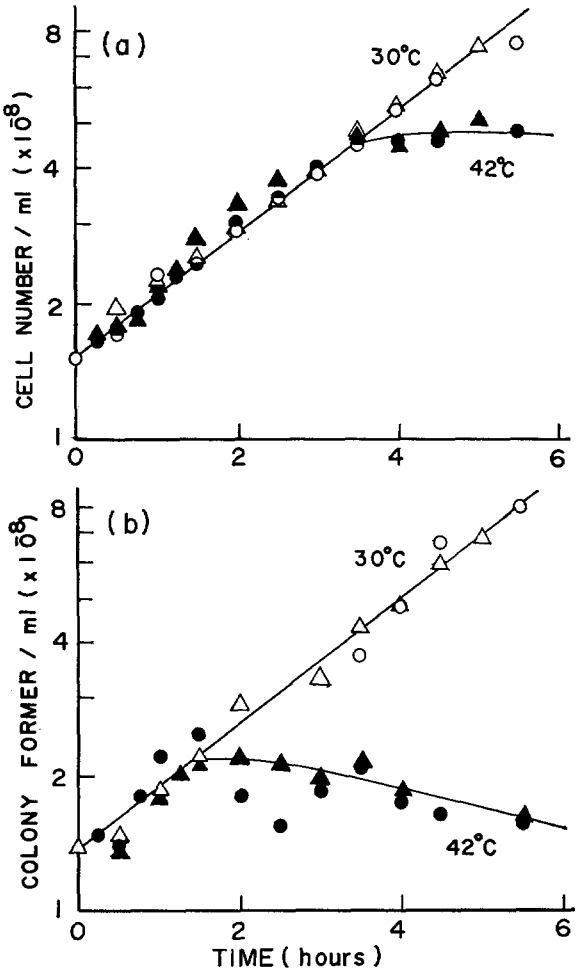


FIGURE 5.—Effect of temperature on cell division and viability of the mutant. A log-phase culture of the mutant (KY2750) grown in medium E-Casamino acids at 30° was divided into two parts and one aerated at 30° (open symbols) and the other at 42° (closed symbols). Aliquots were taken at the times indicated for determination of both total cell number (a) and colony formers (b). The cell number was estimated by a Coulter counter (Model B) at the settings of $T_L = 20$, A = ¼ and I = ½. Colony formers were determined by plating appropriate dilutions of each sample on PG agar followed by incubation at 30° for 2 days. Experiment 1 (○, ●); Experiment 2 (△, ▲).

DNA synthesis observed in the presence of chloramphenicol did not vary appreciably when the length of previous exposure to 42° varied between 1 and 4 hrs. Thus, the initiation capacity does not seem to accumulate during incubation at the restrictive temperature, in contrast to the recent observations with another DNA initiation mutant CT28 (SCHUBACH, WHITMER and DAVERN 1973). Despite the rapid recovery of DNA synthesis observed, cell division hardly occurred when a mutant culture that had been kept at 41° for 4 hrs was returned to 30° and

TABLE 2

*Growth of bacteriophages in the mutant bacteria**

Time (min)	T4D		lambda	
	30°	42°	30°	42°
0	2.6×10^3	1.9×10^3	6.1×10^6	4.6×10^5
30	3.6×10^5	8.0×10^5	——	——
90	——	——	5.0×10^6	8.7×10^7
120	——	——	5.5×10^8	——

* Log-phase cultures of the mutant (KY2750 or KY2900 for experiments with T4D or λ, respectively) in PG medium were shaken at 30° or 42° for 90 min and were infected with T4D (m.o.i. = 1.6) or λ (m.o.i. = 1.1). After standing for 5 (for T4D) or 15 min (for λ) at each temperature to allow for adsorption, a portion was diluted 10^{-4} (for T4D) or 10^{-2} (for λ) into prewarmed PG medium and was further shaken at each temperature. Samples were taken at the times indicated, treated with chloroform, and the phage yields determined with strain W3350 as indicator bacteria.

incubated further for as long as 10 hrs. Apparently, the prolonged incubation at the restrictive temperature irreversibly inactivated the capacity of the mutant cells to carry out further divisions. This may be contrasted to what has been found with other DNA initiation mutants.

In another experiment, a mutant culture that had been incubated at 41° for

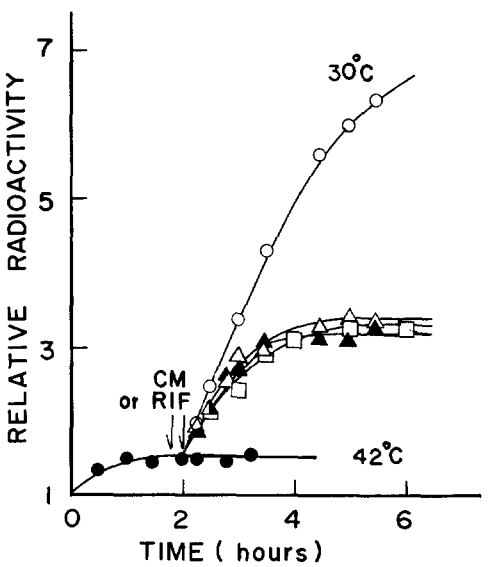


FIGURE 6.—The recovery of DNA synthesis in the mutant at low temperature. A log-phase culture (1×10^8 cells/ml) of the mutant (KY2901) grown at 30° in medium E-Casamino acids containing ^{3}H-thymine was transferred to 42° at time 0 and was aerated for 2 hr. The culture was then divided into five parts, one kept at 42° (●——●), whereas the others were returned to 30°. ○——○, control at 30°. Chloramphenicol (CM, 150 μg/ml) was added 15 min before (▲——▲) or at the time of the shift (△——△) to the low temperature. Rifampicin (50 μg/ml) was added at the time of the temperature shift (□——□). The radioactivity at time 0 was 4,160 counts/min.

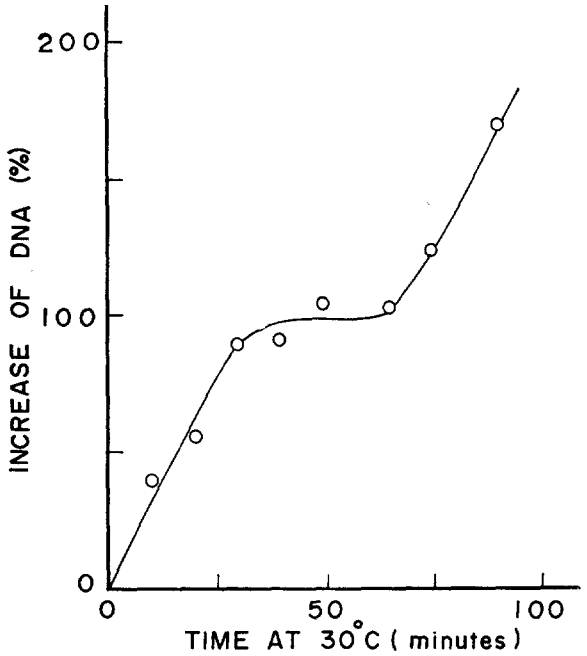


FIGURE 7.—Residual DNA synthesis at 41° as a function of the length of pulse exposure to low temperature. A culture of the mutant (KY2750) was grown in medium E-Casamino acids containing ^{3}H-thymidine for several generations to about 1.5×10^{8} cells/ml at 30°. The culture was shifted to 41°, shaken for 90 min, and then returned to 30° for further incubation. Aliquots of the culture were taken at the times indicated on the abscissa, and were shaken at 41° for an additional 120 min. Radioactivity in acid-insoluble fraction was determined as described in MATERIALS AND METHODS and is expressed as percent increase over the value (1,050 counts/min) at the time of the shift to the low temperature.

90 min to allow cessation of DNA synthesis was exposed to a low temperature (30°) and then returned to 41° again. Under these conditions, certain fraction of cells underwent apparently one cycle of DNA replication. The amount of DNA synthesized, however, varied depending upon the length of the pulse exposure to the low temperature. As seen in Figure 7, the doubling of DNA in total population took place when the cells were kept at 30° for 30 to 65 min. It thus appears that about 30-min incubation at 30° is required for the majority of cells to become capable of initiating a new cycle of DNA replication. The exposure to the low temperature for 75 min or longer resulted in more than doubling of the DNA, presumably due to the second round of DNA replication. The generation time of this mutant under these conditions was about 100 min at 30°. It was further shown that chloramphenicol or dinitrophenol added to the medium during the pulse incubation at 30° had little effect on the subsequent resumption of DNA replication at 42° (Figure 8).

All these results clearly indicate that the effect of high temperatures on DNA synthesis is reversible and that the resumed synthesis after exposure to 30° does not depend on protein or RNA synthesis or other energy-requiring processes

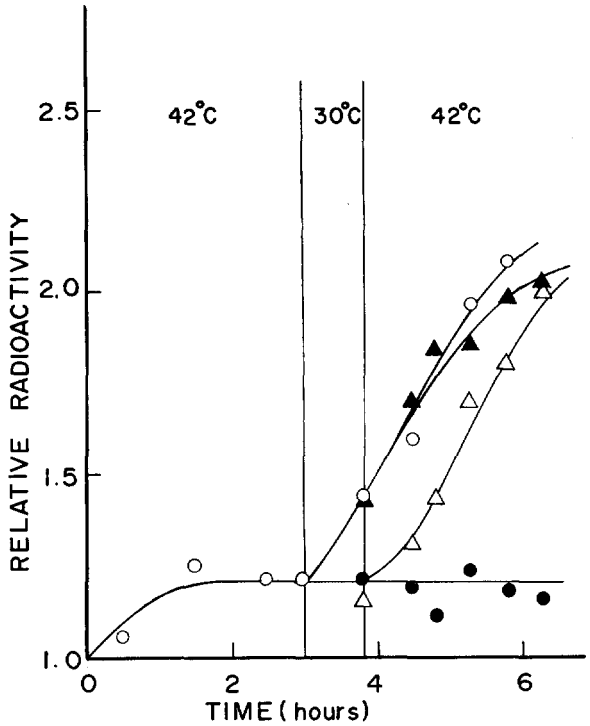


FIGURE 8.—Effects of chloramphenicol and dinitrophenol on the recovery of DNA synthesis. A log-phase culture (1×10^8 cells/ml) of the mutant (KY2750) grown at 30° in medium E-Casamino acids containing ^{3}H-thymidine was transferred to 42° and was shaken further for 3 hr. The culture was divided into four parts, with one kept at 42° throughout the experiment (●——●), while the others were transferred to 30° and shaken for 50 min with no addition (○——○) or with an addition of 150 μg/ml of chloramphenicol (▲——▲) or 2×10^{-3} M dinitrophenol (△——△). Each culture was then diluted 20-fold in the same prewarmed medium with no drug and was shaken further at 42°. The radioactivity at time 0, was 4,750 counts/min.

during incubation at 30°. Moreover, the fact that the amount of DNA doubled during recovery in the presence of chloramphenicol (Figure 6), as well as the data presented in Figure 7, suggest that the residual DNA synthesis at high temperatures tends to synchronize the replication cycle, perhaps at the stage of initiation.

DNA synthesis following amino acid starvation: To further substantiate the effect of temperature on the initiation of DNA replication, the mutant cells were first starved for amino acids at 30°, and the recovery of DNA synthesis upon re-addition of amino acids was examined at 30° and 42°. As shown in Figure 9, DNA synthesis resumed at 30° after some lag, but did not resume at 42°. Since amino acid starvation presumably permits the completion but not the initiation of DNA replication in *E. coli*, these results are also taken to indicate that the mutant cannot initiate a new round of DNA replication cycle at high temperatures.

Specific regions of the chromosome replicated early during recovery at 30°:

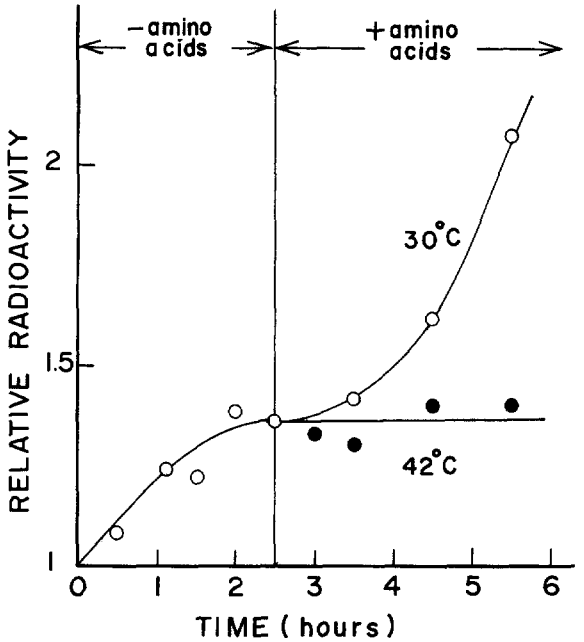


FIGURE 9.—Effect of temperature on DNA replication after amino acid starvation in the mutant. A mutant culture (KY2901) in medium E-Casamino acids containing ^{3}H-thymine was shaken at 30° to about 1.5 × 10^{8} cells/ml. The culture was filtered through a sterile Millipore filter (HA 0.45 μ), washed and resuspended in medium E containing ^{3}H-thymine but lacking all amino acids. After shaking for 150 min at 30°, the culture was divided into two parts, each shaken at 30° or 42°. Amino acids (casamino acids plus tryptophan) were added to both cultures immediately after temperature equilibration. There was no increase in optical density during the amino acid starvation in this experiment. The radioactivity at time 0 was 3,020 counts/min.

The following experiments were carried out to see whether the regions of DNA replicated early during recovery at 30° are fixed or randomly distributed over the entire chromosome in the mutant cell population. The mutant cells at the initial phase of the recovery at 30° after a previous exposure to 41° were pulse-labeled by ^{3}H-thymidine for 10 min, followed by further incubation for 15 min with unlabeled thymidine. The culture was then shifted to 41°, shaken for 90 min, and the cells were harvested by filtration and finally incubated in the medium containing ^{2}H-glucose and $^{15}NH_4Cl$ at 30°. Samples were taken at intervals and DNA's extracted by phenol were analyzed by CsCl density gradient equilibrium centrifugation. As a control, a log-phase culture of the mutant was pulse-labeled with ^{3}H-thymidine at 30° and was treated in exactly the same way as for the experimental culture. Figure 10 summarizes the result of such experiments, indicating clearly that the transfer of ^{3}H-labeled DNA to the hybrid density is much faster for the experimental than for the control culture. These results suggest that the portions of DNA labeled at the initial phase of the recovery at 30° are not randomly distributed but are restricted to certain regions of the chromosome. The data are consistent with the hypothesis that the mutant is affected at the initiation or termination step of the DNA replication cycle.

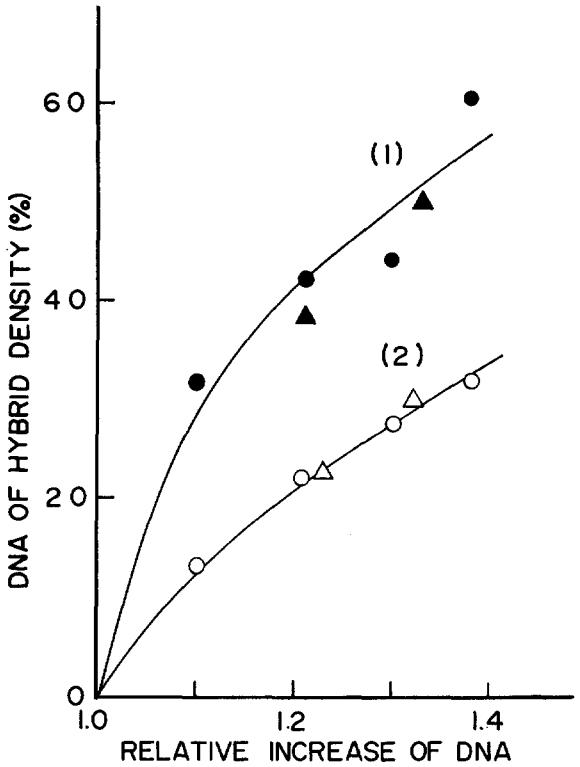


FIGURE 10.—CsCl density gradient centrifugation of DNA pulse-labeled at the log phase or at the initial phase of the resumed synthesis. (1) A log-phase culture of the mutant (KY2750) grown at 30° in medium E-Casamino acids was exposed to 41° for 90 min, returned to 30° and was pulse-labeled with ^{3}H-thymidine (20 $\mu c/8$ μg/ml) for 10 min (between 5 and 15 min of incubation). Cells were transferred to a non-radioactive medium, shaken for 15 min at 30°, exposed again to 41° for 90 min, and finally transferred to the medium containing $^{15}NH_4Cl$ (2 mg/ml), ^{2}H-glucose (2 mg/ml) and an acid hydrolysate of *E. coli* cells that had been grown on ^{2}H-glucose and $^{15}NH_4Cl$. During incubation at 30°, aliquots were taken at 30, 60, 80, and 100 min, cells were collected, and DNA's extracted by phenol were centrifuged in CsCl density gradients. (2) As a control, a portion of the same log-phase culture used above was pulse-labeled with ^{3}H-thymidine at 30° for 10 min, transferred to a non-radioactive medium and incubated at 41° for 90 min. The culture was then returned to 30° for 30 min, exposed again to 41° for 90 min, and finally incubated at 30° in the $^{2}H^{15}N$-medium. Aliquots were taken, DNA's extracted and centrifuged in the same way as above. The percent of DNA of hybrid density was calculated from the radioactivity profile of each sample ($1–2 \times 10^4$ counts/min) and was plotted against total DNA as determined by the parallel experiment. Circles and triangles represent the results of two independent experiments.

Effect of temperature on the envelope proteins: To investigate the possible alteration of envelope proteins in the mutant, cells were labeled by radioactive L-arginine and the envelope fractions prepared were analyzed by SDS-polyacrylamide gel electrophoresis. When a mixture of wild-type and mutant cells, each labeled with ^{14}C- or ^{3}H-arginine at 42°, was analyzed by this method, a striking difference was noted between the proteins labeled by ^{14}C and ^{3}H (Figure 11). One of the major peaks present in the wild-type preparation is almost

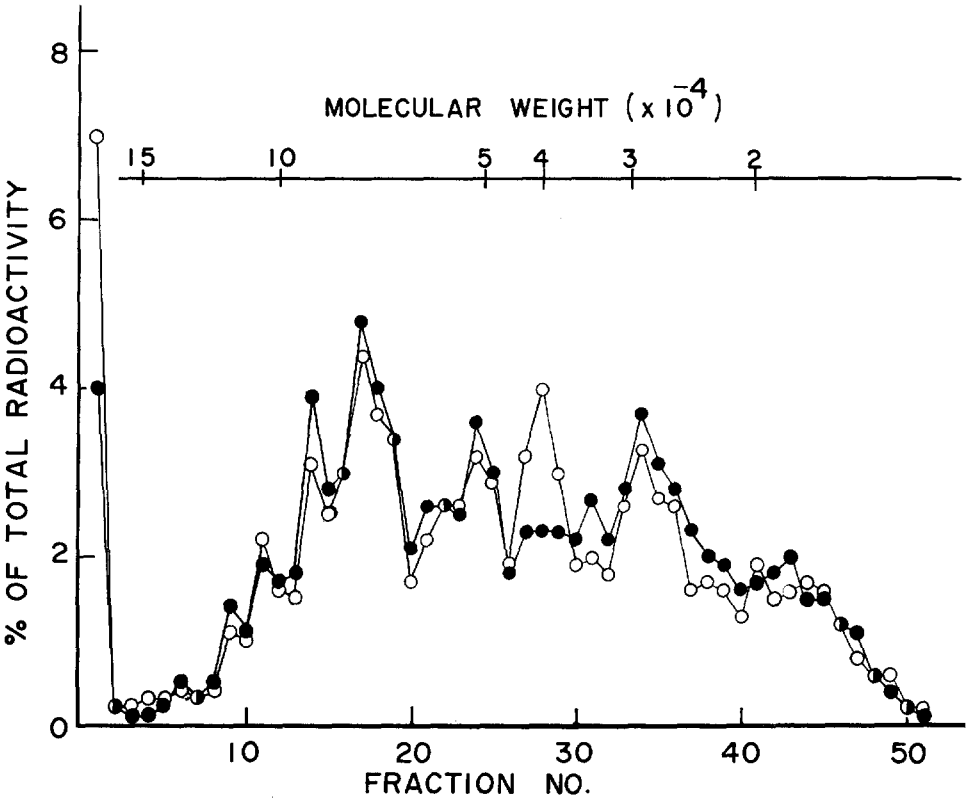


FIGURE 11.—SDS-gel electrophoresis of envelope proteins. Wild-type (KY2750) cultures were grown at 30° to 3×10^8 cells/ml in medium E supplemented with 0.1% Casamino acids, 20 μg/ml of each amino acid required and 2 μg/ml of thiamine. Both cultures were transferred to 42° and were labeled with 0.8 μc/ml of ^{14}C-arginine (wild type, O——O) or 4 μc/ml of ^{3}H-arginine (mutant, ●——●) for the period that allowed doubling of the optical density. The two cultures were mixed before harvesting cells and the envelope fractions were prepared and analyzed by SDS-gel electrophoresis essentially as described by INOUYE and GUTHRIE (1969). The sample contained 11,400 (^{3}H) and 2,800 (^{14}C) counts/min of radioactivity. The molecular weight scale indicated has been calculated from the protein profiles of RNA polymerase and 50 S ribosomes of *E. coli* run simultaneously with other gels.

missing in the mutant preparation. Such a difference was not detected when cells were grown at 30°. This component, having molecular weight of about 40,000, probably corresponds to "Y protein" (INOUYE and GUTHRIE 1969) or "MP40" (LAZDUNSKI and SHAPIRO 1973) that are known to disappear when DNA synthesis is inhibited (INOUYE and PARDEE 1970; LAZDUNSKI and SHAPIRO 1973). Thus, no specific alteration of envelope proteins associated with the *dnaP* mutation could be detected by these experiments.

Genetic mapping of the mutation: Preliminary mapping of the temperature-sensitive PEA-resistance mutation was performed by conjugation between the mutant KY2750 (F⁻ *thr*, *leu*, *mal*, *xyl*, *str*, Pea-R/Ts) and the wild-type strain CS101 (Hfr *thr*$^+$, *leu*$^+$, *mal*$^+$, *xyl*$^+$, *str*$^+$, Pea-S/Ts$^+$). Among the Xyl$^+$ Str-R

TABLE 3

Transduction mapping of the dnaP18 mutation by phage P1

Experiment*	Selection	Unselected marker†					Number of transductants obtained
		tna	*phoS*	*cya*	*dnaP*	*metE*	
I	*ilv*+	1	1	.	0	.	2
		0	1	.	1	.	4
		0	1	.	0	.	15
		0	0	.	1	.	18
		0	0	.	0	.	61
						Total	100
II	*ilv*+	.	.	.	1	1	2
		.	.	.	1	0	43
		.	.	.	0	1	4
		.	.	.	0	0	43
						Total	92
III	*ilv*+	.	.	1	1	1	1
		.	.	1	1	0	19
		.	.	1	0	1	5
		.	.	1	0	0	49
		.	.	0	1	0	1
		.	.	0	0	1	1
		.	.	0	0	0	29
						Total	105

* Experiment	Donor	Recipient
I	KY2750 (*tna*+ *phoS*+ *ilv*+ *dnaP18*)	BC73 (*tna phoS ilv dnaP*+)
II	KY9115 (*ilv*+ *dnaP18 metE*+)	AB2277 (*ilv dnaP*+ *metE*)
III	KY2750 (*ilv*+ *cya*+*dnaP18 metE*+)	GP1 (*ilv cya dnaP*+ *metE*)

† 1 and 0 represent markers from donor and recipient strains, respectively. The *dnaP* character was scored by streaking cells from overnight cultures on PG agar followed by incubation at 30° and 42° to determine temperature sensitivity.

or Mal$^+$ Str-R recombinants tested, some were found to be both temperature-independent (Ts$^+$) and PEA-sensitive (Pea-S), whereas none of the Thr$^+$ Leu$^+$ Str-R recombinants tested inherited these characteristics from the Hfr parent. Further mapping experiments were performed by transduction using phage P1, and the results obtained are summarized in Table 3. It can be seen that the temperature sensitivity of the mutant is due to a mutation or mutations occurring between *cya* and *metE*. We tentatively designate this gene as *dnaP*, since it seems to represent a novel gene essential for DNA synthesis in *E. coli*. The probable gene order in this region of the chromosome is: *tna–phoS–ilv–cya–dnaP–metE*. As was expected from the location of *dnaP*, the episome F14 was found to carry the wild-type allele of this gene, *dnaP*$^+$. When F14 was transferred to the mutant KY2750, the resulting merodiploid strain carrying both *dnaP18* and *dnaP*$^+$ was found to be capable of growing at 42° in PG medium, as is the haploid wild-type strain. This shows that the *dnaP18* mutant allele is recessive to *dnaP*$^+$. The F$^+$ derivative of the mutant was still temperature-sensitive, indicating that the function of the *dnaP*$^+$ gene cannot be supplied or complemented by the F$^+$ factor itself.

TABLE 4

*Properties of temperature-independent revertants**

Strain	Phenethyl alcohol (0.2%)	Deoxycholate (0.5%)	Rifampicin (8 μg/ml)	Number obtained
Wild type (KY2053)	S	R	R	..
Mutant (KY2750)	R	S	S	..
Revertants	S	R	R	9
	S	R	S	12
	S	S	R	1
	S	S	S	1
	R	R	S	1
	R	S	S	2
			Total	26

* Spontaneous revertants independently obtained from mutant KY2750 were examined for their sensitivity to the drugs at the concentrations indicated. The sensitivities were determined by measuring optical density of the culture after 48 hr (phenethyl alcohol) or 4 hr (deoxycholate or rifampicin) at 30° in PG medium.

Temperature-independent reversion: Further evidence that a single mutation at the *dnaP* locus is responsible for both PEA resistance and temperature sensitivity was obtained by studying temperature-independent revertants spontaneously obtained from the mutant. As shown in Table 4, many of the revertants underwent simultaneous alterations toward the wild-type phenotype with respect to the sensitivity to PEA, DOC or rifampicin. Effects of PEA concentration on the growth of some of the revertants are included in Figure 1. These results are consistent with the notion that the *dnaP18* mutation primarily affects the structure of some membrane component and that this membrane alteration is responsible for all the mutant characteristics observed.

When an F^+ factor was introduced into the mutant by conjugation, the resulting F^+ strain underwent reversion to the temperature independence at a frequency about one hundred times higher than the original F^- strain. Whether this effect of the F^+ factor on the reversion frequency is due to the integration of the episome into the chromosome (NISHIMURA *et al.* 1971) has not been investigated.

Electron microscopic observation of the mutant: To study the morphological changes of the mutant cells upon exposure to high temperatures, electron microscopic observations were made on thin sections of the mutant as well as the wild-type bacteria grown at 30° or 42°. It was revealed that the majority of mutant cells become long filaments after 4-hr incubation at 42°, and septum formation was completely lacking in those filaments. Many of the cells at this stage exhibited nucleoid structures that appeared to be highly dispersed and DNA fibrils that appeared less condensed than those of mutant cells grown at 30° or at 42° for 2 hr (compare d with b and c, or g with f in Figure 12) or in the wild-type bacteria grown at 30° or 42° (data not shown). After 6-hr incubation at 42°, the structure of the cytoplasmic membrane became much less conspicuous, and the electron density of the cytoplasm became markedly reduced (Figure 12e). These obser-

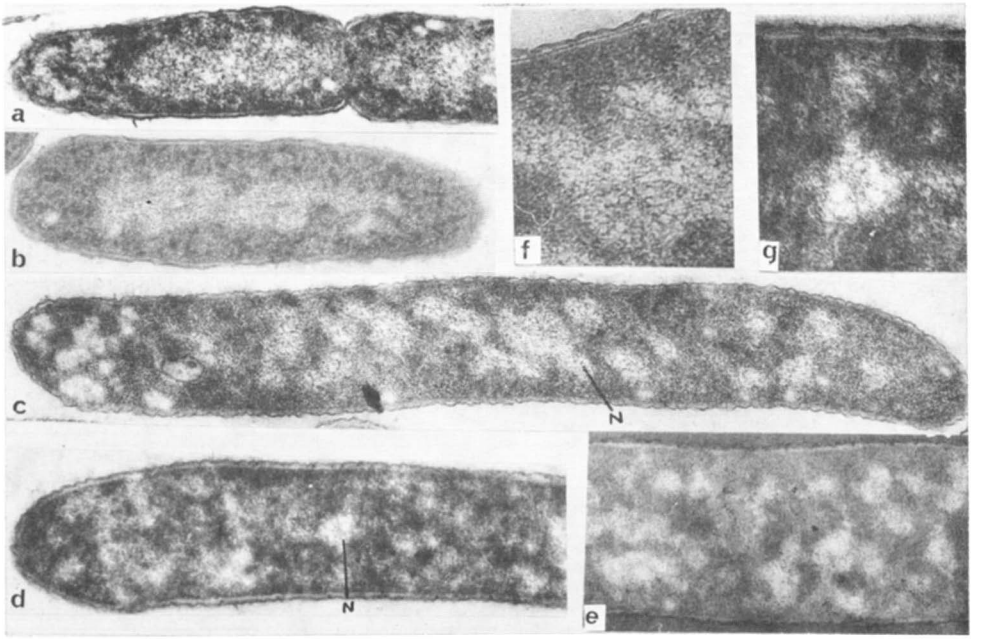


FIGURE 12.—Electron micrographs of thin sections of the mutant (KY2750) grown in PG medium at 30° or 42°. Cells were fixed and embedded by the method of RYTER and KELLENBERGER (1958) with minor modifications. a, control (30°). A sequence of changes that appears to take place at 42° is represented by b, c, d and e. The portions of c and d indicated by arrows were further magnified to give f and g, respectively. N: nucleoids. a–e, × 35,000; f,g, × 80,000.

vations may explain the irreversible loss of viability of the mutant cells under these conditions.

DISCUSSION

The temperature-sensitive mutation (*dnaP18*) described in this paper may be regarded as a mutation primarily affecting the structure and function of some essential component of the bacterial membrane, and DNA replication and cell division become arrested at high temperatures as the result of this membrane alteration. Firstly, the mutant was selected for its resistance to PEA, whose primary site of action appears to be on the membrane (SILVER and WENDT 1967); and PEA resistance of certain *E. coli* strains has been shown to involve an alteration in the membrane properties (YURA and WADA 1968). Secondly, the mutant was found to be altered in sensitivities to DOC and to rifampicin. The DOC sensitivity has been correlated with alterations of the bacterial surface structure (NAGEL DE ZWAIG and LURIA 1967; HIROTA, MORDOH and JACOB 1970), and the higher sensitivity of the mutant to rifampicin presumably reflects the increased permeability to the drug (SCHLEIF 1969). Finally, all these mutant properties can often be lost simultaneously by reverse mutations to temperature independence (Table 4), suggesting that they are due to a single gene mutation.

When the mutant culture grown at 30° was transferred to 42°, DNA synthesis

stopped after some residual synthesis, whereas RNA and protein synthesis continued exponentially for at least two or three generations. Even after the complete cessation of DNA synthesis, however, the mutant cells supported the growth of phage lambda as well as T4 at 42° (Table 2), suggesting that the normal functioning of the *dnaP*$^+$ gene is not required for growth of these phages. The normal burst size of lambda after infection at 42° also suggests that the effect of the mutation does not involve the general damage on DNA synthesis such as depletion of the deoxyribonucleotide pools. The F$^+$ factor introduced into the mutant cell failed to reverse the temperature-sensitive phenotype of the mutant, indicating that the F$^+$ factor cannot supply the *dnaP* function. The question of whether replication of the F$^+$ factor itself is arrested at high temperatures like that of the chromosomal DNA has not so far been investigated.

As to the stage of the chromosomal replication affected by the *dnaP18* mutation, available data do not permit clear distinction among various possibilities. However, several lines of evidence listed below suggest that replication becomes arrested at or near the initiation of the cycle when the mutant cells are exposed to high temperatures. (1) The amounts of residual DNA synthesis at 42° and during amino acid starvation are nearly the same (Figures 6 and 9). (2) After alignment of the DNA at 30° by amino acid starvation, re-addition of amino acids at 42° did not permit further synthesis of DNA (Figure 9). (3) After prior inhibition of DNA replication at 42°, the amount of DNA doubled upon return to 30° in the presence of chloramphenicol or rifampicin (Figure 6). (4) The quantitative analysis of dependence of the residual DNA synthesis on the length of pulse exposure to low temperature (Figure 7) suggested that the incubation at the high temperature tends to synchronize the replication cycle at the stage of initiation or termination. (5) The residual cell division at 42° reached the level (about threefold) that may be expected for an initiation-defective mutant (Beyersmann, Schlicht and Schuster 1971). In addition, the results of the density-shift experiments presented (Figure 10) and the capacity of the mutant cells to permit replication of independent replicons such as phage lambda and T4 at the restrictive temperature are at least consistent with this interpretation. Many of the characteristics of the mutant described here are similar to what have been reported for other *dna* mutants defective in the initiation of DNA replication (Kohiyama 1968; Hirota, Ryter and Jacob 1968; Hirota, Mordoh and Jacob 1970; Carl 1970; Abe and Tomizawa 1971; Beyersmann, Schlicht and Schuster 1971; Wechsler and Gross 1971; Wolf 1972; Schubach, Whitmer and Davern 1973).

The *dnaP18* mutation has been mapped between *ilv* and *metE* on the *E. coli* chromosome, the frequency of co-transduction with *ilv* being about 20%. The DNA initiation mutant CRT46 carries a *dnaA* mutation closely linked to *ilv* but on the opposite side of *ilv* from *metE* (Hirota, Mordoh and Jacob 1970). Another mutation studied by Abe and Tomizawa (1971) is also located close to the CRT46 mutation (Abe, personal communication). The relation between *dnaP* and other mutations located between *cya* and *metE*, including *rep* (Calendar *et al.* 1970), *uvrD* (Ogawa, Shimada and Tomizawa 1968), *uvr502* (Smirnov

and SKAVRONSKAYA 1971), *mutU* (SIEGEL 1973), and *pdeB* (HORIUCHI and NAGATA 1973), also remains obscure at the present time, although the normal sensitivity of the present mutant to ultraviolet light tends to preclude a close relationship with any of these mutants.

The available evidence discussed above leads us to a specific suggestion on the possible nature of the present mutation and its effect on the chromosomal DNA replication in *E. coli*. Thus, the *dnaP18* mutation presumably affects the structure and function of some membrane component of the cell in such a manner that further rounds of DNA replication are prevented upon shift to high temperatures. In view of the results presented in Figure 8 on the recovery of DNA replication by a pulse exposure to the low temperature, as well as the recessive nature of the mutation, the *dnaP* gene product might represent an enzyme that constitutes one of the membrane proteins in *E. coli*. This might be related to a "PEA-sensitive" protein, postulated by LARK and LARK (1966), that is required for the initiation of DNA replication.

The present results of electron microscopic observation suggest that the nucleoids in the *dnaP18* mutant cells become disperse and the DNA fibrils less condensed as they cease to synthesize DNA at high temperatures. Whether these observations indeed reflect a unique property of the mutant or represent the results of other indirect nature might be worth investigating in the future. In this connection, the previous observations with the *dnaA* mutants indicate that the nucleoids are often found in the central region of the elongated cells (KOHIYAMA *et al.* 1966; HIROTA, RYTER and JACOB 1968).

In addition to the well-known effect of PEA on the initiation of DNA replication in *E. coli*, strong inhibition of phospholipid synthesis was reported recently that might be related to the membrane alteration brought about by PEA (NUNN and TROPP 1972). The formation of active dimers of alkaline phosphatase from inactive monomers—which seems to take place at the membrane—is also inhibited by PEA (TRIBHUVAN *et al.* 1970). Moreover, a higher concentration of PEA (0.5%) causes the release of DNA from the DNA-membrane complex (MASKER and EBERLE 1972). Although the precise mechanism of action of PEA on DNA replication is not clear at present, further analyses of PEA-resistant mutants, including the one described here, should offer a profitable approach to the general problems of the role of cellular membrane in DNA replication in bacteria.

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