JOURNAL OF BACTERIOLOGY, Dec. 1974, p. 1227-1237
Vol. 120, No. 3
Printed in U.S.A.
Copyright © 1974 American Society for Microbiology

Replication of the Bacteriocinogenic Plasmid Clo DF13 in Thermosensitive *Escherichia coli* Mutants Defective in Initiation or Elongation of Deoxyribonucleic Acid Replication

E. VELTKAMP AND H. J. J. NIJKAMP

Department of Molecular Genetics, Biological Laboratory, Free University, Amsterdam-Buitenveldert, The Netherlands

Received for publication 2 October 1974

The replication of the bacteriocinogenic plasmid Clo DF13 has been studied in the seven temperature-sensitive *Escherichia coli* mutants defective in deoxyribonucleic acid (DNA) replication (*dnaA*-*dnaG*). Experiments with *dna* initiation mutants revealed that the replication of the Clo DF13 plasmid depends to a great extent on the host-determined *dnaC* (*dnaD*) gene product, but depends slightly on the *dnaA* gene product. The synthesis of Clo DF13 plasmid DNA also requires the *dnaF* and *dnaG* gene products, which are involved in the elongation of chromosomal DNA replication. In contrast, the Clo DF13 plasmid is able to replicate in the *dnaB* and *dnaE* elongation mutants at the restrictive temperature. When de novo protein synthesis is inhibited by chloramphenicol in wild-type cells, the Clo DF13 plasmid continues to replicate for at least 12 h, long after chromosomal DNA synthesis has ceased, resulting in an accumulation of Clo DF13 DNA molecules of about 500 copies per cell. After 3 h of chloramphenicol treatment, the Clo DF13 plasmid replicates at a rate approximately five times the rate in the absence of chloramphenicol. Inhibition of protein synthesis by chloramphenicol does not influence the level of Clo DF13 DNA synthesis at the restrictive temperature in the *dna* mutants, except for the *dnaA* mutant. Chloramphenicol abolishes the inhibition of Clo DF13 DNA synthesis in the *dnaA* mutant at the nonpermissive temperature. Under these conditions, Clo DF13 DNA synthesis was slightly stimulated in the first 30 min after the temperature shift, and continued for more than 3 h at an almost uninhibited level.

The cloacinogenic plasmid DF13 (Clo DF13) is a non-essential, extrachromosomal deoxyribonucleic acid (DNA) molecule that determines the production of the extracellular antibiotic protein cloacin DF13. The aim of our work is to study the replication and regulation of replication of the Clo DG13 plasmid. The Clo DF13 plasmid orginates from *Enterobacter cloacae* (36); however, *Escherichia coli* can also serve as a host. The nontransmissible Clo DF13 plasmid is a relative small circular DNA molecule (molecular weight 6×10^6) with a contour length of 3.0 ± 0.2 μm (39) and is present in *E. coli* cells to the extent of about 10 copies per cell (23). The Clo DF13 DNA directs the synthesis of at least four messenger ribonucleic acid (RNA) species and eight proteins (22, 24). The function of the protein cloacin DF13 is known: it abolishes protein synthesis of sensitive cells by inhibiting the binding of formylmethionine-transfer RNA to the messenger RNA-30*S* ribosome complex (14). de Graaf et al. (15) have shown that this inhibition of protein synthesis is caused by a specific cleavage of 16*S* ribosomal RNA near its 3′ terminus. Recently, one other Clo DF13-specific protein has been purified and characterized as the immunity substance (14; Kool and Nijkamp, manuscript in preparation). With respect to replication, Clo DF13 shows, like other nontransmissible bacteriocinogenic plasmids, a unique dependence on DNA polymerase I (7, 12, 20, 21, 41). DNA polymerase II and III seem not to be essential for the replication of these plasmids (12, 41). We have recently shown that de novo synthesis of chromosomal and Clo DF13-specific proteins is not required for the replication of the Clo DF13 plasmid, whereas de novo synthesis of RNA, and specifically Clo DF13 RNA, is involved in Clo DF13 DNA replication (40). To determine which bacterial functions are involved in the replication of Clo DF13 DNA, the multiplication of Clo DF13

was studied in various *E. coli* strains carrying temperature-sensitive mutations for DNA synthesis. Temperature-sensitive mutants of *E. coli* that are defective in DNA replication have been isolated (3, 18, 28, 33, 42, 43, 47), and the mutations have been placed in seven distinct locations on the *E. coli* chromosome, designated as *dnaA*, *-B*, *-C*, *-D*, *-E*, *-F*, *and -G* (42). Genetic evidence that the *dnaC* and the *dnaD* genes are identical has been reported by Wechsler, using in vivo complementation tests (43).

With regard to the inhibition of chromosomal DNA synthesis at the nonpermissive temperature, the *dna* mutants fall into two classes. The first class of mutants appears to be defective in the initiation of chromosome replication (*dnaA*, *-C, and -D*); they are able to complete rounds of replication at the restrictive temperature. In the other class of mutants, *dna* elongation mutants, DNA synthesis ceases immediately when the organism is placed at the nonpermissive temperature (*dnaB*, *-E*, *-F*, *and -G*). (For a review of the properties of the various temperature-sensitive *dna* mutants see reference 17).

Little is known about the nature of the defective gene products of these thermosensitive DNA replication mutants. The *dnaE* gene product has been identified as DNA polymerase III, the DNA polymerase directly involved in chromosomal DNA replication (9, 29), whereas the *dnaF* gene product has been characterized as ribonucleotide reductase (8). The gene products of *dnaB*, *-C*, (*-D*), and *-G* have been purified, but their enzymatic function in DNA synthesis is unknown (44, 45). With respect to their dependence on host-specified functions, there are remarkable differences between the various extrachromosomal DNA elements. The replication of Col E1 DNA depends on the *dnaA* and *dnaC* gene products, but does not seem to require the elements that are defective at the restrictive temperature in *dnaB* and *dnaE* mutants (10, 11, 13). In contrast, several large plasmids such as Col V, Col Ib, Hly, and the P1-like DNA of *E. coli* 15 are not able to replicate in *dnaB* and *dnaE* mutants (12, 13).

In addition, it has been demonstrated that *dnaA* and *dnaF* mutants can support the growth of bacteriophage ϕX174 at the restrictive temperature, whereas bacteriophage ϕX174 cannot grow in temperature-sensitive *dnaB*, *-C*, *-D*, *-E*, and *-G* mutants at the nonpermissive temperature (25, 27, 37).

Inhibition of protein synthesis by chloramphenicol (CAP), blocks the synthesis of chromosomal DNA (1); however, de novo protein synthesis appeared not to be required for the replication of the Clo DF13 and Col E1 plasmids (4, 40). Recently, it has been demonstrated by Goebel (11) that the inhibition of Col E1 DNA synthesis in the *dnaA* mutant at 43 C is abolished by CAP. This paper reports a study on the replication of the Clo DF13 plasmid in the seven hitherto known *E. coli* mutants defective in the initiation or elongation of chromosomal DNA replication. The ability of Clo DF13 DNA to replicate at 43 C in these mutants, in the presence as well as in the absence of the protein synthesis inhibitior CAP, was examined.

MATERIALS AND METHODS

Bacterial strains. The characteristics and origins of *E. coli* strains used in this study are summarized in Table 1. The nomenclature of genetic markers follows the proposals of Demerec et al. (6) and Taylor and Trotter (38).

Media. Nutrient broth contained (percent, weight/volume): yeast extract (Oxoid), 0.5; NaCl, 0.5; and nutrient broth no. 2 (Oxoid, 0.80 Super nutrient broth contained nutrient broth (above) supplemented with 0.1% (wt/vol) glucose and 30 μg of thymine per ml. Phosphate-buffered minimal medium contained (percent, weight/volume): NH_4Cl, 0.1; KH_2PO_4, 0.15; Na_2HPO_4, 0.35; Casamino Acids (Difco), 0.5; $MgSO_4$, 0.02; glucose, 0.2%; and thiamine-hydrochloride 0.02. It was supplemented, unless otherwise stated, with 10 μg of thymine per ml. Demineralized water was used as solvent.

Conjugal transfer of the Clo DF13 plasmid. The bacteriocinogenic factor DF13 was transferred to the seven temperature-sensitive DNA replication mutants by using S941 HfrR4 harboring the Clo DF13 factor as donor strain. Conjugations were carried out as described previously (41), and the mating mixtures were plated on selective medium. The temperature-sensitive recombinants were examined for the presence of the Clo DF13 plasmid by determining cloacin production according to the double-layer method of Stouthamer and Tieze (36).

Determination of the number of Clo DF13 copies per cell. Strain JC411TS214, temperature-sensitive for DNA polymerase I, was made cloacinogenic in our laboratory (23). The kinetics of segregation of the DNA polymerase I-dependent Clo DF13 plasmid from the cloacinogenic strain JC411 (strain N3165) and the estimation of the number of Clo DF13 copies per cell were as described by Durkacz and Sherratt (7).

Growth and labeling conditions. For radioactive labeling of DNA, the seven temperature-sensitive DNA replications mutants harboring the Clo DF13 plasmid were grown at 30 C to a density of about 3×10^8 cells per ml. The cultures were then shifted to 43 C, and at various times after the temperature shift samples were removed and labeled for 40 min with 10 μCi of [*methyl*-^{3}H]thymidine per ml. After the labeling, the reaction was stopped by the addition of 100 μg of KCN per ml, followed by rapid chilling on ice. Labeled cells were harvested by centrifugation for 15 min at $10{,}000 \times g$.

Preparation of cleared lysates. Lysates were prepared by the procedure of Clewell and Helsinski (5). It

TABLE 1. *Bacterial strains*

Strain	Relevant Properties	Origin or Reference
Escherichia coli		
S941	HfrR4 (Clo DF13)	Tieze et al. (39)
CRT46	F^- *thr leu thy thi ilv lacY mal str*^{R} *dnaA*	Hirota et al. (18)[a]
CRT266	F^- *thr leu thy thi str*^{R} *dnaB*	Y. Hirota
Q91	F^- *leu thy str*^{R} *dnaC*	Carl (3)[b]
Q93	F^- *leu thy str*^{R} *dnaD*	Carl (3)[b]
Q94	F^- *thr leu thy thi str*^{R} *dnaE*	Wechsler and Gross (42)[b]
Q96	F^- *thr leu thy thi str*^{R} *dnaF*	Wechsler and Gross (42)[b]
Q97	F^- *thr leu thy thi str*^{R} *dnaG*	Marinus and Adelberg (28)[b]
JC411 TS214	F^- *leu thy his arg met str*^{R} *polA*$_{ts214}$	Kingsbury and Helinski (21)[c]
N3165	F^- *leu thy his arg met str*^{R} *polA*$_{ts214}$ (Clo DF13)	Kool et al. (24)
DG76	F^- *thr leu str*^{R}	Wolf (47)[d]
Kelbsiella edwardsii		
S15	Sensitive to cloacin DF13	Tieze et al. (39)

[a] Obtained from W. Goebel.
[b] Obtained from M. B. Yarmolinsky.
[c] Obtained from D. J. Sherratt.
[d] Obtained from B Wolf.

essentially involves the lysis of ethylenediaminetetraacetic acid-lysozyme spheroplasts with Brij-58 (final concentration 1%, wt/vol) as detergent. Centrifugation of the crude lysate was performed for 30 min at 48,000 × *g* at 4 C in an MSE 75 or Beckman L5-65 ultracentrifuge. DNA in the supernatant fraction (cleared lysate) was fractionated by cesium chloride-ethidium bromide gradient centrifugation as described previously (41). The percentage of the Clo DF13 DNA released by this technique is 88% or more both at 30 C and 43 C.

Preparation of Sarkosyl lysates. Cells were harvested and suspended in 1 ml of 25% sucrose-0.05 M tris(hydroxymethyl)aminomethane (pH 8.0) and lysed by the method of Clewell (4).

Dye-buoyant density centrifugation. Cesium chloride-ethidium bromide gradients were prepared as described previously (41). Centrifugation was performed in an SW6X5 rotor of an MSE 75 or Beckman L5-65 ultracentrifuge for 60 h at 120,000 × *g* at 15 C. At the end of the run, the gradients were removed from the top of the tube with a saturated $CsSO_4$ solution, and 100-μliter fractions were collected either directly onto Whatman filter disks or in small vials, from which samples were taken for the determination of radioactivity. Fractions containing supercoiled Clo DF13 DNA were pooled from the dye-buoyant density gradients. Cesium chloride and ethidium bromide were removed as described previously (40).

Sucrose density gradients. Linear sucrose density gradients (5 to 20%, wt/vol) were prepared in TES buffer [0.03 M tris(hydroxymethyl)aminomethane, 5 mM ethylenediaminetetraacetic acid, and 0.05 M NaCl, pH 8.0]. After centrifugation in an SW50.1 rotor at 50,000 rpm for 120 min in a Beckman L5-65 ultracentrifuge, 100-μliter fractions were collected from the top of the tube onto Whatman filter disks.

Counting of radioisotopes. Radioactive samples were collected onto Whatman 3MM filter disks and precipitated with trichloroacetic acid as described previously (41). Filters were dried and counted in vials containing 10 ml of a toluene-based scintillation fluid (41) in a liquid scintillation counter (Nuclear-Chicago Mark I or II).

Chemicals. CAP was obtained from Sigma Chemical Co. Pronase (B grade, free from nucleases) and ethidium bromide were from Calbiochem. Dowex H^+ (type 50WX 8, 50 to 100 mesh) was obtained from Serva. [*Methyl*-^{3}H]thymidine (specific activity 40,000 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. Ribonuclease-free sucrose was from Serva and autoclaved before use. The sources of all other chemicals used were as described previously (2).

RESULTS

Replication of Clo DF13 DNA in temperature-sensitive dna initiation mutants. To study replication of the Clo DF13 DNA in *dna* initiation mutants, the temperature-sensitive *E. coli* mutants CRT46*dnaA*, Q91*dnaC*, and Q93*dnaD* were first made cloacinogenic. The conjugal transfer of the Clo DF13 factor, which does not exhibit a sex factor activity, was promoted by *E. coli* S941 HfrR4 harboring the Clo DF13 plasmid. Temperature-sensitive recipients that produced cloacin were isolated and used in further experiments. Cultures of *E. coli* CRT46 (Clo DF13), Q91(Clo DF13), and Q93 (Clo DF13) were grown at 30 C in phosphate-buffered minimal medium to a density of about 3 × 10^8 cells per ml. The cultures were then divided in several equal portions. One part of each culture was kept at 30 C; the others were shifted to 43 C. DNA was labeled for 40 min

with 10 μCi of [*methyl*-^{3}H]thymidine per ml at various times after the temperature shift.

^{3}H-labeled DNA was isolated from the cultures by the lysozyme-Brij 58 technique. The resultant cleared lysates were analyzed in cesium chloride-ethidium bromide gradients. By this procedure supercoiled Clo DF13 DNA is separated from the chromosomal DNA and from the open circular Clo DF13 DNA (41). In most cases the fractions containing supercoiled Clo DF13 DNA were pooled from the dye-buoyant density gradients and then analyzed on neutral sucrose gradients. There was no indication of an appreciable amount of open circular plasmid DNA under these conditions. The amount of ^{3}H radioactivity incorporated into supercoiled Clo DF13 DNA synthesized at the restrictive temperature was determined and compared with the amount of radioactivity incorporated into Clo DF13 DNA in an equal number of exponential-phase cells at 30 C. The amount of radioactivity incorporated into supercoiled Clo DF13 DNA at 30 C was taken as 100%. The results shown in Fig. 1 demonstrate that the synthesis of Clo DF13 DNA decreases in the temperature-sensitive initiation mutants at the restrictive temperature. In the *dnaA* mutant CRT46, Clo DF13 DNA synthesis decreased slowly, suggesting that several new cycles of replication of this plasmid can be initiated at the restrictive temperature (Fig. 1A).

The incorporation of [^{3}H]thymidine into Clo DF13 DNA at the restrictive temperature in the *dnaC* and *dnaD* mutant is quite different from that in the *dnaA* mutant (Fig. 1B, C). The synthesis of Clo DF13 DNA declined rapidly in these mutants and roughly followed the reduction in the incorporation of [^{3}H]thymidine into chromosomal DNA. From these results, we conclude that the replication of the Clo DF13 plasmid depends to a great extent on the host-determined *dnaC* (*dnaD*) product, but depends slightly on the *dnaA* product, since the synthesis of Clo DF13 DNA does not immediately cease in the *dnaA* mutant upon a shift to the nonpermissive temperature.

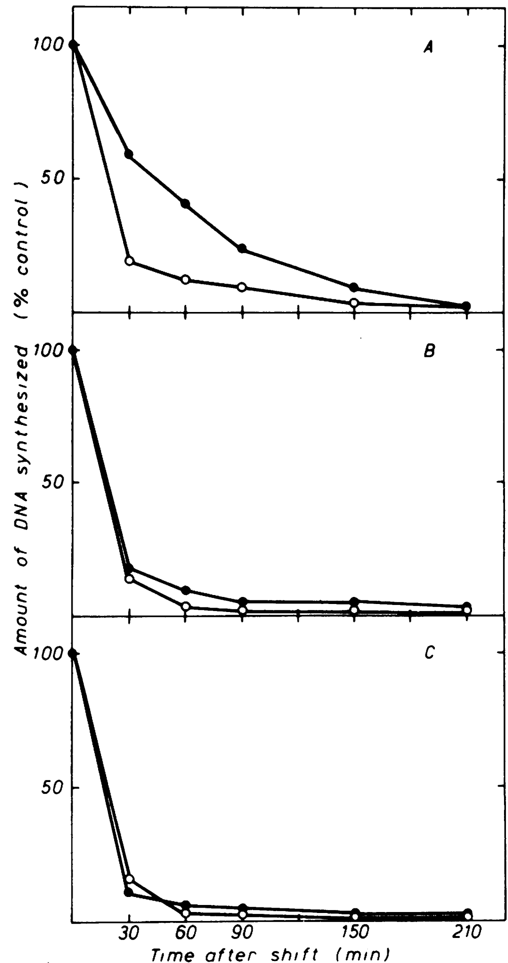

FIG. 1. *Incorporation of [^{3}H]thymidine into chromosomal DNA and Clo DF13 plasmid DNA at 43 C in the dna initiation mutants. Exponential-phase cultures of CRT46, Q91, and Q93, all harboring the Clo DF13 plasmid, were shifted to 43 C and labeled for 40 min with [^{3}H]thymidine at the indicated times. ^{3}H-labeled DNA was isolated and analyzed by cesium chloride-ethidium bromide and as described in Materials and Methods. The amount of DNA synthesized at 43 C is expressed as a percentage of that synthesized at 30 C under the same conditions (set as 100%). (A) DNA synthesis in the dnaA mutant CRT46 (Clo DF13). (B) DNA synthesis in the dnaC mutant Q91 (Clo DF13). (C) DNA synthesis in the dnaD mutant Q93 (Clo DF13). Symbols: ○, chromosomal DNA; ●, Clo DF13 plasmid DNA.*

Replication of Clo DF13 in temperature-sensitive dna elongation mutants. The synthesis of Clo DF13 DNA in the temperature-sensitive *dna* elongation mutants, *E. coli* CRT266*dnaB*, Q94*dnaE*, Q96*dnaF*, and Q97*dnaG*, was examined. To study replication of the plasmid, the same procedure was followed as described in the previous section. The *dna* elongation mutants were first made cloacinogenic by conjugation with *E. coli* S941 HfrR4 (Clo DF13) as donor strain. Cultures of the *dna* elongation mutants, all harboring the Clo DF13 plasmid, were grown in minimal medium at 30 C. At a density of about 3×10^8 cells per ml, the cultures were divided into several parts and shifted to 43 C. DNA was labeled, isolated, and analyzed in cesium chloride-ethidium bromide and sucrose gradients. In contrast to the synthe-

sis of chromosomal DNA, the replication of Clo DF13 DNA does not cease at the restrictive temperature in the temperature-sensitive *dnaB* mutant CRT266 (Fig. 2A). Replication of Clo DF13 DNA in this mutant continued at a reduced level for at least 2 h at 43 C. Clo DF13 DNA synthesized at the nonpermissive temperature in the *dnaB* mutant sedimented in sucrose gradients predominantly as 23.5*S*, representing supercoiled Clo DF13 DNA (Fig. 3A). The synthesis of Clo DF13 DNA continues also in the *dnaE* mutant Q94 at the restrictive temperature (Fig. 2B). As described previously (41), the incorporation of [^{3}H]thymidine into DF13 DNA in this mutant continued during the first 2 h after the temperature shifted from 30 to 43 C. Clo DF13 DNA synthesized under these conditions sedimented in sucrose as 18.5*S* (open circular Clo DF13 DNA) and 23.5*S* (supercoiled Clo DF13 DNA) material (Fig. 3B). At the permissive temperature open circular and covalently closed Clo DF13 DNAs were also synthesized at values of 18.5*S* and 23.5*S* (41), respectively. Thus, in this respect, Clo DF13 DNA synthesized at 43 C in the *dnaB* and *dnaE* mutants does not differ from Clo DF13 DNA synthesized in wild-type cells. The replication of the Clo DF13 plasmid in the thermosensitive *dnaF* and *dnaG* mutants ceased immediately upon a temperature shift from 30 to 43 C (Fig. 2C, D). The experiments described indicate that the replication of the Clo DF13 plasmid depends on the *dnaF* and *dnaG* gene products but does not seem to require the *dnaB* and *dnaE* gene products.

Influence of CAP on Clo DF13 replication in wild-type and dna mutant strains. It has been shown previously that the cloacinogenic plasmid Clo DF13 replicates in *E. coli* cells and minicells in the presence of the protein synthesis inhibitor CAP (40). Before studying the effect of CAP on the Clo DF13 replication in *dna* mutants, we examined further the influence of CAP on the Clo DF13 replication in wild-type cells. Wild-type cells were treated for 12 h with CAP (final concentration 150 μg/ml) and the rate of replication and the number of Clo DF13 copies per cell were estimated. We have shown previously that the Clo DF13 plasmid requires DNA polymerase I for its replication (41); the Clo DF13 factor is unable to replicate at the restrictive temperature (43 C) in strain JC411 (Clo DF13) with a temperature-sensitive DNA polymerase I (strain N3165). Cell divisions of strain JC411 (Clo DF13) occur at the nonpermissive temperature and result in the generation of non-cloacinogenic cells. If the Clo DF13 DNA continues to replicate in the presence of CAP, then plasmid DNA molecules accumulate, resulting in more Clo DF13 copies per cell than in non-CAP-treated cells, and more cell divisions must occur at 43 C before the first plasmid-free cells arise. The kinetics of loss of the Clo DF13 plasmid from CAP-treated and untreated JC411 cells are shown in Fig. 4. In a non-CAP-treated culture of strain JC411 (Clo DF13), no

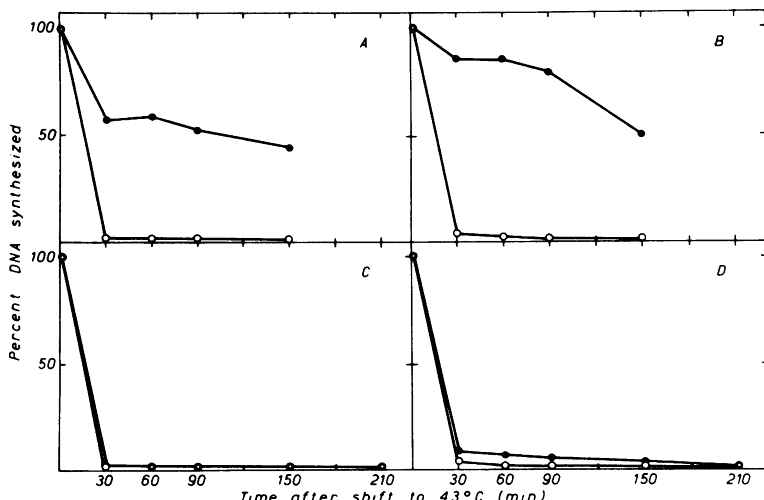

Fig. 2. *Incorporation of [^{3}H]thymidine into chromosomal DNA and Clo DF13 plasmid DNA at 43 C in the dna elongation mutants. DNA was labeled and isolated from the cells as described in the legend to Fig. 1. The percentages are defined in the legend to Fig. 1. (A–D) Amount of DNA synthesis at 43 C in CRT266dnaB (Clo DF13), Q94dnaE (Clo DF13), Q96dnaF (Clo DF13), and Q97dnaG (Clo DF13), respectively. Symbols:* O, *chromosomal DNA;* ●, *Clo DF13 plasmid DNA.*

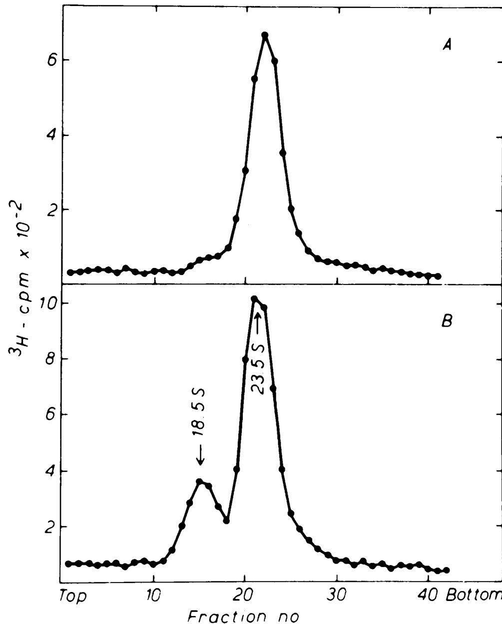

FIG. 3. *Sucrose gradient analysis of supercoiled Clo DF13 DNA synthesized at 43 C in E. coli CRT266dnaB (A) and Q94dnaE (B). Cultures of both dna elongation mutants were shifted to 43 C and labeled after 60 min of incubation at 43 C. DNA was isolated and separated in cesium chloride-ethidium bromide gradients. Fractions containing supercoiled Clo DF13 DNA were subsequently analyzed in neutral sucrose gradients (see Fig. 2). S values are related to the following markers: φX174 (18.4S) and 17S and 26S ribosomal ribonucleic acid.*

plasmid-free cells were observed until the third or fourth generation at 43 C (Fig. 4; see also reference 24). In a culture of strain JC411 (Clo DF13) treated with CAP for 12 h before the shift to the nonpermissive temperature, at least seven or eight cell divisions at 43 C must occur before non-cloacinogenic cells arise. To analyze these data further, we plotted the values from Fig. 4 as described by Durkacz and Sherratt (7).

The slopes in Fig. 5 were drawn from least square fit values. These lines have an intercept on the ordinate of $\log_e(M_o/N_o)$ in which M_o/N_o stands for the average value of Clo DF13 plasmid copies per cell at the moment of the shift to 43 C. The intercepts on the ordinate give a value of about 10 Clo DF13 copies per cell for non-CAP-treated culture, whereas CAP-treated culture contains about 580 Clo DF13 copies per cell. Data from other identical experiments give an average value between 480 and 600 Clo DF13 copies per cell.

Another method of determining the effect of CAP on the number of Clo DF13 copies per cell involved isotopic labeling experiments. To an exponential-phase culture of strain JC411 (Clo DF13) growing in nutrient broth medium containing 0.1% glucose and 10 μCi of [^{3}H]thymidine per ml, 150 μg of CAP was added per ml, and samples were removed at 0, 3, 6, 9 and 12 h. Lysates were prepared by the Sarkosyl procedure and centrifuged to equilibrium in cesium chloride-ethidium bromide density gradients. The gradients were fractionated and the radioactivity was determined. After 3 h of CAP treatment the level of chromosomal DNA remained constant and the amount of ^{3}H radioactivity incorporated into supercoiled Clo DF13 DNA could be related to the amount of incorporation into chromosomal DNA. Figure 6 shows the ratios of plasmid DNA to chromosomal DNA after 3, 6, 9, and 12 h of CAP treatment of JC411 (Clo DF13) cells. After 3 h of exposure to CAP, replication of Clo DF13 DNA occurred linearly for several more hours and then leveled off.

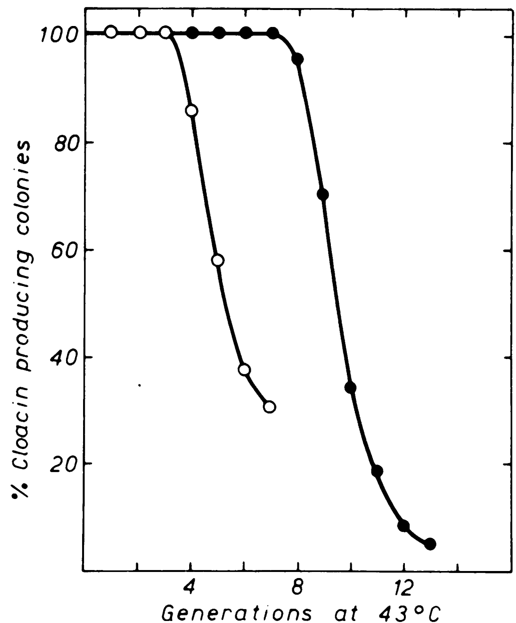

FIG. 4. *Kinetics of loss of the Clo DF13 plasmid from CAP-treated and untreated JC411 polA ts 214 (Clo DF13) cells (strain N3165) at 43 C. Bacteria were grown at 30 C to a density of 10⁸ cells per ml. The cultures were shifted to 43 C and grown for one generation, measured by absorbance increase. The cultures were then diluted 1:1 into prewarmed medium and grown for one more generation at 43 C. This protocol was followed for 14 generations at 43 C. At the intervals indicated, samples were removed, plated and grown at 30 C, and the resulting colonies were tested for the presence of the Clo DF13 plasmid by the double-layer technique. Symbols: ○, JC411 (Clo DF13); ●, JC411 (Clo DF13) treated for 12 h with CAP before the shift to 43 C.*

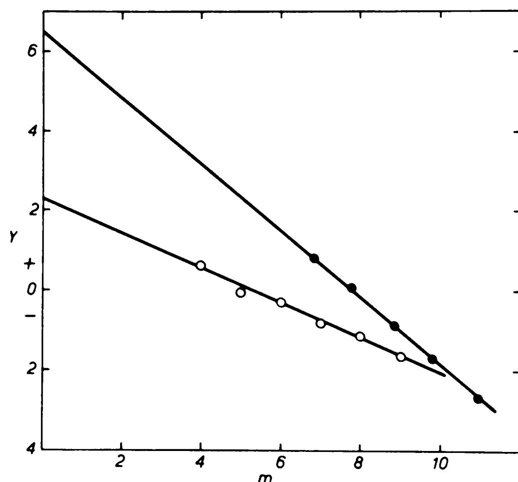

FIG. 5. *Data in Fig. 4 replotted to determine the number of Clo DF13 plasmid copies per cell at the moment of the shift to 43 C.* $Y = \log_e \log_e (1/1 - F_m) \cdot F_m$ *stands for the fraction of cells that contain at least one plasmid after m generations at 43 C. Symbols:* O, *untreated JC411 (Clo DF13);* ●, *CAP-treated JC411 (Clo DF13).*

After 12 h of CAP exposure, the incorporation of [³H]thymidine into Clo DF13 DNA approached a level that represents about 79.2% of the level of chromosomal DNA. In the absence of CAP, 1.7% of cellular DNA was isolated as supercoiled Clo DF13 DNA, which corresponds, as calculated from the segregation kinetics experiments, to about 10 Clo DF13 copies per cell. These results indicate that JC411 (Clo DF13) cells, treated for 12 h with CAP, contain on the average 47 times more Clo DF13 plasmids per cell than non-CAP-treated cloacinogenic JC411 cells, corresponding to about 470 copies per cell. From the results obtained with both methods, we conclude that when de novo protein synthesis is inhibited by CAP the Clo DF13 plasmid continues to replicate for at least 12 h, resulting in an accumulation of Clo DF13 DNA molecules of about 500 copies per cell.

In nutrient broth medium, cells have a normal doubling time at 30 C of 50 min. A net doubling of Clo DF13 DNA per cell in one generation corresponds to the synthesis of about 10 copies per 50 min. From these data an average rate of 0.2 Clo DF13 copies per min can be calculated. The rate of Clo DF13 replication in the presence of CAP was estimated by the method of Clewell (4). The rates were calculated from the slope of the line in Fig. 6; the difference between the number of Clo DF13 copies at two arbitrarily chosen time points was determined and divided by the time elapsed between the two points. After CAP treatment, the replication rate of Clo DF13 DNA increased, resulting in a maximal average rate of 1.0 copies per min. This implies that the rate of replication in the presence of CAP is approximately five times the rate of replication in the absence of CAP.

We wanted to determine whether inhibition of de novo protein synthesis by CAP affected the Clo DF13 DNA synthesis at 43 C in temperature-sensitive *dna* mutants. It has been demonstrated recently that CAP restores the synthesis of Col E1 DNA at 43 C in *dnaA* mutants (11). To study Clo DF13 plasmid replication in the presence of CAP, exponential-phase cultures of the *dna* initiation and *dna* elongation mutants were shifted to 43 C and labeled after various periods of time. CAP was added at the moment of the temperature shift from 30 to 43

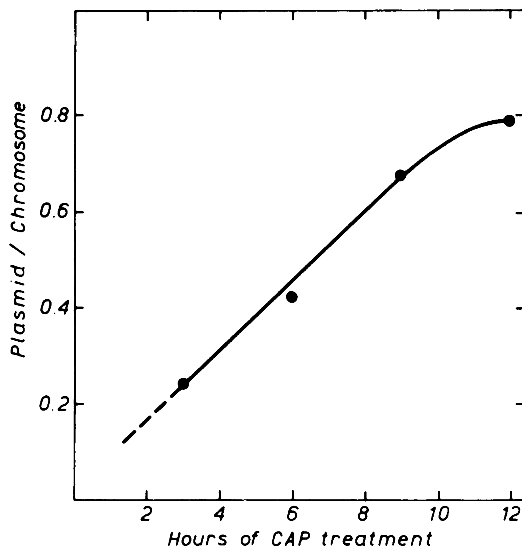

FIG. 6. *Synthesis of Clo DF13 DNA during treatment with CAP. A culture of E. coli JC411 (Clo DF13) was grown at 30 C in nutrient broth medium containing [³H]thymidine. CAP was added to the culture, and at the indicated times samples were removed and lysed by the Sarkosyl procedure. Lysates were centrifuged to equilibrium in cesium chloride-ethidium bromide gradients. The ratio of plasmid DNA to chromosomal DNA was estimated on the basis of the areas enclosed in two peaks of the dye-buoyant density gradients. After 3 h of CAP exposure, replication of chromosomal DNA ceased, and it is reasonable to assume that the level of chromosomal DNA remained constant, in which case the amount of Clo DF13 plasmid DNA can be measured relative to the level of chromosomal DNA. After 3 h of CAP treatment, the ratio of Clo DF13 DNA (138,075 counts/min) to chromosomal DNA (600,172 counts/min) is 0.23.*

C at a final concentration of 5 μg/ml. ^{3}H-labeled DNA was isolated and analyzed as described. As shown in Fig. 7A, addition of CAP led to a considerable increase in the level of Clo DF13 synthesis in the *dnaA* mutant CRT46 (Clo DF13). Clo DF13 DNA synthesis was stimulated in the first 30 min after the temperature shift and continued for more than 3 h at an almost uninhibited level. Higher concentrations of CAP (150 μg/ml) did not influence this pattern significantly. In contrast, there was no stimulatory effect by CAP on the Clo DF13 DNA synthesis in the *dnaC* mutant Q91 at 43 C (Fig. 7B). Inhibition of protein synthesis by CAP did not influence the level of Clo DF13 DNA synthesis at the restrictive temperature in the *dna* elongation mutants (figures not shown). The results obtained with the different temperature-sensitive DNA replication mutants are summarized in Table 2.

DISCUSSION

The replication of the cloacinogenic plasmid Clo DF13 has been studied in seven thermosensitive *dna* mutants of *E. coli*. In the *dnaA* initiation mutant, synthesis of Clo DF13 DNA decreased slowly at 43 C; however, replication of the Clo DF13 plasmid stopped immediately in the *dnaC* and *dnaD* initiation mutants upon a shift to 43 C. These data indicate that the synthesis of Clo DF13 DNA depends to a great extent on the *dnaC* (*dnaD*) gene product, but depends slightly on the *dnaA* gene product. Recently it has been demonstrated by Goebel (11) that the replication of the Col E1 plasmid depends strictly on the *dnaA* gene product, whereas Col E1 DNA synthesis decreases slowly at 43 C in the *dnaC* mutant. Thus, with regard to the replication in *dna* initiation mutants, the Clo DF13 plasmid clearly differs from the Col E1 plasmid. The Clo DF13 plasmid does not require the *dnaB* function for its replication. There are data that suggest that the defect in the *dnaB* mutant involves an alteration in the cell membrane (19, 35). A possible explanation for the observation that Clo DF13 DNA replicates at 43 C in the *dnaB* mutant could be that a membrane replication site(s) involved in Clo DF13 DNA synthesis differs from that involved in the replication of chromosomal DNA. Another possibility is that the Clo DF13 plasmid as phage P1 (Yarmolinsky, personal communication) determines a product analogous in function to the protein determined by the *dnaB* gene of its host, *E. coli*.

With respect to replication, there is a remarkable difference between transmissible plasmids, such as Col V, Col Ib, and P1-like DNA, and nontransmissible plasmids, such as Clo DF13, Col E1, and minicircular DNA, of *E. coli* 15. Whereas transmissible plasmids seem to require the *dnaB* gene product for their replication, Clo DF13, like Col E1 and minicircular DNA of *E. coli* 15, replicates at 43 C in the *dnaB* mutant (13; this report). In addition, the Clo DF13 plasmid depends for its replication on DNA

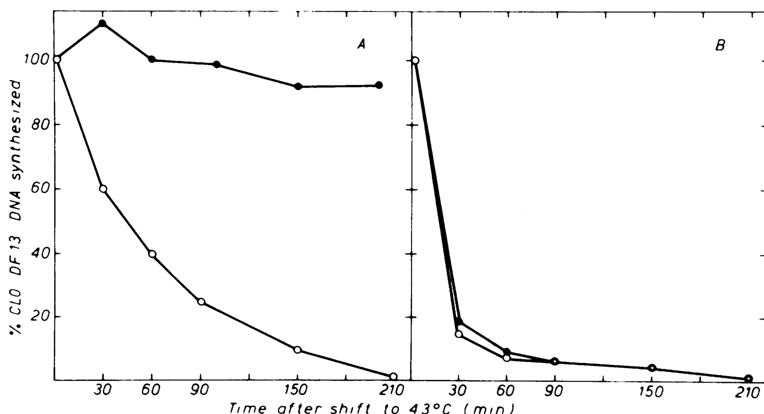

FIG. 7. *Synthesis of Clo DF13 DNA in the dna initiation mutants at 43 C in the presence of CAP. Cultures of E. coli CRT46dnaA (Clo DF13) and Q91dnaC (Clo DF13) were grown at 30 C to a density of 3 × 10⁸ cells per ml. The cultures were then shifted to 43 C, and CAP at a final concentration of 5 μg/ml was added at the moment of the shift. Isolation of Clo DF13 plasmid DNA was the same as described in the legend to Fig. 1. (A) Clo DF13 DNA synthesis at 43 C in CRT46 (Clo DF13) in the presence (●) and absence (○) of CAP. (B) Clo DF13 DNA synthesis at 43 C in Q91 (Clo DF13) in the presence (●) and absence (○) of CAP. The percentage Clo DF13 DNA synthesized is expressed as a percentage of that synthesized at 30 C in the absence of CAP (set as 100%, corresponding to 48,253 counts/min).*

polymerase I, whereas DNA polymerase III, which is the replicase of chromosomal DNA and DNA of transmissible plasmids, seems not to be essential (12, 41, this report). The replication of the Clo DF13 plasmid requires the function of ribonucleotide diphosphate reductase, which has been shown to be the protein defective in *dnaF* mutants (8). The replication of the Clo DF13 factor also depends on the *dnaG* gene product. Lark (26) has presented data indicating that this product is involved in the elongation process of chromosomal DNA replication, possibly by controlling the initiation of the discontinuously synthesized DNA segments in the daughter strand. Inhibition of protein synthesis by CAP blocks the replication of chromosomal DNA (1); however, de novo protein synthesis appeared not to be required for the replication of the Clo DF13 plasmid. When wild-type cells were treated with CAP, the Clo DF13 plasmid continued to replicate for at least 12 h, resulting in an accumulation of the number of Clo DF13 plasmids per cell. Estimations of the number of Clo DF13 copies per cell show that cells treated with CAP for 12 h contain about 500 copies per cell, whereas untreated cells contain only 10 Clo DF13 copies per cell. After 3 h of CAP treatment, the Clo DF13 plasmid replicated at a rate approximately five times the average rate that would be required for a net doubling of Clo DF13 DNA per cell in one generation.

An explanation for this stimulatory effect of CAP could be that the indirect inhibition of chromosomal DNA replication makes more replicating enzymes accessible to Clo DF13 DNA. The Clo DF13 plasmid depends for its replication on DNA polymerase I (41). This replicase is normally present to the extent of about 400 molecules per cell (31); therefore it is difficult to imagine that this enzyme is the rate-limiting factor. Another possibility is that the replication of the Clo DF13 plasmid is negatively regulated. Several investigators have proposed that the regulation of DNA synthesis is negatively controlled (2, 30, 32). If initiation of Clo DF13 DNA synthesis were under negative control by a replication repressor, then inhibition of protein synthesis by CAP treatment would block the synthesis of the repressor leading to a derepressed situation with an increased frequency of initiation. According to Clewell (4), the Col E1 DNA also replicates at an increased rate in the presence of CAP, whereas it has been demonstrated by Sherratt and Helinski (34) that the replication time in CAP is not significantly different to that in exponentially growing

TABLE 2. *Replication of Clo DF13 DNA in temperature-sensitive dna mutants of Escherichia coli*

Strain[a]	Properties	Clo DF13 DNA synthesis	
		43 C, −CAP	43 C, +CAP
DG76	Wild type	+	+
CRT46	*dnaA*	−	+
CRT266	*dnaB*	+	+
Q91	*dnaC*	−	−
Q93	*dnaD*	−	−
Q94	*dnaE*	+	+
Q96	*dnaF*	−	−
Q97	*dnaG*	−	−

[a] The characteristics and origins of the bacterial strains are described in Table 1.

cells. These data can be explained by assuming that in the presence of CAP the frequency of initiation of Col E1 DNA synthesis is increased. The synthesis of Clo DF13 DNA is restored by CAP in the *dnaA* mutant at the restrictive temperature. Whereas in wild-type cells the Clo DF13 plasmid replicates in the presence of CAP at a rate five times the rate under non-CAP conditions, the rate of Clo DF13 DNA synthesis in the *dnaA* mutant at 43 C in the presence of CAP does not differ much from the rate of Clo DF13 DNA synthesis in this mutant at 30 C in the absence of CAP. Apparently rate-limiting factors are still present. The function of the *dnaA* gene product is still unknown. Our results, however, indicate that the *dnaA* gene product is not acting in a direct, positive way on the initiation of Clo DF13 DNA replication. As suggested by Goebel (11), the *dnaA* gene product could act as an "antirepressor" substance, removing the repressor from the origin. However, there is little experimental evidence at present to suggest which model, the negative control or positive control model, is correct for the regulation of Clo DF13 replication. Studies are in progress to get more insight in the regulation of Clo DF13 replication.

ACKNOWLEDGMENTS

We are grateful to M. B. Yarmolinsky, Y. Hirota, W. Goebel, B. Wolf, and D. J. Sherratt for supplying bacterial strains and to A. J. Kool for his interest and suggestions. The excellent technical assistance of C. A. M. Hazenberg and L. Meijers-van Moppes throughout this work is very much appreciated.

This investigation was supported by the Netherlands Foundation for Chemical Research (SON), with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

LITERATURE CITED

1. Bazaral, M., and D. R. Helinski. 1970. Replication of a

bacterial plasmid and an episome in *Escherichia coli*. Biochemistry **9**:399-406.

2. Blau, S., and J. Mordoh. 1972. A new element in the control of DNA initiation in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **69**:2895-2898.
3. Carl, P. L. 1970. *Escherichia coli* mutants with temperature-sensitive synthesis of DNA. Mol. Gen. Genet. **109**:107-122.
4. Clewell, D. B. 1972. Nature of Col E1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667-676.
5. Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Nat. Acad. Sci. U.S.A. **62**:1159-1166.
6. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**:61-76.
7. Durkacz, B. W., and D. J. Sherratt. 1973. Segregation kinetics of colicinogenic factor Col E1, from a bacterial population temperature-sensitive for DNA polymerase I. Mol. Gen. Genet. **121**:71-75.
8. Fuchs, J. A., H. O. Karlström, H. R. Warner, and P. Reichard. 1972. Defective Gene product in a *dnaF* mutant of *Escherichia coli*. Nature N. Biol. **238**:69-71.
9. Gefter, M. L., Y. Hirota, T. Kornberg, J. A. Wechsler, and C. Barnoux. 1971. Analysis of DNA polymerase II and III in mutants of *Escherichia coli* thermosensitive for DNA synthesis. Proc. Nat. Acad. Sci. U.S.A. **68**:3150-3153.
10. Goebel, W. 1970. Studies on extrachromosomal elements: Replication of the colicinogenic factor Col E1 in two temperature-sensitive mutants of *Escherichia coli* defective in DNA replication. Eur. J. Biochem. **15**:311-320.
11. Goebel, W. 1974. Studies on the initiation of plasmid DNA replication. Eur. J. Biochem. **41**:51-62.
12. Goebel, W., and H. Schrempf. 1972. Replication of the minicircular DNA of *E. coli* 15 is dependent on DNA polymerase I but independent on DNA polymerase III. Biochem. Biophys. Res. Commun. **49**:591-601.
13. Goebel, W., and H. Schrempf. 1972. Replication of plasmid DNA in temperature-sensitive DNA replication mutants of *Escherichia coli*. Biochem. Biophys. Acta **262**:32-41.
14. Graaf, F. K. de., and P. Klaasen-Boor. 1974. Purification and characterization of the cloacin DF13 immunity protein. FEBS Lett. **40**:293-297.
15. Graaf, F. K. de., H. G. D. Niekus, and J. Klootwijk. 1973. Inactivation of bacterial ribosomes *in vivo* and *in vitro* by cloacin DF13. FEBS Lett. **35**:161-165.
16. Graaf, F. K. de., R. J. Planta, and A. H. Stouthamer. 1971. Effect of a bacteriocin produced by *Enterobacter cloacae* on protein synthesis. Biochem. Biophys. Acta **240**:122-137.
17. Gross, J. D. 1972. DNA replication in bacteria. Curr. Top. Microbiol. Immunol. **57**:39-74.
18. Hirota, Y., J. Mordoh, and F. Jacob. 1970. On the process of cellular division in *Escherichia coli*. III. Thermosensitive mutants of *E. coli* altered in the process of DNA initiation. J. Mol. Biol. **53**:369-387.
19. Inouye, M., and J. Guthrie. 1969. A mutation which changes a membrane protein of *E. coli*. Proc. Nat. Acad. Sci. U.S.A. **64**:957-961.
20. Kingsbury, D. T., and D. R. Helinski. 1970. DNA polymerases as a requirement for the maintenance of the bacterial plasmid colicinogenic factor E1. Biochem. Biophys. Res. Commun. **41**:1538-1545.
21. Kingsbury, D. T., and D. R. Helinski. 1973. Temperature-sensitive mutants for the replication of plasmids in *Escherichia coli*: requirement for deoxyribonucleic acid polymerase I in the replication of the plasmid Col E1. J. Bacteriol. **114**:1116-1124.
22. Kool, A. J., and H. J. J. Nijkamp. 1972. Proteins synthesized by a noninduced bacteriocinogenic factor in minicells of *Escherichia coli*. Mol. Gen. Genet. **115**:314-323.
23. Kool, A. J., and H. J. J. Nijkamp. 1974. Isolation and characterization of a copy mutant of the bacteriocinogenic plasmid Clo DF13. J. Bacteriol. **120**:569-578.
24. Kool, A. J., M. S. van Zeben, and H. J. J. Nijkamp. 1974. Identification of messenger ribonucleic acids and proteins synthesized by the bacteriocinogenic factor Clo DF13 in purified minicells of *Escherichia coli*. J. Bacteriol. **118**:213-224.
25. Kranias, E. G., and L. B. Dumas. 1974. Replication of Bacteriophage ϕX174 DNA in a temperature-sensitive *dnaC* mutant of *Escherichia coli* C. J. Virol. **13**:146-154.
26. Lark, K. G. 1972. Genetic control over the initiation of the synthesis of the short deoxy-nucleotide chains in *E. coli*. Nature N. Biol. **240**:237-240.
27. Manheimer, J., and N. Truffaut. 1974. Growth of Bacteriophage ϕX174 in *Escherichia coli* strains carrying temperature-sensitive mutations for DNA initiation. Mol. Gen. Genet. **130**:21-27.
28. Marinus, M. G., and E. A. Adelberg. 1970. Vegetative replication and transfer replication of deoxyribonucleic acid in temperature-sensitive mutants of *Escherichia coli* K-12. J. Bacteriol. **104**:1266-1272.
29. Nüsslein, V., B. Otto, F. Bonhoeffer, and H. Schaller. 1971. Functions of DNA polymerase III in DNA replication. Nature N. Biol. **234**:285-286.
30. Pritchard, R. H., P. T. Barth, and J. Collins. 1969. Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. **19**:263-297.
31. Richardson, C. C., C. L. Schildkraut, H. V. Aposhian, and A. Kornberg. 1964. Enzymatic synthesis of deoxyribonucleic acid. XIV. Further purification and properties of deoxyribonucleic acid polymerase of *Escherichia coli*. J. Biol. Chem. **239**:222-232.
32. Rosenberg, B. H., L. F. Cavalieri, and G. Ungers. 1969. The negative control mechanism for *E. coli* DNA replication. Proc. Nat. Acad. Sci. U.S.A. **63**:1410-1417.
33. Schubach, W. H., J. D. Whitmer, and C. I. Davern. 1973. Genetic control of DNA initiation in *Escherichia coli*. J. Mol. Biol. **74**:205-221.
34. Sherratt, D. J., and D. R. Helinski. 1973. Replication of Colicinogenic factor E1 in *Escherichia coli*. Properties of newly replicated supercoils. Eur. J. Biochem. **37**:95-99.
35. Siccardi. A., B. Shapiro, Y. Hirota, and F. Jacob. 1971. On the process of cellular division in *Escherichia coli*. IV. Altered protein composition and turnover of the membranes of thermosensitive mutants defective in chromosomal replication. J. Mol. Biol. **56**:475-490.
36. Stouthamer, A. H., and G. A. Tieze. 1966. Bacteriocin production by members of the genus *Klebsiella*. Antonie van Leeuwenhoek J. Microbiol. Serol. **32**:171-182.
37. Taketo, A. 1973. Sensitivity of *Escherichia coli* to viral nucleic acid. VI. Capacity of *dna* mutants and DNA polymerase-less mutants for multiplication of ϕA and ϕX174. Mol. Gen. Genet. **122**:15-22.
38. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* strain K-12. Bacteriol. Rev. **36**:504-524.
39. Tieze, G. A., A. H. Stouthamer, H. S. Jansz, J. Zandberg, and E. F. J. van Bruggen. 1969. A bacteriocinogenic factor of *Enterobacter cloacae*. Mol. Gen. Genet. **106**:48-65.
40. Veltkamp, E., W. Barendsen, and H. J. J Nijkamp. 1974. Influence of protein and ribonucleic acid synthesis on

the replication of the bacteriocinogenic factor Clo DF13 in *Escherichia coli* cells and minicells. J. Bacteriol. **118**:165–174.

41. Veltkamp, E., and H. J. J. Nijkamp. 1973. The role of DNA polymerase I, II and III in the replication of the bacteriocinogenic factor Clo DF13. Mol. Gen. Genet. **125**:329–340.
42. Wechsler, J. A., and J. D. Gross. 1971. *Escherichia coli* mutants temperature-sensitive for DNA synthesis. Mol. Gen. Genet. **113**:273–284.
43. Wechsler, J. A., V. Nüsslein, B. Otto, A. Klein, F. Bonhoeffer, R. Herrmann, L. Gloger, and H. Schaller. 1973. Isolation and characterization of thermosensitive *Escherichia coli* mutants defective in deoxyribonucleic acid replication. J. Bacteriol. **113**:1381–1388.
44. Wickner, S., J. Berkhower, M. Wright, and J. Hurwitz. 1973. Studies on *in vitro* DNA synthesis. Purification of the *dnaC* gene product from *Escherichia coli*, Proc. Nat. Acad. Sci. U.S.A. **70**:2369–2373.
45. Wickner, S., M. Wright, and J. Hurwitz. 1973. Studies on *in vitro* DNA synthesis. Purification of the *dnaG* gene product from *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **70**:1613–1618.
46. Wright, M., S. Wickner, and J. Hurwitz. 1973. Studies on *in vitro* DNA synthesis. Isolation of the *dnaB* gene product from *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **70**:3120–3124.
47. Wolf, B. 1972. The characteristics and genetic map location of a temperature-sensitive DNA mutant of *E. coli* K12. Genetics **72**:569–593.