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Direction of Deoxyribonucleic Acid Replication in *Escherichia coli* Under Various Conditions of Cell Growth

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The direction of chromosome replication in a temperature-sensitive initiation mutant of *Escherichia coli* (CT28) is shown autoradiographically to be bidirectional. This mode of replication persists even when the rate of replication is reduced by slow growth in succinate minimal medium or in the presence of chloramphenicol. Therefore, although the rate of replication can be affected by certain physiological stimuli, the topology of replication need not be.

Over a wide range of growth conditions with generation times between 22 and 50 min, it is believed that the overall rate of bacterial chromosome replication is regulated by the frequency of initiation and not by the rate of deoxyribonucleic acid (DNA) synthesis at each replication fork (2, 6, 7, 16, 24). Although the rate of DNA synthesis appears to be independent of the rate of growth at these generation times, as the generation time increases beyond 50 min, so does the transit time for replicating forks (3, 9, 12, 23). An increase in the transit time for chromosome replication could be accounted for by a decrease in the rate of polymerization and/or a decrease in the initiation frequency for Okazaki fragment (3). Whereas it has been shown that a limited supply of certain DNA precursors decreases the rate of DNA synthesis (5, 14, 15, 17, 20), it is still not clearly understood which of these two possibilities is actually affected.

This investigation is designed to test an alternative explanation for the slower rates of chromosome replication which are associated with slow rates of cell growth. Steady-state [^{3}H] thymine incorporation studies, along with DNA autoradiography, are employed to determine if slower rates of replication are due to a switch in the topology of replication from bidirectional to unidirectional. In these experiments, slow rates of cell growth are achieved by substituting for glucose the low-energy yielding carbon sources succinate and aspartate.

A third physiological condition is achieved with the use of chloramphenicol at a concentration which inhibits gross cellular protein synthesis. The rate of DNA synthesis under this condition has been shown to be approximately one-half that seen in the untreated cells (1, 13).

MATERIALS AND METHODS

Bacterial strains. CT28 is a reversible temperature-sensitive DNA initiation mutant derived from the *Escherichia coli* K-12 F$^-$ strain AB1157. At 42 C, CT28 completes ongoing rounds of DNA replication but fails to initiate new rounds (22). CT28 carries a temperature-sensitive defect in addition to that affecting DNA initiation; however, its effect is not apparent at 25 C.

Media. The minimal medium used in these experiments was that described by Hershey (8). The medium was buffered with 0.05 M phosphate buffer, pH 7.1, and was supplemented with 60 μg of arginine, histidine, leucine, threonine, and proline/ml, 0.2 μg of thiamine/ml, and 2 μg of thymine/ml. As a source of carbon, 10% solutions of glucose and sodium succinate (sterilized separately) were added to the medium to a final concentration of 0.2%. Aspartate minimal medium was prepared by adding 0.2 g of aspartic acid to 100 ml of the supplemented minimal medium, which was then sterilized.

Determination of cell division times. Division times were determined spectrophotometrically with a Beckman model DB spectrophotometer set at a wavelength of 550 nm. Cells were maintained for at least 10 generations in each type of medium at a temperature of 25 C. At intervals, 3-ml samples were taken from a well-aerated culture and the turbidity was measured as a function of absorbance (550 nm). Absorbance values for stationary-phase cultures were also determined and used as a measure of yield for each type of medium.

Radioactive determination of DNA synthesis. Cells from glucose, succinate, and aspartate cultures were transferred to their respective minimal media, which contained 2.2 μg of [^{3}H]thymine/ml (New England Nuclear Corp., Boston, Mass.) at a specific activity of 0.37 Ci/mmol. After seven generations in each type of radioactive minimal medium, the cultures, which had a density of approximately 10^8 cells/ml, were transferred to the restrictive tempera-

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ture (i.e., 42 C). At intervals after the shift to 42 C, 20-μl samples were taken and dispensed into 4-ml portions of cold 5% trichloroacetic acid containing 1 mg of thymine/ml. Samples were filtered onto two glass fiber filters (Millipore Corp.) and assayed by the procedure described by Schubach et al. (22).

Labeling of cells for autoradiography. Cells were grown in each type of minimal medium at 25 C to a concentration of 10^8 cells/ml. The cells were harvested by centrifugation and suspended in fresh medium at a concentration of 2×10^8 cells/ml. DNA replication in each case was terminated at 42 C for 90 min. To a 0.1-ml sample of each terminalized cell culture was added 0.1 ml of the respective minimal medium containing 4.3 μg of [^{3}H]thymidine/ml (13.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.). The cultures were incubated at 25 C to initiate synchronous rounds of chromosome replication for 12.5, 15, and 20 min for glucose, succinate, and aspartate minimal media, respectively. After each interval 2-μl aliquots of the labeled cells were transferred to 0.2 ml of the appropriate media containing 1.05 μg of [^{3}H]thymidine/ml (57 Ci/mmol). Each aliquot of labeled cells was pulsed with the high-specific-activity radioactive media (final specific activity, 54.8 Ci/mmol) for 5 min except for the glucose culture, which was labeled for 2.5 min. In the case where chromosomes were radioactively labeled in the presence of chloramphenicol, glucose-grown cells were terminalized at 42 C for 74 min and then radioactively labeled with the following modifications. The 0.1 ml of low-specific-activity [^{4}H]thymidine medium contained 300 μg of chloramphenicol/ml while the 0.2 ml of high-specific-activity [^{3}H]thymidine medium contained 150 μg of chloramphenicol/ml. The terminalized cells were reinitiated in the low-specific-activity medium for 10 min at 25 C, after which 2 μl of the cells was transferred to the high-specific-activity medium for 2.5 min at 42 C. As a control, a terminalized culture of glucose-grown cells was reinitiated at 25 C and labeled for 12.5 min with only the low-specific-activity label. In all instances, labeling of the chromosomes was quickly stopped by the addition of a 2-ml solution of cold 0.01 M tris(hydroxymethyl)aminomethane, 0.01 M ethylenediaminetetraacetate, 0.01 M KCN (pH 8.1) containing unlabeled "carrier" cells at a concentration of 10^8 cells/ml.

Preparation of labeled cells for DNA autoradiography. Following the procedure described by Prescott and Kuempel (18), the labeled cells were washed twice by centrifugation and resuspended in fresh 0.01 M tris(hydroxymethyl)aminomethane, 0.01 M ethylenediaminetetraacetate, and 0.01 M KCN, pH 8.1. The cells were converted to spheroplast by treatment with 100 μg of lysozyme/ml (Calbiochem) at 0 C for 30 min. By the technique of Lark et al. (11), chromosomes were spread by mixing a 20-μl drop of spheroplast with an equal volume of 3% sodium dodecyl sulfate, 0.01 M tris(hydroxymethyl)aminomethane, 0.01 M ethylenediaminetetraacetate, pH 8.1, and after 10 min, by pulling the mixture down a glass slide with a stainless steel rod. The slides had been previously coated with a subbing solution of 0.1% gelatin and 0.01% chrome alum. After the lysing mixture had dried, the chromosomes were fixed by dipping the slides into solutions of cold 5% trichloroacetic acid, 70% ethanol, and 100% ethanol, in that order.

Preparation of autoradiographs. The slides were coated with Ilford L4 photographic emulsion (Ilford, Essex, England) which had been diluted 1:1 with glass-distilled water. After an exposure period of 45 to 60 days at 5 C, the autoradiographs were developed for 4 min at 20 C in Kodak D19 developer, stopped for 10 s in 1% acetic acid, and fixed with Kodak rapid fix for 2.5 min. The autoradiographs were photographed with a Zeiss automatic photomicroscope camera on Kodak high-contrast copy film.

RESULTS

Rate of cell growth. The results of growth rate determinations for glucose-, succinate-, and aspartate-grown cultures of CT28 are shown in Table 1. It can be seen that the generation times for CT28 grown in succinate and aspartate minimal media are considerably longer than the glucose-grown culture. However, unlike the results obtained by Urban and Lark (23), the aspartate cultures are found to have a generation time slightly shorter than the succinate cultures, although aspartate is clearly shown to be the poorest carbon source in terms of the yield.

Kinetics of DNA synthesis. Before succinate- and aspartate-grown cells of CT28 were prepared for autoradiography, steady-state labeling studies were performed to ensure that the terminalization and reinitiation characteristics of this strain were retained under these growth conditions, as well as to verify the reduced rate of replication previously reported by others (9, 21, 23). As shown in Fig. 1B and C, succinate and aspartate cultures of CT28 continue to terminalize and reinitiate chromosome replication as observed in glucose cultures (Fig. 1A). From extrapolating the slopes of the curves shown in Fig. 1A, B, and C, it can be seen that the time required for the completion of reinitiated rounds of replication at the slower growth rates is approximately 60 min while in glucose

TABLE 1. *Generation time*

Carbon source	Time at 25 C (min)	Yield[a] (%)
Glucose	180	100
Succinate	288	50.6
Aspartate	264	13.2

[a] In this instance, yield is defined in terms of the absorbancy at 550 nm ($A_{550\,nm}$) for stationary-phase cultures of CT28 instead of grams (cells) per milliliter of culture. The $A_{550\,nm}$ values for glucose, succinate, and aspartate stationary-phase cultures are 1.24, 0.63, and 0.16, respectively.

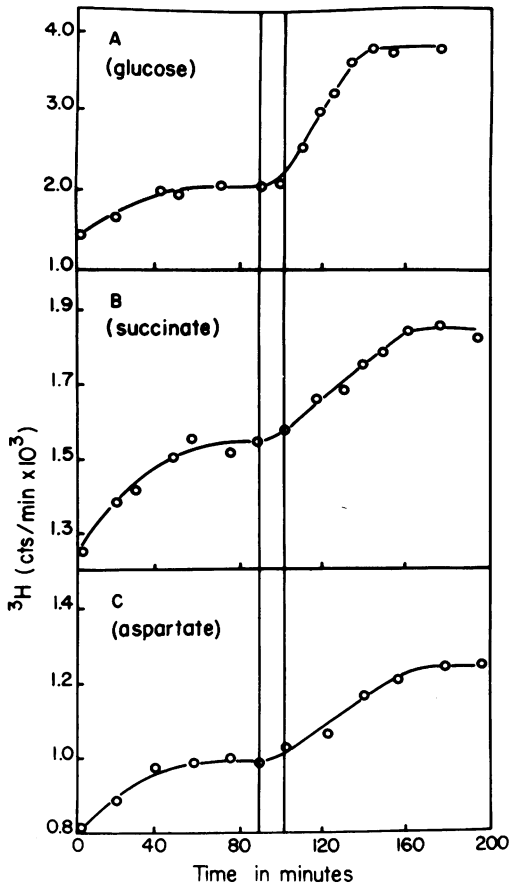

FIG. 1. *Kinetics of DNA synthesis in glucose, succinate, and aspartate cultures. CT28 was grown from an initial concentration of 10^6 cells/ml to a final concentration of 10^8 cells/ml in 1-ml cultures of glucose (A), succinate (B), and aspartate (C) minimal medium, supplemented with [^{3}H]thymine/ml (0.3 Ci/mmol). Each culture was placed at the restricted temperature (42 C) for 90 min to terminalize ongoing rounds of replication. After this period at 42 C, replication was reinitiated by a 10-min period at the permissible temperature (25 C), after which the cultures were placed back at 42 C to allow new rounds of replication to terminalize once again. Samples were taken as indicated and assayed by the procedure described in Materials and Methods.*

cultures this process is shown to take only 40 min. These data clearly show that the rate of chromosome replication decreases at slower growth rates.

Another distinct feature of chromosome replication in succinate and aspartate cultures is the increment of residual DNA synthesis that occurs as the cells are transferred to the restrictive temperature. In glucose cultures this increment amounts to 39%, whereas it is only 22.5% for succinate and 23% for aspartate cultures. According to Pritchard and Zaritsky (20), the increment in residual DNA synthesis can be related to the average frequency of initiation per replication fork transit time (n) by the expression: percentage of residual DNA synthesis $= (2^n \times n \times ln\ 2)/(2^n - 1) - 1 \times 100$. The increments in residual DNA synthesis correspond to an average of 1.0, 0.6, and 0.6 initiations per chromosome for glucose, succinate, and aspartate cultures, respectively (Fig. 1A-C). These data can be interpreted as suggesting that only about 60% of the cells in a slow-growing culture are synthesizing DNA at any instant. A similar conclusion has been drawn by Maaløe and Kjeldgaard (16) based on whole-cell autoradiography of slow-growing cultures of *E. coli*.

After a 10-min period at 25 C, it can be seen that the amount of DNA synthesis which is reinitiated in succinate and aspartate cultures is only 20 and 25%, respectively, as compared to the 95% increase observed in the glucose culture. This discrepancy may reflect the capacity of CT28 to synthesize initiator protein under these stringent conditions of growth (22).

Kinetics of DNA synthesis in the presence of chloramphenicol. As mentioned earlier, CT28 has the potential to reinitiate new rounds of chromosome replication in the presence of chloramphenicol at concentrations which inhibit further production of initiator protein (22). When chloramphenicol is added to cultures of terminalized cells (final concentration, 150 μg/ml) just before new rounds of replication are reinitiated by a 10-min shift to the permissive temperature, a decrease in rate of DNA synthesis can be observed (Fig. 2, curve B). This decreased rate results in an increase in chromosome replication time from 45 min to 83 min. The additional decrease in the DNA synthesis rate observed for the control and chloramphenicol-treated cells left at 25 C after reinitiation (curves C and D) is presumed to be due to the large temperature difference between permissive and restrictive temperatures.

Whereas the control cells which were reinitiated by a 10-min period at 25 C show a 99% increase in DNA synthesis (curve A), DNA synthesis in the chloramphenicol-treated cells (curve B) increased to only 80%. This reduced level of synthesis may be due to the premature terminalization of DNA replication which has been shown to occur in the presence of various concentrations of chloramphenicol (10). However, curve D (chloramphenicol-treated cells at

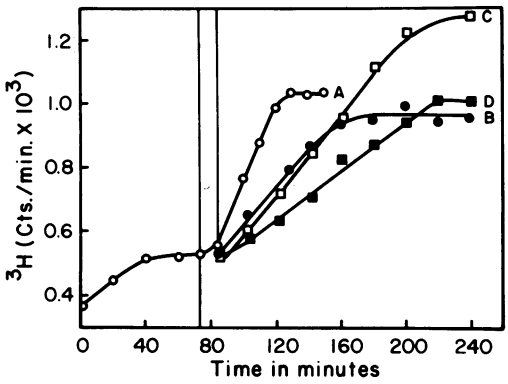

FIG. 2. *Kinetics of DNA synthesis in the presence of 150 μg of chloramphenicol/ml. CT28 was grown from an initial concentration of 10^6 to 10^8 cells/ml in a 4-ml culture of glucose minimal medium supplemented with [^{3}H]thymine/ml (0.37 Ci/mmol). The culture was placed at 42 C for 74 min to terminalize ongoing rounds of chromosome replication. The culture was then split into four equal subcultures (A, B, C, and D), two of which (B and D) received 0.01 ml of chloramphenicol (15 mg of chloramphenicol/ml prepared in glucose minimal medium). DNA replication in subcultures A and B was reinitiated by a 10-min period at 25 C while subcultures C and D were left at 25 C after reinitiation. Subculture A, without chloramphenicol; subculture B, with chloramphenicol; subculture C, without chloramphenicol; subculture D, with chloramphenicol.*

25 C) shows no significant reduction in residual synthesis as compared to the control subculture (curve A).

Autoradiography of differentially labeled chromosomes. To inquire whether or not the increased times for a round of replication shown in Fig. 1 and 2 involve a switch to unidirectional replication, chromosomes were differentially labeled with different specific activities of [^{3}H]thymidine according to the rationale of Prescott and Kuempel (18). By their procedure, chromosomal origins of CT28 were first labeled with 13 Ci of [^{3}H]thymidine per mmol for a short period followed by another short period of labeling with 54 Ci of [^{3}H]thymidine per mmol. This labeling scheme represents a differential in specific activities of 4.2. The autoradiographic patterns expected from unidirectional and bidirectional replication are diagrammatically represented in Fig. 3A, and B.

Autoradiographs of chromosome origins labeled under each experimental condition are displayed in Fig. 4 and 5. Control-labeled chromosomes (i.e., glucose cultures labeled with low-specific-activity [^{3}H]thymidine only) show no regions of high-grain density (Fig. 4A). Glucose (Fig. 4B) and succinate-grown cultures (Fig. 5A), as well as chloramphenicol-treated cultures (Fig. 5B), display grain-density patterns that are clearly consistent with the bidirectional mode of replication shown in Fig. 3B. Although a number of autoradiographic experiments have been performed on aspartate culture of CT28, interpretable autoradiographic figures for this condition have yet to be found.

Of those interpretable figures observed, approximately 30% displayed only one region of high grain density similar to that pattern shown in Fig. 3A. The proportion of these figures did not vary significantly from one experimental condition to another.

Measurements of high grain density length, which are referred to as "pulse" lengths, are presented in Table 2. All measurements were made on interpretable autoradiographic figures bearing two regions of high grain density (Fig. 3B). Since the differences in pulse lengths represent different periods of radioactive labeling as well as variations in the rate of DNA synthesis, these measurements cannot be compared until they are converted to replication rates (i.e., pulse lengths in micrometers per labeling period).

Assuming that the maximum pulse lengths represent chromosome replication at its maximum rate, these values along with their respective labeling periods can be used to calculate relative replication rates whose differences can be compared. Such a comparison of the values given in Table 2 reveal that while the replication rates for succinate and chloramphenicol-treated glucose cultures are similar, they are considerably slower than the rate determined for glucose cultures.

DISCUSSION

Autoradiographic evidence displayed in Fig. 4 and 5 demonstrates that chromosome replication in glucose, succinate, and chloramphenicol-treated cultures of CT28 proceeds bidirectionally and therefore does not support the hypothe-

FIG. 3. *A diagrammatic representation of the grain-density patterns that would be expected to appear in autoradiographs of the E. coli chromosome as a result of (A) unidirectional and (B) bidirectional replication. According to the labeling schedule described in the text, the heavy line segments represents DNA strands which have incorporated high-specific-activity label and the thin lines represent single DNA strands that were labeled with low-specific-activity label.*

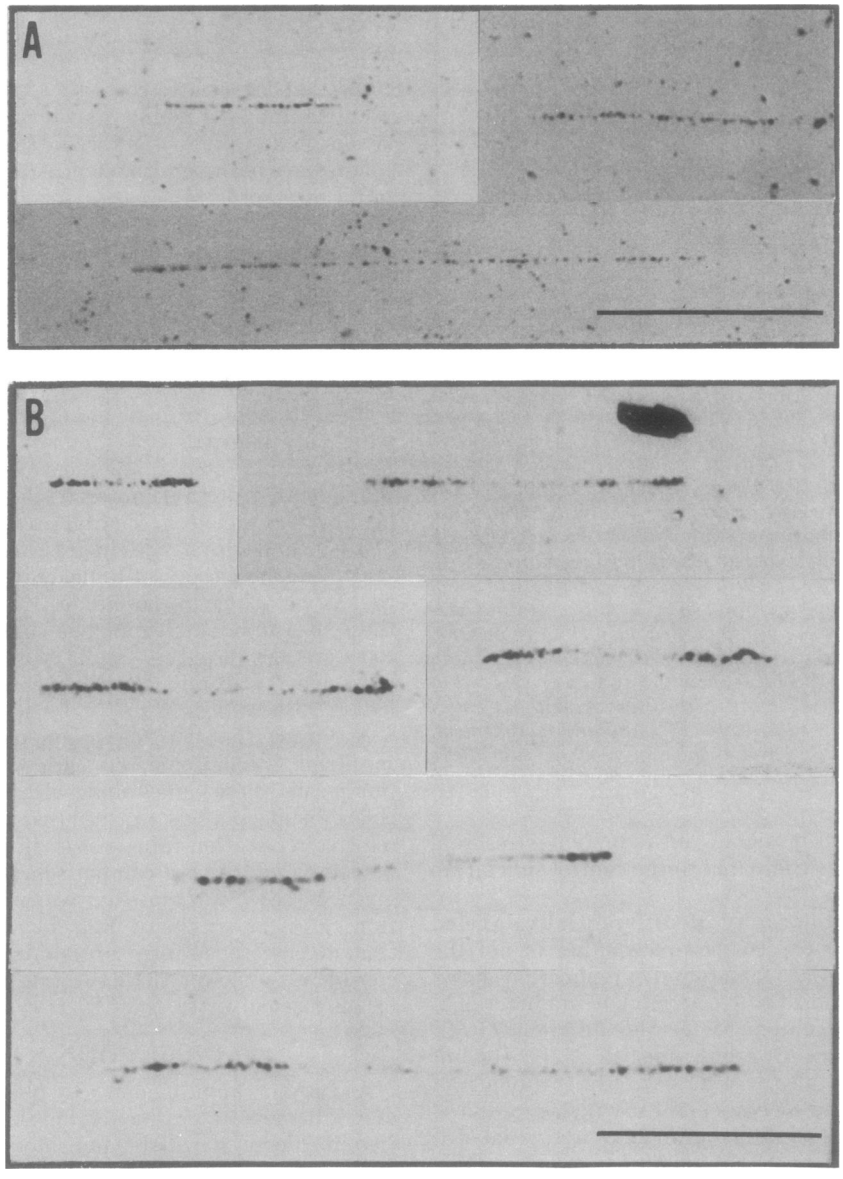

FIG. 4. *Autoradiographs of the origin region of the E. coli chromosome. (A) Control-labeled glucose cultures; (B) differentially labeled glucose cultures.*

sis that the topology of chromosome replication can be modulated by these physiological stimuli. However, since approximately 30% of all interpretable autoradiographs observed displayed only one region of high grain density (as illustrated in Figure 3A), the possibility that some chromosomes replicated unidirectionally cannot be completely ruled out. Since the proportion of these autoradiographic figures did not vary significantly from one experimental condition to the next, an equally plausible explanation would be that they represent chromosome fragments having one region of high grain density missing. The proportion of these fragments may reflect the degree of shear damage associated with the chromosome spreading technique. Furthermore, it should be mentioned that these findings may not be applicable to exponentially grown cells. Since chromosome synchronization is known to affect the cellular concentration of initiation factors (22), there may be other factors affecting the direction of

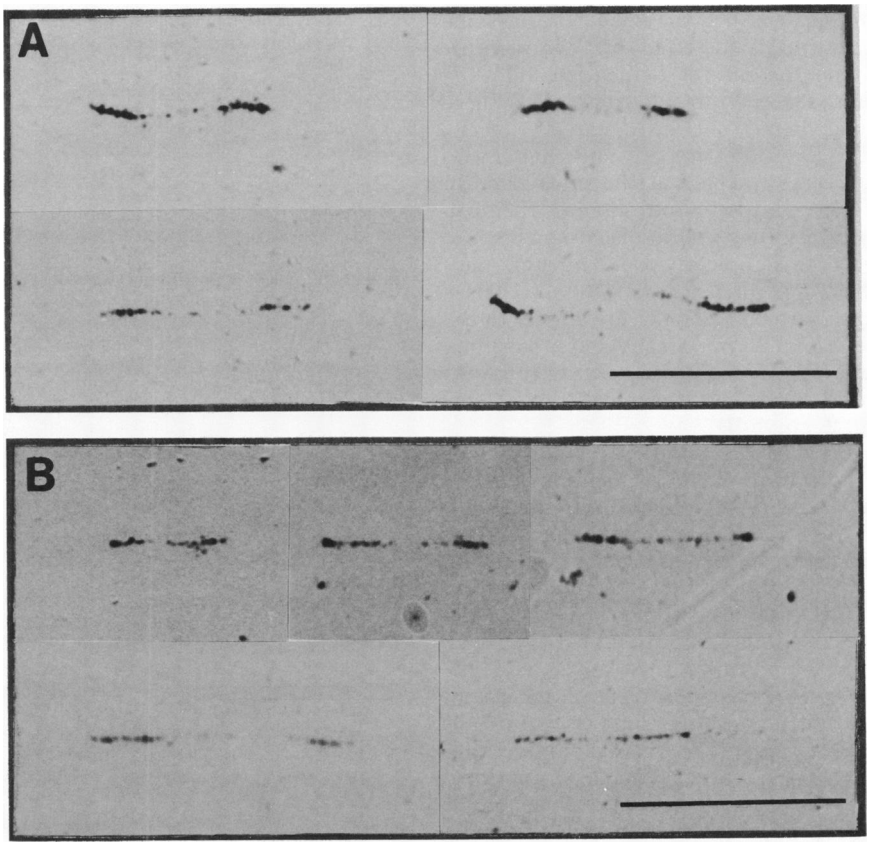

FIG. 5. *Autoradiographs of the origin region of the E. coli chromosome. Differentially labeled (C) succinate and (D) chloramphenicol-treated glucose cultures.*

TABLE 2. *Pulse lengths*

Carbon source	No. of interpretable figures observed	Avg length (μm)	Maximum length (μm)	Rate of replication (μm/min)[a]
Glucose	98	16.2	34	13.6
Succinate	91	10.5	20	4.1
Glucose and chloramphenicol	58	10.5	17	6.8

[a] Replication rates were calculated from maximum lengths and apply to only one replication fork.

replication which are not normally found in unsynchronized cultures of bacteria.

Since the control-labeled chromosomes (Fig. 4A) displayed no regions of high grain density, the possibility that these regions might be due to folding of DNA strands is unlikely. However, the twofold variation in pulse lengths observed in each experimental condition suggests that these regions could be fragmented or stretched. If stretching of DNA strands was the cause for the variation in pulse lengths, this would be revealed by an inverse relationship between the number of silver grains per micrometer of DNA and the length of the pulsed region. Such a quantitative analysis was not feasible in this instance because of the high density of silver grains in the pulsed region. The fact that length variations could be seen between two pulsed regions on the same autoradiographic figure can also be interpreted as being due to stretching or fragmentation.

The reduced rate of replication observed in succinate and aspartate cultures as compared to glucose cultures is consistent with the findings of others (9, 12, 23). Furthermore, the slower rates of replication calculated from pulse lengths for succinate and chloramphenicol-treated cultures are analogous with the reduced rate determined from the steady-state DNA labeling studies (Fig. 1B and 2, curve B). Unfortunately, due to the lack of certainty

concerning the length of the *E. coli* chromosome (19, 21), as well as the effect of a temperature hysteresis on the rate of replication following reinitiation (23), the rates determined by these two means should not be equated directly. However, it is interesting to note that if two replication forks traveled at the rates given in Table 2 for glucose- and chloramphenicol-treated cultures, they would take 40 and 80 min, respectively, to completely replicate a chromosome with a contour length of 1100 μm. These replication times correspond very closely to the times determined by steady-state labeling, which were 40 min for glucose cultures (Fig. 1A) and 83 min for chloramphenicol-treated cultures (Fig. 2, curve B).

We conclude in this report that while the rate of DNA replication is shown to be reduced in succinate- and chloramphenicol-treated cultures, the direction of DNA replication remains unchanged. Our conclusions are not in complete agreement with those of Chandler et al. (4), who have found the rate of chromosome replication to be nearly constant in cultures of *E. coli* K-12 having generation times between 22 and 220 min. Although we have observed an increase in the replication fork transit time in cultures of CT28 with generation times of 264 and 288 min, it should be noted that this increase is not proportional to the attendant increase in generation time. We feel that this situation may reflect the refractory nature of the DNA replicating machinery in *E. coli*.

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