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Characterization of a Temperature-sensitive Mutant of *Bacillus subtilis* Defective in Deoxyribonucleic Acid Replication

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In this paper we present a preliminary characterization of a temperature-sensitive mutant of *Bacillus subtilis* which appears to be defective in deoxyribonucleic acid (DNA) replication at high temperature. When log-phase cells of the mutant were transferred from 30 to 45 C, protein synthesis and ribonucleic acid synthesis continued more or less normally for several hours, whereas DNA synthesis continued at a normal rate for only 20 to 30 min and then was drastically reduced. The amount of DNA synthesized prior to this reduction corresponded approximately to the amount of DNA synthesized under conditions of protein synthesis inhibition by the parent or mutant strain. After 1 hr of growth at high temperature, cells of the mutant showed a pronounced drop in viable count. After 30 or 60 min of growth at high temperature, DNA synthesis could be restored by lowering the temperature. A longer period of growth at 45 C led to a loss of reversibility of DNA synthesis. Spores of the mutant synthesized no DNA when germinated at high temperature, although an outgrowing cell appeared. When spores were germinated at low temperature until DNA synthesis began, and then were transferred to high temperature, macromolecular synthesis continued as the log-phase transfer experiments described above.

Although an extensive literature now exists concerning biophysical and biochemical aspects of deoxyribonucleic acid (DNA) replication, very little is currently known concerning the mechanism of DNA replication in vivo. The analysis of temperature-sensitive phage mutants defective in DNA replication has provided considerable insight in this area (2, 3, 12). Temperature-sensitive mutants of *Escherichia coli* and of *Saccharomyces cerevisiae* defective in DNA replication have also been described (4, 6, 7).

We have undertaken a study of the DNA replication process in *Bacillus subtilis* by analysis of temperature-sensitive mutants defective in DNA replication. Characterization of the first such mutant we have isolated (mutant 168ts-134) constitutes the subject of this report.

MATERIALS AND METHODS

Bacteria. The parent strain (designated 168ts$^+$) is the indole$^-$ thymine$^-$ mutant of *B. subtilis* strain 168. Mutant 168ts-134 is a temperature-sensitive mutant derived from 168ts$^+$.

Isolation of mutant 168ts-134. During attempts to develop an enrichment procedure for temperature-sensitive mutants defective in DNA synthesis, similar to that of Bonhoeffer and Schaller (1), we observed that *B. subtilis* 168ts$^+$ is more rapidly killed by incubation with 5-bromouracil (BU) than by the absence of thymine. We have used this observation to try to enrich for the desired mutants by the following procedure. Cells of 168ts$^+$ were grown overnight at 30 C in enriched minimal medium, washed free of thymine, and resuspended at about 10^8 colony-forming units per ml in the presence of 20 μg of BU per ml. The culture was incubated at 45 C for 3 hr, at which time it had dropped about 2 log units in viability. (A thymine starved culture showed little if any loss of viability at this time.) The culture was then washed free from BU and resuspended at 30 C in enriched minimal medium containing 20 μg of thymine per ml. After overnight growth at 30 C, the culture was diluted and plated on yeast-tryptone (YT) agar. The colonies which arose after incubation overnight at 30 C were replica-plated on prewarmed (45 C) YT plates. The replicas were examined after 6 hr of incubation at 45 C. Colonies present on the 30 C master plate but absent on the 45 C replica were picked and streaked for further testing.

Media. Minimal medium consisted of grams (per liter): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 14.0; KH_2PO_4, 6.0;

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sodium citrate·$2H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; glucose, 5.0; and Casamino Acids, 0.1. Enriched minimal medium consisted of minimal medium with 10.0 g of Casamino Acids plus 3.0 g of L-glutamic acid and 3.0 g of L-asparagine per liter. L-Tryptophan at a final concentration of 20 μg/ml and thymine at a final concentration of 10 μg/ml were added as required.

Complex media for plating consisted of YT agar containing (grams per liter): tryptone, 10.0; yeast extract, 5.0; and agar, 15.0 (all Difco products).

Growth of cells. Bacterial populations were grown as liquid tube cultures with aeration. The growth temperatures used were 30 and 45 C. Scattered-light measurements with an EEL nephelometer (Evans Electroselenium Ltd., Halstead, Essex, England) served as a measurement of protein synthesis.

Kinetics of DNA and ribonucleic acid (RNA) synthesis. The incorporation of ^{14}C-thymine into cold 5% trichloroacetic acid-insoluble components was determined as a measure of DNA synthesis. Similarly, ^{14}C-uracil incorporation was used as a measure of RNA synthesis. Samples (0.1 ml) were withdrawn from the cultures into 10 ml of cold 5% trichloroacetic acid. After 1 hr, the acid-insoluble components were collected on HA filters (Millipore Corp., Bedford, Mass.) and washed with additional cold 5% trichloroacetic acid. The dried filters were counted in a Nuclear-Chicago gas-flow counter.

Viability. The viable count was determined by plating on YT agar and incubating at 30 C. Standard plating techniques were used.

Morphology. Cells were sampled into 1% formaldehyde. Wet mounts were examined with a Reichert phase-contrast microscope fitted with a Wild high-resolution oil immersion objective.

Spore studies. Phase-bright spores were obtained according to the method described by Yoshikawa (14). After germination, cell outgrowth was observed in a microscope. DNA synthesis was followed by ^{14}C-thymine incorporation as described above.

RESULTS

In the experiment presented in Fig. 1, we have examined the kinetics of DNA synthesis (^{14}C-thymine incorporation), scattered-light increase, and change in viable-cell count upon transferring cultures of 168ts$^+$ and 168ts-134 from 30 to 45 C. The cultures were grown at 30 C in medium containing ^{14}C-thymine and were transferred to 45 C in medium of the same specific activity of ^{14}C-thymine with or without 40 μg of chloramphenicol per ml. Figure 2 gives the results of an experiment in which incorporation of ^{14}C-uracil was studied as a measure of RNA synthesis. In this experiment, label was added to the cells at the time of transferring to 45 C. Details of the experimental procedures are given in the legends to the figures.

Figures 1 and 2 show that mutant 168ts-134 synthesized RNA and protein at a rate similar to the parent strain for at least 1 hr after transfer to 45 C. On the other hand, DNA synthesis proceeded at a normal rate for only 20 to 30 min and was thereafter severely reduced. The amount of

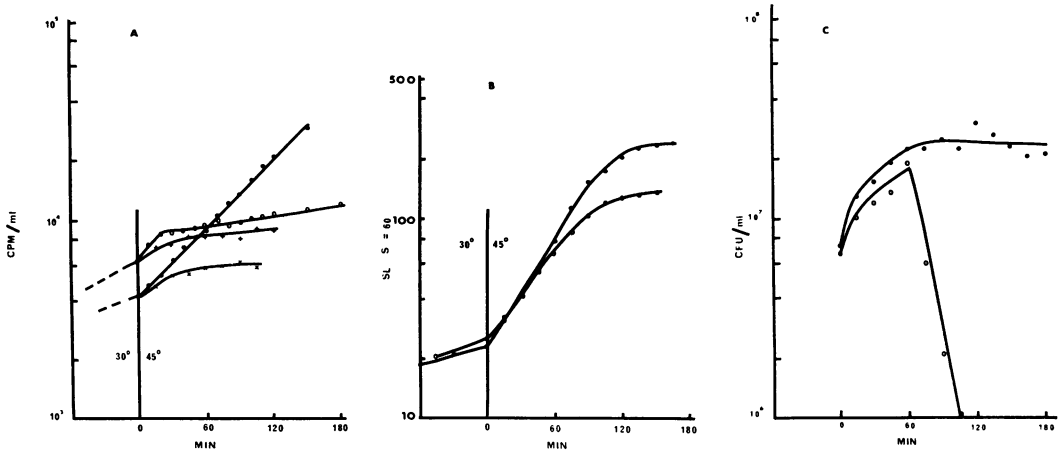

FIG. 1. *Kinetics of DNA synthesis, scattered-light increase, and cell viability upon transfer of 168ts$^+$ and 168ts-134 from 30 to 45 C. Log-phase cultures grown at 30 C in enriched minimal medium containing 0.085 μc of thymine-2-^{14}C and 10 μg of ^{12}C-thymine per ml were divided into three portions and transferred to 45 C. One portion was used for scattered-light readings. The second was sampled at various times for acid-precipitable radioactivity and viable count. The third portion, which received 40 μg of chloramphenicol per ml at time zero was sampled at various times for acid-precipitable counts only. (A) ^{14}C-thymine incorporation into acid-precipitable material (counts per min per milliliter of sample). (B) Scattered-light readings relative to a standard of 60. The standard corresponds to about 2 × 10^7 colony-forming units per ml of log-phase cells growing at 30 C in enriched minimal medium. (C) Viable count: ●, 168ts$^+$; ○, 168ts-134; ×, 168ts$^+$ with 40 μg of chloramphenicol per ml; +, 168ts-134 with 40 μg of chloramphenicol per ml.*

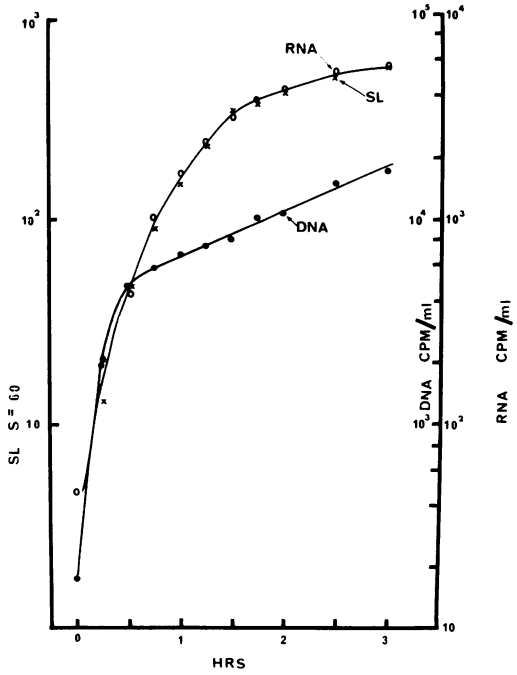

FIG. 2. *Kinetics of DNA, RNA, and scattered-light increase upon transfer of 168ts-134 from 30 to 45 C. A log-phase culture grown at 30 C in enriched minimal medium was divided into three portions and transferred to 45 C at time zero. One portion received 0.170 μc of thymine-2-^{14}C per ml at time zero. The second portion received 0.050 μc of uracil-2-^{14}C per ml at time zero. Both were sampled at various times for acid-precipitable radioactivity. The third portion was used for scattered-light (SL) readings. The scattered-light data are plotted as the increase in scattered light so as to be directly comparable to the RNA data.*

DNA synthesis at 45 C by 168ts-134 prior to the reduction in rate represented approximately a 40% increase over the amount present at the time of transfer to 45 C. This increase was approximately the same as that observed when either the mutant or the parent strain was transferred to 45 C in the presence of chloramphenicol to inhibit protein synthesis. The viable count data (Fig. 1) show that the number of viable cells in the culture of 168ts-134 increased somewhat during the 1st hr after transfer to 45 C and then decreased dramatically. The failure of 168ts$^+$ to increase in viable count after the 1st hr is due to the formation of chains.

In the experiment presented in Fig. 3, we have examined the effect of tryptophan deprivation on the residual DNA synthesis by 168ts-134 at 45 C. The mutant strain was grown at 30 C in the presence of ^{14}C-thymine, washed, and resuspended in the same medium without tryptophan. The culture was divided into two portions which were transferred to 45 C. One portion received tryptophan immediately. At 60 min, tryptophan was again added to a portion of the tryptophan-starved culture.

In the presence of tryptophan, 168ts-134 showed, as before, a two-phase incorporation curve with a sharp reduction in the rate of DNA synthesis at 25 min after transfer to 45 C. Tryptophan deprivation had little, if any, effect on the first phase of DNA synthesis at 45 C. However, it completely abolished the low level of continued synthesis observed after 30 min. Addition of tryptophan at 60 min immediately restored this low rate of DNA synthesis.

Figure 4 presents data on the reversibility of the inhibition of DNA synthesis in the mutant. A culture was grown at 30 C in enriched minimal medium containing ^{14}C-thymine, transferred to 45 C, and sampled at intervals for acid-precipitable radioactivity and cell viability. In addition,

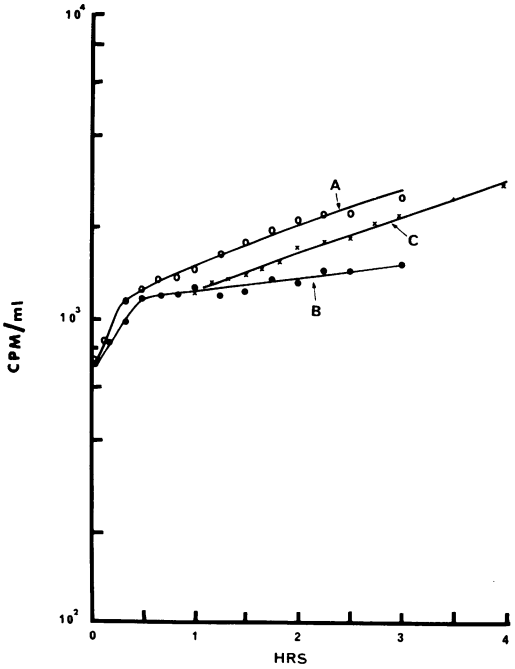

FIG. 3. *Effect of tryptophan starvation on DNA synthesis by 168ts-134 at 45 C. Log-phase cells grown at 30 C in minimal medium containing ^{14}C-thymine as in Fig. 1 were washed free from tryptophan by filtration, resuspended in the same medium without tryptophan, and divided into two portions, A and B, which were transferred to 45 C. Portion A received 20 μg of tryptophan per ml; B received no tryptophan. After 60 min, 20 μg of tryptophan per ml was added to a portion of B (tube C). Each tube was sampled at intervals for acid-precipitable radioactivity.*

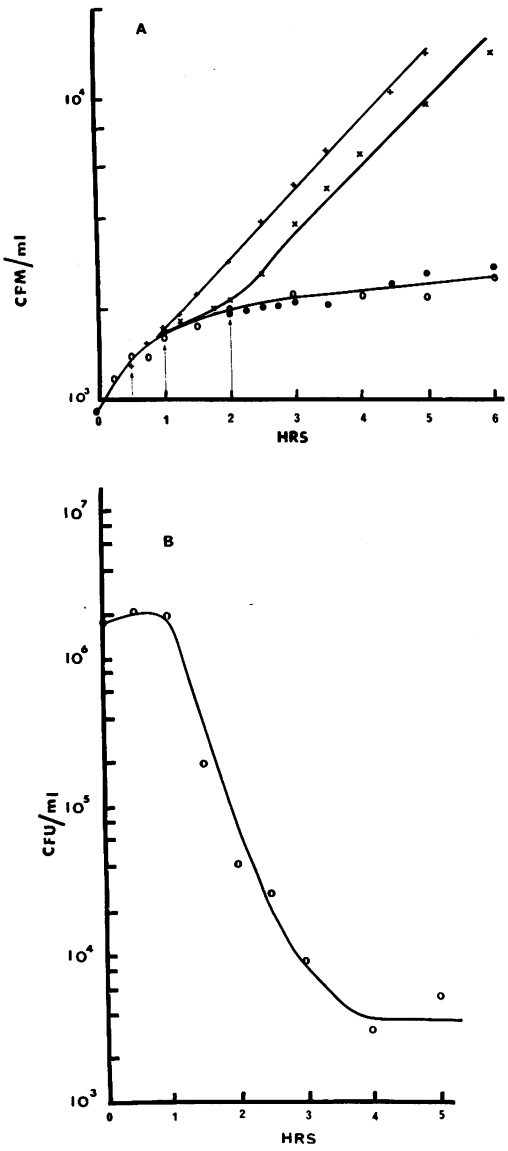

FIG. 4. *Reversibility of inhibition of DNA synthesis in 168ts-134. Log-phase cells grown at 30 C in enriched minimal medium containing ^{14}C-thymine as in Fig. 1 were transferred to 45 C at time zero and sampled at various times for acid-precipitable radioactivity and viable count. At 30, 60, and 120 min, as indicated by the arrows, samples were transferred to 30 C and assayed at intervals for acid-precipitable radioactivity. (A) ^{14}C-thymine incorporation. (B) Viable count.*

portions of the culture were returned to 30 C after 30, 60, and 120 min at 45 C, and were sampled in the same way. The results show that temperature reversal after 30 min permitted immediate resumption of DNA synthesis at the normal 30 C rate. Reversal at 60 min led to similar recovery after a lag of about 30 min. Temperature reversal after 2 hr at 45 C, by which time there had been extensive decrease of viable count (Fig. 4B), permitted no detectable recovery of DNA synthesis.

The loss of viable count which occurred when 168ts-134 was incubated for more than 1 hr at 45 C was at least partially inhibited by the addition of chloramphenicol, 80 μg/ml (Fig. 5). The appearance of the cells after 4 hr of incubation with and without chloramphenicol at 45 C is presented in Fig. 6. Mutant 168ts-134 showed peculiar irregularities and swellings, which were also typical of thymine-requiring cells after prolonged thymine starvation. The morphological abnormalities did not occur after incubation at high temperature in the presence of chloramphenicol. The temperature-sensitive character of mutant 168ts-134 was not dependent on the thymine requirement, since several thymine-independent revertants still showed the same temperature sensitivity.

The synthesis of DNA during germination and outgrowth of spores of 168ts-134 has been ex-

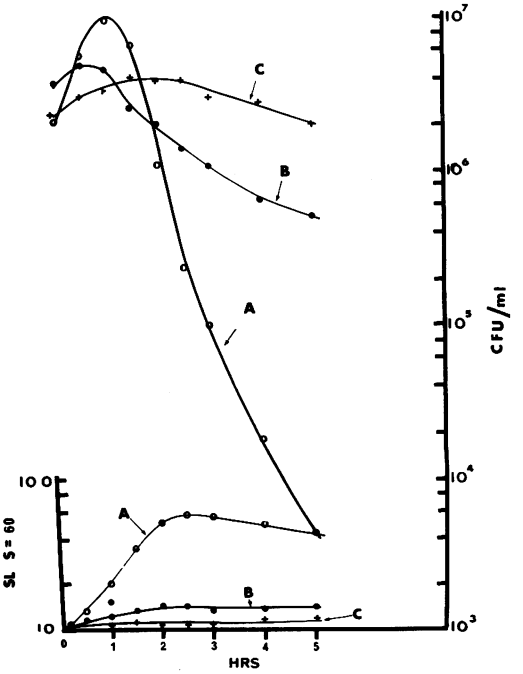

FIG. 5. *Effect of chloramphenicol on viability of 168ts-134. A log-phase culture of 168ts-134 grown at 30 C was divided into three portions: A received no additions and was transferred to 45 C; B received 80 μg of chloramphenicol per ml and was transferred to 45 C; C received 80 μg of chloramphenicol per ml and was incubated at 30 C. Each tube was sampled at various intervals for viability (main figure) and scattered light readings made (lower insert).*

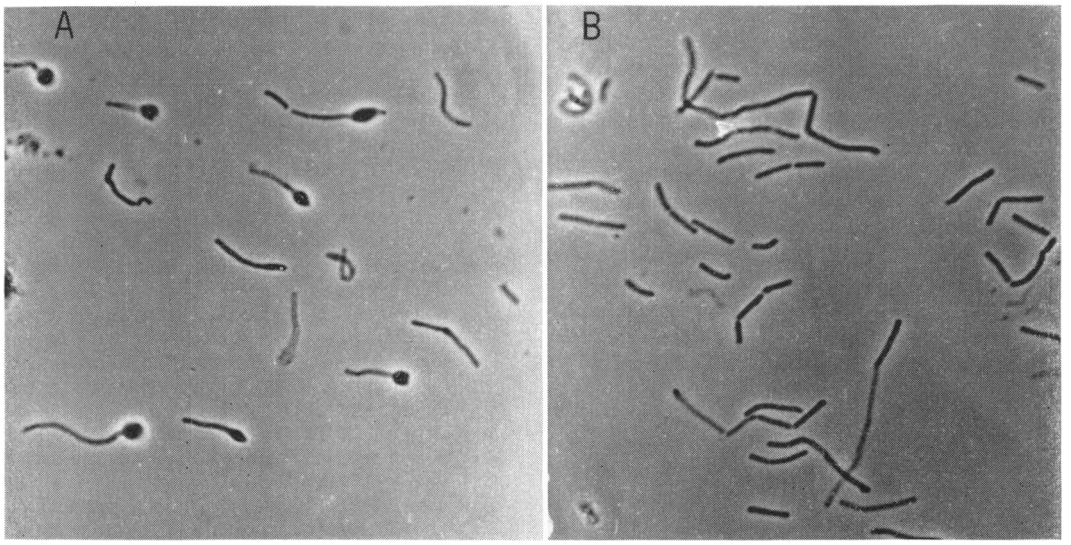

FIG. 6. *Photomicrograph of 168ts-134 after incubation for 4 hr in enriched minimal medium with and without chloramphenicol. (A) Sample from tube A of Fig. 5 at 4 hr. (B) Sample from tube B of Fig. 5 at 4 hr.*

amined. Spores were germinated at 30 and 45 C in enriched minimal medium with ^{14}C-thymine in the presence or absence of chloramphenicol. In addition, samples were withdrawn from the culture at 30 C (without chloramphenicol) and transferred to 45 C with or without chloramphenicol. These experiments were similar in design to those of Yoshikawa (14). The results (Fig. 7) demonstrate that 168ts-134 synthesized no DNA when germinated at high temperature. Outgrowing cells could, however, be observed in the microscope. When germinated at low temperature, DNA synthesis commenced after 3 to 4 hr and transfer of a sample of cells to 45 C with and without chloramphenicol at 5 hr gave incorporation curves similar to those for log-phase transfers (Fig. 1). However, in this case, the level of incorporation with chloramphenicol present was clearly somewhat lower than when it was absent.

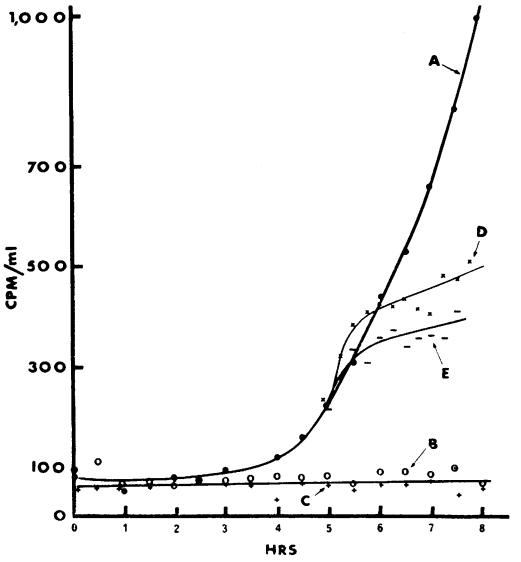

FIG. 7. *Kinetics of DNA synthesis during germination of spores of 168ts-134. Spores were germinated in enriched minimal medium containing 0.175 μc of thymine-2-^{14}C and 10 μg of ^{12}C-thymine per ml at 30 and 45 C in the presence and absence of 40 μg of chloramphenicol per ml. After 5 hr, samples were withdrawn from the 30 C culture (without chloramphenicol) and transferred to 45 C in the presence and absence of chloramphenicol. Each tube was sampled at various intervals for acid-insoluble radioactivity: (A) 30 C from time zero; (B) 45 C from time zero; (C) 30 C plus 40 μg of chloramphenicol per ml from time zero; (D) 30 C from time zero transferred to 45 C at 5 hr; (E) 30 C from time zero transferred to 45 C plus 40 μg of chloramphenicol per ml at 5 hr.*

DISCUSSION

The experiments reported above indicate that the temperature-sensitive lesion in mutant strain 168ts-134 involves the replication of DNA. When cells of this strain are shifted to 45 C, bulk RNA and protein synthesis continue at a substantial rate for several hours. Increases about 10-fold over the amounts present at the time of transfer to 45 C have been observed. DNA synthesis, on the other hand, proceeds normally for only about 25 to 30 min; thereafter, synthesis continues at a much reduced rate (Fig. 1, 2, and 3).

The per cent increase in DNA at 45 C prior to sharp reduction in rate of synthesis ranged in our

experiments from about 30 to 60%. In a randomly growing population of cells, an increment of DNA content of about 40% is expected when initiation is prevented, provided all chromosomes in the culture are replicating with a single fork (8–10). Inhibition of protein synthesis by amino acid deprivation or addition of chloramphenicol is believed, at least in some cases, to allow completion but prevent reinitiation of chromosome replication (11). The point of some significance is that the amount of DNA synthesized by 168ts-134 prior to the rate change in any given experiment is always similar to, though possibly somewhat more than, the amount of synthesis which takes place upon inhibition of protein synthesis in the parent strain or the mutant strain. This observation suggests that the lesion may allow completion of the round of replication which is underway at the time of transfer to 45 C and severely inhibit, though not entirely prevent, the initiation of any further rounds of DNA synthesis.

The fact that spores of 168ts-134 are unable to synthesize any DNA when germinated at 45 C is consistent with this interpretation. Some additional support is provided by preliminary autoradiographic analysis of the incorporation of ^{3}H-thymine pulses given to cells of 168ts-134 at various times after transfer to 45 C (Mendelson, *unpublished data*). These studies indicate that the fraction of unlabeled cells increases progressively after transfer to 45 C as would be expected if an increasing proportion of the cells have completed the round of replication and are unable to start a new one. The autoradiographs also suggest that the low rate of DNA synthesis observed at 45 C is due in part to a proportion of cells carrying out an abnormal type of synthesis. It may be noted that the loss of viability which begins to occur after 1 hr at 45 C may be caused by the induction of phage PBSX, since we have observed the appearance of PBSX in the media during this period (5, 13).

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