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A Bacteriophage of *Bacillus subtilis* Which Forms Plaques Only at Temperatures Above 50 C

III. Inhibition of TSP-1-Specific Deoxyribonucleic Acid Synthesis at 37 C and 45 C[1]

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The effect of temperature on phage-specific deoxyribonucleic acid (DNA) synthesis was studied in TSP-1-infected *Bacillus subtilis*. This was facilitated by selectively inhibiting host DNA synthesis with 6-(*p*-hydroxyphenylazo)-uracil. The results indicated that TSP-1 DNA synthesis did not continue at 37 C and was immediately shut down after transfer to this temperature. Incubation at 45 C greatly reduced TSP-1 DNA synthesis. Phage-specific DNA synthesis could resume at 53 C, however, when the infected culture was returned to 53 C after a 2-min incubation period at 37 C. The results suggest that the inhibition of phage DNA synthesis at 37 C is reversible. Since infected cultures returned to 53 C after 2 min at 37 C could not complete the replicative cycle, the irreversible inhibition of yet another intermediate step was suggested.

The subtilisphage, TSP-1, was isolated from soil, and its physical and chemical properties have been described (5). It is a unique bacteriophage in that it will only form plaques in a temperature range of 50 to 55 C. This rigorous temperature requirement is partially due to the absence of phage receptor sites on cells grown at 37 and 45 C, and also as a result of the rapid inactivation of replicating phage at temperatures below 50 C (6).

Studies on phage deoxyribonucleic acid (DNA) synthesis in vivo should be greatly facilitated if bacterial DNA synthesis could be reduced or selectively inhibited during the course of phage DNA synthesis. These conditions can be attained in *Bacillus subtilis* with either nalidixic acid or 6-(*p*-hydroxyphenylazo)-uracil (HPUra). Gage and Fujita (4) reported that nalidixic acid will not inhibit *B. subtilis* DNA synthesis. Although SP01 DNA synthesis occured under these conditions, nalidixic acid decreases the rate of SP01 DNA synthesis and may delay its onset. The second inhibitor, HPUra, was first shown to inhibit DNA synthesis in gram-positive bacteria by Brown and Handschumacher (3). HPUra can inhibit DNA synthesis over a very broad concentration range (0.005 mM to 1.0 mM) but has no effect on protein or ribonucleic acid (RNA) synthesis or on cell mass increase. Interestingly, this compound exerts its inhibitory effect only while in the oxidized state (red) and not in the reduced form (colorless). Subsequently, Brown (2) demonstrated that HPUra selectively inhibited bacterial DNA synthesis without affecting phage DNA synthesis. He was able to obtain phage DNA synthesis in *B. subtilis* infected with the thymine-containing phages, SP3 and SP02C$_1$, and with the hydroxymethyluracil-containing phage SP8 in the presence of HPUra. Moreover, neither the pattern nor the rate of phage DNA synthesis was affected by this inhibitor. Since HPUra does not appear to inhibit or alter phage DNA synthesis in any way, it was used to isolate phage DNA synthesis in vivo. In this manner, the effect of incubation temperature on TSP-1-specific DNA synthesis could be more easily assessed.

MATERIALS AND METHODS

Bacterial strains and bacteriophage used. All the experiments described below were done with *B. subtilis* A26, ura$^-$ met$^-$, and TSP-1.

Growth conditions. *B. subtilis* A26 was grown in semidefined medium (CH medium) composed of minimal salts (9), 1% casein hydrolysate (enzymatic hydrolysate, Calbiochem), and 1% glucose, supplemented with uracil (5 μg/ml). Cultures were incubated in

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a reciprocating shaker-water bath (New Brunswick Scientific Co.). Temperature shifts were made by transferring a culture at 53 C to a second reciprocating shaker-water bath at 37 C (6).

Measurement of DNA synthesis. DNA synthesis was measured as the incorporation of radioactive uracil into alkali-stable counts per minute. Uracil was used because it serves as a precursor for thymine in *B. subtilis* and is readily incorporated into *B. subtilis* and phage DNA (8). The method used to measure uracil incorporation into DNA was essentially the one described by Roodyn and Mandel (7) for *B. cereus*. *B. subtilis* A26 was grown at 53 C in CH medium in 60-ml amounts to a turbidity of 55 Klett units (red filter, 640 to 700 nm). At this time, uracil-6-^3H (New England Nuclear) and HPUra were added to final concentrations of 4 μCi/ml and 0.5 mM, respectively. The culture was then subdivided into six 10-ml cultures and further incubated at 53 C in 125-ml Erlenmeyer flasks for another 10 min before addition of TSP-1 at a multiplicity of infection (MOI) of 10. One-half-milliliter samples were taken at 5-min intervals and pipetted directly into equal volumes of 1 N NaOH. The samples were then incubated at 37 C for 18 to 24 hr, and the residue was collected on nitrocellulose filters (triacetate metricel type GA-6, 25 mm, 0.45 μm pore size; Gelman Instruments) and washed with 15 ml of demineralized water at room temperature. The membranes were then placed in scintillation vials, dried at room temperature overnight, and counted in a Beckman Instruments 200B liquid scintillation counter using Spectrafluor (Amersham/Searle)-toluene as a scintillator. Roodyn and Mandel (7) have demonstrated that this procedure specifically measures total DNA synthesized. In those experiments in which host DNA synthesis was not inhibited, the same procedures were employed, but HPUra was not added.

Preparation of HPUra stock solutions. HPUra (U.K. patent 876,601) was synthesized and generously provided by Bernard Langley of Imperial Chemical Industries, Ltd. Stock solutions of HPUra (20 mM) were prepared in 0.05 N NaOH, autoclaved, and stored at room temperature.

Preparation of TSP-1 antiserum. Antiserum was prepared in rabbits by the procedure described previously (6).

RESULTS

Characteristics of TSP-1 DNA synthesis at 53 C. The pattern of TSP-1 DNA synthesis in the absence of HPUra is illustrated in Fig. 1. In this experiment, a 25-ml culture of *B. subtilis* A26 was grown in CH medium at 53 C until it reached a turbidity of 55 Klett units (about 10^7 bacteria/ml). At this time (T = −10 min), uracil-6-^3H was added to a concentration of 4 μCi/ml, and the culture was divided into two 12-ml subcultures. Incubation was continued at 53 C, and TSP-1 was added (MOI = 10) 10 min later (T = 0 min) to one of these cultures. DNA synthesis in the uninfected cells was typically linear (Fig. 1) for the duration of the experiment, whereas DNA synthesis in the infected culture was radically different. The rate of synthesis in the infected culture

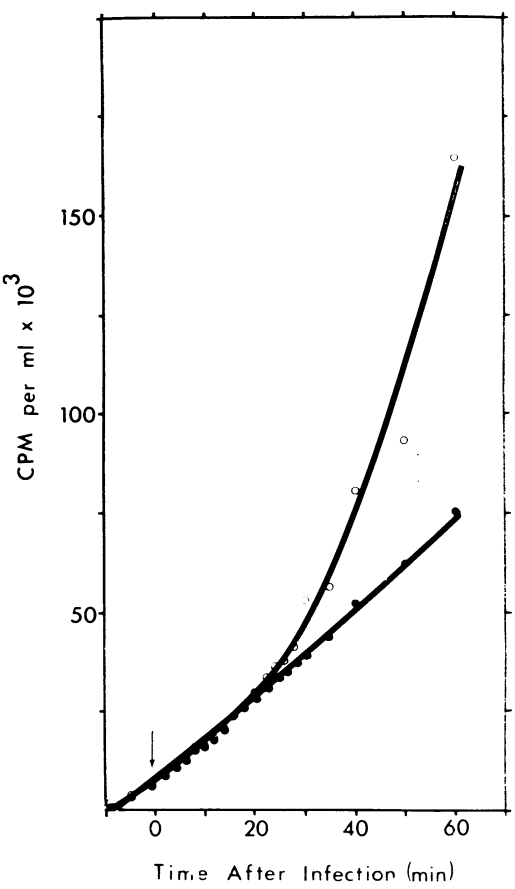

FIG. 1. *DNA synthesis in the absence of 6-(p-hydroxyphenylazo)-uracil. Uracil-6-^3H (4 μCi/ml) was added at T = 10. TSP-1 was added at the time indicated by the arrow. Infected cultures* (○) *and uninfected cultures* (●).

was linear for the first 15 min after infection when a sudden increase in rate was observed. Characteristically, phage DNA synthesis occurs at a faster rate than bacterial DNA synthesis, and this burst in DNA synthesis probably represents phage DNA synthesis. This was readily confirmed by doing the same experiment but in the presence of HPUra. These results are shown in Fig. 2. It appears from these results as though phage DNA synthesis occurs at a rapid rate in the presence of HPUra, although the early rate may be somewhat slower. Host DNA synthesis, however, was completely inhibited. It is apparent from these two experiments that TSP-1 DNA synthesis starts about 15 to 20 min after infection. Likewise, the rate of phage DNA synthesis is not reduced by the presence of HPUra.

Effect of temperature shifts from 53 to 37 C and 45 C on TSP-1 DNA synthesis. It is clear from the previous experiments that TSP-1 DNA synthesis

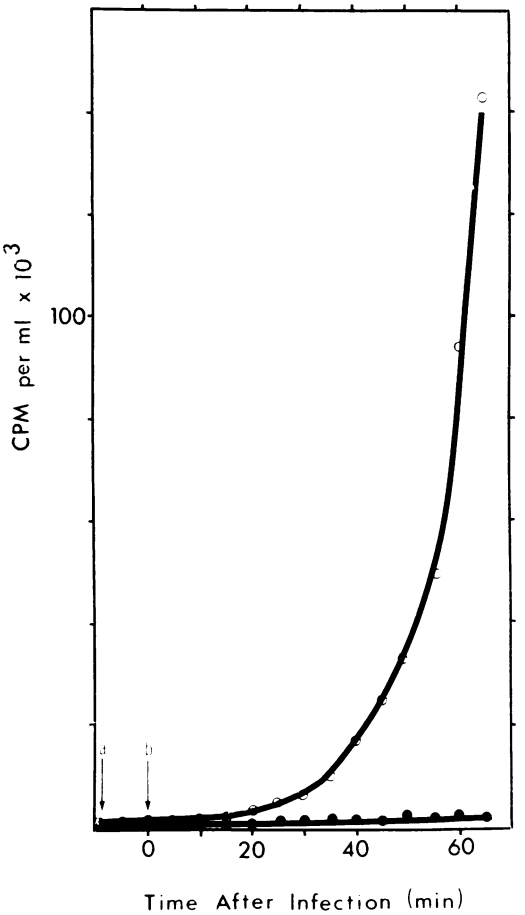

FIG. 2. *TSP-1 DNA synthesis after addition of 6-(p-hydroxyphenylazo)-uracil (0.5 mM). Uracil-6-^{3}H (4 μCi/ml) and HPUra were added at T = −10 min (a), and TSP-1 (MOI = 10) was added at T = 0 (b). Infected culture (○), uninfected culture (●).*

is unaffected by HPUra. The presence of this inhibitor allowed us to study only TSP-1 DNA synthesis, since bacterial DNA synthesis was completely inhibited. We indicated previously (6) that replicating TSP-1 was rapidly inactivated by a temperature shift from 53 C to 37 C. One possible explanation for this effect is that TSP-1 is a thermophilic bacteriophage. This would imply that the replicative enzymes are not functional at temperatures below 50 C. To determine if this was indeed the case, bacteria were infected at 53 C and then shifted to 37 C at 15, 30, and 45 min after infection. The synthesis of TSP-1 DNA was followed in each case as previously described. The results in Fig. 3 demonstrate that DNA synthesis stops within 5 min after shifting to 37 C at 15 and 30 min. When shifted to 37 C at 45 min, DNA synthesis continues at a rapid rate for approximately 10 min. Transfer to 45 C at 30 min after infection resulted in a drastic reduction of phage DNA synthesis. After 30 min at 45 C (60 min postinfection), only about 15% as much DNA was synthesized when compared to the control.

TSP-1 DNA synthesis in infected cells at 53 C subjected to a transient (2-min) incubation at 37 C. As previously reported (6) TSP-1 infections could be aborted if infected cells were transferred from 53 to 37 C for 2 min and returned to 53 C. Furthermore, the infection could be aborted only if this shift occurred at any time up to 30 to 35 min after infection. We were interested, therefore, in determining if such a transient incubation would inhibit phage-specific DNA synthesis. This experiment was done in the same way as the previous temperature shift experiments, except that 0.1 ml of phage antiserum was added to prevent reinfection, and the culture was returned to 53 C. The results (Fig. 4) indicate that a transient incubation at 37 C will not irreversibly stop TSP-1 DNA

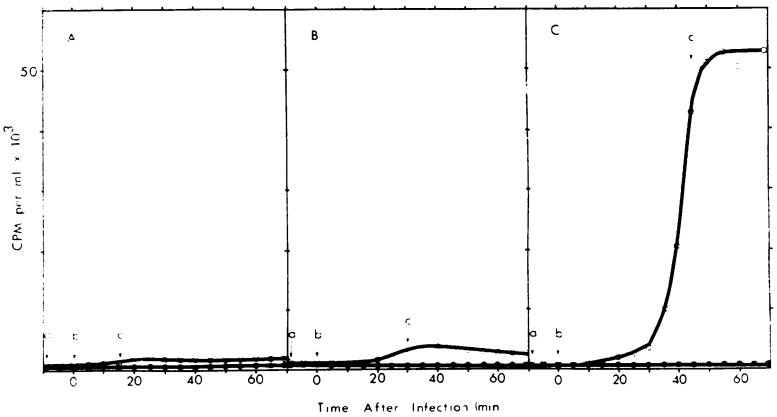

FIG. 3. *Effect of transfer from 53 to 37 C on TSP-1-specific DNA synthesis. Uracil-6-^{3}H (4 μCi/ml) and 6-(p-hydroxyphenylazo)-uracil (0.5 mM) were added to all cultures at T = −10 min (a). TSP-1 was added at T = 0 min (b), and the cultures were transferred to 37 C at the times indicated by (c). A, B, and C were transferred to 37 C at 15, 30, and 45 min after infection, respectively. Symbols: infected cultures (○), uninfected cultures (●).*

synthesis. Phage DNA synthesis, although somewhat delayed, continued after the transfer if the transient incubation was made at 15 min (Fig. 4B) after infection. At 30 min after infection, a 2-min incubation period at 37 C resulted in a marked reduction in DNA synthesis (Fig. 4C). By 45 min after infection, a 2-min incubation period at 37 C appeared to cause an immediate degradation of DNA (Fig. 4D).

DISCUSSION

In the experiments described above, we were able to explore the temperature requirements of a phage-specific function in TSP-1 infections of *B.*

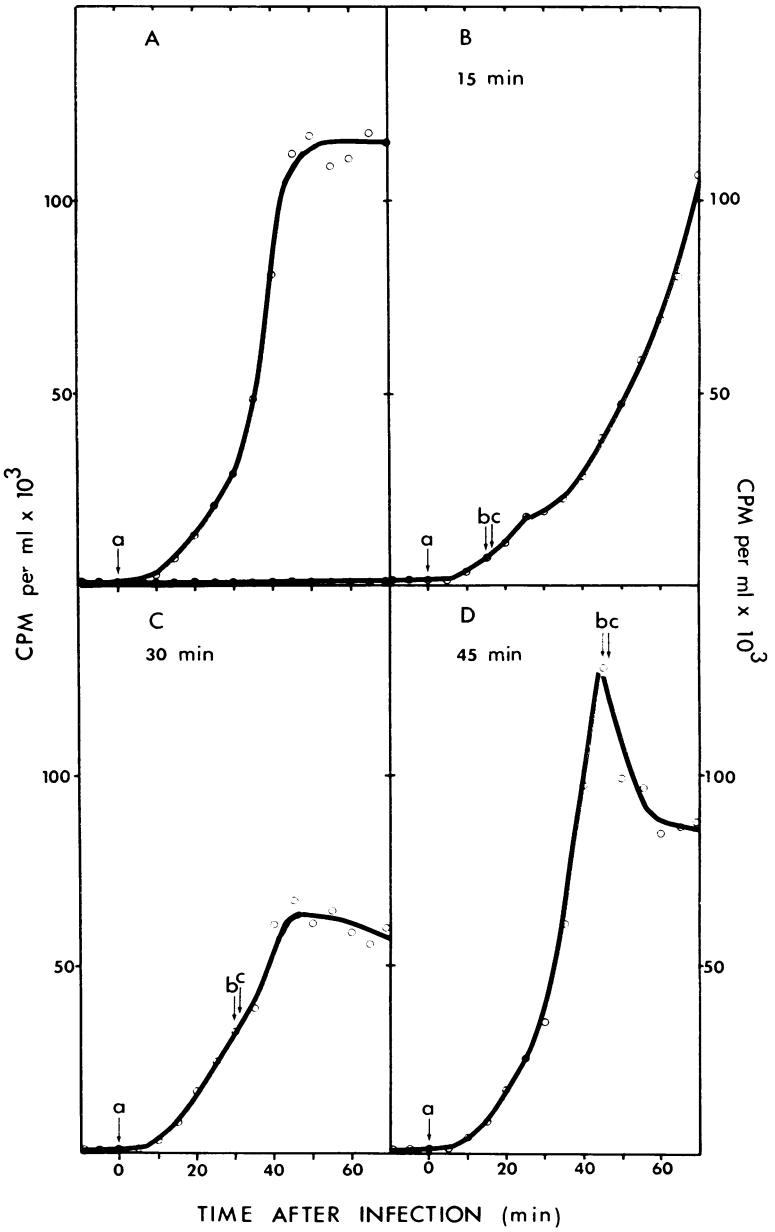

FIG. 4. *Effect of a transient (2-min) incubation at 37 C on TSP-1-specific DNA synthesis. B. subtilis A26 was incubated in CH medium at 53 C and infected with TSP-1 (MOI = 1.0) at time (a); transferred to 37 C at time (b); phage antiserum was added, and the culture was returned to 53 C at time (c). Uracil-6-³H (4 μCi/ml) and 6-(p-hydroxyphenylazo)-uracil (0.5 mM) were added at T = −10 min. Key: infected control kept at 53 C (A), infected culture transferred to 37 C at T = 15 min (B), T = 30 min (C), T = 45 min (D). Symbols: (○) infected cultures, (●) uninfected control transferred to 37 C for 2 min and returned to 53 C.*

subtilis. This is an important consideration since TSP-1 is unable to form plaques on *B. subtilis* at temperatures below 50 C (5). We have demonstrated that phage receptor sites are absent or inaccessible on *B. subitilis* growing at 37 or 45 C (6). The absence of phage receptor sites by itself, however, cannot account for the observed temperature-dependent inactivation of replicating TSP-1. This may be due to an absolute requirement by TSP-1 for the higher temperatures for replication. One possibility is that TSP-1 is a thermophilic bacteriophage. That is, its intermediate replicative steps will not occur at temperatures below 50 C because its replicative enzymes are not functional at this temperature. One of the key intermediates in phage replication is phage DNA synthesis. By using HPUra we were able to study the effect of temperature on TSP-1 DNA synthesis. Our experiments clearly demonstrate that TSP-1 DNA synthesis will not take place at 37 C and is greatly reduced at 45 C. This would strongly suggest that one or more of the enzymes involved in DNA synthesis of this phage are probably thermophilic.

In addition to the demonstrated inhibition of TSP-1-specific DNA synthesis at 37 and 45 C, some other element of phage replication is sensitive to the lower temperature. The results of the experiments in which phage DNA synthesis was measured after a transient (2-min) incubation period at 37 C were quite unexpected (Fig. 4). Earlier studies had indicated that a similar incubation would prevent infected cells from lysing after they were returned to 53 C. However, a 2-min pulse to 37 C did not irreversibly stop phage DNA synthesis, although some alteration in the pattern was evident. Since phage replication is a highly integrated process, these results suggested that the shift to 37 C for 2 min interfered with the proper sequence of events needed to get phage replication by destroying an essential intermediate of TSP-1 replication which is required during the initial 30 min of infection. Apparently, this intermediate is not directly required for phage DNA synthesis. One possibility is that this intermediate is essential for the synthesis of phage-specific proteins or RNA. A function essential for assembly could also be involved.

The inhibition of TSP-1 DNA synthesis could be due to the presence of a thermophilic phage DNA polymerase. This is a very interesting possibility as it would provide a useful experimental system for the study of DNA replication. Our data cannot be used to draw conclusions as to the mechanism of phage DNA inhibition at 37 or 45 C. This determination will have to await the identification of the enzymes involved and the evaluation of temperature changes on their kinetic parameters and conformational state.

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