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

II. Reduction of TSP-1-Specific Receptor Sites on Cells Grown at 37 C or 45 C, and the Temperature-Dependent Inactivation of Replicating Phage[1]

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The adsorption of subtilisphage TSP-1 to *Bacillus subtilis* W168 was tested with cells grown at 37, 45, and 53 C. Kinetic data, electron micrographs, and quantitative measurement of the per cent phage adsorbed all indicated that irreversible adsorption occurs only with cells grown at 53 C. If TSP-1 was allowed to infect cells at 53 C, subsequent shifts to 37 C inhibited phage replication and resulted in the inactivation of plaque-forming units.

The physical and chemical characteristics of the subtilisphage, TSP-1, have recently been described (8). It was isolated from soil, contains double-stranded deoxyribonucleic acid (DNA) without a thymine analogue, and is morphologically similar to other subtilisphages (3, 5, 10). It differs dramatically, however, from other *B. subtilis* bacteriophages in that it will form plaques only at temperatures above 50 C. Clear plaques are formed on any of the *B. subtilis* strains that grow above 50 C, and on *B. licheniformis* 9945A above 50 C. This rigorous restriction to temperatures above 50 C (to an upper limit of 55 C) may be a reflection of the interaction of one or more factors, acting singly or in concert to inhibit plaque formation below 50 C. These factors include: (i) a temperature-sensitive restriction mechanism which is inactivated above 50 C (6); (ii) the absence of phage receptor sites below 50 C, due to changes in cell wall structure (4); and (iii) TSP-1 may have intermediate replicative enzymes which require the higher temperature for optimal activity.

As an initial step in identifying which of these factors may account for this stringent temperature requirement, we have asked the following questions: (i) will TSP-1 adsorb to *B. subtilis* W168 growing at temperatures below 50 C, and (ii) if adsorption is a limiting factor, can TSP-1 replication continue below 50 C once phage adsorption and DNA injection have occurred at 53 C? We approached the first question by analyzing the kinetics of phage adsorption with experiments similar to the classic experiment of Hershey and Chase (7), by measuring the amount of phage which would adsorb to *B. subtilis* cells growing at various temperatures and by examining negative stains of TSP-1-infected *B. subtilis* with the electron microscope. To answer the second question, the initial steps in phage replication, phage adsorption, and DNA injection were allowed to occur at 53 C. The infected cultures were then transferred to 37 C to determine if phage replication could be completed at this temperature.

MATERIALS AND METHODS

Bacterial strains and bacteriophage used. All of these experiments were done with TSP-1 infecting *B. subtilis* W168, a prototrophic transformant of *B. subtilis* 168, unless otherwise noted.

Growth conditions. All bacterial cultures were grown in Difco Brain Heart Infusion (BHI) broth medium. Broth cultures were incubated at 53, 45, or 37 C in reciprocating shaker-water baths (New Brunswick Scientific Co.). Plaque counts were done on BHI agar (1.5%) by the agar overlay technique (1) with 0.1 ml of a *B. subtilis* W168 spore suspension (10^8 spores per ml) as the inoculum for the lawn. Serial dilutions were made in BHI broth, and the overlay was BHI soft agar (0.65%).

Preparation of TSP-1 antisera. Anti-TSP-1 antisera was prepared in rabbits with TSP-1 suspensions puri-

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fied as previously described (8). Primary inoculations consisted of 0.25 ml of a TSP-1 suspension (10^{12} plaque-forming units) emulsified in an equal volume of complete Freund's adjuvant (Difco) and injected into the footpad. This was followed by two 0.5-ml subcutaneous injections of TSP-1 at 10-day intervals. Rabbits were bled by cardiac puncture 6 to 8 weeks after the primary inoculation. The sera were collected and heated for 30 min at 36 C to inactivate complement. Antisera obtained by this procedure had *K* values of at least 400.

Temperature shift experiments. Bacteria were incubated in 300-ml Nephelo flasks (side arm = 14 by 130 mm; Bellco, Vineland, N.J.) containing 40 ml of BHI in a reciprocating shaker-water bath at 53 C. The bacterial growth was monitored with a Klett-Summerson colorimeter with a red filter (640 to 700 nm) and incubated until the culture reached a turbidity of 75 Klett units (equivalent to 5×10^7 bacteria per ml). At this time, TSP-1 was added to the desired cultures at a multiplicity of infection (MOI) of 1. The shift to a lower temperature was made by transferring the desired flask to a second reciprocating shaker-water bath at the appropriate temperature Under these conditions, a culture transferred from 53 to 37 C, for example, reached 37 C within 1.5 to 2.0 min after transfer. In those experiments which involved the transfer from 53 to 37 C, followed by a return to 53 C, 0.1 ml of undiluted phage antisera was added shortly after the shift to 37 C.

Radioactive labeling of TSP-1 DNA. TSP-1 was labeled with ^{32}P-orthophosphate (285 Ci/mg; International Chemical and Nuclear Corp.). The radioactive phosphate was added to a culture of *B. subtilis* W168 growing in BHI to a final concentration of 3 μCi/ml 2 hr before addition of TSP-1. The labeled phage were purified by the polyethylene glycol-sodium chloride procedure previously described (8).

Kinetics of TSP-1 adsorption and DNA injection. The kinetics of TSP-1 adsorption and DNA injection were studied by a procedure similar to that of Hershey and Chase (7). Phage labeled with ^{32}P were added to late log-phase culture of *B. subtilis* W168 (approximately 10^8 bacteria per ml) growing at 53 C at a MOI of 10. Samples (3 ml) were taken at 5-min intervals and divided into two fractions as follows: (i) 0.5 ml was filtered immediately onto nitrocellulose filters (25 mm diameter; 0.45 μm pore size; Millipore Corp.) and washed with 15 ml of minimal salts (7); (ii) the remaining 2.5 ml was homogenized in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.), equipped with a 5.0-ml microattachment, for 60 sec at a setting of 5. A 0.5-ml sample of this homogenate was then filtered and washed. This homogenization procedure was sufficient to remove any adsorbed phage particles as judged by removal of TSP-1 particles labeled with ^{14}C-isoleucine. The filters were placed in scintillation vials, dried, and counted with Spectrafluor (Amersham/Searle)-toluene in a Beckman Instruments 200B liquid scintillation counter.

Electron microscopy. TSP-1 was added to cultures of *B. subtilis* W168 (MOI = 50) growing at 53 or 37 C in BHI. After 10 min of further incubation, 10 ml of the culture was centrifuged in a clinical centrifuge (International Equipment Co., Needham, Mass.) at maximum speed (ca. 3,000 $\times$ *g*) for 10 min. The pellet was suspended in 5 ml of 10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-10^{-3} M $MgSO_4$ buffer, *p*H 7.4. A drop of the suspended pellet was mixed with an equal amount of 2% phosphotungstic acid-2% normal rabbit serum, *p*H 7.0, and examined on Formvar-coated grids in a Phillips 300E electron microscope operating at 80 kv.

RESULTS

Adsorption of TSP-1 to B. subtilis W168. TSP-1 was labeled with ^{32}P, purified, and added to *B. subtilis* W168 growing under various temperature conditions. If adsorption was not accompanied with DNA injection, the cell-associated radioactivity would be removable by homogenization. In contrast, if adsorption was followed by DNA injection, the radioactivity would be retained after homogenization.

It is apparent from the results of these experiments that TSP-1 will adsorb efficiently to 53 C grown cells and that DNA injection accompanies this adsorption (Fig. 1A). Adsorption at 53 C occurred rapidly, and a plateau was reached by 5 min after infection. A comparable situation does not prevail at 37 C, however (Fig. 1B). In this case, TSP-1 adsorbed to 37 C cells, but not to the degree observed with 53 C grown cells, and only 20% of the label remained after homogenization. These results suggest two alternative explanations: (i) *B. subtilis* grown at 37 C does not have TSP-1 receptors on its surface, and (ii) *B. subtilis* grown at 37 C has the proper receptors, but DNA injection will not occur because it is a thermophilic process. To analyze this possibility, *B. subtilis* was grown at 53 C, shifted to 37 C 10 min before addition of TSP-1 (MOI = 10), and the kinetics of phage adsorption and DNA injection were determined. The results show that adsorption and DNA injection take place under these conditions, indicating that TSP-1 DNA injection is not a thermophilic process (Fig. 1C). Collectively, these results can be interpreted to indicate that TSP-1 adsorption to 53 C cells is specific, whereas adsorption to 37 C cells is not. In this context, specific adsorption is defined as that adsorption which leads to DNA injection. These experiments suggest that specific phage receptors are not present on cells grown at 37 C and that any adsorption to 37 C cells is probably nonspecific. It is also clear that phage DNA injection can occur at 37 C if the proper phage receptors are present.

To confirm this suggestion, *B. subtilis* W168 grown at 37 and 53 C were infected with TSP-1 (MOI ca. 50), negatively stained, and examined by electron microscopy. These micrographs clearly indicated that TSP-1 does not irreversibly attach

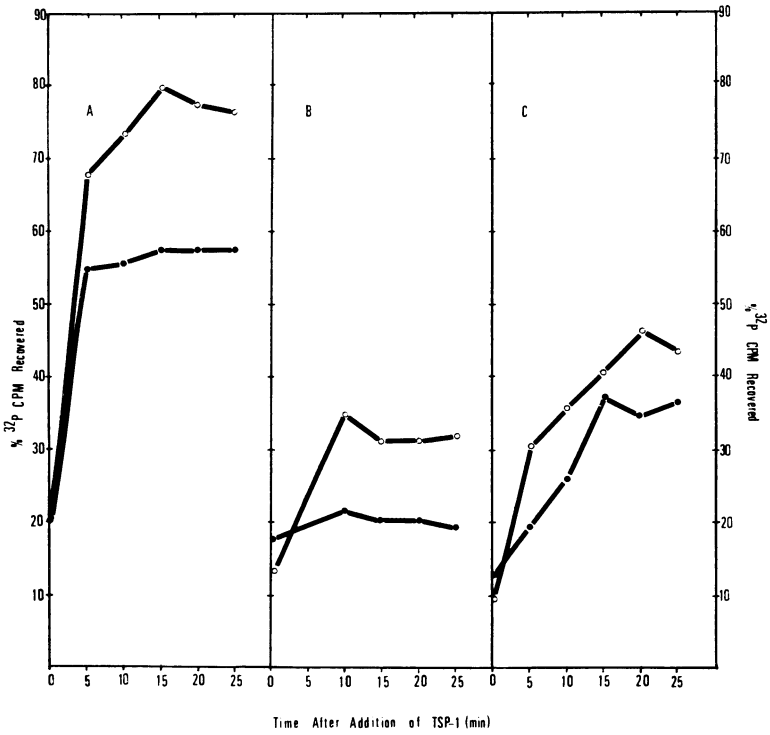

FIG. 1. *Kinetics of TSP-1 adsorption and DNA injection. Bacillus subtilis W168 was grown to late log phase (10^8 cells/ml) and ^{32}P-labeled TSP-1 was added (MOI = 10) to cells grown at 53 C (A), 37 C (B), and 53 C cells shifted to 37 C 10 min before TSP-1 addition (C). Cell-associated radioactivity after (●) and before (○) homogenization.*

to *B. subtilis* W168 cells grown at 37 C, but does attach to 53 C grown cells.

An additional experiment was done to verify the absence of phage receptor sites at 37 C. In this instance, TSP-1 was added to *B. subtilis* W168 growing at 37 C (MOI = 1.0) and incubated at 37 C for an additional 5 or 10 min before the culture was transferred to 53 C and monitored turbidimetrically for the development of lysis. If TSP-1 did not adsorb due to the absence of phage receptor sites, it should be rapidly neutralized by TSP-1 antiserum, and the culture would be protected from infection. These results clearly show that this is the case, for in all cultures to which antiserum was added the cultures did not manifest any evidence of phage infection (Fig. 2). All cultures to which nonspecific serum was added lysed about 60 to 70 min after transfer to 53 C.

Estimation of per cent phage adsorbed to B. subtilis W168 growing at 53 and 45 C. A simple method to quantitate the number of bacteriophage particles which adsorb to a suitable host is to add a few drops of $CHCl_3$ to the bacteria-bacteriophage suspension and then to assay the resultant aqueous supernatant fluid for surviving infectious centers (2). This procedure actually measures the number of particles that are irreversibly adsorbed to bacteria, since it kills uninfected as well as infected bacteria but has no effect on unadsorbed particles. The number of adsorbed phage can then be determined by subtracting the titer obtained with $CHCl_3$ treatment (i.e., free phage), from the titer obtained without $CHCl_3$ treatment (i.e., free phage plus infected bacteria). However, when this experiment was done with TSP-1 and *B. subtilis* W168 (MOI = 1.0) at 53 C, the number of infectious centers obtained with or without $CHCl_3$ was identical. Moreover, if one compares these titers with the titer calculated from the initial phage inoculum, it is apparent that about 95% of the input phage is inactivated very soon after addition of TSP-1 (Fig. 3B). This coincidence in phage titers indicates that infected cells exposed to temperatures below 50 C will not form plaques. This loss in phage may be due to the temperature change which accompanies dilution and sample plating by the agar overlay technique. To test the possibility that extracellular phage particles may be inactivated by a rapid temperature shift, a TSP-1 suspension was incu-

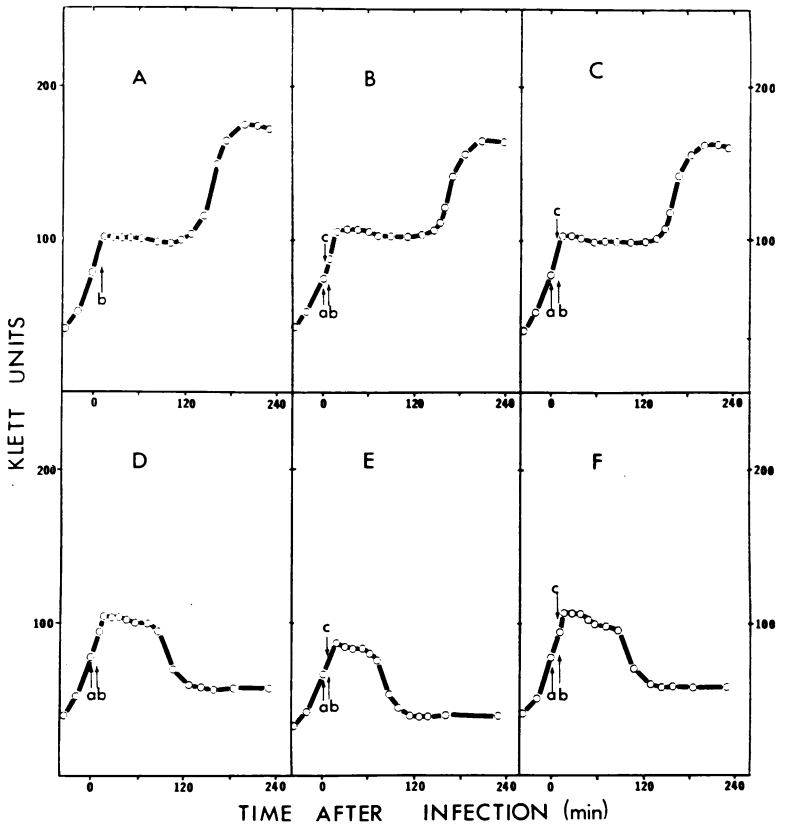

FIG. 2. *Susceptibility of TSP-1 to neutralization by phage antiserum if TSP-1 is added to B. subtilis W168 growing at 37 C. TSP-1 added at time (a) (MOI = 1.0), transfer to 53 C at time (b), and antiserum or nonspecific serum added at time (c). Key: uninfected culture transferred to 53 C at T = 5 min (A); infected culture transferred to 53 C at T = 5 min, antiserum added immediately before transfer to 53 C (B); infected culture transferred to 53 C at T = 10 min, antiserum added immediately before transfer to 53 C (C); infected culture transferred to 53 C at T = 5 min (D); infected culture transferred to 53 C at T = 5 min, nonspecific serum added immediately before transfer (E); infected culture transferred to 53 C at T = 10 min, nonspecific serum added immediately before transfer to 53 C (F).*

bated at 53 C, rapidly transferred to 37 C, and assayed at various times before and after transfer. The results of these experiments indicated that TSP-1 particles per se are not inactivated by a rapid temperature change since there was no loss of input after transfer to 37 C. It would appear then that replicating phage are sensitive to a drop in temperature, and as a consequence plaques will not be formed from infected bacteria. One can then conveniently measure TSP-1 adsorption by assaying an infected culture without $CHCl_3$ treatment and comparing this titer with the one estimated from the phage inoculum. Repeated experiments indicate that TSP-1 adsorption to *B. subtilis* and *B. licheniformis* 9945A occurs rapidly, with 90 to 95% input phage adsorbed after 2 min at 53 C. This loss of input phage, however, is not evident if one adds TSP-1 to *B. subtilis* incubated at 45 C (Fig. 3A) or 37 C. In this case, the phage titers obtained with or without $CHCl_3$ are reduced by about 40% of the estimated phage titer. There is also no evidence of phage replication (Fig. 3A) comparable to the burst of new phage particles observed at 53 C (Fig. 3B). Addition of TSP-1 to cells incubated at 37 C gives the same results as the cells at 45 C (*data not shown*), indicating that irreversible phage adsorption and injection are reduced under these circumstances.

Temperature-dependent inactivation of replicating TSP-1. The results presented above demonstrate that TSP-1 specific receptor sites are either not present or are not accessible on *B. subtilis* W168 cells grown at 37 C. In addition, it appears adsorbed phage can be inactivated by decreasing the temperature of incubation. These observations imply that, although adsorption is a barrier to

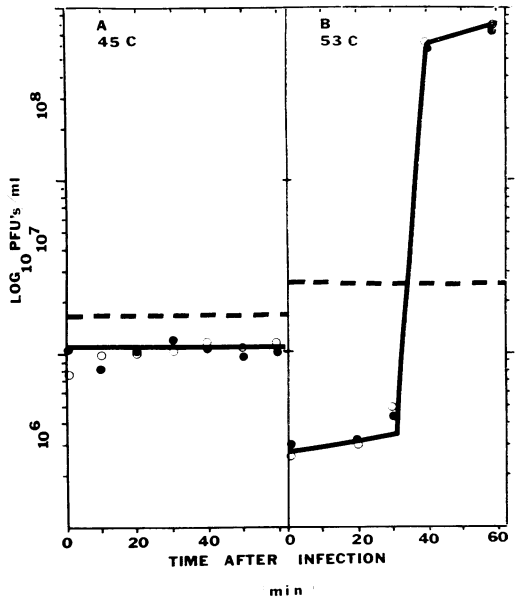

FIG. 3. *One-step growth curves of TSP-1 done on B. subtilis W168 growing at 45 C (A) and 53 C (B). The dashed line represents the phage titer calculated from the phage inoculum. Phage titers obtained with $CHCl_3$ (●) and without $CHCl_3$ (○). PFU = plaque-forming units.*

TSP-1 replication below 50 C, it is not the only one. To define more clearly this apparent temperature-dependent inactivation and its effect on phage replication, we undertook several experiments in which TSP-1 was added to *B. subtilis* at 53 C (to allow adsorption), transferring the infected culture to 37 C at various times after infection and determining if the lytic cycle could be completed at this temperature. We employed two indexes of replication: (i) the turbidimetric measurement of host cell lysis and (ii) the measurement of new phage synthesis by infectious center assays.

In the first experiment, TSP-1 was added to *B. subtilis* W168 (MOI = 1.0) at 53 C. The culture was transferred to 37 C immediately after infection, and the number of infectious centers was determined with or without $CHCl_3$ treatment at various times after infection. These titers were compared with the calculated input phage titer and plotted against time. Over 95% of the phage inoculum was destroyed, and the surviving phage titer was maintained for the duration of the experiment. These results indicate that replicating phage were destroyed and that further phage adsorption, injection, and replication did not occur at 37 C. In contrast, infected cultures at 53 C showed an identical loss of input phage but was characterized by a burst of new phage particles about 30 min after infection (Fig. 3B).

To determine if it was possible to inactivate replicating TSP-1 at various times after infection, the effects of a temperature shift from 53 to 37 C on the turbidity of infected and uninfected *B. subtilis* W168 were observed. Ten flasks of BHI broth were inoculated with *B. subtilis* W168 and incubated at 53 C until they reached Klett 75 (5×10^7 bacteria per ml). TSP-1 was then added (MOI = 1.0) to nine of these flasks. At 5-min intervals through 35 min, cultures were transferred to 37 C along with the uninfected control. In this case, it was possible to determine at what time the temperature requirement was overcome. Those infected flasks which were transferred to 37 C at less than 30 min after infection continued to increased in turbidity at about the same rate as the uninfected control culture. Flasks transferred at times after 30 min eventually lysed, suggesting that the requirement for 53 C was overcome after 30 min at 53 C. However, this experiment only measured changes in turbidity and did not indicate if phage replication was actually taking place, even in those cultures transferred to 37 C after 30 min at 53 C. We tested this directly by transferring samples of an infected culture to 37 C at various times after infection, incubating them at 37 C for 1 hr and then assaying for infectious centers. The titers of the transferred cultures were compared to the titers of the mother culture at the time of transfer. If the transferred culture showed a higher titer than the mother culture at the time of transfer, then one could conclude that in that sample replication had continued after transfer to 37 C. The results of one of these experiments are shown in Fig. 4 and confirm that infected cultures must be maintained at 53 C for at least 40 min after infection to obtain replication at 37 C in this experiment.

To determine how long an incubation at 37 C was required to obtain the irreversible inactivation of replicating phage, infected cultures at 53 C were transferred to 37 C for 2-min intervals at various times after infection, phage antiserum was added, and the culture was returned to 53 C. This time interval was used because it is just sufficient to bring the temperature of the medium from 53 to 37 C. The results demonstrate that 2 min at 37 C is sufficient to prevent the lysis of infected cultures provided that the exposure at 37 C occurs during the first 35 min after infection.

DISCUSSION

The data presented above demonstrate that there are at leat two barriers to TSP-1 replication at temperatures below 50 C. One obstacle is the absence of phage receptor sites on *B. subtilis* grown at 37 C or 45 C. This observation is

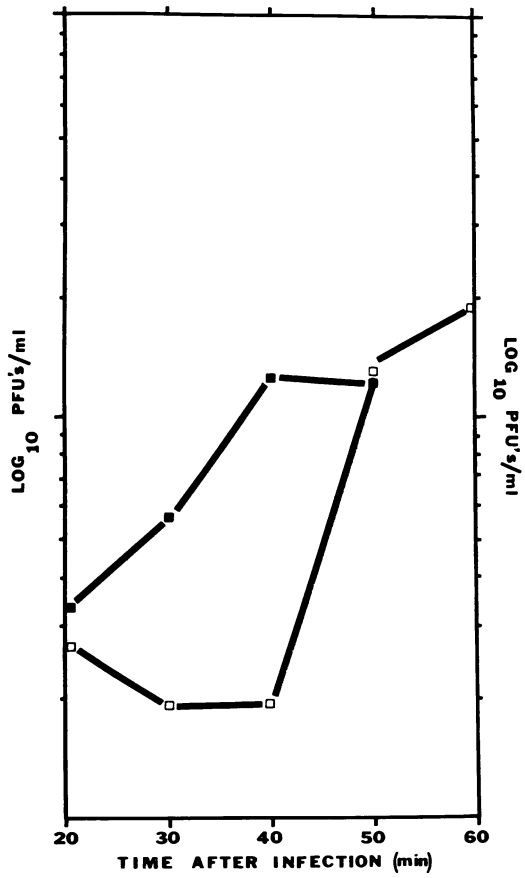

FIG. 4. *Effect of transfer from 53 to 37 C on TSP-1 replication in B. subtilis W168. At T = 0, TSP-1 was added (MOI = 5); after 20 min of incubation at 53 C, 3.0-ml samples were removed and incubated at 37 C for 1 hr. At the end of the 37 C incubation period, these samples were assayed for TSP-1. The phage titers after 1 hr at 37 C (□) were compared to the phage titers in the original culture at the time of transfer (■) to 37 C.*

strongly supported by evidence obtained through four different experiments. Kinetic data indicate that phage adsorption and phage DNA injection occurred only with cells grown and incubated at 53 C, or to a lesser extent, with cells that had been grown at 53 C until shortly (10 min) before phage addition and then transferred to 37 C. Phage DNA injection was shown not to be a thermophilic event since it could occur with 53 C grown cells incubated at 37 C. Electron micrographs of infected cells grown at 53 and 37 C confirmed the absence of irreversible TSP-1 adsorption. Additional evidence was provided by quantitative measurements of the per cent phage adsorbed to cells growing at 53, 37, or 45 C. The disappearance of TSP-1 receptor sites may be another manifestation of the cell wall changes observed by Dul and McDonald (4) in *B. subtilis* growing at 53 and 37 C. They observed a thicker cell wall and a greater thermal resistance in *B. subtilis* incubated at 53 C. Additionally, the thermal resistance in 37 C grown cells can be induced by antibiotics which inhibit the final cross-linking of the peptidoglycan. It is possible that the phage receptor site for TSP-1 is associated with this non-cross-linked peptidoglycan. Young et al. have shown that the specificity for phage receptor sites resides in the glucosylation of the polyglycerol teichoic acid (11). Perhaps the absence of a cross-linked peptidoglycan is required for the suitable exposure of phage receptors on the teichoic acid. Another possibility is that the teichoic acid present in cells grown at 53 C is qualitatively different from that at 37 C.

Apparently the absence of TSP-1 receptor sites on 37 C grown cells is not the only encumbrance to phage replication at 37 C. Our results reveal a temperature-dependent inactivation of replicating TSP-1. This becomes evident when one allows TSP-1 to adsorb to cells growing at 53 C, shifts these to 37 C, and observes the culture for evidence of TSP-1 replication. These results clearly indicate that if infected cells are transferred to 37 C during the initial 30 to 35 min of infection, the inhibition of mature phage particle production occurs. This time period is similar to the observed latent period of TSP-1 in *B. subtilis* (8). One can offer at least two explanations for this effect. The inhibition could be due to the presence of a temperature-sensitive restriction mechanism. Evidence has been presented for such a system in *B. subtilis* 168 by Gwinn and Lawton (6). They observed that two serologically identical phages, SP-10 and SP-20, formed plaques on *B. subtilis* W23 S^r but not on *B. subtilis* 168. These bacteriophages could form plaques on *B. subtilis* 168, however, if the bacteria were incubated at 53 C for 10 min before being infected at 37 C. Furthermore, their evidence suggested a temperature-sensitive repressor, as they were able to rescue phage markers from infected *B. subtilis* 168 as late as 3 hr after infection. If this restriction mechanism plays a role in the inhibition of TSP-1 replication at 37 C, then preincubation at 53 C should be sufficient to allow phage replication at 37 C. This does not occur in TSP-1 infections. The alternative explanation for this temperature-dependent inactivation may be that TSP-1 is truly a thermophilic bacteriophage. That is, its phage-specific enzymes are inactive below 50 C. If this is the case then either phage-specific DNA, ribonucleic acid, or protein synthesis might be temperature-sensitive.

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