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

I. Physical and Chemical Characteristics of TSP-1[1]

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Bacteriophage TSP-1 was isolated from soil in a search for phage which would form plaques on *Bacillus subtilis* W168 at 53 C. It forms clear plaques only at temperatures from 50 to 55 C. Approximately 95% of the free phage adsorb after 2 min at 53 C. The lytic cycle is between 55 and 60 min long with a burst size of about 55 particles per infected bacterium. The phage was shown to contain double-stranded deoxyribonucleic acid with a base composition of 44.7% guanine plus cytosine. This deoxyribonucleic acid does not contain a base analogue for thymine and has a molecular weight estimated at 56×10^6 daltons.

Bacillus subtilis can grow over a very broad temperature range with optimal growth occurring between 28 and 40 C (*Bergey's Manual*, 7th ed.) However, many strains are able to grow above 50 C to an upper limit of 55 C and this ability is transformable (13). Phage TSP-1 was isolated in our laboratory from a soil sample in a search for bacteriophage which would form plaques on *B. subtilis* strain W168 at the higher growth temperatures (50 to 55 C). We subsequently observed that TSP-1 would not form plaques on *B. subtilis* W168 at 37 C. This paper describes some of the physical, chemical, and biological properties of this unusual bacteriophage.

MATERIALS AND METHODS

Bacterial strains and bacteriophage used. Bacterial strains and bacteriophage used are listed in Table 1.

Growth conditions. All bacterial cultures were grown on Difco Brain Heart Infusion (BHI) broth medium incubated at 37 or 53 C in reciprocating shaker-water baths (7). Plaque counts were done on BHI agar by using the soft agar overlay method (1). The basal layer contained 1.5% agar, whereas the overlay contained 0.65% agar.

Bacteriophage propagation and concentration. TSP-1 was routinely propagated on *B. subtilis* W168 or W23 55^+. Bacterial growth was monitored with a Klett-Summerson colorimeter with a 640- to 700-nm (red) filter. When the culture reached a colorimeter reading of 75 (ca. 5×10^7 bacteria per ml), TSP-1 was added at a multiplicity of infection (MOI) of about five phage particles per bacterium and incubated at 53 C until lysis was completed (ca. 90 min). The lysate was cooled to 5 C and polyethylene glycol (PEG; molecular weight of 6,000; Matheson, Coleman and Bell) was added to a final concentration of 13% (w/v). After stirring for 30 min at 5 C, 5 M NaCl was added to the lysate-PEG mixture to a final concentration of 0.5 M NaCl. After 5 min of additional incubation at 5 C, the diffuse precipitate which formed was collected by centrifugation at $10,000 \times g$ for 20 min. The supernatant fluid was discarded and the precipitate was gently suspended to 5% of the original volume in 10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-10^{-3} M $MgSO_4$ buffer, pH 7.4 (Tris-Mg buffer). The phage was then banded in 70% CsCl (w/v, solution density about 1.5 g/cc) by using a type SW50L rotor at 45,000 rev/min for 18 hr in a Beckman model L2-65B ultracentrifuge. The TSP-1 band, which appeared in the lower third of the tube, was collected and dialyzed overnight against 1,000 ml of Tris-Mg buffer. This procedure yielded phage suspensions of about 5×10^{12} plaque-forming units (PFU) per ml with more than 75% recovery. These purified lysates were examined by electron microscopy and contained no phage morphologically different from TSP-1. Bacteriophage T2 was propagated on *Escherichia coli* 15T, harvested, and purified in the same manner.

Bacteriophage nucleic acid extraction. TSP-1 nucleic acid was extracted as described by Freifelder (9). The phage suspension (0.1 ml) was mixed with 0.5 ml of 10^{-2} M PO_4-10^{-3} M ethylenediaminetetraacetate, pH 7.8 (PO_4-EDTA buffer), and 0.1 ml of neutralized 7.5 M $NaClO_4$. After 20 min at room tem-

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TABLE 1. *Characteristics of the organisms used*

Strain	Characteristics	Source
Bacillus subtilis W168	Prototrophic transformant of 168, grows above 50 C	Our laboratory
B. subtilis W23	Prototrophic strain of 23, will not grow above 50 C	Our laboratory
B. subtilis W23 55^+	Spontaneous mutant of W23 which will grow above 50 C	Our laboratory
B. licheniformis 9945A	Facultative thermophile	R. Altenbern
B. stearothermophilus B	Obligate thermophile	G. Saunders
B. stearothermophilus 2184	Obligate thermophile	G. Saunders
Escherichia coli 15 T	Requires thymine, methionine, arginine, and tryptophan	T. S. Matney
T2	Coliphage	T. S. Matney
TSP-1	Subtilis phage	Our laboratory

perature, 0.5 ml of PO_4-EDTA buffer was added. The nucleic acid was precipitated with 95% ethanol, collected, and dissolved in 3.0 ml of 0.15 M NaCl plus 0.015 M Na_3 citrate (1 × SSC). The deoxyribonucleic acid (DNA) concentration in these solutions was about 125 μg/ml, with an optical density (OD) ratio of 0.6 OD_{280nm}/OD_{260nm}.

Chemical analyses of the nucleic acid. The diphenylamine test for deoxyribose was performed by the method of Clark (3). Melting point (T_m) determinations were done with a Hitachi-Perkin-Elmer spectrophotometer equipped with an insulated cuvette chamber attached to a Temptrol 151 temperature regulator (Precision Scientific Co.). The DNA was hydrolyzed with crude deoxyribonuclease (EC3.1.4.5., Worthington Biochemical Corp.) and phosphodiesterase I (EC3.1.4.1., Worthington Biochemical Corp.) (4). The resulting 5'-deoxyribonucleotides were separated by descending paper chromatography in an isopropanol-hydrochloride-water (170:41:29, v/v/v) solvent (20). The nucleotides were located on the paper with an ultraviolet lamp (260 nm). Known 5'-deoxyribonucleotides were obtained from Calbiochem.

Electron microscopy. Phage particles were negatively stained with 2% phosphotungstic acid and 2% serum (*p*H 7.0) on parlodion-covered grids and observed with a Phillips 300E electron microscope operating at 60 kv.

Radioactive labeling of phage DNA. TSP-1 was labeled with thymidine-*methyl*-^3H (2 to 30 Ci/mmole) or ^{32}P-orthophosphate (285 Ci/mg) obtained from the International Chemical and Nuclear Co. The desired radioactive compound was added 2 hr before addition to TSP-1 to a culture of *B. subtilis* W23 55^+ in BHI to a final concentration of 3 μCi/ml. The labeled phage were purified from the lysate as described above. T2 DNA was labeled by growing *E. coli* 15 T in BHI with 3 μCi of thymidine-*methyl*-^3H/ml for 2 hr before addition of phage and harvesting of the lysate as described above.

Molecular-weight determinations of TSP-1 DNA. Molecular-weight measurements were done by the method of Burgi and Hershey (2), as used to determine the molecular weight of PBS-1 DNA (11), with the modification that the length of the sedimentation run was increased from 3 to 3.5 or 4 hr. Four-drop fractions were collected directly into scintillation vials containing 1-inch (ca. 2.54 cm) squares of Whatman no. 1 filter paper. The fractions were then dried and counted using Spectrafluor (Amersham/Searle)-toluene in a 200B liquid scintillation counter (Beckman Instruments, Inc.).

Burst size determination. The average burst size of TSP-1-infected *B. subtilis* W168 was estimated by one-step growth and single-cell burst experiments as described by Adams (1). Samples for one-step growth experiments were diluted in BHI at room temperature, plated with 0.1 ml of *B. subtilis* W168 spores (~10^8 spores/ml), and incubated at 53 C for 18 to 24 hr. To determine the average burst size by the single-cell burst procedure, TSP-1 was added to *B. subtilis* W168 (MOI 0.6), diluted with BHI maintained at 53 C to a concentration of 1 bacterium per ml. The final dilution was made into 150 ml of BHI. From this dilution, 118 separate 1.0-ml samples were incubated at 53 C for 60 min. After this incubation, 2 ml of BHI agar (1.0%) and 0.1 ml of *B. subtilis* W168 spores were added. The samples were plated on BHI agar plates and incubated at 53 C for 18 to 24 hr.

RESULTS

Bacteriophage morphology. Over 50 bacteriophage particles were measured and examined morphologically. TSP-1 has a polygonal head, roughly hexagonal in outline, a tail with a contractile sheath, and a baseplate with several projections present. The head is approximately 90 nm at its widest diameter, and the tail is about 200 nm long. An examination of particles with contracted sheaths suggested that the apparent tail flexibility was lost after this event, and the contracted sheath exposed a rigid central core roughly 10 nm in width. These morphological features are illustrated in Fig. 1.

Host range of TSP-1. The ability of TSP-1 to form plaques on several hosts at 37 and 53 C was used to define its host range. TSP-1 was unable to form plaques at 37 C on any of the four *B. subtilis* strains tested. One strain, *B. subtilis* W23, will not grow above 50 C, but a mutant (W23

55^+) is able to grow above 50 C. Strains W23 55^+ and W168 and two other *B. subtilis* strains tested which grow above 50 C were sensitive to TSP-1, but only at the higher growth temperatures (50 to 55 C). TSP-1 formed plaques on *B. licheniformis* 9945A only above 50 C and did not form plaques on two *B. stearothermophilus* strains tested at 53 C. Attempts to form plaques at 37 C

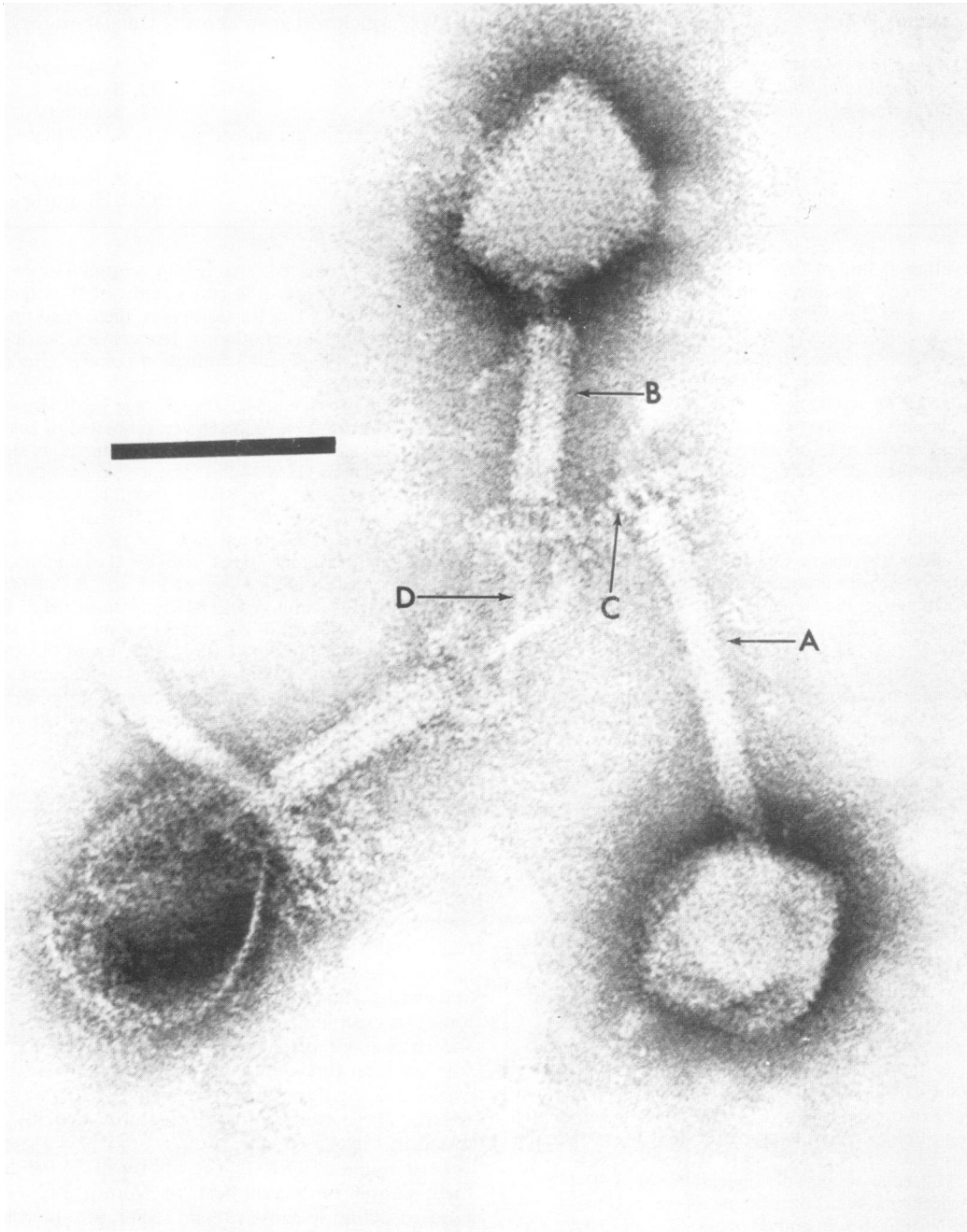

FIG. 1. *A negative stain of TSP-1 purified as described in the text. Note the long flexible tail (A), the contractile sheath (B), the pitchfork-like baseplate with its projections (C), and the rigid central core (D). Bar represents 100 nm.*

with as many as 10^{10} PFU per plate were unsuccessful.

Growth characteristics of TSP-1. Adsorption of TSP-1 to susceptible bacteria was determined by measuring the number of PFU per milliliter remaining after treatment of an infected culture with a few drops of $CHCl_3$. This procedure, which killed infected as well as uninfected bacteria, had no affect on unadsorbed bacteriophage. Adsorption occurred rapidly with more than 95% of the input phage inactivated by $CHCl_3$ after 2 min at 53 C. The lytic cycle was analyzed by monitoring the turbidity of infected cultures and performing one-step growth experiments. The results, illustrated in Fig. 2, are representative of the lytic cycle of TSP-1 in *B. subtilis* W168. The turbidity of an infected culture increased at about the same rate as an uninfected culture (data not shown) for about 50 min. At this point, cell lysis started and was completed by 90 min after infection. One-step growth curves reveal that the number of PFU per milliliter increases sharply after 30 min and begins to level off after 40 min.

These experiments revealed an intriguing aspect of TSP-1 replication, namely, the temperature-dependent inactivation of replicating phage. This is evident when the input phage titer is compared to the experimentally obtained titer. We repeatedly

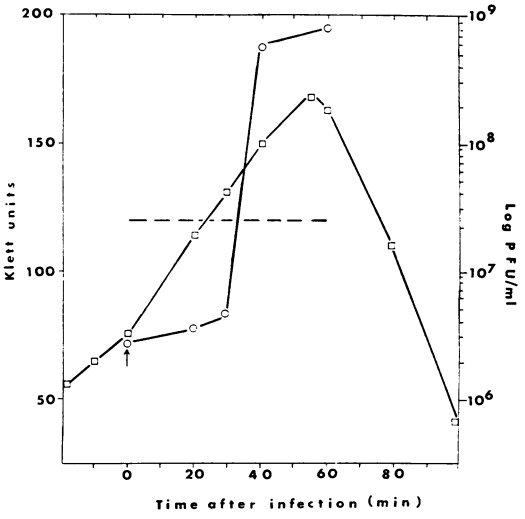

FIG. 2. *An experiment describing the lytic cycle of TSP-1 in Bacillus subtilis W168. Arrow indicates the time of addition of TSP-1 at a multiplicity of infection of approximately 1.0. The turbidimetric measurements are shown (□) and compared with the number of plaque-forming units (PFU) per milliliter at various times after infection (○). Dashed line represents the input phage titer. At the time of phage addition, there were approximately 5.0 × 10^7 colony-forming units of B. subtilis W168.*

observed at least a 95% inactivation of input phage whenever TSP-1 was added to a culture of *B. subtilis* growing at 53 C. This inactivation is probably due to an environmental change introduced by the phage assay procedure. One environmental change that could account for these results is the drop in temperature which occurs during sample dilution and as the soft agar overlay solidifies. Moreover, only the intracellular, replicating phages are sensitive to a reduction in temperature since (i) bacteria-free, TSP-1 phage suspensions are not inactivated by similar changes in temperature and (ii) the total phage titers (i.e., infected bacteria plus free phage) are essentially the same as those obtained after treatment with $CHCl_3$. As a result of this effect, it is impossible to determine the number of infected cells because they will not form plaques. With this in mind, and knowing how many cells are present in the infected culture, the burst size of TSP-1 can be estimated as approximately 30 to 50 particles per infected bacterium (Fig. 2). The one-step growth experiments indicate that the phage eclipse period lasts about 30 to 35 min. The rise period is about 15 min and phage replication appears to be complete 45 to 50 min after infection, with rapid cell lysis occurring shortly afterwards. The average burst size of TSP-1 was also determined by single-cell burst analysis. The results of this determination (Table 2) indicate an average burst size of about 55 particles per infected bacterium. This observed estimate is in close agreement with the calculated average burst size and falls slightly above the burst size estimated from the one-step growth experiments.

Temperature range of TSP-1. The temperature range necessary for plaque formation was determined by preparing equivalent sets of phage assay plates and incubating them at various

TABLE 2. *Single-cell burst size analysis of TSP-1 infections*

Determination	No.
No. of plates without plaques	86
No. of plates with plaques	32
Total no. of plates	118
Total no. of plaques	2,058
Average no. of infected bacteria per plate[a]	0.32
Calculated total no. of infected bacteria	37.2
Calculated burst size[b]	55.3
Observed total no. of infected bacteria	37.8
Observed burst size[b]	54.7

[a] Determined by the Poisson formula.

[b] Total number of plaques per total number of infected bacteria.

temperatures between 37 and 55 C. The results demonstrated that the efficiency of plating declines significantly below 51 C. Plaques formed at 51 C are also more variable in size. At temperatures below 50 C, plaques were not evident.

Bacteriophage nucleic acid. The characterization of TSP-1 nucleic acid was done with material extracted from purified phage preparations to avoid any contamination by DNA present in defective PBSX particles (15). TSP-1 nucleic acid gave a positive reaction with the diphenylamine test for deoxyribose When heated, it displayed the abrupt hyperchromic shift characteristic of double-stranded DNA molecules. The average of five separate extractions and T_m determinations with TSP-1 DNA was 87.9 ± 0.5 C. Each T_m was converted to the per cent guanine plus cytosine (%GC) by using the relationship described by deLey (6) [%GC = 2.44 (T_m − 69.4)]. The average of these calculations was 44.7% GC.

Several *B. subtilis* bacteriophages have been reported to contain uracil or 5-hydroxymethyluracil replacing thymine in their DNA (12, 14, 16, 18). The presence of these bases can alter the relationship between the %GC derived by T_m and buoyant density determinations (15, 18). The results of an experiment, shown in Table 3, in which TSP-1 DNA was hydrolyzed and the resulting nucleotides were identified by descending paper chromatography indicated that TSP-1 DNA does not contain any unusual bases in detectable amounts.

Estimate of the molecular weight of TSP-1 DNA. We estimated the molecular weight of TSP-1 DNA by cosedimenting ^{32}P-labeled TSP-1 DNA with ^{3}H-labeled T2 DNA. T2 DNA was used as a standard because its molecular weight has been well characterized (10, 17). The results of three different sedimentations are presented in Table 4. The molecular weight of TSP-1 DNA, calculated from these data, corresponds to approximately 56 × 10^6 daltons.

TABLE 3. *R_F values obtained from a hydrolysate of TSP-1 deoxyribonucleic acid*

Controls[a]	R_F	Control[b] mixture	R_F	TSP-1 DNA[c] hydrolysate	R_F
5′-dGMP	0.25	5′-dGMP	0.25	Spot 1	0.25
5′-dAMP	0.31	5′-dAMP	0.37	Spot 2	0.32
5′-dCMP	0.64	5′-dCMP	0.62	Spot 3	0.65
5′-dUMP	0.77	5′-dUMP	0.75		
5′-dTMP	0.85			Spot 4	0.84

[a] A 10-μliter amount of each nucleotide (0.02 M) was run separately. Abbreviations: dGMP, deoxyguanosine monophosphate; dAMP, deoxyadenosine monophosphate; dCMP, deoxycytidine monophosphate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.

[b] A 40-μliter amount of a nucleotide mix containing a 0.005 M solution of dGMP, dAMP, dCMP, and dUMP.

[c] A 50-μliter amount of TSP-1 DNA hydrolysate.

DISCUSSION

The foregoing results describe a new bacteriophage of *B. subtilis*, which we call TSP-1. Morphologically, TSP-1 shares many characteristics with other *B. subtilis* bacteriophages. The long tail and hexagonal head are also found in bacteriophages SP-8 (5), SP-50 (8), and AR-1, AR-2, and AR-3 (19). TSP-1, by comparison, appears to be slightly smaller than SP-8 (5). Although SP-8 and TSP-1 are similar morphologically, they are also distinguishable on this basis. SP-8 has a longer head, a thinner tail, and a different baseplate. Additionally, SP-8 does not form plaques at 53 C, thereby providing another distinguishing characteristic between these two phages.

The nucleic acid found in TSP-1 was identified as double-stranded DNA with a base composition of 44.7% GC. Similar base ratios have been reported for other *B. subtilis* bacteriophages, particularly SP α, SPx, φ1, φ14, SP-8, SP-82, and SP-01 (14). Hydrolysates of TSP-1 DNA indicated that it did not contain an analogue for thymine. Several other *B. subtilis* bacteriophages also have been reported which do not contain thymine analogues (14). The molecular weight of TSP-1 DNA was estimated at approximately 56 × 10^6 daltons, thereby comparing favorably with the reported molecular weights of other *B. subtilis* phages of similar size and morphology. The molecular weight of SP-8 DNA, for example, has been reported to be 69 × 10^6 daltons. Because TSP-1 is slightly smaller than SP-8, a smaller molecular weight DNA would be expected.

Certainly the most unusual property of TSP-1

TABLE 4. *Molecular weight of TSP-1 DNA*[a]

Expt no.	Length of run (hr)	D_2/D_1[b]	Mol wt of TSP-1 DNA
8-14-70	3.5	1.28	57.5 × 10^6
8-18-70	4.0	1.31	54.0 × 10^6
9-6-70	4.0	1.29	56.3 × 10^6

[a] Less than 2.5 μg of DNA was sedimented in each case.

[b] Sedimentation distances (D) are related to molecular weight as follows (10): $D_2/D_1 = (M_2/M_1)^{0.38}$.

is the restricted temperature at which it forms plaques. This requirement for temperatures above 50 C may be the reflection of the interaction of one or more of three distinct processes: (i) TSP-1 may be unable to adsorb and inject its DNA into cells grown below 50 C, (ii) a temperature-sensitive restriction mechanism may be present which prevents phage replication below 50 C but is inactive above 50 C, and (iii) TSP-1 may be a thermophilic bacteriophage unable to replicate below 50 C. These possibilities will be considered further in an accompanying paper.

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