Characterization of Two Lytic Bacteriophages of *Bacillus thuringiensis* Isolated from Soil

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ABSTRACT

Two lytic bacteriophages for *B. thuringiensis* designated as TV-1 and TV-2 were isolated from a soil treated with the host becterium as an insecticide. TV-1 phage has a head with a hexagonal outline measuring approximately 40 nm in diameter. The tail sheath measures about 110 × 15 nm when extended and 45 × 15 nm when contracted. TV-2 phage has a head with a similar hexagonal outline but a flexible tail with no contractile sheath. The head of TV-2 phage is approximately 45 nm in diameter and the tail measures about 160 × 7 nm.

Other properties of these two phages were also determined. They included host range sensitivity, one-step growth characteristics, sensitivity to ultraviolet light, ultrasonic effect and thermal inactivation.

INTRODUCTION

A considerable attention has been paid in recent years for the study of *Bacillus thuringiensis* bacteriophages since the pioneer work of Yoder and Nelson (15) who isolated two phages active on *B. thuringiensis* var. *thuringiensis*, *B. anthracis*, *B. cereus* var. *mycoides*, and *B. laterosporus*. Various other phages were isolated from different sources which showed cross-reactions between *B. thuringiensis* and *B. cereus* (7,8,10,13) as well as *B. subtilis* varieties (1). The Lysogenic phages for *B. thuringiensis* var. *galleriae* (6) were also found to bear a certain resemblance to previously reported virulent phages of *B. thuringiensis* (4,5).

The purpose of this investigation was to characterize two indigenous soil bacteriophages lytic for *B. thuringiensis*. The significance of these phages in soil treated with the bacterium was reported previously (12).

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MATERIALS AND METHODS

Bacteria

B. thuringiensis var. *thuringiensis* Berliner in the form of spore-crystal concentrate (Thuricide 9OT) obtained from the International Mineral and Chemical Corp. was used to prepare a pure culture stock of the organism as described previously (12). *B. thuringiensis* var. *galleriae* BH 11 and *B. thuringiensis* var. *entomocidus* BH 12 were provided by G. M. Boush, Department of Entomology, University of Wisconsin. All other prototrophs and auxotrophic mutants listed in Table 1 were obtained from the culture collection of H. O. Halverson, Molecular Biology Department, University of Wisconsin. The cultures were maintained on Eugon agar slants (Difco) at 4°C, and were transferred once every 3 months.

Media and growth of bacteria

Unless stated otherwise, the bacteria were grown in Difco nutrient broth made 0.002 M with respect to Ca^{2+}. Broth cultures were incubated in a Metabolyte G77 shaker water bath (New Brunswick Scientific Co., New Brunswick, N. J.) set at 25°C and 250 rev./min.

Preparation of bacteriophage stocks

Pure stocks of TV-1 and TV-2 phages originally isolated from soil (12) were used to produce high-titre phage lysates by confluent lysis on agar plates (2) followed by two

Table 1 *Bacillus* strains and some auxotrophic mutants, used in these experiments.

Strain number	Strain	Mutation deficiency
BH-1	*B. thuringiensis* var. *thuringiensis*	—
BH-11	*B. thuringiensis* var. *galleriae*	—
BH-12	*B. thuringiensis* var. *entomocidus*	—
1679	*B. cereus*, USDA 201	—
1678	*B. cereus* var. *mycoides*	—
1677	*B. cereus* var. *terminalis*	—
1682	*B. cereus* var. *terminalis*	lys
1683	*B. cereus* var. *terminalis*	pur
1684	*B. cereus* var. *terminalis*	lys, DPA
1685	*B. cereus* var. *terminalis*	pyr
1676	*B. polymyxa*	—
1680	*B. subtilis*	—
1681	*B. megaterium*, USDA 234	—
1691	*B. licheniformis*	—
1688	*B. licheniformis*	val
1689	*B. licheniformis*	lys
1690	*B. licheniformis*	arg, pur
1692	*B. licheniformis*	leu
1693	*B. licheniformis*	ile
1694	*B. licheniformis*	gly
1695	*B. licheniformis*	met
1696	*B. licheniformis*	his
1697	*B. licheniformis*	gly, ser, his

cycles of low (12,000 g for 30 min) and high (40,000 g for 2 hr) speed centrifuging. Resuspension of the bacteriophage pellets was effected by overlayering with the appropriate buffer or medium and standing for 16 hr to allow bacteriophage diffusion. Final suspensions contained 10^9 to 10^{11} pfu/ml.

Electron microscopy

A drop of phage suspension was applied to a Parlodion and carbon coated copper grid and allowed to remain for 1 min. Excess fluid was drawn off with filter paper, and a drop of 2% phosphotungstic acid solution adjusted to pH 6.8 with NaOH was added to the grid. Excess fluid was again drawn off with filter paper, and the grid was allowed to air dry. The grids were examined in an Hitachi HU-11E electron microscope at an accelerating voltage of 50 kv. The magnification was calibrated using a carbon grating replica (Ernest F. Fullman, Inc., Schenctady, N.Y.).

Host range

The host range of the phages were determined by adding 0.1 ml of a bacterial culture at the logarithmic phase containing approximately 10^8 cells/ml and 0.02 ml of a suitably diluted phage preparation to a tube of melted soft agar made 0.002 M with respect to Ca^{2+}. The contents were mixed and poured on to the surface of solid agar base plates. The host was considered sensitive when clearly defined plaques on the bacterial lawn were formed. Doubtful cases were repeated for verification.

One-step growth experiments.

The latent period and average burst size were determined by Adams one-step growth method (2). The multiplicity of infection (M.O.I.) was held to 1.0 or less and adsorption was allowed to proceed for 5 min while the infected culture was incubated in the shaker water bath at 25°C and 250 rev./min.

Phage sensitivity to medium's pH.

Samples of nutrient broth (Difco) medium were adjusted to pH 2.5–8.5 with 1N–HCl or 1N–NaOH, and placed in sterile tubes in 4.5 ml quantities. An amount of 0.5 ml of TV-1 phage containing 8×10^6 pfu/ml was added to each tube. The tubes were stirred and incubated for 24 hr at 25°C. A sample of 0.1 ml from each tube was used for plating.

Phage sensitivity to ultrasonic vibration.

Three-ml samples of tris-phage suspensions containing 3×10^5 and 8×10^4 pfu/ml of TV-1 and TV-2 phages respectively were subjected to 30 sec bursts of acoustic energy from a Biosonic II (Bronwill Scientific, Rochester, N.Y.) as described by Kropinski and Warren (9). The samples were then diluted in tris buffer and plated.

Phage inactivation by u.v. light irradiation.

One-ml samples of tris-phage suspension containing 5×10^4 pfu/ml of TV-1 phage were placed in Falcon plastic Petri plates of 5 cm diameter and irradiated at a distance of 37 cm with a General Electric 15 W germicidal lamp (principle wave-length at 2575A°) with continuous stirring. The irradiated samples were then diluted in tris buffer

and plated under yellow, nonphotoreactivating light. The plates were foil-shielded and incubated at 25°C.

Thermal inactivation

Tubes measuring 1.2 × 18 cm that contained 9.9 ml of nutrient broth were placed in constant temperature water bath at 65°C. After temperature equilibration for 10 min, 0.1 ml of the appropriate phage suspension was added to each tube to give approximately 10^7 to 10^8 pfu/ml. At regular intervals, 0.2 ml samples were removed to small test tubes chilled in an ice-bucket. All the samples were plated at the end of the experiment.

RESULTS AND DISCUSSION

Morphology and size

Electron microscopy showed the phages to be of two distinct morphological types. Phage VI-1 (Figs. 1 and 2) has a head with a hexagonal outline, a tail with a contractile sheath, and a base-plate with tail fibers attached. The head measures approximately 40 nm in diameter. The tail sheath measures approximately 110 × 15 nm when extended and about 45 × 15 nm when contracted. The diameter at one end of the tail is often seen to narrow and then widen to form a 'knob' (Fig. 2). In cases where the head is empty, the knob is seen to be inserted into the head. The subunits in the sheath appear to be arranged in a helix (Fig. 1). This arrangement can not be seen in contracted sheaths (Figs. 1 and 2).

Phage TV-2 had a head with a hexagonal outline and a flexible tail with no contractile sheath (Figures 3 and 4). The outline of the head could be interpreted as representing either an icosahedron or an octahedron. The head is approximately 45 nm in diameter. The tail measures approximately 160 × 7 nm. The tail exhibits a number of cross striations with a periodicity of about 3 nm.

Phage TV-1 belongs to Bradley's morphological group A, While phage TV-2 belongs to morphological type B (3). Both phages, however, differ in dimensions from related bactoriophages reported previously (1,4,5,6,11,14).

Host range

While *B. thuringiensis* var. *thuringiensis*, *B. thuringiensis* var. *entomocidus*, and *B. thuringiensis* var. *galleriae* were able to give a lytic response to infection with TV-1 phage, only the former two varieties were lysed by TV-2 phage. The latter variety was found to be resistant to infection by TV-2 phage even on repeated tests with increasing concentrations of Ca^{2+} and Mg^{2+} in the assay mixtures.

The ability of the two phages to form plaques on other prototrophic bacilli and some of their auxotrophic mutants (Table 1) was investigated. All tested strains were shown to be insensitive to lysis by these two phages. No attempt was made, however, to correlate a biochemical mutation of any host bacterium with susceptibility to phage infection.

One-step growth characteristics.

The latent period, eclipse time, and average burst size for TV-1 bacteriophage, were about 36 min, 44 min, and 67 pfu/bacterium respectively (Figure 5). A relatively similar

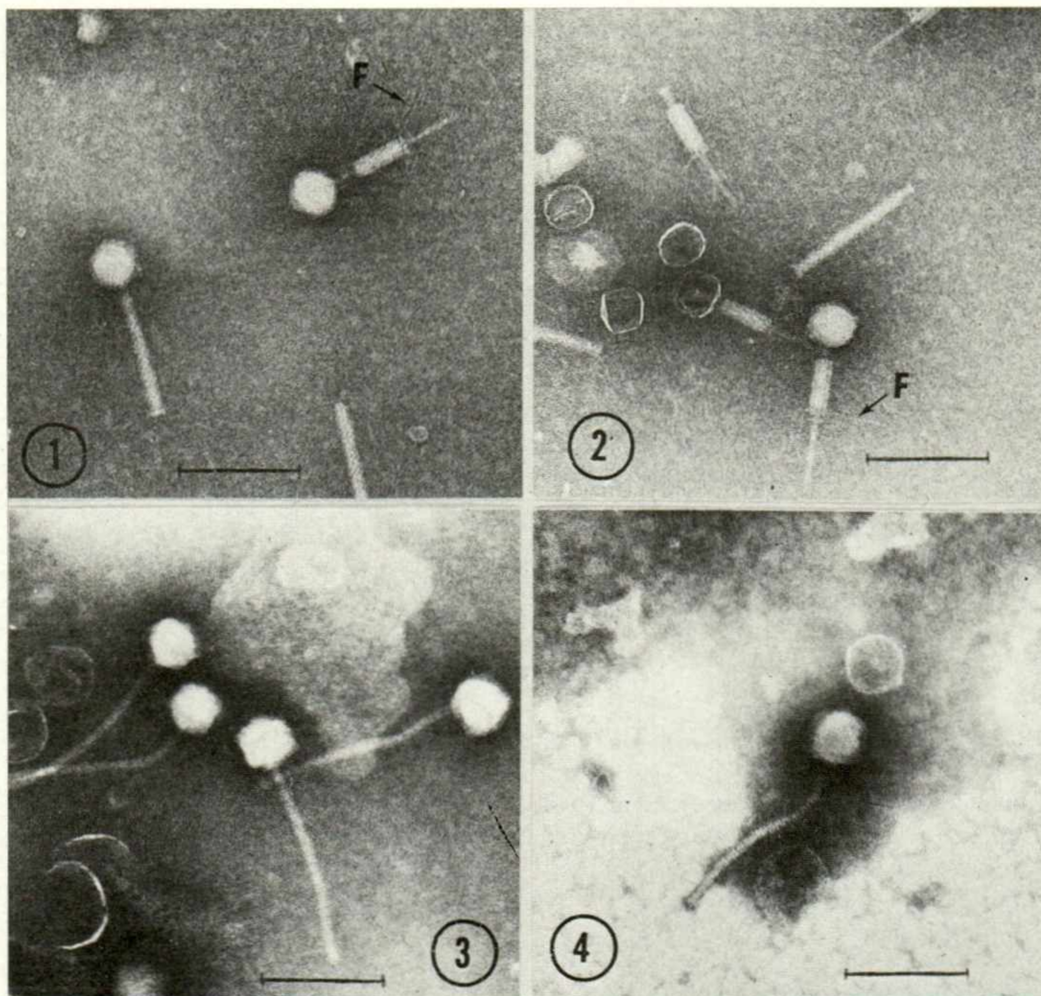

Fig. 1. Phage TV-1. Scale marker in all micrographs represents 100 nm. Tail fibers (F) can be seen attached to the base-plate of the contracted sheath. The apparent helical arrangement of subunits in the sheath can be seen. 200,000 ×.

Fig. 2. TV-1 showing the expanded proximal end of the tail which inserts into the head. 200,000 ×.

Fig. 3. Phage TV-2. The hexagonal outlines of the heads and cross-striations of the non-contractile tails are evident. 200,000 ×.

Fig. 4. Phage TV-2. The head gives the appearance of an octahedron. The distal end of the tail appears slightly expanded. 200,000 ×.

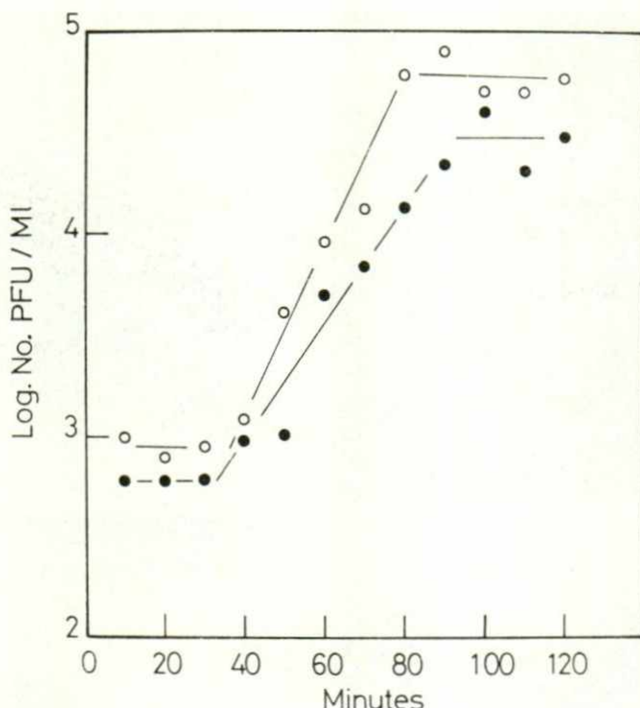

Fig. 5. One-step growth curves of TV-1 (○——○) and TV-2 (●——●) bacteriophages.

latent period (33 min) was observed for TV-2 phage. The eclipse time and the average burst size for the latter phage were rather different being 59 min and 50 pfu/bacterium respectively. The latent periods of both phages were relatively shorter than those reported for GV phages (5). However, their burst sizes were similar to those of GV-5 and GV-2 phages.

Sensitivity to medium's pH

This phage was relatively stable between pH 6.0 and pH 8.5 (Fig. 6). A rapid decline in numbers of infective centers was observed as the medium pH was decreased to pH 4.1. Phage TV-2 was also found to be stable at a pH range of 6.0–8.5.

Ultrasonic inactivation

Figure 7 shows that 1.33 percent of TV-1 phage population survived sonication for 1 min. The lethal effect of this treatment on TV-2 phage was relatively smaller. About 15.33% of TV-2 phage particles were maintained after 1 min of sonication. These results closely resemble the sensitivity patterns reported for *P. acidovorans* ϕW-14 a^+ phage and coliphage T1 (9). During the following 9 minutes of exposure, both TV-1 and TV-2 phages were gradually inactivated by this process.

Sensitivity to ultraviolet light

Multihit kinetics were observed for the inactivation of TV-1 bacteriophage. The 99% inactivation, calculated from the exponential region of the curve, was about 22 sec (Fig. 8).

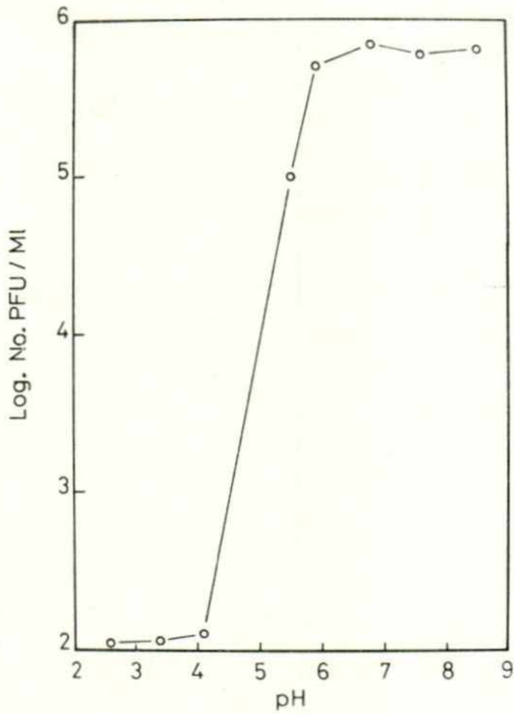

Fig. 6. pH inactivation of TV-1 bacteriophage.

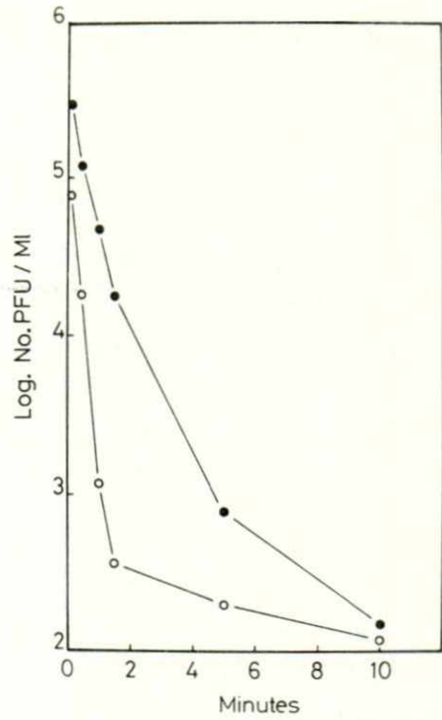

Fig. 7. Sonic sensitivity of TV-1 (○——○) and TV-2 (●——●) bacteriophages.

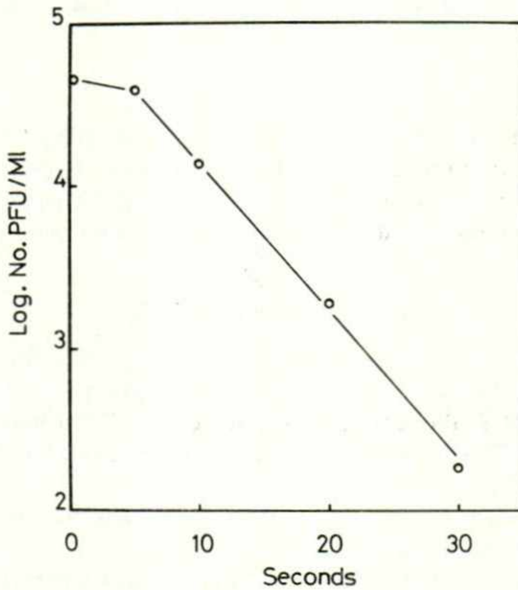

Fig. 8. Ultraviolet light inactivation of TV-1 bacteriophage.

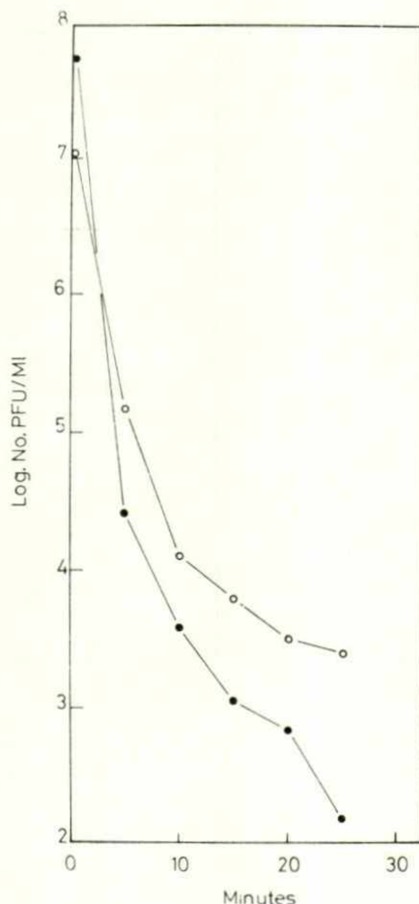

Fig. 9. Thermal inactivation of TV-1 (○ —— ○) and TV-2 (● —— ●) bacteriophages.

Thermal inactivation

TV-2 was more thermolabile than TV-1 phage. A mere 0.05 percent of TV-2 population survived heating for 5 min at 65°C (Fig. 9). A 100-fold higher survival percentage, however, was noted for TV-1 phage for the same exposure time and temperature. Both inactivation curves declined gradually thereafter, but at a slower rate for TV-1 phage.

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تشخيص نوعين من الفيروسات الخاصة بالبكترية باسلس ثرنجيينسس والمعزولة من التربة

صالح محسن صالح ز جى ، ام ٠ بوش ، جى ٠ ال ٠ بيت

المستخلص

عزل نوعين مختلفين من الفيروسات الخاصة بتلك البكترية من التربة وقد ظهر بان النوع الاول (ت٠ف ـ ١) ذو رأس سداس الشكل قطره حوالى ٤٠ نانومتر وذنب بابعاد ١١٠ × ١٥ نانومتر عندما يكون ممتد أو ٤٥ × ١٥ نانومتر عندما عندما يكون متشنجا ٠ أما النوع الثانى (ت٠ف ـ ٢) فله رأس سداسى مشابه للنوع الاول ولكنه ذو ذيل مرن ٠ وقد بلغ معدل قطر الرأس حوالى ٤٥ نانومتر بينما كانت ابعاد ذيله حوالى ١٦٠ × ٧ نانومتر ٠

وقد درست الخواص الاخرى لهذين الفيروسين كحساسية البكترية المختلفة لها وطبيعة نمو كل منها وحساسيتها للاشعة فوق البنفسجية وتاثير الموجات فوق الصوتية عليها وتاثرها بالحرارة ٠