Characteristics of ϕ T, the Temperate Bacteriophage Carried by *Bacillus megaterium* 899a

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Phage T was the only phage observed in lysates of *Bacillus megaterium* 899a induced with mitomycin C, 0.35 μ g/ml. The phage adsorbed slowly to its host in nutrient agar, giving rise to plaques of varying sizes and turbidity. Only clear plaques were observed when the phage and host cells were preincubated in an adsorption buffer and plated under optimum conditions. Plaque turbidity was caused by either the addition of 0.5×10^{-2} to 1.0×10^{-2} M CaCl_2 to the phage assay medium, or by raising the incubation temperature to 34 C. Phage T purified on a CsCl gradient had a density of 1.48 g/ml in CsCl and the extracted phage DNA had a buoyant density in CsCl of 1.6975 g/ml, equivalent to 38.2% guanine plus cytosine. The phage was rapidly inactivated at 75 C and was unstable in the presence of chloroform at 4 C, but it was stable in buffer stored in ice. When stage I sporulating cells were induced with mitomycin C, phage were carried into spores which when germinated lyse with the release of ϕ T. The burst size on induction of early-log vegetative cells was 52, whereas the burst size of induced T_0 sporulating cells, diluted in fresh medium, was 47 for a sporulating strain and 140 for an asporogenous mutant. A typical phage T had a long, noncontracting tail 240 nm long, 9 to 11 nm wide, with a repeating disk unit along the tail, 4 nm in size center to center. The tail ended in a small disk (15 nm wide) which is presumably for attachment to the host. The hexagonal head measures 68 by 57 nm and is composed of donut-shaped units 9 nm in diameter.

Two plaque types have been observed from lysates of *Bacillus megaterium* 899a and were considered due to two phage, ϕ T and ϕ C (8) (14). However, turbid and clear plaque types were not stable on repeated transfer in peptone (15). The phage from *B. megaterium* 899 lysates had also been designated as ϕ M1 (7), but different plaque types were not this time observed.

This investigation of ϕ T was begun in connection with a study of the effect of sporulation on the burst size of temperate phage in *B. megaterium* after mitomycin C induction. Our studies do not support the suggestion from the literature of multiple lysogeny in *B. megaterium* 899a. New data is presented for a further description of the bacteriophage.

MATERIALS AND METHODS

Bacterial strains and cultivation. *B. megaterium* 899a was kindly sent to us by J. T. Wachsman along with *B. megaterium* KM (T Tr U),⁻ which was used as a ϕ T indicator strain (11). An asporogenous strain *B. megaterium* 899a Sp⁻-1, which was blocked early in sporulation, was readily selected.

The growth and sporulation medium (22) was altered for *B. megaterium* 899a so that it contained only $\frac{1}{2}$ of the 1.47% solution of CaCl_2 used previously. Phage T was found to be stable at 4 C in a buffer containing 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.2), 5×10^{-3} M MgCl_2 , and 100 μ g of peptone/ml (Difco), and the adsorption medium for ϕ T was the above buffer containing 5×10^{-4} M CaCl_2 and 100 μ g of Casitone/ml.

Phage assay. The phage assay was similar to that used previously (11), but the media were changed so that in an overlay medium the bottom layer was nutrient agar (Difco, 1.5% agar) with 2×10^{-3} M MgCl_2 , and the top layer consisted of 4 ml of sloppy agar ($\frac{1}{3}$ nutrient agar plus $\frac{2}{3}$ nutrient broth (Difco) with 2×10^{-3} M MgCl_2), 1.0 ml of phage suspension and 0.2 ml of a suspension (10^6 bacteria/ml) of the indicator strain. The indicator bacteria were grown overnight on nutrient agar. The plates were incubated for 36 h at 29 C.

Phage induction. The method was similar to that used previously (11); 0.35 μ g of mitomycin C Nutritional Biochemical Co. (NBC)/ml was the minimum concentration which produced full lysis of the culture.

Phage purification. The phage was pelleted in the Beckman model L ultracentrifuge with a type 30 rotor at $78,000 \times g$ for 2 h and resuspended in phage buffer. Purification by two cycles of differential centrifuga-

tion was followed by concentration in a CsCl gradient. A 1-ml amount of phage suspension was layered on a discontinuous gradient formed in a 5-ml tube by layering 1.3 ml of each solution of CsCl having densities of 1.7, 1.5, 1.3 g/ml. After centrifugation for 1 h at $100,000 \times g$ in a SW50 rotor, the tube contents were scanned at 254 nm in a gradient analyzer (ISCO model D with UA₂ UV analyzer), and specific fractions were recovered. The density of each fraction was determined from the refractive index, and then the fractions were dialyzed against phage buffer. Optical cross-section (9) was determined with a Gilford spectrophotometer model 2400.

Buoyant density of DNA. DNA was extracted from purified ϕ T in standard saline citrate buffer (SSC) (pH 7.0) (12) by extraction with phenol (4) buffered with SSC. DNA was extracted from log-phase cells (12) of *B. megaterium* 899a. Equilibrium density gradient centrifugation in CsCl was determined with a Spinco model E ultracentrifuge (19), with an internal standard of *B. subtilis* DNA having a density of 1.7133 g/ml.

Electron microscopy. Phage T preparations were examined by potassium phosphotungstate (PTA) negative staining (2), except that ϕ T was suspended in phage buffer and mixed with 1% PTA, pH 6.9. Uranyl negative staining was carried out similarly with a 1% aqueous solution of uranyl acetate, and molybdate negative staining with a 1% aqueous solution of ammonium molybdate in ammonium acetate buffer, pH 7.2.

Average burst size. Sporulation medium was inoculated with vegetative cells from an overnight culture on nutrient agar to give an optical density at 650 nm (OD_{650}) of 0.05 in a Bausch and Lomb Spectronic 20 with a 1.0-cm cell. Early log-phase cells were induced at an OD_{650} of 0.18. The cell concentration was determined in a Petroff Hauser bacterial counting chamber viewed with a phase contrast microscope, and ϕ T was assayed 30 min before and 60 min after the beginning of phage lysis, which occurred 90 min after induction. It was reported (5), and we have also noted, that there was no increase in cell numbers after induction. The average burst size was estimated to be the increase in ϕ T titer divided by the number of lysed cells.

Trapping of induced ϕ T in spores of *B. megaterium* 899a. Mitomycin C (0.35 μ g/ml) was added to a sporulating culture of *B. megaterium* 899a at a time before the spore septum had formed, stage I (6). The resulting spores were harvested in buffer, 0.1 M NaCl, 0.01 M K₂HPO₄ (pH 7.0), and 10^{-3} M CaCl₂, heat shocked at 75 C for 15 min, and then titered for infectious centers using the phage assay.

Heat stability of ϕ T. One-half milliliter amounts of a suspension of purified ϕ T (10^8 phage/ml) in phage buffer were added to preheated tubes in a 75 C water bath. At specified times a tube was removed and cooled on ice, and the phage was diluted in ice-cold phage buffer and assayed.

Sensitivity to chloroform. A 5-ml amount of a suspension of purified ϕ T (10^8 phage/ml) in buffer was shaken with three drops of chloroform. A companion tube received no chloroform, and both tubes were stored at 4 C for 12 h and then assayed.

RESULTS

Some phage T characteristics are summarized in Table 1.

Plaque morphology. Lysates of induced *B. megaterium* 899a first assayed in an overlay agar consisting of $\frac{1}{2}$ nutrient agar and $\frac{1}{2}$ nutrient broth, with 2×10^{-3} CaCl₂ and MgCl₂ in both top and bottom layers, gave plaques of variable morphology. They were large, 1.5 mm, and small, 0.3 mm, both turbid and clear, and thus were quite similar to the mutant plaque types reported earlier (15). Representatives of each plaque type were picked off, suspended in nutrient broth, and reassayed. It was found that each plaque type gave rise with this method of assay to the original mixture of plaque types. A second round of cloning gave the same results. The plaque types therefore did not breed true.

Conditions of the phage assay were then optimized and the system was used as reported here. Secondly, the phage were preincubated at 29 C for 20 min with the host strain in phage adsorption buffer before being assayed. These last two changes resulted in an increase (60%) in titer efficiency and, more importantly, all plaques were clear and uniform in size. It appeared that the different plaque types noted originally were due to the plating phenomenon of slow adsorption in nutrient agar. Only one

TABLE 1. Summary of some characteristics of ϕ T

Characteristic	Determination
Stability in phage buffer:	
4 C	Excellent
75 C	Rapidly inactivated
4 C with CHCl ₃	Sensitive
Avg burst size ^a 899a sp ⁺	52/cell
Avg burst size ^a 899a sp ⁺	140/cell
Buoyant density of ϕ T	1.48 g/ml
Buoyant density of ϕ T DNA	1.6975 g/ml
Base composition of ϕ T	38.2% G+C
DNA ^b	
Average dimensions:	
Head	
Width	57 nm
Length	68 nm
Capsomeres	9 nm
Tail	
Width	9-11 nm
Length	240 nm
Terminal disk width	15 nm
Disk units ^c	4 nm
Type ^d	Long, noncontracting

^a After mitomycin C induction.

^b By calculation (16).

^c Center to center spacing.

^d Bradley (15).

plaque type, clear, was present, and this was borne out by examination of phage preparations in an electron microscope (EM) where only one phage type was seen.

All plaques were turbid when 0.5×10^{-2} to 1.0×10^{-2} M CaCl₂ was added to the assay medium in the presence or absence of equal quantities of MgCl₂. This turbidity caused the plaque center to be milky but did not seriously interfere with plaque counting. Turbid plaques were also obtained by raising the incubator temperature to 33 to 35 C. This type of turbidity was due to bacterial growth in the plaque centers and made enumeration difficult. For this reason the incubation temperature was carefully controlled at 29 C.

Phage stability. Phage T was rapidly inactivated at 75 C in phage buffer, where more than 10^8 phage/ml was inactivated in 2 min (Table 2). Under the conditions of our test, the phage titer fell by four logs in the presence of chloroform. Long-term stability in bacterial lysate at 4 C is poor, as a titer of 6.0×10^8 phage/ml fell to 4.7×10^8 phage/ml in 30 days. However, the phage is stable in the phage buffer.

Average burst size. The burst size was 52; however, if cultures grown in sporulation medium to time T₀ (16) were induced and diluted in fresh medium, then the burst size was found to be 47 for the sporeforming strain and 140 for the asporogenous mutant.

Carrying of induced ϕ T in spores. The induced form of ϕ T was carried in spores of *B. megaterium* 899a, and these spores, when germinated, lysed and released ϕ T. This takes place in 90% of spores only if induction is carried out at an optimum time (stage 1) in sporulation. That is, 10% of the spores gave rise to centered plaques (noninduced phage) when spores were examined by the phage assay. This occurred in a manner analogous to ϕ e infection in sporulating cells of *Bacillus subtilis* (18). In fact, if ϕ T is used as lytic phage and *B. megaterium* KM Sp⁺ as the host, then infection and carrying of phage into spores occurs at stage 1 of sporulation, as it did for the induced phage in the lysogenic strain. Details of this will be published separately.

Phage purity. The purified ϕ T of titer $4.7 \times$

10^{10} /ml had an OD₂₆₀ of 0.277 which gave an optical cross-section of 0.59×10^{-11} cm². This lies in the range for purified phage, although we did note a few pieces of cell wall as impurities.

Phage Density. Figure 1(a) shows the optical density scan of purified ϕ T on a CsCl gradient, from which the density of the phage was determined to be 1.48 g/ml. Figure 1(b) shows the

FIG. 1. Optical density (OD) scans of CsCl gradients with the density plot given by the broken line, and ϕ T by the sharp peak shown in (a). Gradient (b) contained a 25 $\times$ concentrated lysate of induced *B. megaterium* 899a. Peak A contained empty ϕ T, $\rho = 1.29$ g/ml, and peak B contained viable ϕ T, $\rho = 1.48$ g/ml.

TABLE 2. Heat stability of ϕ T

Elapsed time (s)	ϕ T assay (stage/ml)
0	1.53×10^8
15	1.41×10^8
30	0.57×10^8
60	0.02×10^8
120	0

optical density scan of concentrated *B. megaterium* 899a lysate on a CsCl gradient where ϕ T was identified as the small peak B, and peak A contained ϕ T with unfilled heads, with a density of 1.28 g/ml. These peaks did not indicate pure components. Figure 1(a) showed that ϕ T was stable in the CsCl gradient.

Buoyant density of ϕ T DNA. Figure 2(a) shows the OD₂₆₁ scan of DNA from ϕ T and standard at equilibrium in CsCl. The density of ϕ T DNA was calculated to be 1.6975 g/ml. Figure 2(b) shows the OD₂₆₁ scan of an equal mixture of ϕ T and *B. megaterium* 899a DNA with the standard. The mixed peak is broader than ϕ T DNA alone but is not resolved from the bacterial DNA. It is concluded that the densities of DNA of ϕ T and *B. megaterium* 899a are very close. The buoyant density of mixed DNA was calculated to be 1.6990 g/ml. The density of the DNA from a strain of *B. megaterium*, strain unnoted, was reported as 1.697 g/ml (17).

Phage morphology. Measurements of the dimensions of ϕ T were taken from the PTA negative stains seen in Fig. 3 and 4. The repeated disk unit on the tail was measured in Fig. 5 and 7, and the units in the phage head were obtained from Fig. 6, an ammonium molybdate negative stain. These results are in Table 1.

FIG. 2. Optical density (OD) scans of CsCl gradients run to equilibrium in a Spinco model E ultracentrifuge. (a), Peak A contained ϕ T DNA and peak B the *B. subtilis* DNA ($\rho = 1.7313$ g/ml) used as a standard. (b), Peak A contained a mixture of ϕ T DNA and DNA from *B. megaterium* 899a, and peak B the *B. subtilis* standard DNA.

DISCUSSION

The variable plaque morphology of phage from induced lysates of *B. megaterium* 899a, reported earlier (8, 15), was shown to be controlled, with an increase in titer efficiency, by changes in the plating medium and by preadsorption of phage to host. One plaque type was present and one phage type was seen in EM preparations, and so there was no evidence for more than one phage in the lysate of *B. megaterium* 899a induced under our conditions.

Phage T was unstable; however, this is characteristic of many *Bacillus* phages (23). In another communication (submitted for publication) we have described how ϕ T was unstable under some conditions when drying in PTA negative stains, and how this may be avoided. Chloroform could not be used to stabilize ϕ T suspensions, but stability was achieved by storing the phage in packed ice. Dimethyl sulfoxide added to phage lysates did not stabilize them; but we did not freeze the lysates as has been suggested (23). We have described conditions which allow the phage to be stored and used for a variety of purposes. Phage T was readily inactivated by heat and we have made use of this property in demonstrating ϕ T carried in spores after suitable induction. The phage was liberated during growth reaching titers of about 10^7 /ml, and these phage must be removed from the harvested spores or they will add to the estimate of spores as infective centers. This removal was done by first heating the spores to 75 C for 15 min. Phage which were added to spores, and then heat treated, were not recovered by the phage assay.

The density of ϕ T, 1.48 g/ml, was comparable to the density of ϕ of *Escherichia coli*, which is the same morphological type as ϕ T, and has been reported (21) as 1.47 to 1.52 g/ml. The buoyant density of ϕ T DNA, 1.6975 g/ml, was substituted in the equation relating buoyant density and base composition of DNA (17), giving a base composition of 39.2% guanine plus cytosine (G+C). This is close to the base composition of *B. megaterium* strains, 34 to 38% G+C, compiled by Hill (10).

Lytic phage may be carried in spores of *Bacillus* spp. (18), and ϕ PB S-1, a pseudolysogenic noninducible phage, may be carried into spores of *B. subtilis* (20). Our results appear to be the first reported instance of a temperate phage being induced during sporulation and being carried in the induced form into the spores of a *Bacillus* sp., and are likely to be analogous to those reported for ϕ e (18).

B. megaterium 899a grows in chains of variable length composed of rod-shaped units, each

FIG. 3. PTA-negative stain of ϕT . The arrow indicates the phage tail terminal disk. $\times 112,000$.

FIG. 4. PTA-negative stain of ϕT showed empty and filled hexagonal heads. $\times 194,000$.

FIG. 5. Ammonium molybdate negative stain of ϕT . The arrow indicates the repeating tail units. $\times 194,000$.

FIG. 6. Ammonium molybdate negative stain of ϕT . The arrow indicates the donut-shaped capsomeres of the phage head. $\times 194,000$.

FIG. 7. Uranyl acetate negative stain of ϕT . The arrow indicates the repeating disk-like units on the tail. The phage head is positively stained. $\times 145,000$.

usually made up of a number of cells ranging from 2 to 8. This presents some difficulty in determining the number of cells contributing to a burst of phage determined by a dilution method, particularly when the culture does not lyse fully. Therefore, the method used to estimate the average burst size is reasonable in these circumstances. The burst size of ϕ T from *B. megaterium* 899a undergoing spontaneous phage production has been reported as 45 (3), and is comparable to our result of 52 for ϕ T production after induction with mitomycin C. The burst size of ϕ T from the host *B. megaterium* S, a nonsporulating strain, has been reported as 105 (7). We have shown a burst size of 140 in *B. megaterium* 899a sp⁻¹ after induction. Furthermore, many incomplete ϕ T have been detected in bacterial lysates (Fig. 1(b)). We are continuing with this aspect of the investigation to determine if ϕ T production was restricted in some way by sporulation in the lysogenic host.

B. megaterium phage were previously visualised in metal-shadowed preparations in an EM (7, 13), and examples of the more recent technique of PTA negative staining appear not to be available. No photograph of ϕ M-1 of *B. megaterium* 899a was published, but the dimensions from a palladium-shadowed EM preparation were reported as a head diameter of 62 nm and a tail length of 290 nm (7). Our results indicate a hexagonal head of 68 by 57 nm and a tail 240 nm long which is substantially in agreement with the previously published measurement. In addition, the phage T tail was 9 to 11 nm wide with a repeating disk unit along the tail of 4 nm, ending in a terminal disk 15 nm wide, and the phage head was composed of donut-shaped capsomeres with 9-nm diameters.

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