JOURNAL OF BACTERIOLOGY, Jan., 1965
Vol. 89, No. 1
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Induction and Properties of a Temperate Bacteriophage from *Bacillus stearothermophilus*

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Received for publication 27 June 1964

ABSTRACT

WELKER, N. E. (University of Illinois, Urbana), AND L. LEON CAMPBELL. Induction and properties of a temperate bacteriophage from *Bacillus stearothermophilus*. J. Bacteriol. **89**:175–184. 1965.—*Bacillus stearothermophilus* 1503-4R growing at 55 C was induced to lyse either when 0.05 μg/ml of mitomycin C was added or when it was exposed to ultraviolet light for 30 sec. Lysis of the induced cultures occurred 45 to 60 min after induction. Phage were assayed on *B. stearothermophilus* 4S giving turbid plaques 0.05 to 0.3 cm in diameter. Noninduced cultures of 1503-4R spontaneously produced one phage per 2.8×10^6 bacterial cells. The optimal temperature for phage production and assay was found to be 55 C. *B. stearothermophilus* 1503-4R was immune to the isolated temperate phage (TP-1) and to a clear-plaque mutant phage (TP-1C), even when tested at phage mutliplicities of 100. TP-1 and TP-1C phage were identical morphologically having a head 65 mμ in diameter and a tail 240 mμ long and 12 mμ wide. TP-1C phage deoxyribonucleic acid (DNA) had an $S_{20,w}$ value of 24.1 and a calculated molecular weight of 1.21×10^7. DNA base compositions of TP-1 and TP-1C phage were identical (42% guanine + cytosine), but differed significantly from those of the lysogenic or indicator strains of *B. stearothermophilus* (50% guanine + cytosine). No unusual bases were detected in either the bacterial or phage DNA.

During a study of the synthesis of α-amylase by *Bacillus stearothermophilus* (Welker and Campbell, 1963*a*, *b*), it was found that growing cultures of this organism began to lyse 45 to 60 min after the addition of the antibiotic mitomycin C (Hata et al., 1956). This suggested that this strain of *B. stearothermophilus* was lysogenic, and that, upon addition of mitomycin C, a temperate phage was induced which resulted in lysis of the culture. The induction of phage and bacteriocins by mitomycin C has been reported previously (Otsuji et al., 1959; Levine, 1961; Iijima, 1962; Korn and Weissbach, 1962; Sutton and Quadling, 1963). This paper describes the induction of a lysogenic strain of *B. stearothermophilus* and some of the properties of the temperate phage.

MATERIALS AND METHODS

Organisms. The organism used in this study was an obligately thermophilic strain of *B. stearothermophilus* (1503-4R) employed previously (Welker, 1963; Welker and Campbell, 1963*a*, *b*). *B. stearothermophilus* strain 4S served as the indicator organism for the induced phage.

Media. The two liquid media used were the MCH medium of Welker and Campbell (1963*a*), which is a minimal medium supplemented with 0.1% casein hydrolysate, and TY medium composed of Trypticase, 20 g; yeast extract, 5 g; $FeCl_3 \cdot 6H_2O$, 7 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; and $MgSO_4 \cdot 7H_2O$, 15 mg; per liter of distilled water. After sterilization at 121 C for 15 min, the media were adjusted to *p*H 7.3 with sterile 10% KOH. Fructose (sterilized separately) served as the carbon source at a concentration of 0.5%. These media are designated MCHF and TYF.

Trypticase agar (medium TY minus yeast extract plus 2% agar) was used as the plating medium (40 to 45 ml per glass petri plate). Prior to use, the plates were incubated at 55 C for 12 hr and then at room temperature for 24 hr to remove excess moisture.

Preparation of inocula. All growth experiments with liquid media were carried out at 55 C in a New Brunswick gyrotory water bath shaker (model G-76) at a speed of 133 rev/min, describing a 0.5 in. diameter circle.

A 250-ml Erlenmeyer flask containing 20 ml of TYF medium was inoculated with 12-hr cells from a Trypticase agar plate and shaken for 2 hr. The cells were removed by centrifugation, and, for experiments involving TYF medium, the cells

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were washed once with 5 ml, and suspended in 4 ml, of TYF medium. For experiments using MCHF medium, the cells were washed once with 5 ml, and suspended in 4 ml, of the M buffer described by Welker and Campbell (1963*a*). Cells prepared in the above manner are referred to as TYF-log phase cells or MCHF-log phase cells, and were used as the inocula for all growth experiments.

Viable-cell counts. To determine the number of viable cells, samples were removed from the growth flasks and diluted in the appropriate growth medium; 0.1 ml of the diluted cells was then mixed with 3 ml of prewarmed (50 C) soft agar (2% Trypticase-1% agar) in capped tubes (13 × 100 mm). After mixing thoroughly, the contents of each tube were poured onto the surface of a Trypticase agar plate.

Induction of lysis. Induction experiments were performed in a 250-ml nephelometer flask, containing 20 ml of either TYF or MCHF medium. The flasks were inoculated with 0.1 ml of TYF-log phase cells or 0.2 ml of MCHF-log phase cells, respectively. Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 mμ. After induction, growth was followed until lysis was complete, or until the cultures entered the stationary phase of growth.

Induction by ultraviolet light was accomplished by placing the contents of the experimental flask in a sterile petri dish, and exposing the cells (while being agitated) to a General Electric germicidal lamp at a distance of 50 cm. The irradiated cells were placed in a sterile nephelometer flask, wrapped in aluminum foil, and returned to the shaker.

Detection of phage in induced lysates. The procedure used permitted the detection of phage or bacteriocins. Twenty-four strains of *B. stearothermophilus* and 41 unclassified obligately thermophilic bacteria (isolated in this laboratory) were tested for their ability to serve as indicator organisms. Cultures of each strain were prepared by inoculating capped tubes (20 × 150 mm), containing 5 ml of TYF medium, with a loopful of cells from a 12-hr Trypticase agar plate. The tubes were incubated for 2 hr at 55 C on a rotary shaker. A 0.5-ml sample of the cells was then mixed with 3 ml of Trypticase-soft agar (supplemented with 0.015 M $CaCl_2$), and spread evenly over the surface of a Trypticase agar plate. The mitomycin C- or ultraviolet light-induced lysate of *B. stearothermophilus* 1503-4R was centrifuged to remove cell debris; 0.1 ml of the lysate was dropped onto the seeded bacterial lawns, and spread by tipping the plate. The plates were incubated at 55 C for 12 hr. This procedure is called the drop-assay for phage.

Forty of the strains tested showed general areas of lysis corresponding to the area covered by the lysate. In control experiments, mitomycin C (0.05 or 0.1 μg/ml) did not lyse any of the strains tested. With the exception of one strain, none of the lysed areas could be propagated by transfer to fresh plates seeded with the corresponding bacterial strain. Transfer of the lysed area on *B. stearothermophilus* 4S to fresh seeded plates of this strain gave rise to turbid plaques. These plaques were picked and purified by further propagation on fresh lawns of this strain. The phage, after passage through strain 4S, was assayed by the conventional agar overlay method (Adams, 1950) by use of the soft agar and plating media described above. The phage isolated from mitomycin C-induced lysates was designated TP-1. A clear-plaque mutant, which appeared on subsequent transfers of TP-1 on strain 4S, was isolated and designated TP-1C.

A phage endolysin is responsible for the areas of lysis occurring on the other strains. This enzyme has been purified, and a study of its properties will be reported separately.

Purification of phage. TP-1 and TP-1C phage were purified as follows. The starting inoculum was prepared by inoculating a 2,800-ml Fernbach flask, containing 1 liter of TYF medium, with 3 to 4 loopfuls of cells of *B. stearothermophilus* 4S from a 12-hr Trypticase agar plate. The flask was shaken on a rotary shaker at 55 C for 6 hr. This culture was used to inoculate (100 ml per flask) six 2,800-ml Fernbach flasks, each containing 1 liter of TYF medium. After incubation at 55 C for 1 hr on a rotary shaker, TP-1 or TP-1C phage were added to a final concentration of 1.0×10^7 plaque-forming units (PFU) per ml. The flasks were incubated for an additional 7 hr, and then the cells were removed by centrifugation. TP-1C lysates contained 2.0 to 7.0×10^{10} PFU/ml, and TP-1 lysates contained both TP-1 (5.0 to 6.0×10^7 PFU/ml) and TP-1C (2.5 to 3.0×10^8 PFU/ml). See the section on properties of phage TP-1 for explanation.

Lysozyme, ribonuclease, and deoxyribonuclease (50 μg/ml each) were added, and the lysates were stirred for 2 hr at ambient room temperature and placed at 4 C overnight. The lysates were brought up to ambient room temperature, and the phage were precipitated by the addition, with stirring, of ammonium sulfate to a saturation of 70%. The mixture was placed at 4 C for 18 to 20 hr to allow the precipitate to settle.

The clear supernatant fluid was siphoned off; the precipitate was collected by centrifugation and suspended in 150 ml of 0.01 M potassium phosphate–0.05 M NaCl–0.001 M $MgCl_2$, *p*H 7.0 buffer (PNM-buffer). The larger particles were removed by centrifugation at 10,000 rev/min (Servall, SS-34 rotor) for 30 min. The suspended phage were dialyzed against 12 liters of PNM-buffer for 48 hr at 4 C.

The phage were collected by centrifugation at 17,500 rev/min for 2 hr and the pellets were gently suspended in 10 to 15 ml of PNM-buffer. The larger particles were removed by centrifugation at 10,000 rev/min for 30 min. The supernatant fluids contained 95% of the starting PFU of phage.

The phage were further purified by banding in a cesium chloride (density, 1.50 g/cc) density gradient (Meselson, Stahl, and Vinograd, 1957). After 20-hr centrifugation at 30,000 rev/min in a Spinco model L centrifuge (rotor no. SW39), the

layer of phage was removed with a pipette and dialyzed against several changes of 0.15 M NaCl–0.015 M trisodium citrate, *p*H 7 (SSC).

Preparation of phage for electron microscopy. A suspension of purified phage was mixed with an equal volume of 1% phospho-tungstic acid solution adjusted to *p*H 7.0 (Brenner and Horne, 1959). A droplet of this mixture was placed on a carbon-coated Formvar grid, and air-dried. Purified phage, in SSC, were also shadowed with uranium trioxide. The grids were examined in a Siemens Elmiskop I, and electron micrographs were made at initial magnifications of 20,000 and 40,000 ×.

Isolation of deoxyribonucleic acid (DNA). Bacterial DNA was isolated from log-phase cells growing in TYF medium by the method of Marmur (1961). Phage DNA was isolated by shaking purified phage with equal volumes of water-saturated phenol (Kirby, 1957) for 30 min on a wrist-action shaker at 4 C (phenol was first adjusted to *p*H 7.0 with 1 N NaOH). The layers were separated by centrifugation, and the aqueous layer, containing the nucleic acids, was removed. Residual phenol was removed by dialysis against several changes of SSC. DNA was isolated by use of the procedure of Marmur (1961), starting with the last 95% ethanol precipitation step. Both bacterial and phage DNA preparations were dialyzed for 16 hr at 4 C against 500 ml of SSC. Purified bacterial and phage DNA gave typical absorption ratios at 260:230:280 mμ of 1.0:0.48:0.54.

Buoyant density (ρ) measurements. Portions of the dialyzed DNA samples were centrifuged to equilibrium in a Spinco model E analytical ultracentrifuge at 44,770 rev/min at 25 C for 18 to 24 hr in approximately 5.7 M CsCl–0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.5) with N^{15}-labeled *Pseudomonas aeruginosa* DNA (ρ 1.742 g/cc) as a reference standard. The banded DNA was photographed by use of ultraviolet-absorption optics. Photographs were traced with a Joyce-Lobel double-beam recording microdensitometer. Buoyant densities and base compositions were calculated according to the equations of Sueoka (1961).

Thermal denaturation measurements. Thermal denaturation (Tm) of the dialyzed DNA samples was followed in an Optica spectrophotometer equipped with a heated cuvette holder and temperature-control unit. Base compositions were calculated by use of the relationship of Marmur and Doty (1962), per cent guanine + cytosine (G + C) = (Tm − 69.3)2.439.

Chemical determination of DNA base composition. Bacterial or phage DNA was precipitated with 2 volumes of 95% ethanol, and 0.5- to 1.5-mg samples were dissolved in 0.5 ml of 90% formic acid. The DNA was then transferred to a 1-ml glass bulb and sealed under reduced pressure. The sample was hydrolyzed at 175 C for 30 min and cooled, and the hydrolysate was taken to dryness under reduced pressure. The hydrolysate was dissolved in 0.1 to 0.2 ml of 0.1 N HCl, and 0.05 ml was used for chromatography on Schleicher and Schuell no. 598 paper with isopropanol-concentrated HCl-water (171:41:39, by volume) as the solvent. The bases, located with an ultraviolet lamp, were eluted with 5 ml of 0.1 N HCl, and the concentration was determined from the measured absorbance and their molar extinction coefficients (Bendich, 1957). The bases were measured against corresponding filter paper blanks eluted in parallel.

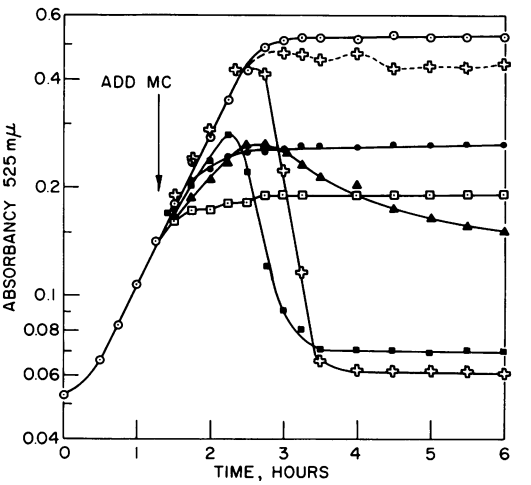

FIG. 1. *Effect of mitomycin C concentration on growth of Bacillus stearothermophilus 1503-4R. Mitomycin C (MC) was added, as indicated by arrow, to cultures growing at 55 C in MCHF-medium. Control, ⊙; 5 μg/ml, ⊡; 1 μg/ml, ●; 0.5 μg/ml, ▲; 0.1 μg/ml,; ■ 0.05 μg/ml, ——+——; 0.01 μg/ml, – – – + – – –.*

Determination of sedimentation coefficients ($S_{20,w}$). The $S_{20,w}$ of purified TP-1C phage (8 × 10^{11} particles) in SSC was determined in a Spinco model E ultracentrifuge with Schlieren optics at a rotor speed of 29,500 rev/min using a 12-mm centrifuge cell, fitted with a Kel-F centerpiece. The $S_{20,w}$ of purified TP-1C phage DNA (24 μg/ml) in SSC was determined by use of ultraviolet optics and a rotor speed of 35,600 rev/min.

Chemicals. Mitomycin C was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Actinomycin D was obtained through the courtesy of C. A. Stone, Merck Institute for Therapeutic Research, Westpoint, Pa. 8-Azaguanine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Phenethyl alcohol and aminopterin were obtained from J. T. Wachsman, and chloramphenicol and puromycin from S. Spiegelman.

RESULTS AND DISCUSSION

Induction of cells growing in MCHF medium. Induction studies were initially carried out in MCHF medium. Figure 1 shows that mitomycin C (MC), at concentrations of 0.05 or 0.1 μg/ml, induced lysis of growing cultures 60 to 75 min after its addition. The rate of growth was not

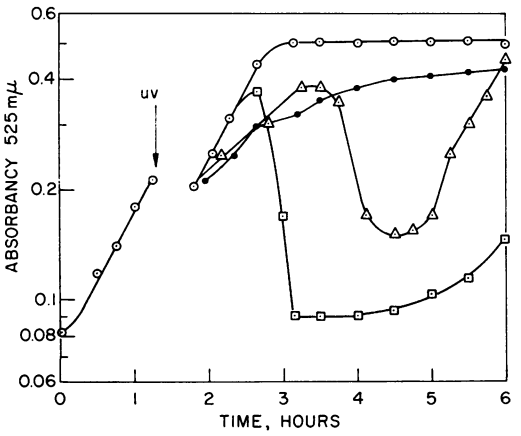

FIG. 2. *Effect of ultraviolet irradiation on growth of Bacillus stearothermophilus 1503-4R. Cultures growing at 55 C in MCHF-medium were exposed to a General Electric germicidal lamp at a distance of 50 cm. The break in the growth curves corresponds to the time the cultures were removed from the rotary shaker. Exposure time: zero control, ⊙; 30 sec, ⊡; 180 sec, △; 300 sec, ●.*

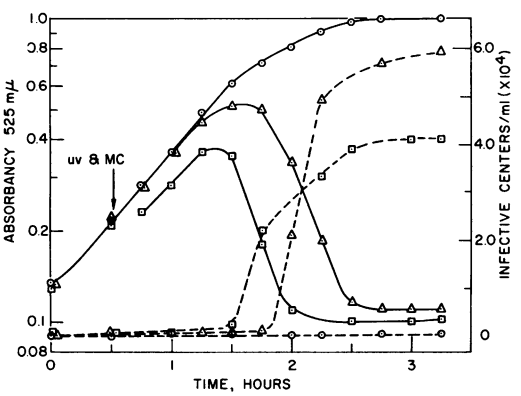

FIG. 3. *Development of infective centers in non-induced and in mitomycin C- or ultraviolet-induced cultures of Bacillus stearothermophilus 1503-4R. Induction was carried out, as indicated by the arrow, on cultures growing at 55 C in TYF medium. Phage were assayed at 55 C on B. stearothermophilus 4S, by the drop-assay described in the text. Solid line, growth; dashed line, infective centers; noninduced culture, ⊙; mitomycin C-induced culture, △; UV-induced culture, ⊡.*

affected by 0.05 μg/ml of MC, but higher concentrations (0.1 to 5.0 μg/ml) either inhibited growth or decreased the rate of growth. MC at concentrations less than 0.05 μg/ml did not induce lysis or inhibit growth.

The induction of lysogenic bacteria by ultraviolet light has been well established (Lwoff, 1953). Low doses (30 sec) of ultraviolet light also induced lysis of growing cultures of *B. stearothermophilus* 1503-4R, with lysis occurring 60 to 75 min after ultraviolet exposure (Fig. 2). Higher doses of ultraviolet light resulted in either incomplete lysis (180 sec) or conversion to linear growth (300 sec); doses less than 30 sec had no effect on growing cultures.

Several agents have been shown to induce lysogenic bacteria (Lwoff, 1953). All of these agents appeared to have one thing in common: interruption of DNA synthesis. We, therefore, tested other compounds which are known to inhibit the synthesis of DNA and RNA, as possible inducing agents. Lysis of growing cultures occurred almost immediately after the addition of 0.5 or 1.0 μg/ml of actinomycin D. Phenethyl alcohol (0.1 or 0.25%) also caused an immediate lysis of growing cultures. Actinomycin D is a polypeptide antibiotic which inhibits RNA synthesis presumably by binding the DNA primer (Kahan, Kahan, and Hurwitz, 1963). Phenethyl alcohol has been reported to selectively inhibit DNA synthesis in gram-negative bacteria (Berrah and Konetzka, 1962). Several attempts to isolate phage from the actinomycin D- or phenethyl alcohol-induced lysates were unsuccessful. This fact coupled with their almost immediate effect on growing cultures suggests that these agents induce lysis by a mechanism different from that of MC or ultraviolet light.

Aminopterin has been shown to induce lysogenic bacteria (Ben-Gurion, 1962). This compound at levels of 0.05 to 10 μg/ml did not induce lysis of strain 1503-4R. Chloramphenicol, puromycin, and 8-azaguanine over a concentration range of 0.01 to 10 μg/ml also failed to induce lysis of this strain.

Induction of cells growing in TYF medium. Cells growing in TYF medium were induced by MC (0.05 μg/ml) or ultraviolet light (4 to 6 min) with lysis occurring 45 to 60 min after exposure to the inducing agent. The longer ultraviolet light exposure time required to induce lysis was probably due to the protective effect of the more complex medium. The production of phage from induced (MC and ultraviolet light) and non-induced cultures growing in TYF medium is shown in Fig. 3. Increased phage production did not begin in the induced cultures until the onset of lysis. Induction with MC resulted in the production of a greater number of phage than did induction by ultraviolet light. This may be due to a secondary or indirect effect of ultraviolet light on host or phage DNA synthesis. The non-induced cultures spontaneously produced one phage per 2.8×10^6 bacterial cells.

A comparison of phage production by induced and noninduced cells growing in TYF and MCHF

media is shown in Table 1. Cells (induced or non-induced) growing in TYF medium produced a greater number of phage than those growing in MCHF medium.

Survival curves of *B. stearothermophilus* 1503-4R after induction with MC and ultraviolet ight are shown in Fig. 4. Of the cells, 98 and 93%

TABLE 1. *Production of phage by induced and non-induced cultures of Bacillus stearothermophilus 1503-4R growing in TYF and MCHF media**

Inducing agent†	PFU/ml‡	
	TYF	MCHF
Mitomycin C.....	6.0×10^4	2.7×10^4
Ultraviolet.....	4.0×10^4	1.4×10^4
None.....	1.0×10^2	7.0×10^1

* TYF = Trypticase-yeast extract-fructose; MCHF = minimal medium-casein hydrolysate-fructose.

† Mitomycin C = 0.05 μg/ml; ultraviolet irradiation = 5 min in TYF or 30 sec in MCHF at 50 cm.

‡ Phage assayed at 55 C on *B. stearothermophilus* 4S by the drop-assay described in the text.

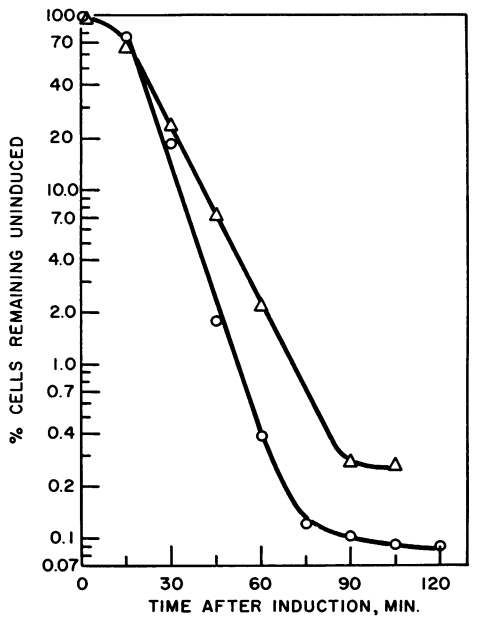

FIG. 4. *Survival of Bacillus stearothermophilus 1503-4R after treatment with mitomycin C or ultraviolet irradiation. Cultures growing at 55 C in TYF medium were exposed to mitomycin C (0.05 μg/ml) or ultraviolet irradiation (5 min at 50 cm) when the absorbancy was 0.2. Samples were removed at the indicated times, diluted in TYF, and plated for viable cells. MC, ○; UV, △.*

TABLE 2. *Effect of growth temperature on induction of Bacillus stearothermophilus 1503-4R by mitomycin C*[a]

Temp	Growth rate[b]	Time elapsed before lysis[c]	PFU/ml[d]
C	*k*	*min*	
45	0.22	240	0
50	0.31	90	2.0×10^4
55	0.42	45	6.0×10^4
65	0.48	30	4.1×10^4

[a] Cultures, growing at 55 C in TYF medium, were induced with mitomycin C (0.05 μg/ml) when they reached an absorbancy of 0.2.

[b] Growth rate constant is expressed as $dA/dt = kA$, where A is the absorbancy of the culture and t is the time expressed in hours.

[c] Time measured from the addition of mitomycin C until the beginning of culture lysis.

[d] Plaque-forming units per ml. Phage assayed at 55 C on *B. stearothermophilus* 4S by the drop-assay described in the text.

were induced 45 min after exposure to MC or ultraviolet light respectively.

Effect of carbon source on induction by MC. When glycerol (1.0%) or glucose (0.5%) was substituted for fructose as the carbon source in TYF medium, cell lysis began 60 min after MC induction compared with 45 min in TYF medium. This is probably due to the slower growth rate (k) with glycerol (k = 0.20) and glucose (k = 0.23) than with fructose (k = 0.41). Fewer phage were produced with these carbon sources than with fructose: glycerol, 2.7×10^4 PFU/ml; glucose, 3.8×10^4 PFU/ml; fructose, 6.0×10^4 PFU/ml.

Effect of growth temperature on MC induction and phage yield. Table 2 compares the rate of growth, time of onset of lysis, and phage production of MC-induced cells growing in TYF medium as a function of the growth temperature. Cells growing at 45 C showed only partial lysis after MC induction, and phage were not detected. Though cells growing at 65 C had a higher growth rate and a shorter induction time than cells growing at 55 C, fewer phage were detected at 65 C. This may be due to the thermal sensitivity of the phage at the higher temperature. This suggestion is supported by the observation that, when phage produced at 55 C were assayed at 65 C, no plaques were formed (Table 4).

Properties of phage TP-1. When 0.1 ml of an MC-induced lysate of 1503-4R was spread on the indicator strain 4S, the plaques produced ranged from 0.05 to 0.3 cm in diameter and contained a phage-resistant colony in the center. A clear-plaque mutant (TP-1C) appeared with a frequency of 1 per 155 turbid plaques when a

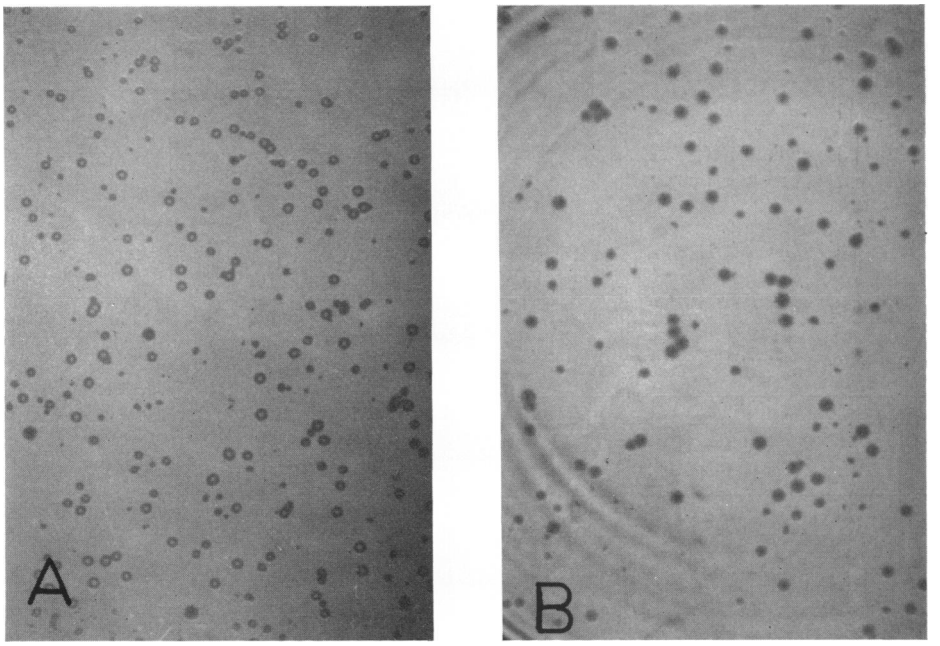

FIG. 5. *Plaque morphology of TP-1 and TP-1C phage. Phage were assayed at 55 C on Bacillus stearothermophilus 4S by the agar overlay method described in the text. A, TP-1; B, TP-1C.*

TABLE 3. *Effect of $CaCl_2$ concentration on the formation of plaques by TP-1 phage on Bacillus stearothermophilus 4S*

$CaCl_2$	PFU/ml* (× 10^9)
M	
—	2.8
0.010	2.9
0.015	5.5
0.020	4.4
0.050	4.3

* Plaque-forming units per ml. Phage assayed at 55 C using the agar overlay method described in the text.

TABLE 4. *Effect of temperature on plaque formation by TP-1 phage on Bacillus stearothermophilus 4S*

Temp	PFU/ml* (× 10^7)
C	
45	0
50	3.3
55	5.4
60	3.7
65	0

* Plaque-forming units per ml. Phage assayed at the temperature indicated by the agar overlay method described in the text.

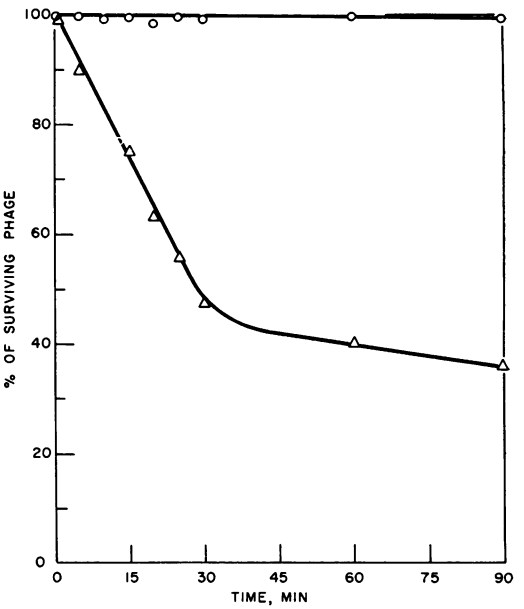

FIG. 6. *Inactivation of TP-1 phage at 65 C. One ml of TP-1 phage (2.5 × 10^{10} PFU/ml) in a screw-cap tube was placed in a constant-temperature water bath at 65 C. A duplicate phage sample was placed at 55 C as a control. At the indicated times, samples (0.01 ml) were removed, diluted in TYF and assayed at 55 C, in duplicate, on Bacillus stearothermophilus 4S; 55 C control, ○; 65 C, △.*

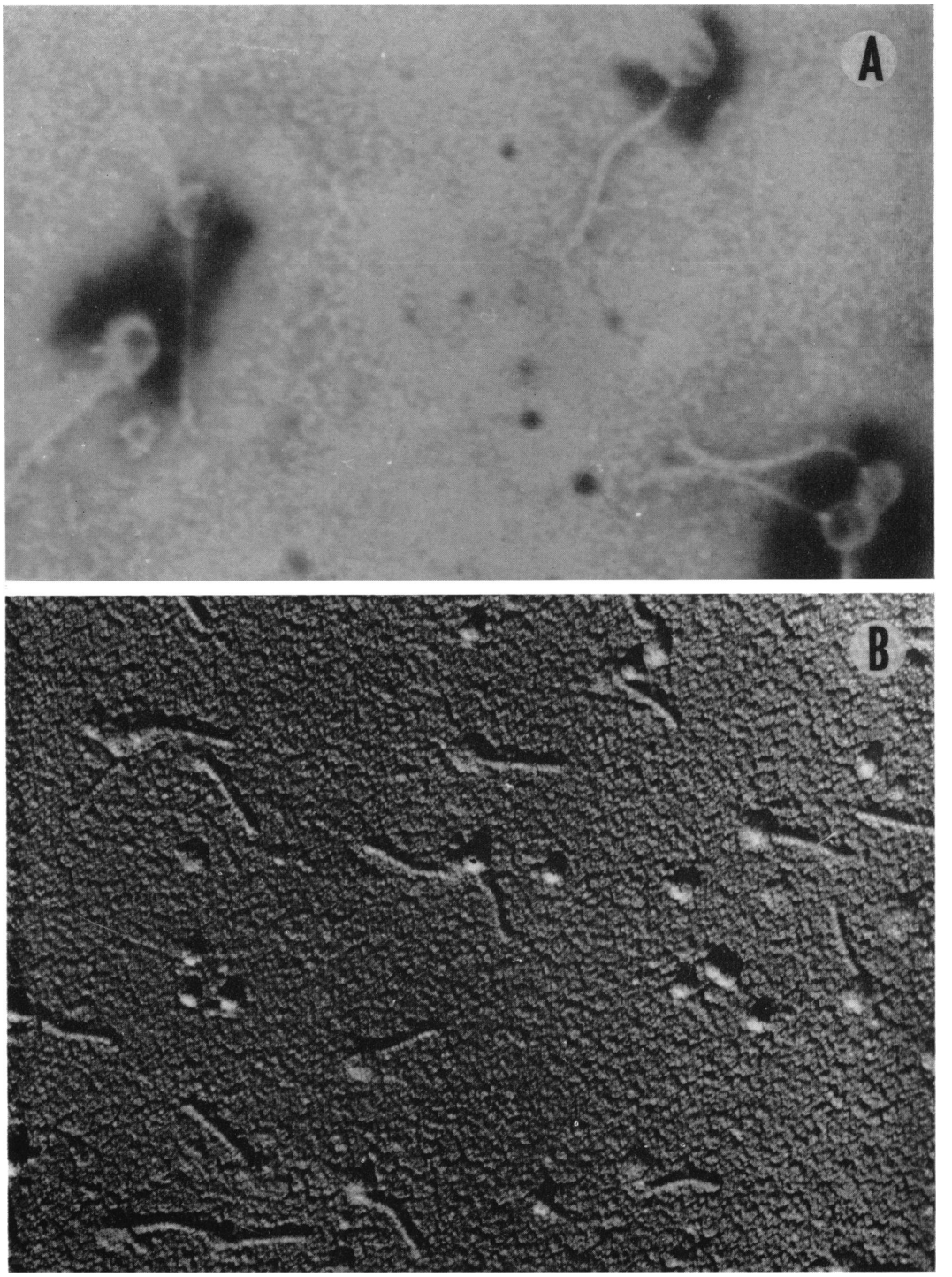

FIG. 7. *Electron micrographs of TP-1C phage. A, phosphotungstic acid negative stain × 152,000; B, uranium trioxide shadow-cast × 59,800.*

TABLE 5. *Deoxyribonucleic acid base composition of Bacillus stearothermophilus and phage deduced from buoyant density and thermal denaturation (Tm) measurements*

DNA	Tm	Guanine + cytosine*	Buoyant density	Guanine + cytosine†
	C	%	g/cc	%
B. stearothermophilus				
1503-4R	89	49	1.713	50
4S	89	49	1.713	50
Phage				
TP-1	—	—	1.706	42
TP-1C	86	41	1.706	42

* Base compositions from Tm measurements were calculated by the equation of Marmur and Doty (1962): % guanine + cytosine = (Tm − 69.3)2.439.

† Base compositions were calculated from buoyant density values according to the equations of Sueoka (1961).

single turbid plaque was picked and plated back on strain 4S. Figure 5 compares the plaque morphology of purified TP-1 and TP-1C phage on strain 4S.

To test the temperate nature of phage TP-1, a phage-resistant colony was picked from the center of a plaque and purified from free phage by single-colony passages on Trypticase agar. The final isolate, designated strain 4S(TP-1), was inoculated into 20 ml of TYF medium in a 250-ml flask and grown for 10 hr at 55 C on a rotary shaker. The cells were removed by centrifugation, and the supernatant liquid was tested for phage by assay on the sensitive indicator strain 4S. Plaques [one phage per six 4S(TP-1) cells] were produced, indicating that the phage-resistant cells isolated from the plaque had been lysogenized by TP-1. This also indicated that the prophage association with 4S(TP-1) was less stable than with strain 1503-4R, and that phage were liberated spontaneously. Plaques were not produced by TP-1 or TP-1C on the lysogenized strain 4S(TP-1). Strain 1503-4R was also immune to TP-1 and TP-1C, even when tested at high phage multiplicities (multiplicity of infection of 100), further supporting the conclusion that this strain is lysogenic. Attempts to cure strain 1503-4R by ultraviolet irradiation (40 min) were unsuccessful.

The addition of 0.015 M $CaCl_2$ to the soft agar doubled the number of plaques on strain 4S (Table 3) presumably due to a Ca^{++} requirement for phage adsorption. Calcium has been shown previously to be required for adsorption of other phages for *B. stearothermophilus* (White, Georgi, and Militzer, 1955; Shafia and Thompson, 1962, 1964). Other compounds tested had no effect on the number of plaques formed: glucose, 0.1 to 0.5%; fructose, 0.1 to 0.5%; yeast extract, 0.01 to 0.5%; and NaCl, 0.005 to 0.2 M.

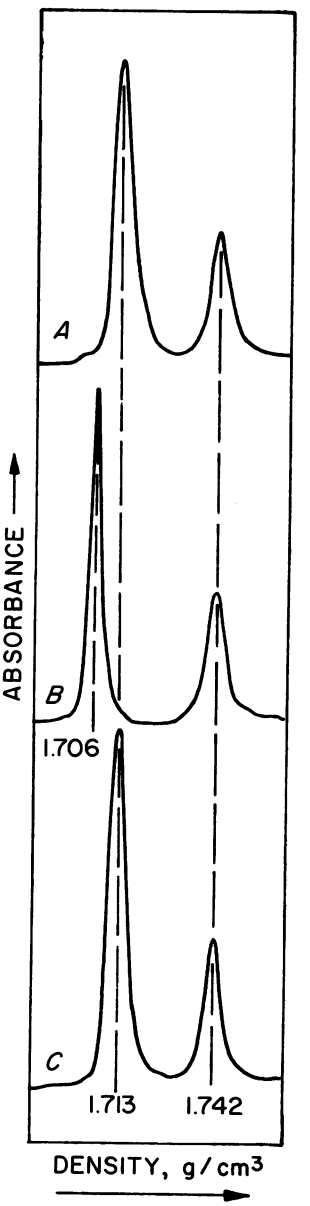

FIG. 8. *Microdensitometer tracings of deoxyribonucleic acid (DNA) of Bacillus stearothermophilus and TP-1C phage. DNA was equilibrated in a CsCl density-gradient formed by centrifugation at 44,770 rev/min at 25 C. A, B. stearothermophilus 1503-4R; B, TP-1C phage; C, B. stearothermophilus 4S. The band at the right in each case is N^{15}-labeled Pseudomonas aeruginosa DNA to provide a density reference.*

The incubation temperature significantly affected the number of plaques formed by TP-1 on strain 4S (Table 4). TP-1, when suspended in TYF medium at 65 C, lost 52% of the PFU in the first 30 min, but an identical sample at 55 C did not undergo thermal inactivation in 120 min (Fig. 6). These data along with those of Tables 2 and 4 show that 55 C was the optimal temperature for the production and assay of TP-1. Identical results were obtained with the clear plaque mutant TP-1C.

Phage morphology and size. Electron micrographs of TP-1 and TP-1C phage show the usual differentiation into head and tail components with heads approximately 65 mμ in diameter and tails 240 mμ long and 12 mμ wide (Fig. 7). The tails are relatively long and flexuous, and terminal knobs of indefinite shape were observed in some preparations. Morphologically, TP-1 and TP-1C resemble the bacteriophages of *Bacillus cereus* described by Dawson, Smillie, and Norris (1962). TP-1C has a sedimentation coefficient, $S_{20,w}$, of 226.

DNA-base composition. Purified TP-1C phage DNA had a sedimentation coefficient, $S_{20,w}$, of 24.1 and an estimated molecular weight of 1.21×10^7 calculated according to the equation: $S_{20,w} = 0.08\ M^{0.35}$ (Burgi and Hershey, 1963). Because only a small amount of TP-1 phage DNA could be isolated from induced lysates, we were unable to make these measurements on TP-1 DNA. However, a sufficient amount of TP-1 DNA was isolated to determine its buoyant density, and the value obtained was identical to that of TP-1C DNA.

The buoyant density, Tm, and per cent guanine + cytosine (G + C) values for bacterial and phage DNA are presented in Table 5. Microdensitometer tracings of the banded DNA are shown in Fig. 8. The per cent G + C values calculated from Tm and buoyant density measurements agree and are similar to those obtained from chemical determinations (Table 6). These data and the fact that the sum of the purine bases is close to the sum of the pyrimidine bases (Table 6) indicate that no significant quantities of unusual bases are present in either the bacterial or phage DNA.

The value of 42% G + C obtained for TP-1 phage DNA differs significantly from that of the DNA (50% G + C) of the lysogenic and indicator strains of *B. stearothermophilus*. This is the second example known of a temperate phage that has a DNA base composition which differs from that of its host. Marmur and Cordes (1963) reported that the transducing phage PBS2 has a DNA base composition different from its host *B. subtilis*.

The value of 50% G + C obtained for the DNA of the two strains of *B. stearothermophilus* differs from that (46% G + C) reported by Marmur (1960) for strain 194. We, therefore, determined the DNA base composition of 12 other strains by buoyant density and Tm measurements, and obtained values from 50 to 52% G + C. We also confirmed the value obtained by Marmur (1960) for the DNA base composition (46% G + C) of strain 194, but this strain does not show the physiological or biochemical characteristics of authentic strains of *B. stearothermophilus*.

TABLE 6. *Base composition (mole %) of Bacillus stearothermophilus, and TP-1C phage deoxyribonucleic acid**

Base	*B. stearothermophilus*		Phage TP-1C
	Strain 4S	Strain 1503-4R	
Adenine....	23.8†	23.7	28.1
Thymine...	24.2	24.3	28.6
Guanine...	26.2	25.9	21.5
Cytosine...	25.9	25.9	21.8

* DNA was precipitated with ethanol and then hydrolyzed with 90% formic acid at 175 C for 30 min. The hydrolysate was evaporated and chromatographed on Schleicher and Schuell no. 598 with isopropanol-concentrated HCl-water (171:41:39 by volume) as a solvent. The bases were eluted with 5 ml of 0.1 N HCl, and their concentration was determined from the measured absorbance and molar extinction coefficients (Bendich, 1957).

† Results are expressed as mole per cent. Numbers are the average of three separate determinations.

ACKNOWLEDGMENTS

We are grateful to Alix Bassel for preparation of the electron micrographs.

This study was supported in part by a contract (NR-108-330) between the Office of Naval Research, Biochemistry Branch, Department of the Navy, and the University of Illinois, and by a grant (GB-4) from the National Science Foundation.

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