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ISOLATION AND PRELIMINARY CHARACTERIZATION OF BACTERIOPHAGE $\Phi\mu$-4

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ABSTRACT

SHAFIA, FRED (University of Nebraska, Lincoln), AND T. L. THOMPSON. Isolation and preliminary characterization of bacteriophage $\Phi\mu$-4. J. Bacteriol. **87**:999–1002. 1964.—Bacteriophage $\Phi\mu$-4 was isolated from lysogenic *Bacillus stearothermophilus* NU strain 4, and was propagated in strain 10 of the same species. The phage was extremely host-specific. One of the 23 strains of thermophiles screened for susceptibility supported phage replication. Plaque-forming efficiency of $\Phi\mu$-4 depended on the agar medium employed and the temperature of incubation. Generally, media which resulted in restricted growth of the host cells on agar plates were most satisfactory for plaque formation. A latent period of 35 min was terminated in cell lysis and release of about 175 plaque-forming units (PFU) per infected cell. The phage particles, propagated in cells cultured in Trypticase (BBL)-yeast extract-dextrose-calcium chloride broth, were routinely removed from the lysate by 0.6 ammonium sulfate saturation in the cold. Phage purification was by ultracentrifugation followed by sucrose density-gradient. The phage particles appeared spherical, in electron micrographs. Particle size determination by electron micrographs indicated a diameter of less than 100 A.

Although several reports have been published on lytic phage-cell systems in thermophiles (White, Georgi, and Militzer, 1955; Hirano, 1961; Onodera, 1961), no attempt has been made to determine the presence of temperate phage in thermophilic microorganisms.

Preliminary studies on *Bacillus stearothermophilus* NU strain 4 indicated association of a temperate phage with cultures of this strain. The bacteriophage was subsequently isolated and characterized with regard to plaque formation, latent period, burst size, host range, size, thermal decay, and ionic requirement for proliferation.

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MATERIALS AND METHODS

Cultures. Strains of *B. stearothermophilus* were grown in a broth containing 2% Trypticase (BBL), 0.3% yeast extract, and 0.1% dextrose. Cultures were incubated in shake flasks at 65 C.

Phage isolation. Cultures of strain 4 grown for 18 hr at 65 C were clarified by centrifugation and Millipore (type HA, pore diameter 0.45 μ) filtration. Dilutions of the combined filtrates were assayed for phage. $\Phi\mu$-4 was obtained by the single plaque isolation method.

Phage assay. Particles were assayed by a modification of the overlaying method of Adams (1950), in which the overlayer contained 1 ml of 1% soft agar, 0.5 ml of strain 10 culture (10^8 cells per ml), and 0.5 ml of phage suspension. The base layer contained 0.75% Trypticase, 0.3% yeast extract, 0.1% dextrose, 0.1% calcium chloride, and 1.5% agar. Assay plates were incubated at 50 C for 18 hr.

Phage mass culture. Exponentially growing host cells were diluted in 20-fold dilution of Trypticase broth to 5×10^7 cells per ml. Phage particles were introduced to a multiplicity of 50 PFU per cell. Calcium chloride was added to a final concentration of 0.1%. Portions (1 liter) of the preparation, in 2-liter bottles, were vigorously aerated by sparging. The temperature of the suspension was maintained at 65 C, and incubation continued for 3 hr or until lysis occurred. The lysate was clarified by centrifugation at $30,000 \times g$ for 10 min.

Phage concentration. Phage particles were removed from clarified lysates by 0.4 to 0.6 ammonium sulfate saturation in the cold without pH adjustment. The precipitate was sedimented at $30,000 \times g$ for 15 min. The pellet was taken up in a minimal volume of distilled water, stirred for 20 min, and clarified by low-speed centrifugation. The phage particles were recovered from the supernatant fluid by ultracentrifugation at $40,000 \times g$ for 4 hr in the SW-25 rotor of a Spinco model L centrifuge. Final purification was

by the sucrose density-gradient method of Brakke (1958).

Latent period and burst size. A modification of the method of Ellis and Delbruck (1939) was employed for latent-period and burst-size determination. Exponentially growing host cells and phage particles in a multiplicity of about 1 phage per cell were incubated in a shake flask at 65 C. Samples were removed at intervals and filtered, and the free phage titer in the filtrate was determined. The infected cells from a portion of the cell-phage suspension were diluted in soft agar to obtain a suspension containing 1 infected cell per ml. Portions (1 ml) of the dilution were transferred to each of 100 test tubes and incubated for lysis to occur. Host cells were introduced, and the entire contents were layered on agar plates.

Thermal decay. Dilutions (100-fold) of a phage stock in 0.5% gelatin (pH 6.0), 0.05 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.5), and distilled water (pH 6.5) were incubated at 75 C. Portions were removed at intervals, and viable phage content was determined.

RESULTS

B. stearothermophilus NU strain 4 apparently is a lysogenic organism, because serial single-colony isolations failed to free the culture of phage. Shake cultures of the organism rarely contained over 100 PFU per ml. Attempts to induce activation of prophage by ultraviolet irradiation were unsuccessful.

Upon initial isolation, phage $\Phi\mu$-4 formed plaques which were faint and scarcely discernible. However, subsequent transfer on Trypticase-yeast extract-dextrose agar gave rise to plaques which were of medium size, clear, and with regular edges. Occasionally, minute, clear, plaque-type mutants were observed on assay plates from mass cultures. The titer of this mutant never exceeded over 1% of the parent wild type.

Composition of the medium and incubation conditions affected the final titer of phage produced. Vigorous agitation or aeration of the phage culture at 65 C was imperative for high phage yield. Maximal titer in broth rarely exceeded 5×10^{10} PFU per ml and was not affected by phage-cell ratios. The titer limit was constant whether a multiplicity of 1 or 50 phage per bacterium was initially employed. The final titers were also similar when a 20-fold dilution of the broth was used instead of the usual 2% Trypticase. To minimize the amount of medium carry-over in purification of phage particles, a 20-fold dilution of the broth was employed.

A total of 95% of the phage particles were precipitated from the lysate by 0.6 ammonium sulfate saturation. Phage particles subjected to sucrose density-gradient were recovered from a distinct band in the gradient column within 30 to 40% sucrose level.

Adsorption of $\Phi\mu$-4 followed a first-order reaction. The rate was low, in certain instances, and depended on the state of metabolic activity of the host cells. Normally, 80 to 90% of particles adsorbed to host cells within 10 min of incubation, provided log-phase cells were used. The rate of adsorption diminished to about 5 to 10% during the first 40 min of incubation when cells from the lag or stationary phase of growth were employed. The adsorption rate was not altered significantly at temperatures between 50 and 65 C during the initial 15 min of incubation. However, no adsorption occurred at 40 C during the same length of time.

In exponentially growing cells infected with phage, a latent period of 35 min was followed by lysis of cells and release of about 175 PFU per infected cell.

Strains (22) of *B. stearothermophilus* and 1 strain each of *B. subtilis* and *B. coagulans* screened for susceptibility to $\Phi\mu$-4 did not yield phage progeny.

Neutralizing antibodies prepared by injecting rabbits with purified phage rendered the particles inactive. The antibodies were effective against the phage released from the lysogenic strain as well as against the particles propagated in the host organism.

Ficin and trypsin rapidly inactivated the phage particles. Deoxyribonuclease and ribonuclease, however, had no effect on the infectivity of $\Phi\mu$-4. Particles were most stable in distilled water (Table 1); 50% of phage particles in lysate remained viable after 2 hr at 75 C. However, at 80 C about 95% loss of activity occurred within 5 min. Phage $\Phi\mu$-4 was rather unstable at room temperature, but remained viable, without loss of activity, for 2 years at −27 C.

Incorporation of 0.5% sodium citrate in the assay agar completely inhibited plaque formation of $\Phi\mu$-4. Removal of 0.1% citrate from assay

agar by addition of increasing concentrations of calcium chloride showed an optimal level for divalent ion requirement (Fig. 1).

Platinum-shadowed particles prepared by the method of Hall, Maclean, and Tessman (1959) appeared roughly spherical in electron micrographs. Comparison of shadows cast by phage particles and those cast by 88-mμ latex spheres gave a value of less than 100 A for the diameter of Φμ-4. This value was further supported by complete lack of retention of particles by a 10-mμ pore-size, Millipore (type VF) filter.

DISCUSSION

Ultraviolet irradiation of phage donor cells increased the rate of spontaneous lysis of the cells, but there was no indication of increase in the number of phage particles released. Because this organism also produces a bacteriocin-like substance (*unpublished data*), it is possible that mass lysis of the culture is due to induction of bacteriocin rather than the prophage. It is also speculated that certain ions necessary for induction of prophage were possibly lacking in the medium, as in lysogenic *B. megaterium* which could not be induced to release temperate phage in media deficient in manganese ions (Huybers, 1953).

Plaque-forming ability of the phage depends directly on the composition of the medium used and the temperature of incubation. In a rich medium and at a temperature optimal for the growth of the host cells, Φμ-4 fails to produce discernible plaques, probably owing to rapid growth of the host cells followed by a decline in their metabolic activities, which interferes with phage adsorption (Thompson and Shafia, 1962). However, in a medium which is barely sufficient for growth of cells, visible plaques are formed. The clear plaque mutant observed in mass cultures of Φμ-4 essentially has the same characteristics as does the parent phage. However, it is extremely unstable and spontaneously reverts to the parent wild type (*unpublished data*).

White et al. (1955), working with a lytic thermophilic phage, noted that the titer of particles in broth cultures never exceeded 10^8 PFU per ml. Phage Φμ-4 also exhibits a titer limit, but in this instance the maximal titer occasionally exceeded 10^{10} PFU per ml.

Heat stability of Φμ-4 is not comparable with the thermophilic bacteriophage reported by Onodera (1961), but it resembles, in some respects, the phage studied by White et al. (1955).

TABLE 1. *Thermal decay of phage Φμ-4 at 75 C*

Incubation time (min)	Suspending media (PFU/ml)		
	Gelatin	Tris	Distilled water
0	1.3×10^7	1.4×10^7	1.3×10^7
20	1.3×10^3	1.2×10^6	—
30	3.2×10^2	3.1×10^5	—
60	—	1.1×10^5	1.0×10^7

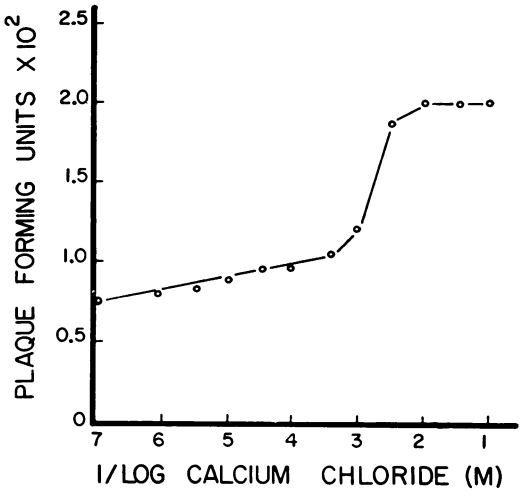

FIG. 1. *Effect of calcium on plaque formation by Φμ-4. Calcium chloride in concentrations indicated was incorporated in 0.1% citrate containing assay agar.*

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