Characterization of Bacteriophage gh-1 for *Pseudomonas putida*¹

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ABSTRACT

LEE, LUCY F. (Michigan State University, East Lansing), AND J. A. BOEZI. Characterization of bacteriophage gh-1 for *Pseudomonas putida*. J. Bacteriol. 92: 1821-1827. 1966.—Bacteriophage gh-1 of *Pseudomonas putida* A.3.12 was isolated and purified by differential centrifugation and diethylaminoethyl (DEAE) cellulose chromatography. An electron micrograph of the phage stained with uranyl acetate revealed a regular hexagonal outline about 50 m μ across with a short wedge-shaped tail attached at one corner of the head. The phage formed 10% as many plaques on *P. putida* C1S as on *P. putida* A.3.12, the organism used in the isolation procedure. No plaques were formed on *P. fluorescens* (ATCC 9712) or *P. aeruginosa*. The latent period of the infectious cycle was 21 min, and the average burst size was 103. The nucleic acid component of gh-1 is double-stranded deoxyribonucleic acid (DNA), with a base composition of 57.0% guanine plus cytosine (G + C) as determined by chemical analysis. The per cent G + C of *P. putida* A.3.12 DNA measured in a similar manner was 63.7%. The buoyant density of phage gh-1 measured by cesium chloride equilibrium centrifugation was 1.45 g/cm³, whereas that of gh-1 DNA, heat-denatured gh-1 DNA, and *P. putida* A.3.12 DNA was 1.716, 1.730, and 1.722 g/cm³, respectively. The per cent G + C of gh-1 DNA and *P. putida* A.3.12 DNA calculated from the buoyant densities was 57.1 and 63.3%, respectively. The sedimentation coefficients, $S_{20,w}^{50}$, of gh-1 and the phenol-extracted gh-1 DNA, measured by the boundary sedimentation velocity method, were 460 and 18.9, respectively. The molecular weight of phenol-extracted gh-1 DNA, calculated by use of the equation of Burgi and Hershey, is 6×10^6 .

The bacteriophages of the genus *Pseudomonas* have not been well characterized. Excepting a few phages of *P. aeruginosa* (11, 13, 20), no phages of other species of *Pseudomonas* have been the subject of a detailed analysis. The present report deals with the isolation and characterization of bacteriophage gh-1 of *P. putida* A.3.12.

MATERIALS AND METHODS

Organisms and growth medium. *P. putida* A.3.12, previously designated *P. fluorescens* (12), was used as the host for bacteriophage gh-1. In the study of host specificity, *P. fluorescens* (ATCC 9712), *P. aeruginosa*, and *P. putida* C1S were used (*P. putida* C1S kindly supplied by I. C. Gunsalus, Univ. Illinois). The organisms were grown at 33 C in a medium containing the following, in grams per liter: yeast extract, 5; glucose, 4; NaCl, 8; (NH₄)₂HPO₄, 6; KH₂PO₄, 3; MgSO₄·7H₂O, 1; and FeCl₃, 0.005.

Bacteriophage gh-1 was assayed by the agar layer technique (1). The top and bottom agar layers contained, in addition to the growth medium, 0.6 and 2.0% agar (Difco), respectively.

Isolation and purification of bacteriophage gh-1. Bacteriophage gh-1 was isolated from a sample taken from the aeration tank at the sewage plant in East Lansing, Mich. The sample was centrifuged to remove debris and filtered through an ultrafine sintered-glass filter. A sample of the filtrate was added to an exponentially growing culture of *P. putida* A.3.12. After incubation overnight, the culture was centrifuged at low speed. A sample of the supernatant fluid was plated out by use of the agar layer technique. A well-isolated plaque of gh-1 was picked and replated several times to ensure genetic homogeneity.

A 6- to 10-liter amount of *P. putida* A.3.12 was grown with vigorous aeration to a density of about 5×10^8 cells per milliliter. Bacteriophage at a multiplicity of about 5 were added. After 3 to 4 hr, the lysed culture was centrifuged at $4,000 \times g$ for 10 min to remove bacteria and cell debris. The titer of the lysates was about 2.5×10^{10} plaque-forming units (PFU) per ml. The virus was collected either by centrifugation at $16,000 \times g$ for 2 hr or by precipi-

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tation with ammonium sulfate at 50% saturation. The preparation was then suspended in buffer containing 0.05 M tris(hydroxymethyl)aminomethane (Tris) at pH 8.0, 0.001 M $MgCl_2$, and 0.2 M NaCl. The suspension was centrifuged at low speed, and the supernatant fraction was loaded on a diethylaminoethyl (DEAE) cellulose column (3 by 17 cm) equilibrated with 0.05 M Tris chloride buffer (pH 8.0) containing 0.2 M NaCl. At this salt concentration, most contaminating material is adsorbed on the column, but the phage is not. The fractions containing gh-1 were concentrated by centrifugation, and the resulting pellets were resuspended in the buffer described above. The yield of plaque-forming units was between 25 and 50%. Purified gh-1 at a concentration of 10^{12} to 10^{13} PFU/ml was stored at 5 C.

Electron microscopy. To prepare for electron microscopy, the purified phage preparation was dialyzed against 0.1 M ammonium acetate at 5 C overnight. A 1% solution of uranyl acetate was used to stain the virus particles (15). Specimens were examined under a Siemens Elmiskop 1 electron microscope at a magnification of 40,000.

One-step growth experiment. The procedure described by Adams (1) was used. The host bacteria were grown at 33 C to a concentration of about 5×10^8 cells per milliliter. Bacteriophage at a multiplicity of 1 to 2 were added. After 5 min for adsorption, the infected culture was diluted into gh-1 antiserum prepared by the method of Adams (1). The concentration of gh-1 antiserum used was sufficient to give 95 to 99% inactivation of the free virus particles in 5 min. After the 5-min incubation period in gh-1 antiserum, the infected culture was diluted. Duplicate samples from the diluted culture were removed every 2.5 min for the next 60 min and assayed for plaque-forming units.

Cesium chloride equilibrium centrifugation of bacteriophage gh-1. Solid cesium chloride (optical grade, obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.) was added to a sample of a purified phage preparation containing 1.2×10^{12} PFU (absorbancy, 260 $m\mu$, of 14 units) to give an initial CsCl concentration of 1.50 g/cm³. The suspension was centrifuged in an SW 39 rotor at 39,000 rev/min in a Spinco model L2 centrifuge at 7 C for 20.5 hr. Fractions were collected from the bottom of the centrifuge tube and analyzed for absorbancy at 260 $m\mu$ and for plaque-forming units. The CsCl concentration of various fractions was determined from refractive index measurements according to the method described by Ifft, Voet, and Vinograd (16).

Purification and characterization of gh-1 deoxyribonucleic acid (DNA). A sample of gh-1 containing 1.0×10^{12} PFU/ml (absorbancy, 260 $m\mu$, of 14.3 units/ml) was suspended in 0.5% sodium dodecyl sulfate (pH 7.3) and then stirred at 5 C for 10 to 15 min. An equal volume of phenol was added, and the mixture was stirred for another 10 min. After a low-speed centrifugation, the aqueous layer was removed. Phenol extraction was repeated, and the purified nucleic acid was dialyzed overnight against 0.01 M Tris chloride (pH 7.3) with 0.1 M KCl, or against SSC (0.15 M NaCl-0.015 M trisodium citrate). For the

analysis of the sedimentation velocity coefficient of gh-1 DNA, the above purification procedure was performed according to the method of Mandell and Hershey (17) to limit degradation due to physical shearing of DNA. DNA preparations from *P. putida* A.3.12 and *Escherichia coli* were purified as described by Armstrong and Boezi (2).

DNA concentrations were calculated from the absorbancy at 260 $m\mu$, by use of an extinction coefficient of 20 cm²/mg. Diphenylamine reactions were carried out by the method described by Burton (8). Orcinol tests were performed according to the procedure of Meibaum (18). Heat denaturation of gh-1 DNA was performed in 10-fold diluted SSC at 100 C for 10 min, followed by quick cooling.

Thermal denaturation profile of DNA. The thermal denaturation profile of DNA was determined by measuring the absorbancy at 260 $m\mu$ at various temperatures. The buffer used in this determination was 0.01 M Tris chloride (pH 7.3) with 0.012 M KCl.

Cesium chloride equilibrium centrifugation of DNA. CsCl equilibrium centrifugation of gh-1 DNA, heat-denatured gh-1 DNA, and *P. putida* A.3.12 DNA was performed according to the procedure described by Schildkraut, Marmur, and Doty (19). The initial concentration of CsCl was 1.710 g/cm³. *E. coli* DNA with a buoyant density of 1.710 g/cm³ was used as the density marker. Centrifugation was performed at 25 C in a Spinco model E analytical ultracentrifuge for 20 hr at 44,700 rev/min. The centrifuge cell was a standard cell fitted with a 1° negative wedge window. Tracings from the ultraviolet absorbancy photographs were made by use of a Joyce-Loebl double-beam recording microdensitometer.

Chemical analysis of base composition. An amount of 2.5×10^{12} PFU of bacteriophage gh-1 was suspended in 0.1 ml of 70% perchloric acid in a small, glass-stoppered test tube. The suspension, after being heated at 100 C for 1 hr with occasional agitation, was diluted to 0.5 ml with water. A black residue was removed by centrifugation. A sample of the hydrolysate was spotted on acid-washed Whatman no. 1 paper. Chromatography was performed by the descending method with isopropanol-HCl-water (65:17:18) as solvent (3). The bases were located by use of an ultraviolet light. After elution with 0.1 N HCl, the isolated bases were identified, and the amount of each was determined from its ultraviolet absorption spectrum by use of a Beckman DU spectrophotometer. Base composition of *P. putida* A.3.12 DNA was determined in a similar manner.

Sedimentation velocity studies of gh-1 and gh-1 DNA. The sedimentation studies of gh-1 and its DNA were carried out by use of the boundary sedimentation velocity method in a Spinco model E analytical ultracentrifuge with ultraviolet optics. The centrifugations were made at 15,220 rev/min for bacteriophage gh-1 and 42,040 rev/min for gh-1 DNA. The centrifuge cell was a standard cell fitted with a Kel F centerpiece. The temperatures of the runs were set variously at 18 to 22 C. After reaching the maximal speed, ultraviolet absorbancy photographs were taken for gh-1 at 4-min intervals and for gh-1 DNA at 8-min intervals. In certain experiments, to avoid shearing,

DNA was added to a partially assembled centrifuge cell by means of a pipette with a large tip opening rather than to a fully assembled cell by use of a syringe equipped with a 22-gauge needle. No difference in sedimentation velocity coefficients for gh-1 DNA was observed with the two methods. The ultraviolet absorbancy photographs were traced with a Joyce-Loebl double-beam recording microdensitometer. In determining S , the position of the boundary was measured from the 50% concentration point. The S^{50} values were corrected to standard conditions.

RESULTS AND DISCUSSION

Bacteriophage morphology. An electron micrograph of gh-1 stained with uranyl acetate is pre-

sented in Fig. 1. The nucleocapsid is of a regular hexagonal outline and about 50 $m\mu$ across. A short, wedge-shaped tail attached at one corner of the head can be seen. Two fibers attached to the wedge-shaped tail are visible on the bacteriophage at the bottom of Fig. 1, as indicated by an arrow. The morphology of gh-1 is similar to that of coliphage T_3 described by Bradley and Kay (5) and T_7 reported by Davison and Freifelder (9).

Plaque morphology and host specificity. Bacteriophage gh-1 forms a clear, smooth plaque, 4 to 6 mm in diameter, on *P. putida* A.3.12. Bacteriophage gh-1 attacks *P. putida* C1S, forming

FIG. 1. Electron micrograph of bacteriophage gh-1 negatively stained with uranyl acetate. $\times 300,000$.

about 10% as many plaques as on *P. putida* A.3.12. No plaques are formed on *P. fluorescens* (ATCC 9712) or on *P. aeruginosa*.

One-step growth experiment. A one-step growth experiment was performed to determine the length of the latent period and the average burst size of the infectious cycle. The results are given in Fig. 2. The latent period is about 21 min. The average burst size, calculated by dividing the number of plaque-forming units present after lysis by that present before lysis, is 103.

Cesium chloride equilibrium centrifugation of gh-1. An analysis of a purified gh-1 preparation by equilibrium centrifugation in CsCl is presented in Fig. 3. Three components are identified by absorbancy measurements: banding at densities of 1.52, 1.50, and 1.45 g/cm³. The material banding at $\rho = 1.52$ g/cm³ was not infectious, whereas the lighter two components were. The infectious material banding at $\rho = 1.45$ g/cm³ was considered to be the intact viral particle. The infectious component banding at $\rho = 1.50$ g/cm³ arose by degradation of the lightest component in the CsCl gradient. The origin of the noninfectious material banding at $\rho = 1.52$ g/cm³ is unknown. Rebanding of the 1.45 g/cm³ density component yielded both the 1.45 and 1.50 g/cm³ density components in the same ratio as they are shown in Fig. 3. Rebanding of the material of $\rho = 1.50$ g/cm³, however, gave a single component of the same density. Furthermore, the $\rho = 1.45$ g/cm³ material was about three times as

FIG. 2. One-step growth experiment of bacteriophage gh-1 on *Pseudomonas putida* A.3.12.

FIG. 3. Cesium chloride equilibrium centrifugation of gh-1. Absorbancy at 260 mμ (solid line) and plaque-forming units (dashed line) are plotted against fraction number. Fraction no. 1 contains material from the bottom of the centrifuge tube; fraction no. 30, from the top of the tube.

infectious per absorbancy unit as the material of $\rho = 1.50$ g/cm³. Assuming that the virus is composed of only protein and of DNA as its nucleic acid component, the percentage of each in the virus may be calculated from the buoyant density (14). Thus, the viral particle banding at $\rho = 1.45$ g/cm³ was composed of 64% protein and 36% DNA.

Characterization of gh-1 nucleic acid as DNA. The nucleic acid of the virus was purified by phenol extraction, and shown to be DNA by a positive diphenylamine reaction, a negative orcinol test, and by its susceptibility to deoxyribonuclease but not to ribonuclease. The ultraviolet spectrum of gh-1 DNA was typical of nucleic acids with a maximal absorbancy at 259 mμ. The ratios of absorbancies at 260 to 280 mμ, and 260 to 230 mμ, were 1.9 and 2.2, respectively.

Thermal denaturation profile of gh-1 DNA. The thermal denaturation profile for gh-1 DNA is given in Fig. 4. The absorbancy of gh-1 DNA remained constant until about 77 C. It rose sharply thereafter and reached maximal by 80 C. The denaturation temperature, T_m , was 79 C. The sharpness of the rise and the extent of the hyperchromic effect (change in absorbancy of 1.34) characterize gh-1 DNA as double strand. The thermal denaturation profiles for *E. coli* DNA and *P. putida* A.3.12 DNA are also presented in Fig. 4. The T_m for the former was 76 C, whereas that of the latter was 82.5 C.

Base compositions of gh-1 DNA and *P. putida* A.3.12 DNA. The base compositions of gh-1 DNA and *P. putida* A.3.12 were determined after

hydrolysis of the DNA preparations in perchloric acid. The results are presented in Table 1. For gh-1 DNA, the mole % adenine equals the mole % thymine, and the mole % guanine (G) equals the mole % cytosine (C). The base composition of gh-1 DNA, expressed as the per cent G + C, was 57.0. The base composition for *P. putida* A.3.12 DNA was 63.7% G + C.

Buoyant densities of gh-1 DNA and *P. putida* A.3.12 DNA. The buoyant densities of gh-1 DNA, heat-denatured gh-1 DNA, and *P. putida* A.3.12 DNA were determined by equilibrium centrifugation in CsCl by use of an analytical ultracentrifuge. The microdensitometer tracings for the three determinations are presented in Fig. 5. In each case, *E. coli* DNA, with a buoyant density of 1.710 g/cm³, was used as the density marker. The buoyant densities of gh-1 DNA, heat-denatured gh-1 DNA, and *P. putida* A.3.12 DNA are 1.716, 1.730, and 1.722 g/cm³, respectively. The base compositions of gh-1 DNA and *P. putida* A.3.12 DNA, calculated from the buoyant densities by the method of Schildkraut et al. (19), were 57.1% G + C for gh-1 DNA and 63.3% G + C for *P. putida* A.3.12 DNA. These results are in good agreement with the results obtained by chemical analysis (Table 1).

FIG. 4. Thermal denaturation profile of gh-1 DNA (○), *Pseudomonas putida* A.3.12 DNA (△), and *Escherichia coli* DNA (●).

TABLE 1. Base composition of gh-1 DNA and *Pseudomonas putida* A.3.12 DNA determined by chemical analysis and calculated from buoyant density measurements

DNA	Mole %				% G + C	Buoyant density	% G + C
	Adenine	Thymine	Guanine (G)	Cytosine (C)			
Phage gh-1.....	21.5	21.5	28.4	28.6	57.0	1.716	57.1
<i>P. putida</i> A.3.12.....	19.6	16.8	32.5	31.2	63.7	1.722	63.3

Sedimentation velocity studies of gh-1 and gh-1 DNA. The sedimentation coefficients of phage gh-1 and gh-1 DNA were determined by the boundary sedimentation velocity method in a Spinco model E analytical ultracentrifuge by use of ultraviolet optics. The sedimentation coefficient, $S_{20,w}^{50}$, of a freshly purified gh-1 preparation (absorbancy, 260 mμ, of 1 unit/ml) suspended in SSC was 460.

In Fig. 6, the sedimentation pattern of phenol-extracted gh-1 DNA is shown. The shape of the boundary, given at various time intervals during centrifugation, is plotted in absorbance units from the microdensitometer tracing, against distance, in arbitrary units, from the center of the rotor. The sedimentation coefficient, $S_{20,w}^{50}$, calculated from data obtained from eight different centrifugation trials on four different DNA preparations was 18.9. Precautions to avoid degradation of gh-1 DNA during the purification procedure (6, 17) and the centrifugation analysis (see Materials and Methods) were carefully followed. The molecular weight, calculated by use of the equation of Burgi and Hershey (7), $S = 0.080 M^{0.35}$, was 6×10^6 .

The gross morphology and sedimentation velocity coefficient of coliphage T₇, reported by Davison and Freifelder (9, 10), are similar to those of gh-1. T₇ has a nucleocapsid of hexagonal outline about 59 to 65 mμ across and a small tail. The $S_{20,w}$ of the virus is 487. T₇ DNA, purified by phenol extraction, has a $S_{20,w}$ of 32.2, which corresponds to a molecular weight of about 24×10^6 . T₇ contains by weight about 45% DNA and 55% protein, with one DNA molecule per phage particle.

Based on these similarities, gh-1 would be expected to contain a single DNA molecule with a molecular weight of about 24×10^6 . However, the molecular weight of phenol-extracted gh-1 DNA was about 6×10^6 . This discrepancy could mean that gh-1, unlike T₇, contains not one DNA molecule per particle, but four per particle. Alternatively, the discrepancy could be explained by assuming that degradation of gh-1 DNA to units of molecular weight of 6×10^6 has occurred

FIG. 5. Cesium chloride equilibrium centrifugation of gh-1 DNA, heat-denatured gh-1 DNA, and *Pseudomonas putida* A.3.12 DNA. *Escherichia coli* DNA is a density marker.

FIG. 6. Sedimentation velocity patterns of phenol-extracted gh-1 DNA.

during the isolation procedure for some unknown reason.

An estimation of the amount of DNA per gh-1 particle based on similarities between it and T₇ must be interpreted with caution. For example, the particle weights and, thus, the amount of DNA per particle may be grossly different. The possibility that gh-1 contains one DNA molecule of molecular weight 6×10^6 per particle has not been ruled out and must be entertained.

The possibility that phenol-extracted gh-1 DNA is an intact circular molecule similar to that described for polyoma DNA (21) and for the intracellular condensed form of λ DNA (4) was considered. Such intact circular molecules can reform the native conformation during the quick cooling process after denaturation by heat. Consequently, no change in sedimentation coefficient or in buoyant density for the heat-treated material is observed. With gh-1 DNA, however,

the sedimentation coefficient of heat-denatured DNA, $S_{20,w}^{50}$, is 37.6, which is about twice that of native DNA. Furthermore, the buoyant density of heat-denatured gh-1 DNA is 0.014 g/cm³ greater than that of native DNA (see Fig. 5). This irreversibility of heat denaturation rules out the possibility that phenol-extracted gh-1 DNA has an intact circular structure, but suggests rather a linear conformation for the molecule.

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