Isolation and Characterization of *Hydrogenomonas facilis* Bacteriophages Under Heterotrophic Growth Conditions

CHRISTINE F. POOTJES, R. B. MAYHEW, AND B. D. KORANT

Department of Microbiology, The Pennsylvania State University, University Park, Pennsylvania

Received for publication 27 August 1966

ABSTRACT

POOTJES, CHRISTINE F. (The Pennsylvania State University, University Park), R. B. MAYHEW, AND B. D. KORANT. Isolation and characterization of *Hydrogenomonas facilis* bacteriophages under heterotrophic growth conditions. J. Bacteriol. 92:1787-1791. 1966.—We have isolated five strains of bacteriophage specific for *Hydrogenomonas facilis*. The host range of the phage is limited to *H. facilis*. Morphologically, the phage particles consist of a head 580 Å in diameter and a short tail 200 Å in length. The particles share a common surface antigen, and all contain deoxyribonucleic acid. The five strains differ from each other in growth characteristics, heat stability, and neutralizing antigens.

Bacteriophage specific for strict autotrophs have not been reported. *Hydrogenomonas facilis* is not a strict autotroph but a facultative autotroph, which can grow with hydrogen gas as an energy source and carbon dioxide as a carbon source, or on an organic medium. A bacteriophage for this organism has been reported (9).

The present report describes the isolation of four additional strains of phage specific for this facultative autotroph. These new isolates, D, R, SH133, and SH6, and the original isolate HF, are characterized under heterotrophic growth conditions. Their growth characteristics, physicochemical properties, and serological relationships are reported.

MATERIALS AND METHODS

Media. Peptone broth, containing 0.4% peptone (Difco) in distilled water, was used for cultivation of *H. facilis* and for phage propagation. Phage assays were made by use of the double agar layer method as described by Adams (1). The base agar layer contained 0.4% peptone and 1.5% agar; the soft agar layer contained 0.4% peptone and 0.7% agar.

Bacterial strains. The original strain of *H. facilis* ATCC 15376 was obtained from R. Burris, Department of Biochemistry, University of Wisconsin. This strain was used in all studies unless otherwise indicated. Additional strains of *H. facilis*, obtained from Diana C. Halaus, Department of Bacteriology, University of California at Berkeley, were: *H. facilis* 133 (isolated as a polymer-decomposer by Fritz Delafield), *H. facilis* ATCC (Halaus), and *H. facilis* R (originated from the laboratory of Sydney Rittenberg). Cultures of *Hydro-*

genomonas H-1, H-6, and H-20 were obtained from Richard Bartha, College of Agriculture, Rutgers, The State University.

Isolation of bacteriophage. We centrifuged 5-ml samples of mud and water from the banks of the Allegheny River in Pittsburgh or from polluted local ponds and streams at $3,000 \times g$ for 10 min to sediment debris. The supernatant fluid was added to 50 ml of peptone broth inoculated with 5 ml of an overnight shaken peptone broth culture of *H. facilis* ATCC 15376 or, in one instance, of *H. facilis* 133. The mixture was incubated overnight on a New Brunswick rotary shaker at 28 C. The next day, the broth was filtered through a type HA membrane filter (pore size, 0.45 μ ; Millipore Filter Corp., Bedford, Mass.). Drops of the filtrate were plated on an inoculated double agar layer plate. Plaques were picked by stabbing with a sterile inoculating needle, the needle was washed in broth, and the phage suspension was replated.

Preparation of high-titer phage stocks. Concentrated phage preparations were obtained by elution of the soft agar layer from 0.4% peptone-agar plates. The phage and bacterial cells were mixed to produce just confluent lysis, as described by Adams (1). The maximal phage titer obtained by this method was 1.2×10^{10} plaque-forming units (PFU) per ml. The phage suspension was further concentrated and purified by differential centrifugation. The lysate was first centrifuged at $3,000 \times g$ for 10 min to remove bacterial debris, and then at $40,000 \times g$ for 3 hr in a Spinco model L centrifuge. The pellets were allowed to resuspend overnight in a small volume of peptone broth or 0.4% ammonium acetate.

Phage sensitivity to heat at 56 and 60 C. Phage suspensions at concentrations of 2×10^8 to 6×10^8 PFU/ml were diluted to 1:100 in prewarmed 0.4%

peptone broth, in a constant-temperature water bath at 56 or 60 C. At given intervals, 0.1 ml was diluted in 9.9 ml of 0.4% peptone broth at room temperature. Duplicate 0.1-ml samples of 10-fold dilutions were plated to assay for surviving plaque-formers (4).

Burst size and single-step growth experiments. The procedures described by Adams (1) were followed. All experiments were conducted in 0.4% peptone broth at 29 C.

Nucleic acid determination. Phage nucleic acid was prepared from a crude lysate containing 10^{10} PFU/ml by direct centrifugation of the phage particles into a layer of neutralized 5 M NaClO₄ as described by Freifelder (5). The nucleic acid was precipitated with ethyl alcohol, collected by centrifugation, and redissolved in saline.

To confirm the chemical analysis, the effect of two inhibitors of viral deoxyribonucleic acid synthesis, 5-fluoro-2'-deoxyuridine (FUDR) (3, 7) and actinomycin D (6, 10), was determined on phage synthesis.

We added 0.1 ml of 5×10^{-4} M FUDR and 0.1 ml of 0.001 M uracil 10 min prior to infection to 0.6 ml of a log-phase culture of *H. facilis* (1.0×10^8) in 0.4% peptone broth. As a control, 0.1 ml of 0.001 M thymidine was added as an inhibitor of the FUDR action. Phage particles were added to give a multiplicity of infection of 1.0. PFU were determined at 20-min intervals over a 65-min period.

Actinomycin D inhibition was measured with the same procedure. The final concentration of the antibiotic was 5 μ g/ml. As a control, the bacterial cells were incubated in the antibiotic medium for 30 min; they were then centrifuged, resuspended in 0.4% peptone broth, and infected with phage.

Electron microscopy. A 0.1-ml amount of a concentrated phage preparation (3.0×10^{11} PFU/ml) was mixed with 0.1 ml of 0.5% phosphotungstic acid for negative staining and with 0.1 ml of polystyrene balls (0.264 μ in diameter) for size determination. Carbon-coated Formvar grids were touched to the surface, allowed to dry, and then examined with an RCA EMU 3-G electron microscope (2).

Serological methods. Before the preparation of antisera, rabbits were bled, and their sera were tested against the phage concerned. After 20 min at 37 C, the plaque counts were unaffected. Subsequently, the rabbits were intravenously injected with a phage preparation containing 6×10^7 to 4×10^8 PFU/ml. A total of 30 ml of the phage suspension was injected in 2.0-ml doses over an average period of 3 months. Final bleedings were made 1 week after the last inoculation. The antisera were stored in 0.5-ml quantities in screw-cap vials at -10 C.

Cross-neutralization tests. Dilutions of the phage preparations and antisera were made in 1% peptone broth supplemented with 0.2% normal rabbit serum (8). The antisera were diluted to give 90 to 99% inactivation of homologous phage in 15 min at 37 C. We added 0.2 ml of a phage suspension of 10^8 PFU/ml to 1.8 ml of diluted serum at 37 C. At 5-min intervals the number of plaque-forming survivors was determined by triplicate platings.

Precipitin tests. Melting-point tubes were half filled by capillary action with undiluted phage antiserum.

The remainder of the tube was filled in the same manner with a phage preparation containing 10^{10} to 10^{11} PFU/ml. A precipitate visible from 15 min to 24 hr after mixture was considered a positive test.

RESULTS

From four geographically different sources, five strains of phage specific for *H. facilis* have been isolated. All sources were polluted and were rich in organic matter. Clear mountain streams did not yield phage strains. The isolated strains have been designated HF, D, R, SH133, and SH6, and their respective sources were the Raritan River, New Brunswick, N.J., a sewage plant pond, State College, Pa., the Allegheny River, Pittsburgh, Pa., and, for both SH-133 and SH-6, a slaughter house stream in Spring Mills, Pa. All strains, aside from SH-133, were enriched with a culture of *H. facilis* ATCC 15376. Strain SH-133 was enriched with *H. facilis* 133.

The host range of these phage isolates is limited to *H. facilis*. No lysis was observed in *Hydrogenomonas* H-1, H-16, or H-20. The pattern of lysis of *Hydrogenomonas* strains is given in Table 1.

Among the phage strains, HF and SH6 have the same host range. SH133 is the only phage strain able to lyse all four of the *H. facilis* strains. This phage was isolated from the same source as SH6, except *H. facilis* 133 was added to the overnight incubation mixture rather than *H. facilis* ATCC 15376.

The growth characteristics of the phage strains in 0.4% peptone broth at 29 C with *H. facilis* ATCC 15376 as host strain are given in Table 2. Latent periods among the phage strains are shown, by one-step growth experiments, to vary from 35 min for SH133 to 65 min for R. R phage, which had the longest latent period, also had the largest average burst size. The average burst sizes for HF, D, SH133, and SH6 are between 20 and 30.

TABLE 1. Host range of *Hydrogenomonas facilis* phages

Phage strain	<i>H. facilis</i> strain				<i>Hydrogenomonas</i> strain		
	ATCC 15376	133	R	ATCC (Hauls)	H-1	H-16	H-20
HF	+	-	-	+	-	-	-
D	+	-	+	+	-	-	-
R	+	-	-	-	-	-	-
SH133	+	+	+	+	-	-	-
SH6	+	-	-	+	-	-	-

TABLE 2. Single-step growth characteristics of *Hydrogenomonas facilis* phage at 29 C

Phage strain	Latent period	Avg burst size
	<i>min</i>	
HF.....	40	20
D.....	45	30
R.....	65	93
SH133.....	35	24
SH6.....	50	28

TABLE 3. Heat stability of *Hydrogenomonas facilis* phage at 56 and 60 C in peptone-water

Phage strain	Time required to attain 99% inactivation	
	At 56 C	At 60 C
	<i>min</i>	<i>min</i>
HF.....	29	13
D.....	45	9
R.....	55	24
SH133.....	450	80
SH6.....	30	10

In the initial phage isolation procedure, the supernatant fluids obtained from centrifuged enriched broth cultures were heated to 56 C for 30 min to kill residual bacterial cells (11). We then became interested in the heat stability of these phage strains. Table 3 shows the relative heat stability of these five phage strains. All strains, except SH133, showed 99% inactivation in less than 1 hr at 56 C, and in less than 30 min at 60 C. Phage SH133 is considerably more heat-stable than the other phage strains.

Nucleic acid determinations indicate that the phage contains deoxyribonucleic acid (DNA). The perchlorate extracted material gave a single peak at 260 μ ; it was insensitive to ribonuclease (5 μ g/ml), but it gave an increase in optical density after deoxyribonuclease (5 μ g/ml) treatment. The diphenylamine test was positive for deoxyribose. Additional studies with two inhibitors of viral DNA synthesis, FUDR (3) and actinomycin D, confirmed this fact. Incorporation of FUDR into 0.4% peptone broth depressed virus synthesis. The addition of thymidine partially reversed the effect. Actinomycin D also depressed phage synthesis.

Electron micrographs of the phage particles show a similar structure for the five strains of phage. The particles are composed of a polyhexagonal head 580 A long. Figure 1 is an electron micrograph of a preparation of phage particles negatively stained with phosphotungstic acid. An enlargement of the rosette of phage

particles attached to a piece of debris (Fig. 2) shows the presence of a short tail structure. This is still more evident in Fig. 3, where a collapsed head and short tail are seen against a background of the stain.

Serological cross-neutralization of all the phage strains with homologous and heterologous phage antisera shows no strong cross-neutralization reactions among any of the phage strains (Table 4). Some cross-neutralization was observed with phage strain D and SH133 phage antiserum (Fig. 4). This cross-neutralization reaction was a reciprocal reaction in that 48% of the phage particles of phage strain SH133 were neutralized by D phage antiserum (Table 4) in 15 min at 37 C. The neutralization velocity constant (*K* value) for each antiserum and homologous phage antigen is in Table 4.

All phage strains showed positive precipitin tests, when concentrated phage particles (10^{10} to 10^{11} PFU/ml) were mixed with homologous and heterologous antisera. Phage preparations containing 10^8 to 10^9 particles did not give a positive reaction. No precipitation was observed with the bacterial cell lysate or with the 0.4% peptone medium. Whole bacterial cell preparations were not agglutinated by these antisera.

DISCUSSION

In nature, phage specific for *H. facilis* are not rare and were therefore readily isolated from polluted waters.

The five phage strains described in this report share some characteristics, but they are not identical. In spite of their similarities, they cannot be divided into subgroups.

All the phage strains have the same morphology, as shown in electron micrographs. They possess a polyhexagonal head and short tail. Precipitin tests indicate that they share a common surface antigen. None of the strains is able to lyse a bacterial species outside the closely related *H. facilis* species. The nucleic acid component of the phage strains is probably DNA.

As can be surmised from the data, these phage strains are not identical. Individual strains vary in their host range on *H. facilis*, and in their burst size, latent period, and heat sensitivity.

Serological cross-neutralization studies show that no relationship among the five strains of phage can be correlated with other characteristics. Limited reciprocal cross-neutralization reactions were observed between SH133 and D phage. These strains, however, differ greatly in host range and heat inactivation, a fact which precludes their being considered identical or more closely related than the other phage strains.

FIG. 1. *Phage HF* negatively stained with phosphotungstate. $\times 55,000$.

FIG. 2. *Phage HF* adsorbed to bacterial debris. Enlargement of a section of Fig. 1. $\times 250,000$.

FIG. 3. Individual phage particle, *HF*, demonstrating phage tail. $\times 230,000$.

TABLE 4. Reaction of antiphage antisera against homologous and heterologous phage for 15 min at 37 C

Serum	K value ^a	Dilution	Fraction of plaque-formers surviving				
			SH6	HF	R	D	SH133
Anti-SH6.....	829	1:3,000	0.014	0.96	1.0	0.89	.92
Anti-HF.....	105	1:500	1.0	0.042	0.72	1.05	.87
Anti-R.....	42	1:100	1.2	1.0	0.023	0.75	.64
Anti-D.....	21	1:100	0.92	0.68	0.87	0.041	.62
Anti-SH133.....	28	1:100	1.1	1.15	1.04	0.42	.063

^a Neutralization velocity constant of each antiserum reacted with homologous phage antigen. Expressed as minute⁻¹.

FIG. 4. Phage neutralization curves for SH133 phage antiserum diluted 1:100. Symbols: $\times$, SH133 phage; $\square$, HF phage; $\triangle$, SH6 phage; $\bullet$, R phage; $\circ$, D phage. Control: $\oplus$, SH133 phage (preinoculation serum).

In conclusion, the phage in this report represent a unique collection of bacterial viruses specific for *H. facilis*. These viruses possess similar morphology, surface antigens, and nucleic acid, and they are able to lyse only *H. facilis*. The strains are not identical in growth characteristics, heat stability, or neutralizing antigens.

ACKNOWLEDGMENTS

We express our appreciation to Harald Schraer for helpful advice and for the use of his electron microscopy laboratory.

This investigation was supported by grant GB-4050 from the National Science Foundation.

LITERATURE CITED

- ADAMS, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
- BRENNER, S., AND R. W. HORNE. 1959. A negative staining method for high resolution electron microscopy of viruses. *Biochim. Biophys. Acta* **34**:103-110.
- COHEN, S. S., J. G. FLAKS, H. D. BARNER, M. R. LOEB, AND J. LICHTENSTEIN. 1958. The mode of action of 5-fluorouracil and its derivatives. *Proc. Natl. Acad. Sci. U.S.A.* **44**:1004-1012.
- FEARY, W. T., E. FISHER, AND N. FISHER. 1964. Isolation and preliminary characteristics of three bacteriophages associated with a lysogenic strain of *Pseudomonas aeruginosa*. *J. Bacteriol.* **87**:196-208.
- FREIFELDER, D. 1965. A rapid technique for the preparation of purified bacteriophage DNA or RNA from crude lysates. *Biochim. Biophys. Acta* **108**:318-319.
- GOLDBERG, I. H., M. RABINOWITZ, AND E. REICH. 1962. Basis of actinomycin action. I. DNA binding and inhibition of RNA polymerase synthetic reactions by actinomycin. *Proc. Natl. Acad. Sci. U.S.A.* **48**:2094-2101.
- HARTMAN, K. U., AND C. HEIDELBERGER. 1961. Fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. *J. Biol. Chem.* **236**:3006-3013.
- JERNE, N. K., AND L. SKOVSTED. 1953. The rate of inactivation of bacteriophage T₄ in specific antiserum: I. Salt effect. II. Cofactor. *Ann. Inst. Pasteur* **84**:73-89.
- POOTJES, C. 1964. Isolation of bacteriophage for *Hydrogenomonas facilis*. *J. Bacteriol.* **87**:1259.
- REICH, E., AND I. H. GOLDBERG. 1964. Actinomycin and nucleic acid function, p. 183-284. *In* J. N. Davidson and W. E. Cohn [ed.], *Progress in nucleic acid research and molecular biology*, vol. 3. Academic Press, Inc., New York.
- WARNER, P. T. J. C. P. 1950. The isolation of the bacteriophages of *Ps. pyocyanea*. *Brit. J. Exptl. Pathol.* **31**:112-129.