Physicochemical and Biological Properties of Fibrous *Pseudomonas* Bacteriophages

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Two fibrous *Pseudomonas* phages, Pf1 and Pf2, have been isolated and characterized. The phages were serologically related and were indistinguishable morphologically when viewed by electron microscopy. Both phages formed minute turbid plaques. A simple method was devised for easy differentiation of such plaques from the bacterial lawn. The infected bacteria continued to grow and to liberate the phages into the medium. The phages were collected from the turbid "lysate" by salting out and were purified by differential centrifugation and density gradient centrifugation. The purified phages were sensitive to ultrasonics and to a proteolytic enzyme, Nagarse. A rapid lysis mutant was obtained from the progeny of plaque-purified Pf2. Pf2 was spontaneously produced by strain P28 of *P. aeruginosa*. Neither acridine orange nor antiserum affected the phage-producing capacity of the bacteria.

Several fibrous phages of *Escherichia coli* have been reported (3). Recently, two fibrous phages infecting other species of bacteria have been isolated: One was active against *Pseudomonas aeruginosa* (9) and another was specific to *Vibrio parahaemolyticus* (8). As reported previously (9), the rod-shaped *Pseudomonas* phage was designated as Pf.

More recently, another fibrous *Pseudomonas* phage was isolated in our laboratory. Therefore, these two phages will be hereafter designated as Pf1 and Pf2, respectively. Physicochemical, biological, and serological characterization of these phages revealed that Pf1 and Pf2 were indistinguishable in many respects. In this paper, characteristics of these fibrous pseudomonas phages are presented.

MATERIALS AND METHODS

Phages. Pf1 was unexpectedly found in a collection of *Pseudomonas* phages which had been isolated in our laboratory. Pf2 was a new isolate from the culture fluid of strain P28 of *P. aeruginosa*, provided by Y. J. Homma, Institute for Medical Science, Tokyo University. Both phages were isolated by use of strain K as host bacterium.

Bacteria. The K strain, a common host bacterium for both phages, was employed throughout the experiments for propagation and assay of phages. To determine the host range of Pf1 and Pf2, 106 strains of *P.*

aeruginosa were examined. These included four male and two female strains (4), provided by T. Watanabe, Keio University. A phage-resistant mutant (ϕ^r) of the K strain, as well as phage-sensitive (ϕ^s) wild type, was used in some experiments.

Phage assay. By use of usual assay procedures, these fibrous phages were observed to form minute, turbid, and, therefore, sometimes hardly recognizable plaques. Since the host bacteria reduced methylene blue into colorless leuco-methylene blue, methylene blue was employed to differentiate such variable plaques. Nutrient Agar plates containing 0.006% methylene blue were used as bottom agar. Preliminary experiments had revealed such a concentration of methylene blue to be most satisfactory (7). The phages were assayed by usual agar-layer technique except that dye-containing plates were utilized.

Purification of phages. The host bacteria, grown in Nutrient Broth to the logarithmic phase, were infected and shaken constantly for an additional 12 to 14 hr at 37 C. Phages in high titer were liberated into the medium, although the cultures never cleared. The phage lysate thus obtained was centrifuged to eliminate bacterial cells or cell debris. The supernatant fluid was saturated with ammonium sulfate and the resulting floating layer of phage was skimmed off and resuspended in 0.01 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.2). After dialysis overnight against the same buffer, the phage was partially purified by alternate cycles of centrifugation (10,000 rev/min for 30 min to sediment debris and 55,000 rev/min for 3 hr to sediment phages). The final pellet was resuspended in the buffer and further purified by density gradient centrifugation. A density gradient solution of sucrose was carefully prepared with 2.35 ml of Tris buffer containing 30% sucrose

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and 2.15 ml of 10% sucrose-Tris buffer. The phage sample in a volume of 0.2 ml was applied to the density gradient solution and centrifuged at 35,000 rev/min (approximately $100,000 \times g$) for 2 hr in a SW-39 Spinco model L centrifuge. Upon completion of the run, the contents were collected from the bottom of the tube; 20 drops were pooled in each fraction. By this procedure, the phage was completely separated from a small amount of contaminating pyocins produced spontaneously by the host bacteria.

Rabbit antisera. Anti-Pf sera were prepared by injecting adult rabbits with 0.5 ml of the purified Pf1 phage (4×10^{10} plaque-forming units/ml) suspended in distilled water containing 100 μ g of streptomycin and 100 units of penicillin per ml. The phage solutions were injected intravenously at 0.5-week intervals for 3 weeks, and the sera were collected 2 weeks after the final injection.

Anti-fd rabbit serum and rabbit serum against fibrous vibrio phage (no. 6) were kindly supplied by T. Tokunaga, National Institute of Health of Japan, and by H. Nakanishi, Public Health Research Institute of Kobe City, respectively.

RESULTS

Plaques of Pf phages. The use of methylene blue gave reproducible and reliable results in plaque formation. After incubation for about 18 hr, the turbid plaques were evidenced as blue dots against white backgrounds, owing to the reduction of methylene blue by the indicator strain. Each plaque was clearly observable and was still visible after an additional incubation period.

A rapid lysis mutant (r) of Pf2 was isolated by this modified method. The plaques formed by this mutant were larger, with more distinct center colonies than the wild type (r^+), as shown in Fig. 1 and 2.

Purification of Pf1 by sucrose density gradient centrifugation. Partially purified Pf1 was subjected to density gradient centrifugation and then fractionation. The host bacteria spontaneously produced a pyocin (pyocin K) which concomitantly contaminated the phage sample. Electron microscopically (Fig. 3), the pyocin K resembled the pyocin R reported by Ishii et al. (5). By this procedure, however, the phage was completely separated from the pyocin, as shown in Fig. 4. The eighth fraction, showing the highest phage activity, contained predominantly fibrous phages when viewed in an electron microscope.

Serological properties. Pf1 was treated with anti-Pf1 and anti-fd sera in the homologous and heterologous systems, respectively. No serological relationship was revealed by cross-neutralization tests (Table 1). As shown in Table 2, Pf2 was easily inactivated by anti-Pf1 serum, whereas neither anti-fd nor anti-no. 6 sera affected its infectivity. Pf1 and Pf2 were thus demonstrated

FIG. 1. Plaques formed by *Pf2r*⁺ (wild type).

FIG. 2. Plaques of *Pf2r* (a rapid lysis mutant). The plaque titration was performed by the usual agar overlay method except that the bottom agar contained 0.006% methylene blue.

to be closely related serologically, but were otherwise quite different from fd and no. 6.

Host ranges. Of 106 strains employed, only 4 strains were found to be sensitive to both Pf1 and Pf2 by the cross-brush method and the agar-over-

FIG. 3. *Pfl* phage and pyocin K. Arrow indicates a pyocin rod.

FIG. 4. Purification of *Pfl* by density gradient centrifugation. Each fraction was assayed for phage with strain K and for pyocin with strain P29. *Pfl* phage was completely separated from the contaminant, pyocin K, which the host strain produced spontaneously. The eighth fraction was composed of highly purified *Pfl*.

lay method. Plating efficiency of both phages on these four strains is shown in Table 3.

Stability of *Pfl*. The phage was sensitive to ultrasonics (thus reflecting its long filamentous structure), whereas infectivity of a small spherical phage ϕ X 174 remained unaffected during the same treatment (Fig. 5). *Pfl* was not affected by treatment with deoxyribonuclease or ribonuclease, but was inactivated by Nagarse, a bacterial proteolytic enzyme (Fig. 6). Trypsin, in high concentration (2 mg/ml), slightly reduced the infectivity (Fig. 6). *Pfl* could be frozen and thawed repeatedly without any appreciable loss of infectivity (Table 4).

Mode of infection. Even when infected with a high multiplicity of phage (Fig. 7), the infected culture never cleared after incubation; instead,

TABLE 1. Cross neutralization test of *Pfl* and *fd*

Phage	Control	After neutralization ^a with	
		Anti- <i>Pfl</i> ^b	Anti- <i>fd</i> ^b
<i>Pfl</i>	1.8×10^{8c} 4.1×10^8	$<1 \times 10^3$	3.9×10^8
<i>fd</i>	3.3×10^9 2.4×10^8	1.7×10^8	$<1 \times 10^3$

^a Incubation at 37 C for 5 min.

^b Homologous system, 1:100 dilution; heterologous system, 1:10 dilution.

^c Plaque-forming units per milliliter.

TABLE 2. Neutralization test^a of *Pf2* phage with antisera of other fibrous phages

Antiserum	Plaque-forming units/ml
<i>Pf2</i> control.....	4.3×10^9
Anti- <i>Pfl</i> , ^a $K = 580$	6.2×10^8
Anti-no. 6, ^a $K = 1,080$	4.0×10^9
Anti- <i>fd</i> , ^a $K = 200$	4.4×10^9

^a Dilution, 1:200; 37 C, 10 min.

TABLE 3. Efficiency of plating of *Pfl* and *Pf2* propagated on strain K to other sensitive strains

Indicator strain	Efficiency of plating (%)	
	<i>Pfl</i>	<i>Pf2</i>
K	100	100
C14	71	111
F23	94	75
P27	0.3	0.5

FIG. 5. Effect of ultrasonics on *Pfl* and ϕ X174 phages. A mixture of *Pfl* and ϕ X174 phages was treated with Kubota ultrasonic generator (10 kc, 200 w). Each sample was assayed for *Pfl* with strain K and for ϕ X174 with *Escherichia coli* C. (A) ϕ X174; (B) *Pfl*.

its turbidity increased. High titers of phages were usually obtained from such turbid lysates. Pyocin produced in the infected culture showed titers similar to those of the uninfected control, indicating that the phage infection did not induce the pyocin production of the host bacteria.

Similar experiments were designed with wild type (r^+) and a rapid lysis mutant (r) of *Pf2*, and with wild type (ϕ^s) and a phage-resistant mutant (ϕ^r) of host strain. The optical-density measurements revealed that all cultures grew continuously but at different rates. There was little difference in growth between the uninfected ϕ^s and the r -infected ϕ^r , whereas r -infected ϕ^s showed greater reduction in growth rate than r^+ -infected ϕ^s . Growth rates started to decrease after 20 min in the r -infected ϕ^s culture and after 40 min in the r^+ -infected ϕ^s culture (Fig. 8). Phage-producing colonies were obtained from the phage-infected culture. Growth of such phage-producing survivors in the presence of antiserum (1:20) overnight resulted in the complete loss of the ability of the colony-formers to produce *Pf2* phage.

Adsorption of Pf2 r^+ and Pf2 r to phage-sensi-

FIG. 6. Effect of enzymes on the infectivity of *Pfl*. Purified *Pfl* was incubated with enzymes at 37 C. A sample of the mixtures was assayed for infectivity at the indicated time. (A) Ribonuclease (Nutritional Biochemicals Corp., Cleveland, Ohio), 0.2 mg/ml; (B) deoxyribonuclease (Nutritional Biochemicals Corp.), 0.2 mg/ml; (C) trypsin (Difco, 1:250), 2 mg/ml; (D) Nagarse (Nagase), 0.2 mg/ml.

TABLE 4. Effect of freeze-thawing on *Pfl* phage^a

No. of times frozen and thawed	Plaque-forming units/ml
0	4.9×10^8
5	3.6×10^8
10	4.2×10^8
20	2.3×10^8

^a Frozen (−60 C) and thawed (37 C).

tive (ϕ^s) and phage-resistant (ϕ^r) host bacteria. Samples of r^+ and r were added to broth (control), or to suspensions of ϕ^s and ϕ^r at a multiplicity of approximately 0.1 for ϕ^s and 0.2 for ϕ^r . After incubation for 15 min at 37 C, the mixtures were diluted 100-fold to prevent further adsorption and were centrifuged to remove cell-associated phages. The supernatant fluid was carefully collected and assayed for the remaining phages. As shown in Table 5, the phages did not adsorb

FIG. 7. Growth of strain K after infection with Pf1. (A) Uninfected control; (B) multiplicity of infection (MOI, 0.1); (C) MOI, 1; (D) MOI, 10. Arrow shows the time of inoculation with the phage.

FIG. 8. Growth of strain K after infection with Pf2. (A) Uninfected $K\phi^*$; (B) $K\phi^*$ infected with Pf2r; (C) $K\phi^*$ infected with Pf2r⁺; (D) $K\phi^*$ infected with Pf2r. The phages were added at a multiplicity of infection of 10 at the time indicated by the arrow.

to phage-resistant cells, whereas, when added to sensitive cells, one-half to one-third of the phages sedimented as cell-associated phages. These results indicated that the resistant mutant had lost phage receptors.

Spontaneous Pf2 production by strain P28. Since Pf2 was obtained from the culture fluid of strain P28, our attention was drawn to the phage-host cell relationship in P28 culture. The effect of antiserum on the phage-producing capacity of the cells was examined first. An autoplague-negative clone of P28 (6) was successively subcultivated in Nutrient Broth with and without antiserum (1:20). After 10 serial passages, the

supernatant fluid of the antiserum-containing culture produced no plaques and retained sufficient antiphage activity, whereas the supernatant from the culture without antiserum contained about 10^4 plaque-forming units/ml of free phages. Ten colonies of the control and 11 colonies of the culture which had undergone 10 passages in the presence of antiserum were picked, subcultured, grown in antiserum-free broth, and examined for their phage production. All of these colonies, whether grown in the presence or absence of antiserum, produced Pf2 plaques. Thus, curing of the cells by antiserum was not achieved.

Strain P28 produced small rod-shaped pyocin (pyocin 28) which was easily induced by the addition of $1 \mu\text{g/ml}$ of mitomycin C (10). Cells of strain P28, grown in Nutrient Broth to about 10^7 cells/ml, were added with $1 \mu\text{g/ml}$ of mitomycin C and incubated at 37°C for an additional 3 hr. The mixture was shaken constantly. Induced cultures had a pyocin titer about 100 times that of uninduced cultures. On the other hand, the Pf2 titer was 10 times lower in induced cultures than in the control cultures (Table 6).

TABLE 5. Adsorption of Pf2 r⁺ and r to $K\phi^*$ and $K\phi^r$

Incubation ^a	Pf2 (plaque-forming units/ml) remaining in the supernatant fluid ^b
Pf2 r ⁺ + broth	5.5×10^7 (100%)
Pf2 r ⁺ + $K\phi^*$ (MOI, 0.1)	3.1×10^7 (56%)
Pf2 r ⁺ + $K\phi^r$ (MOI, 0.2)	5.3×10^7 (96%)
Pf2 r + broth	4.2×10^7 (100%)
Pf2 r + $K\phi^*$ (MOI, 0.1)	1.4×10^7 (33%)
Pf2 r + $K\phi^r$ (MOI, 0.2)	4.1×10^7 (97%)

^a At 37°C for 15 min.

^b Centrifugation: 10,000 rev/min, 10 min, 0 to 2°C .

^c Multiplicity of infection.

TABLE 6. Effect of mitomycin C on pyocin and Pf2 production by strain P28

Condition of incubation	Pyocin and phage titer ^a after 3-hr incubation	
	Pyocin (units) ^b	Pf2 (plaque-forming units) ^c
With mitomycin	10^3	4.4×10^8
Without mitomycin	10	6.1×10^4

^a Supernatant fluids from centrifugation at 10,000 rev/min for 15 min.

^b Expressed by the highest dilution to show the growth inhibition of strain P29.

^c Assayed with strain K.

Attempts were also made to "cure" strain P28 with acridine orange by the method of Hirota (2). However, every colony tested so far continued to produce Pf2.

DISCUSSION

The plaques of Pf phages are minute and turbid. The finding that the host bacteria reduced methylene blue suggested the use of the dye as a means to differentiate plaques from the bacterial lawn. This simple method has increased the accuracy of plaque titration and has facilitated the scoring of plaques. This method has also proved of value in the study of plaque-type mutants and in the search for new *Pseudomonas* phages (including Pf2), some of which might have been overlooked. It may also be applicable to any phage active against bacteria which reduce methylene blue.

Pf phages are not active against the male strains of *P. aeruginosa* used by Holloway and Jennings (4). This is in contrast to the fibrous coli phages which are male-specific (1). Electron micrographs of the indicator bacteria failed to reveal pili or filamentous appendages other than flagella on their cell surfaces. On the other hand, the possibility that the sensitive bacteria are male was examined preliminarily by mating experiments with the female strains used by Holloway and Jennings (4). Drug resistance and nutritional requirement were used as selective markers. The results obtained so far suggest that the K strain has the ability to form recombinants with the female strains (Minamishima, unpublished data).

It should be noted that strain P28 spontaneously produced Pf2 phage. The phage is probably produced from host cells without causing cell lysis. It is of interest that this phage-producing ability was affected neither by antiserum treatment nor by acridine orange treatment

(which is known to eliminate extrachromosomal episomes), whereas phage-producing survivors obtained after infection with Pf1 could easily be "cured" by antiserum treatment.

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