Isolation and Properties of a \textit{Pseudomonas acidovorans} Bacteriophage

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SUMMARY

The lytic bacteriophage $\phi$W-14 was isolated from sewage using \textit{Pseudomonas acidovorans} no. 14 as host. The phage had an icosahedral head some 85 nm. in diameter and a contractile tail some 140 nm. long. $\phi$W-14 formed plaques on only a few strains of \textit{P. acidovorans}. The phage gave biphasic absorption kinetics, with an adsorption constant of $1.9 \times 10^{-9}$ ml./min. The latent period was 60 min. and the burst size was about 300. The burst size was dependent upon culture age. The $k_{\text{ad}}$ for inactivation of $\phi$W-14 was 4.35 min.$^{-1}$. \textit{P. acidovorans} was shown to possess a potent photoreactivating system. The heat of inactivation of $\phi$W-14 was calculated to be 75,700 cal./mole. The phage gave biphasic thermal inactivation kinetics at 55$^\circ$ and 60$^\circ$ but not at 65$^\circ$. The phage mutated spontaneously to a different plaque type. This mutation affected the adsorptive properties, the thermal sensitivity and the burst size of the phage.

INTRODUCTION

There have been relatively few studies of the bacteriophages of non-fluorescent strains of \textit{Pseudomonas}. Phages have been reported for \textit{P. fragi} (Roberts & Doetsch, 1966), \textit{P. stutzeri} (Espejo & Canelo, 1968) and \textit{P. putrefaciens} (Levin & Delisle, 1969). The present report describes the isolation and the properties of a \textit{P. acidovorans} phage.

METHODS

Bacteria. These are listed in Table 1. They were maintained at 4$^\circ$ in standard minimal base stabs with 0.5% yeast extract (Stanier, Palleroni & Doudoroff, 1966), and were transferred once a month.

Media. All media were made up in distilled water. The bacteria were usually grown in mannitol broth (MB) containing, in g./l.: tryptone, 10; yeast extract, 5; NaCl, 5; mannitol, 1. The pH was adjusted to 6.5 before autoclaving. The medium used for adsorption studies (AM) contained, in g./l.: tryptone, 1; yeast extract, 0.5; mannitol, 2.

Growth of bacteria. Unless stated otherwise, cultures were incubated in a Metabolyte G77 shaker water bath (New Brunswick Scientific Co., New Brunswick, N.J.) set at 30$^\circ$ and 250 rev./min.

Titration of phage. Phage was assayed and plaque morphology examined by the agar layer technique (Adams, 1959). The top and bottom layers consisted of MB containing 0.6 and 1.5% agar, respectively.

Isolation of phage. A sample of activated sludge from the Greater Vancouver

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Sewage Processing Plant was centrifuged at 20,000 g for 5 min. A 10 ml. sample of the supernatant fluid and 2 drops of an overnight culture of *P. acidovorans* no. 14 were added to 10 ml. of double strength MB in a 250 ml. Erlenmeyer flask. After incubation for 48 hr without shaking, the culture was centrifuged at 6000 g for 10 min. A sample of the supernatant was diluted and plated with *P. acidovorans* no. 14. A well-isolated plaque was picked with a sterile wire and the phage purified by serial single plaque isolations. The phage was given the designation φW-14.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td><em>P. acidovorans</em></td>
<td>Nos. 14, 29, 114, 146</td>
<td>Dr. R. Y. Stanier, Department of Bacteriology, University of California, Berkeley, Norleucine enrichment, A. Kropinski</td>
</tr>
<tr>
<td></td>
<td>AK-11</td>
<td>Dr. E. F. Lessel Curator of Bacteria, American Type Culture Collection</td>
</tr>
<tr>
<td></td>
<td>15666, 15667</td>
<td>Dr. R. Y. Stanier</td>
</tr>
<tr>
<td></td>
<td>15668</td>
<td>Dr. E. F. Lessel</td>
</tr>
<tr>
<td><em>P. testosteroni</em></td>
<td>Nos. 78, 138</td>
<td>Dr. R. Y. Stanier, Dr. E. F. Lessel, Culture Collection</td>
</tr>
<tr>
<td><em>P. mucidolens</em></td>
<td>ATCC4687</td>
<td>Department of Microbiology, University of British Columbia, Vancouver</td>
</tr>
<tr>
<td><em>P. putrefaciens</em></td>
<td>HAMMER</td>
<td>Department of Microbiology, U.B.C.</td>
</tr>
<tr>
<td><em>P. ovalis</em></td>
<td>ATCC950</td>
<td>Department of Microbiology, U.B.C.</td>
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<td><em>P. taetrolens</em></td>
<td>ATCC4683</td>
<td>Department of Microbiology, U.B.C.</td>
</tr>
<tr>
<td><em>P. synxantha</em></td>
<td>ATCC796</td>
<td>Department of Microbiology, U.B.C.</td>
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<td><em>P. convexa</em></td>
<td>ATCC795</td>
<td>Department of Microbiology, U.B.C.</td>
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<td><em>P. aeruginosa</em></td>
<td>ATCC9027</td>
<td>Department of Microbiology, U.B.C.</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>ATCC9721</td>
<td>Department of Microbiology, U.B.C.</td>
</tr>
<tr>
<td><em>P. fragi</em></td>
<td>ATCC4975</td>
<td>Department of Microbiology, U.B.C.</td>
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</table>

Preparation of high titre lysates. A number of 500 ml. Erlenmeyer flasks each containing 150-200 ml. of MB were inoculated with 10 ml. of an overnight culture of *P. acidovorans* no. 29 and incubated until the cultures reached an optical density at 650 nm. of 1.5 to 2.0, equivalent to 1.0 to 1.3 x 10⁸ cells/ml. Phage φW-14 was added to give a multiplicity of infection (m.o.i.) of about 1, and incubation continued for a further 6 hr. The culture did not clear under these conditions. The lysate was freed of whole cells and debris by centrifugation at 20,000 g for 5 min. This method routinely yielded lysates with titres of 1 to 3 x 10¹¹ p.f.u./ml.

Host range. The host ranges of the phages were determined by spotting drops of high titre lysates (c. 1 x 10¹² p.f.u./ml.) on overlays containing the various organisms, and examining for lysis after overnight incubation at 30°.

One-step growth experiment. The latent period and average burst size for *P. acidovorans* no. 29 were determined by the one-step growth method (Adams, 1959). The infected culture, in MB, was incubated in the shaker water bath at 30° and 250 rev./min. to allow for adsorption.

Electron microscopy. Preparations of phage φW-14 were stained with 2 % phosphotungstic acid, pH 7.2. The grids were allowed to air dry before being examined with a Philips EM-200 electron microscope at an operating voltage of 60 kv. Magnifications, before printing, ranged from 15,000 to 27,800.

Kinetics of adsorption. Adsorption to *P. acidovorans* no. 29 was measured by assaying the unadsorbed phage after removing the bacteria by centrifugation (Adams,
The phage was added at a m.o.i. of 0.01 to cultures at a cell density of $10^8$/ml., in either MB or AM, incubated in the shaker water bath at 30° and 250 rev./min.

**Cell lysis.** Lysis was followed in two ways. In the turbidimetric method, phage was added at a m.o.i. of 5 to 10 ml. of a log. phase MB culture of *P. acidovorans* no. 29 in a 125 ml. sidearm flask. The turbidity of the culture was read at 30 min. intervals in a Klett Summerson Photoelectric colorimeter (Klett Mfg. Co., New York, N.Y.) equipped with a 540 filter. In the one-step growth method, phage was added to 10 ml. of a log phase MB culture ($1 \times 10^8$ cells/ml.) at m.o.i. of 5. After allowing 10 min. for adsorption, more phage was added, again at a m.o.i. of 5. After a further 10 min., the culture was diluted and plated as in the one-step growth experiment.

**Thermal inactivation of phage.** Large tubes (2.2 × 20 cm.) containing 10 ml. of MB were placed in water baths at 50°, 55°, 60° and 65°. After 10 min. for temperature equilibration, phage suspension, generally less than 0.2 ml., was added to each tube to give 1.5 to $3.5 \times 10^5$ p.f.u./ml. (except the tube at 65°, in which the initial titre was $2 \times 10^4$ p.f.u./ml.). At regular intervals, 0.5 ml. samples were removed to small test tubes chilled in an ice-bucket. All the samples were plated at the end of the experiment. The rate constants for thermal inactivation were calculated using the equation of Pollard (1953). The activation energies were calculated using the equation given by Neilands & Stumpf (1958), applying the corrective equation given by Dixon & Webb, (1964).

**Sonic sensitivity of phage.** Quadruplicate 2.8 ml. samples of phage each containing $1 \times 10^6$ p.f.u./ml., were subjected to 30 sec. bursts of acoustic energy from a Biosonic II (Bronwill Scientific, Rochester, N.Y.), equipped with a needle probe. The frequency of ultrasonic output of this instrument is 20 kcyc./sec. ± 400 cyc./sec. A setting of 70 was used throughout, being equivalent to 87.5 W. The samples were chilled in ice-water prior to and during sonication to reduce heat denaturation. Then they were diluted and 0.1 ml. samples plated.

**pH sensitivity of phage.** Samples of MB medium were adjusted to pH 2–12 by the addition of 1M-HCl or 1M-NaOH, and 4.5 ml. amounts were added to test tubes in an ice-water bath. After 15 min., 0.5 ml. of a phage preparation diluted to $3 \times 10^4$ p.f.u./ml. was added to each and the tube shaken vigorously. After a further 30 min., 0.1 ml. samples were plated from each tube.

**Sensitivity of phage to u.v. light.** One millilitre samples of a mixture of coliphage T1 and φW-144a+ in MB (each at $1 \times 10^6$ p.f.u./ml.) were placed in disposable plastic Petri dishes (Millipore Filter Corp., Bedford, Mass.) of 5.0 cm. diameter and irradiated at a distance of 50 cm. with a General Electric 15 W germicidal lamp (principle wavelength at 2575 Å) with continuous stirring by a magnetic stir-bar and stirrer. The irradiated samples were then diluted and plated under yellow, nonphotoreactivating light with *P. acidovorans* no. 29 for φW-144a+ and *Escherichia coli* b for T1. The plates were incubated at 30° and 37°, for φW-144a+ and T1 respectively, in lightproof boxes. The rate constants for inactivation were calculated from the exponential region of the graphed results using the equation of Pollard (1953).

**Photoreactivation of u.v. irradiated phage.** The u.v.-irradiated samples of phage were plated under white light, and the uninverted plates were incubated overnight 30 cm. from a lamp fitted with twin Westinghouse 15 w Cool White or General Electric 15 w Daylight fluorescent bulbs (principle wavelength approximately 5700 Å in both cases). The photoreactivable sector was calculated using the equation of Dulbecco (1950).
Morphology

φW-14 exhibited a regular icosahedral head about 85 nm. across, with a tail some 20 nm. in diameter and 140 nm. long (Fig. 1a). Subunits could be seen in the tails of some of the particles (Fig. 1a). A baseplate at the end of the tail appeared to carry

Fig. 1. (a) φW-14. (b) φW-14, tail detail and empty heads. (c) φW-14 with contracted sheath. (d) Aggregate of φW-14 particles.
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pin-like structures (Fig. 1b). A number of empty heads were observed which retained largely the angular shape of the complete head (Fig. 1b). A few complete particles with empty heads and a great many free tails were observed in the preparation (Fig. 1b, d). The addition of sodium perchlorate, buffered at pH 7 to 9, to a concentration of approximately 0.025 M (Freifelder, 1966), caused contraction of the tail sheath of some particles (Fig. 1c). Some of the contracted particles seemed to have a collar at the juncture of the head and tail. Contraction of the sheath exposed a tail core, and a skirt of projections, originating in the region of the former baseplate, fanned out around the exposed core (Fig. 1c).

Table 2. Host-range of φW-14a+ and derivatives

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lysis by (φW-14a+)</th>
<th>Lysis by (φW-14a)</th>
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</thead>
<tbody>
<tr>
<td>P. acidovorans no. 29</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>P. acidovorans no. 14</td>
<td>+ +</td>
<td></td>
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<tr>
<td>P. acidovorans no. 114</td>
<td>- -</td>
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<td>P. acidovorans no. 146</td>
<td>- -</td>
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<tr>
<td>P. acidovorans AK-11</td>
<td>+ +</td>
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<tr>
<td>P. acidovorans 15666</td>
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<td>P. acidovorans 15668</td>
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<td>P. testosteroni no. 78</td>
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<td>P. fragi 4975</td>
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<td>P. convexa 795</td>
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<tr>
<td>E. coli k12</td>
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<td></td>
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<tr>
<td>E. coli b</td>
<td>- nd</td>
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</tr>
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</table>

*nd = not done.

Phage φW-14 showed a marked tendency to aggregate (Fig. 1d). No bacterial debris appeared to be present to account for this aggregation.

Plaque morphology

When first isolated, φW-14 formed small plaques, about 2 mm. in diameter, with very small, clear centres and turbid haloes, on Pseudomonas acidovorans no. 14. These were designated a+. Later, mutants appeared, designated a, producing slightly smaller plaques with wide, clear centres and indistinct haloes. The a type appeared in lysates prepared with P. acidovorans no. 14, but not in those prepared with P. acidovorans no. 29. Because the a type gave a higher burst size, difficulty was encountered in preparing a high titre lysate of predominantly the a+ type when P. acidovorans no. 14 was used as the host. The a type appeared to be more stable than the a+ type, since reversion was rarely, if ever, observed. The plaques formed on P. acidovorans AK-11 were small and indistinct, so that the efficiency of plating (e.o.p.) could not be determined accurately with this strain.

Host range

φW-14 showed a limited host range, lysing only four of seven different strains of Pseudomonas acidovorans (Table 2). It did not lyse any strains of the related organism, P. testosteroni. φW-14a+ and φW-14a showed identical host ranges, and on each indicator the plaques of the a+ type were haloed and those of the a type were clear. The e.o.p. was 2 to 3 times higher on P. acidovorans no. 29 than on P. acidovorans no. 14 with both the a+ type and the a type prepared on either host strain.
Kinetics of adsorption

In MB, the concentration of unadsorbed phage decreased exponentially with first order kinetics, with adsorption constants of $1.9 \times 10^{-9}$ ml./min. and $4.2 \times 10^{-9}$ ml./min. for $\phi W-14a^+$ and $\phi W-14a$, respectively. Only 60% of $\phi W-14a^+$ appeared to adsorb, while the rate constant for $\phi W-14a$ decreased to $1.5 \times 10^{-9}$ ml./min. after 90% of it had adsorbed. The adsorption rates were the same at 30° and 0°. In low salt medium (AM) the rate constant for $\phi W-14a^+$ increased to $3.0 \times 10^{-9}$ ml./min., with 90% of it adsorbing under these conditions, whereas the rate for $\phi W-14a$ was the same in AM and MB. The rate constant for $\phi W-14a^+$ was decreased to $7.3 \times 10^{-10}$ ml./min. when the phage-host mixture was incubated at 50 rev./min. rather than the normal 250 rev./min.

![Graph](image)

Fig. 2. Effect of cell age on phage development as measured by the one-step growth method. 
“--“, growth of *P. acidovorans* no. 29 at 30° measured in E650 units/ml. “•--•”, average burst size from cell samples taken at specified times and infected with $\phi W-14a^+$.

Fig. 3. Arrhenius plot of the thermal inactivation of $\phi W-14a^+$.

One-step growth experiment

Most of the one-step growth experiments were carried out with *Pseudomonas acidovorans* no. 29 because of the slow adsorption of $\phi W-14$ to *P. acidovorans* no. 14.

The latent period for $\phi W-14a^+$ was about 60 min. and the rise period about 40 min. The burst size, averaged over six experiments, was 300, with a range from 214 to 470. These experiments were performed with cells in the logarithmic phase of growth, i.e. at a density of about $10^9$ cells/ml., diluted to $10^8$ cells/ml. before infection. Subsequently, it was found that the burst size was markedly dependent upon the age of culture (Fig. 2), ranging from 30 for stationary phase cells to about 600 for late log. phase cells. A similar response was observed with *Pseudomonas acidovorans* no. 14. This was not due to effects on the adsorption of the phage, since the capacity of the cells to adsorb the phage remained constant over the growth curve.
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The latent and rise periods for $\phi$W-14a were the same length as those for $\phi$W-14a$^+$, but the burst size was 50% greater.

Attempts to lyse infected cells prematurely with chloroform or lysozyme were unsuccessful.

Lysis inhibition

The turbidity of a culture of Pseudomonas acidovorans no. 29 decreased markedly 30 min. after infection with $\phi$W-14a, and then remained constant. Following infection of the same strain with $\phi$W-14a$^+$, there was a slight decrease in the turbidity of the culture after 30 min., followed by a gradual increase during the next 3 hr. Thus, a culture infected with $\phi$W-14a appeared to be lysis inhibited. However, attempts to demonstrate lysis inhibition by superinfection with $\phi$W-14a$^+$ were unsuccessful. Surprisingly, superinfection decreased the burst size, and it was found subsequently that use of a m.o.i. greater than about 5 led to a reduction in burst size with both $\phi$W-14a$^+$ and $\phi$W-14a.

Thermal inactivation

$\phi$W-14a$^+$ was essentially stable at 50° in MB. At temperatures above this, the rate constants for inactivation were 0.933 min.$^{-1}$, 0.18 min.$^{-1}$ and 1.06 min.$^{-1}$ at 55°, 60° and 65°, respectively. At 55° and 60°, the inactivation curve was biphasic, with some 35% of the particles having some degree of resistance. The resistant fraction appeared to be stable at 55°, but was inactivated slowly at 60°. Using the initial parts of the curves, the heat of activation of $\phi$W-14a$^+$ was calculated to be 75,700 cal./mol. (Fig. 3).

$\phi$W-14a was slightly more thermolabile than $\phi$W-14a$^+$, the activation energy being 62,500 cal./mole. It showed biphasic inactivation at 55° but not at 60°.
Sonic sensitivity

ϕW-14a+, with a $k_s$ value of 1.74 min.$^{-1}$, was slightly more sensitive to sonication than coliphage T1, which gave a $k_s$ value of 1.45 min.$^{-1}$ (Fig. 4). Coliphage S13 was much less sensitive than phage T1, giving a $k_s$ value of 0.12 min.$^{-1}$ (Fig. 3).

pH sensitivity

Both ϕW-14a+ and ϕW-14a were relatively stable between pH 5 and pH 9 (Fig. 4). However, since phage lysates were prepared and stored at pH 6.5 to 7.0, it appeared that ϕW-14a was activated slightly at pH 4–5 (Fig. 5).

Sensitivity to ultraviolet light

Multihit kinetics were observed for the inactivation of ϕW-14a+ and coliphage T1. The rate constants ($k_{ov}$), calculated from the exponential regions of the curves, were 0.60 min.$^{-1}$ for T1, and 4.35 min.$^{-1}$ for ϕW-14a+. The lethal effects of u.v. light were reversed to a considerable extent by irradiation of the overlay plates with white light. Photoreactivable sectors of 0.35 and 0.71 were calculated for T1 and ϕW-14a+, respectively.

Discussion

ϕW-14 falls into Bradley’s morphological classification group A, which contains all phages having contractile tails (Bradley, 1967). Morphologically it resembles other Pseudomonas phages, but it is considerably larger than those reported previously, the heads of which range from 50 to 60 nm in diameter (Lee & Boezi, 1966; Bradley, 1967; Olsen, Metcalf & Todd, 1968). The aggregation of ϕW-14 particles seen in some of the electron micrographs appeared not to involve adsorption to bacterial debris.

ϕW-14 has a very limited host range, with the e.o.p. on the susceptible hosts varying over only a two- to threefold range.

The biphasic adsorption kinetics observed with ϕW-14 are unusual, although phage 7v of P. aeruginosa (Feary, Fisher & Fisher, 1964) and phage χ of E. coli (Schade & Adler, 1967) yield significant numbers of slowly adsorbing particles. The poor adsorption under static conditions remains to be explained.

Compared with other Pseudomonas phages, ϕW-14 has a high burst size. Although it has a long latent period, several P. aeruginosa phages have been reported recently to have latent periods of 65 to 70 min. (O’Callaghan, O’Mara & Grogan, 1969). The decrease in burst size following superinfection is unusual, and cannot be explained at present. Cells of Shigella sonnei gave smaller burst sizes following infection with a large excess of coliphages T4 and T7 (Barry & Goebel, 1951).

Biphasic thermal inactivation curves, like those obtained for ϕW-14, though quite common amongst animal viruses, have not been reported often for bacteriophages. Some 35% of P. aeruginosa phage 7m particles appeared to be more heat resistant than the remainder of the population (Feary et al. 1964). The proportion of heat resistant ϕW-14 particles corresponded to the proportion of slowly adsorbing particles, so that the change from $a_+^+$ to $a$ may involve the adsorption structures of the phage tail.

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REFERENCES


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