Some Properties of *Erwinia amylovora* Bacteriophages

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ABSTRACT

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Eleven *Erwinia amylovora* bacteriophages isolated from aerial parts of apple trees in Michigan orchards during 1975 and 1976 were placed in two groups on the basis of morphology and serology. Group I phages produced two plaque types: (i) PEal(h) plaques were 2–3 mm in diameter surrounded by an expanding, translucent halo; (ii) PEal(nh) plaques were 1–2 mm in diameter without a halo. Group II phages, represented by PEa7, produced 0.5–1.0 mm diameter plaques with an expanding, translucent halo. Phage PEal(h) was polyhedral, 60 nm in diameter with a spikelike tail; PEa7 had an octahedral head, 75 nm in diameter with a rigid, striated tail 135 nm long. Phages PEal(h) and PEal(nh) had a buoyant density of 1.53 g/cc and a sedimentation coefficient of 566 S. Phage PEa7 had a buoyant density of 1.44 g/cc with a sedimentation coefficient of 1037 S. The latent period of PEal(h) was 45 min with a burst size of 50 plaque-forming units (pfu) per productive cell; that of PEa7 was 100 min with 8 pfu per productive cell. Phages PEal(h) and PEal(nh) required 10 min at 55 C for complete thermal inactivation; 10 min at 65 C were required for PEa7. Host ranges for the two phage groups were limited to *E. amylovora* and several *Erwinia herbicola* strains. Phage PEal(h) adsorbed more rapidly to encapsulated than to nonencapsulated strains of *E. amylovora* but PEa7 adsorbed more rapidly to nonencapsulated *E. amylovora* strains.

Additional key words: K-phages, fire blight.

Although *Erwinia amylovora* bacteriophages have been isolated from soil since the 1950s (15), only one strain has been characterized (11). *E. amylovora* phages occasionally have been used to diagnose fire blight or to detect *E. amylovora* (4,7). *Erwinia amylovora* phage-sensitivity may be correlated with virulence (4,11,12) and with bacterial encapsulation (6).

In 1977, we reported the isolation of *E. amylovora* bacteriophages from aerial structures of apple (19), and we now report some of the properties of these phages.

MATERIALS AND METHODS

Media and culture conditions. Bacteria were cultured on 2.0% (w/v) nutrient agar containing 0.5% (w/v) glucose, pH 6.5 (NAG medium). Phages were plated in 100-mm diameter plastic petri dishes using the soft agar overlay method (1). The bottom and top layers were composed of 12–15 ml of NAG and 2.5 ml of 0.7% (w/v) nutrient agar, 0.5% (w/v) glucose, and 0.25% (w/v) yeast extract, pH 6.5, respectively.

Liquid medium consisted of 0.8% (w/v) nutrient broth, 0.5% (w/v) glucose, and 0.25% (w/v) yeast extract, pH 6.5, in distilled water (NBGYE medium). All liquid cultures, unless stated otherwise, were incubated at 22–23 C on a reciprocal shaker (80 oscillations per minute, stroke length 4.0 cm). Rifampin-resistant *E. amylovora* strain 110 rif was used for phage propagation and assay. Quantitative phage assays were performed as described by Adams (1) and measured after 12–36 hr of incubation at 27 C. All bacteria and phage dilutions were made in sterile 0.02 M potassium phosphate buffer (PPB), pH 6.8.

Phage isolation. Phages were isolated as described previously (18). Lysates were obtained from plates with confluent lysis. A 5-ml sample of PPB was added to each plate and the top agar was removed with a glass rod. Chloroform was added to this solution to a final concentration of 1.0% (v/v); the debris was removed by centrifugation for 10 min at 12,100 *g* in a Sorvall RC2-B centrifuge, and the supernatant was filtered (Millipore 0.22-μm pore size). Filtrates were stored over a drop of chloroform at 4 C.

Phage growth and purification. Ten milliliters of a 12-hr culture of *E. amylovora* 110 rif was added to 1 L of NBGYE in a 2-L flask and incubated until A_{525} (Spectronic 20) was 0.06–0.10 (approximately 10^8 colony-forming units [cfu] per milliliter). Phages were added to give a final multiplicity of infection (MOI) of 0.1–1.0. The culture was incubated for 12 hr, chloroform was added to 1.0% (v/v), and the mixture was shaken an additional 5–10 min. Bovine pancreatic DNase (1 μg/ml) and 1.0 μg/ml of beef pancreatic RNase (U. S. Biochemical Corp., Cleveland, OH) were added and incubated in the presence of 10^{-3} M $MgSO_4 \cdot 7\,H_2O$ for 1 hr at 23 C. Debris was removed by centrifugation at 12,100 *g* for 10 min (Sorvall RC2-B) and the supernatant was decanted and stored at 4 C.

Phages were precipitated with 10% (w/v) polyethylene glycol (PEG) 6000 (J. T. Baker Chemical Co., Phillipsburg, NJ) in 0.5 M NaCl as described by Yamamoto (25). The precipitate was pelleted by centrifugation at 12,100 *g* for 20 min, resuspended in PPB, and centrifuged at 3,020 *g* in conical glass tubes for 10 min. The supernatant was centrifuged for 2 hr at 22,000 rpm using a SW30 rotor in a Beckman Model L ultracentrifuge. After the pellets were resuspended overnight in 5.0 ml of PPB at 4 C, the preparation was clarified by low-speed centrifugation. The phage preparation was stored at 4 C over a drop of chloroform. Phages were further purified by centrifugation at 22,000 rpm for 1 hr in a 10–40% (w/v) linear sucrose gradient (4, 7, 7, 7 ml) in 0.1 M potassium phosphate buffer, pH 7.8, using a Beckman SW25.1 rotor. Phages were collected by withdrawing the band with a needle or by collecting 1.0-ml fractions with an ISCO Model D density gradient fractionator while scanning at 260 nm with an ISCO Model UA-4 absorbance monitor. The fractions were diluted with 9.0 ml of PPB and assayed, and the fractions containing maximum phage infectivity were centrifuged for 2 hr at 22,000 rpm. The pellets were resuspended in 2.0 ml of PPB. The preparations were purified further by banding in 50% (w/v) cesium chloride (CsCl) for 18–24 hr at 37,500 rpm in a Beckman SW50.1 rotor.

Host range and plaque morphology. Host range was determined as described previously (18). Plaque morphology was examined by use of the agar overlay method (1). Phage preparations were diluted

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to obtain approximately 50 plaques per plate. The plates were incubated at 27 C and examined during a 12–48 hr period.

Phage thermal inactivation and optimum growth temperature. One-ml samples (approximately 10^7 plaque-forming units [pfu]) of phages PEal(h), PEal(nh), and PEa7 suspended in PPB in thin-walled glass tubes (external diameter, 1.5 cm) were immersed in a water bath at 10° intervals from 25 to 65 C (± 1.0 C). After 10 min, the tubes were transferred to an ice bath and assayed for infectious phage.

Optimum temperatures for infectivity and multiplication of PEal(h) and PEa7 were determined. Aliquots of 1-ml (7×10^7 cfu/ml) of an exponentially growing culture of *E. amylovora* 110 rif were pipetted into test tubes, and the tubes were placed in incubators at 0, 10, 15, 23, 27, or 30 C for 30 min acclimatization. Then 0.1 ml of PEal(h) (12×10^7 pfu/ml) and PEa7 (20×10^7 pfu/ml) were added and the cultures were incubated with shaking at 30-min intervals. After 3 hr, a drop of chloroform was added to each tube and the tubes were stored at 10 C before assay.

One-step growth and adsorption experiments. Growth experiments (one-step) were performed as described for phage PEal (19). The adsorption rates were determined by the chloroform method as outlined by Adams (1).

Electron microscopy. Sucrose density gradient-purified suspensions of PEal(h), PEal(nh), and PEa7 in PPB were placed on parlodion, carbon-coated, copper grids. The grids were stained with 2.0% (w/v) ammonium molybdate, dried, and examined in a Philips 300 transmission electron microscope operating at 60,000 V.

Serology. Phages PEal(h) and PEa7 were purified by two cycles of 10–40% sucrose density gradients, suspended in PPB, and stored at 4 C. The titers were 7×10^{10} pfu/ml and 6×10^8 pfu/ml, respectively. Female, New Zealand white rabbits (3.2–3.6 kg) were injected at 7-day intervals with 2 ml of an emulsified mixture (1:1) of phage and Freund's complete adjuvant (Difco). The first two injections were given intramuscularly into the thighs, whereas the third was given subcutaneously in the dorsal region. Weekly ear bleedings (25 ml) were initiated 2 wk after the final injection. Processed sera were stored frozen at −20 C.

Antisera titers were determined with twofold serial dilutions in PPB containing 10^{-3} M NaCl. Sera were added to the homologous phages at a final titer of 10^7 pfu/ml, allowed to react for 10 min at 23 C, then assayed for infectious phage. A normal serum control was treated similarly. The reciprocal of the dilution at which no significant decline in pfu could be detected was considered the antiserum titer. Neutralization rate experiments with homologous and heterologous antisera (1) were done with phages PEal(h), PEal(nh), and PEa7. The remaining 11 phage isolates were typed by diluting each to 10^7 pfu/ml and mixing with PEal(h) antiserum (dilution 100) and PEa7 antiserum (dilution 50) for 10 min, then assaying for pfu/ml.

Sedimentation coefficients. Sedimentation coefficients for PEal(h), PEal(nh), and PEa7 were determined by comparison with tobacco mosaic virus (TMV) essentially as outlined by Brakke (9). A virus suspension (in PPB) of 1 ml ($A_{260} = 0.7$) was layered on linear 10–40% sucrose gradients (4, 7, 7, 7 ml), in 0.1 M potassium phosphate buffer, pH 7.8, and centrifuged at 20,000 rpm at 4 C in a Beckman SW25.1 rotor. At 20-min intervals during a 140-min period, measurements, to ± 0.5 mm, were made from the meniscus of the gradient to the top of the virus band. Sedimentation distances versus time were plotted and sedimentation rates calculated. Sedimentation coefficients were calculated based on the sedimentation coefficient of TMV at 187 S (14).

Buoyant density. The buoyant densities of PEal(h), PEal(nh), and PEa7 were determined as outlined by Vidaver et al (24). The phages were centrifuged for 25 hr at 37,500 rpm in a Beckman SW50.1 rotor. The density of each 10-drop fraction was determined by direct weighing in 10-μl capillary tubes. The fractions were dialyzed for 12 hr against 0.1 M NaCl at 4 C, brought to 1.0-ml volume, assayed, and the absorbance was read at 260 nm.

RESULTS

Isolation, plaque morphology, and host range. Fourteen phage isolates were chosen for study on the basis of source of isolation and plaque morphology (Table 1). The most common type of plaque was 2–3 mm in diameter and surrounded by an expanding, translucent halo. The second plaque type was 1–2 mm in diameter, lacked a halo, and was never isolated from plant material but was commonly detected in lysates of the halo plaque-producing phages (18). The third plaque type was 0.5–1.0 mm in diameter with an expanding, translucent halo. Phages that produced plaques of the first and second types were placed in group I, represented by phages PEal(h) and PEal(nh); phages producing plaques of the third type were placed in group II, represented by phage PEa7. When the phages were spotted on a mutant of *E. amylovora* 110 rif, resistant to PEal(h), only group II phages produced lysis.

The host range for the 14 phage isolates was essentially limited to strains of *E. amylovora,* as was reported for PEal(19). Strains (130, 150, and 151) of the closely related, yellow saprophyte *Erwinia herbicola* were lysed by PEal(h), PEal(nh), and PEa7 when spotted at titers of 10^9 pfu/ml.

Phage growth and purification. The phage lysates cleared only slightly, even though 10^{10}–10^{11} pfu/ml were produced. The yields after purification were 0.6 and 0.06% for phage PEal(h) and for phage PEa7, respectively (Table 2). Sucrose gradient centrifugation of phages PEal(h), PEal(nh), or PEa7 gave a single, distinct band in the lower half of the gradient (Fig. 1). Phages PEal(h) and PEal(nh) had maximum infectivity and absorbance in fraction 12; maximum infectivity and absorbance for PEa7 occurred in fraction 16.

TABLE 1. *Erwinia amylovora* bacteriophage isolates, plaque morphology, source, and date of isolation from Michigan orchards

	Plaque morphology		Source and date of isolation	
Isolate	Plaque diameter (mm)	Expanding halo	Blighted apple leaves and terminals	Other
PEal(h)	2–3	yes	Paw Paw, 6/17/75	
PEal(nh)	1–2	no		Derived from PEal(h)
PEa2(h)	2–3	yes		Soil, MSU[a], 12/74
PEa2(nh)	1–2	no	Derived from PEa2(h)	
PEa3(n)	2–3	yes		Blighted fruit, MSU, 7/15/75
PEa4(h)	2–3	yes		Newly formed canker, MSU, 7/18/75
PEa5(h)	2–3	yes		Symptomless leaves, MSU, 6/75
PEa6(h)	2–3	yes		Blighted pear leaves, MSU, 7/75
PEa7	0.5–1	yes	Berrien Springs, 6/18/76	
PEa8(h)	2–3	yes	Lawrence, 6/18/76	
PEa12(h)	2–3	yes	Watervliet, 7/22/76	
PEa13(h)	2–3	yes	Paw Paw, 7/22/76	
PEa15	0.5–1	yes	MSU, 8/2/76	
PEa16(h)	2–3	yes	MSU, 8/2/76	

[a]Michigan State University

Maximum infectivity and absorbance for PEal(h) and PEal(nh) occurred in fraction 24 after 25-hr isopycnic centrifugation in 60% CsCl (Fig. 2). The density in this fraction was 1.53 g/cc. Maximum infectivity and absorbance for PEa7 occurred in fraction 6 after 25-hr centrifugation in 40% CsCl (Fig. 2). The density of this fraction was 1.44 g/cc.

Thermal inactivation and optimum growth temperature. Phages PEal(h) and PEal(nh) were completely inactivated by 10-min incubation at 55 C, and PEa7 was completely inactivated by 10-min incubation at 65 C.

Phage PEal(h) multiplied optimally at 15–27 C, yielding approximately a 100-fold increase in pfu; but at 30 C more than a 10^3-fold decrease in pfu occurred (Fig. 3). All remaining group I phage strains, except PEal(nh) and PEa12, multiplied when incubated at 30 C. Phage PEa7 multiplied optimally at 27 C and did not show a dramatic decrease in pfu at 30 C (Fig. 3).

One-step growth and adsorption rate. Phage PEa7 exhibited a latent period of 100 min, a rise period of 35 min, and an average burst size of 8 pfu per productive cell. Phage PEal(h) exhibited a latent period of 45 min, a rise period of 25 min, and an average burst size of 50 pfu per productive cell (Fig. 4).

For 90% adsorption to *E. amylovora* 110 rif, PEal(h) required 10 min and PEa7 20 min (Fig. 5). Contrasting results were obtained when PEal(h) and PEa7 adsorbed to a PEal(h)-resistant strain of 110 rif, *E. amylovora* 110 rif p^r (Fig. 5). Similar results were obtained with *E. amylovora* strains E8, PEal(h)-resistant, and E9, PEal(h)-sensitive (Fig. 5). Phage PEal(h) adsorbed rapidly to encapsulated but not to nonencapsulated strains of *E. amylovora,* and PEa7 adsorbed rapidly to nonencapsulated but slowly to encapsulated strains (Fig. 5).

Electron microscopy. Phages PEal(h) and PEal(nh) are polyhedral, 60 nm in diameter with a spikelike tail (Fig. 6A) and

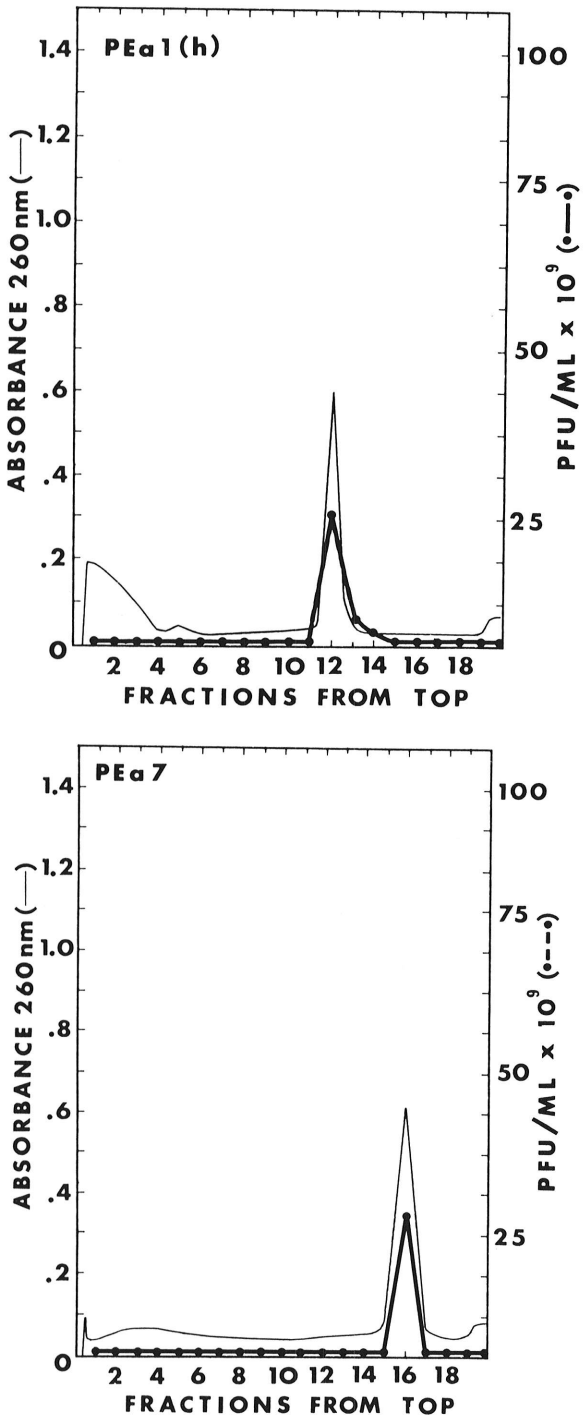

Fig. 1. Sedimentation profiles of bacteriophages PEal(h) and PEa7 in linear 10–40% sucrose density gradients. PFU = plaque-forming units.

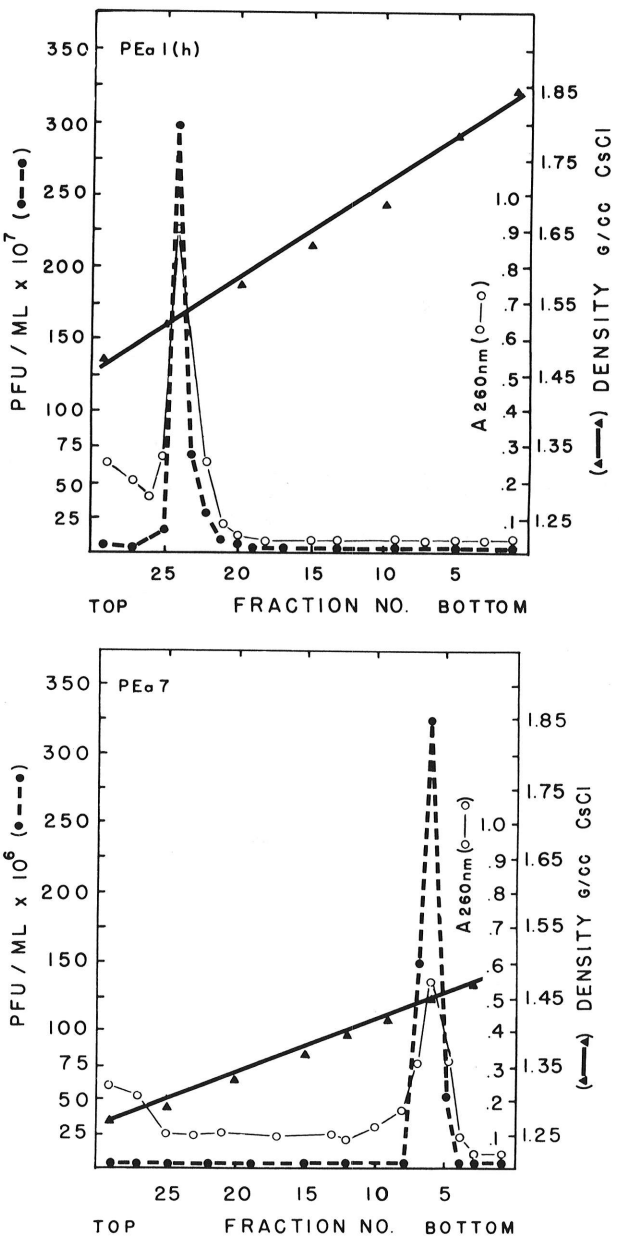

Fig. 2. Isopycnic centrifugation profiles and buoyant densities of bacteriophages PEal(h) and PEa7 in cesium chloride (CsCl). PEal(h) are from a 60% CsCl gradient; those of PEa7 are from a 40% CsCl gradient. PFU = plaque-forming units.

therefore can be placed in Bradley's group C (8). Phage PEa7 (Fig. 6B) has an octahedral head, 75 nm in diameter with a rigid, noncontractile, striated tail, 135 nm long, which fits the criteria of Bradley's group B (8).

Serology. Phage PEal(h) antiserum at a dilution of 1,000 neutralized 97% of its homologous phage within 10 min (Fig. 7), and the velocity constant, K, was 321/ min. Heterologous phages PEal(nh) and PEa7 were 94% neutralized within 5 min (K = 597/min) and 64% neutralized within 30 min (K = 80/min), respectively. Phage PEa7 antiserum, at a dilution of 100, neutralized 93% of its homologous phage within 5 min (K = 56/min) (Fig. 7). For heterologous phages PEal(h) and PEal(nh), 64% and 40%, respectively, were neutralized after 30 min giving an approximate K = 8/min for both. Serologic typing of the 11 remaining isolates resulted in the neutralization of all group I phages with PEal(h) antiserum but not with PEa7 antiserum; PEa7 antiserum neutralized only group II phages. Normal sera had no significant effect on the phages.

Sedimentation coefficients. Estimates of sedimentation coefficients for PEal(h) and PEal(nh) were 566 S and for PEa7 1037 S.

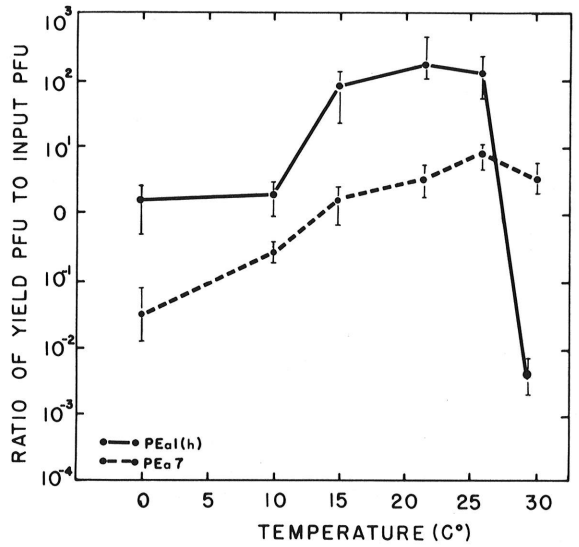

Fig. 3. Multiplication of *Erwinia amylovora* phages PEal(h) and PEa7 at 0–30 C temperatures. Bars indicate the range of three samples. PFU = plaque-forming units.

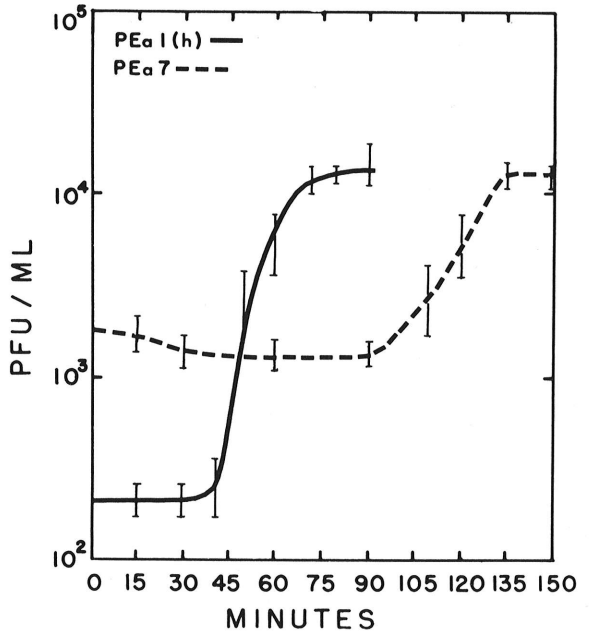

Fig. 4. One-step growth curves for *Erwinia amylovora* bacteriophages PEal(h) and PEa7. Bars indicate the range of three samples. PFU = plaque-forming units.

DISCUSSION

The 14 *E. amylovora* phages in our study were divided into two groups. Phage PEal(h) was typical of group I and PEa7 was typical of group II. Except for plaque morphology, halo and nonhalo-producing phages of group I were similar. Several other phage-bacterial combinations produce expanding, translucent halos that are the result of enzymatic removal of the bacterial capsule (2,5, 13,23). A similar phenomenon may cause the halo surrounding the plaque produced by the *E. amylovora* phages. Failure of PEal(nh)-type phages to produce a halo may relate to nonsynthesis of the enzyme or synthesis of a nonenzymatically active form, as was reported for a *Pseudomonas aeruginosa* phage-2 mutant PDP1 (5).

Specificity of PEal(h) for encapsulated strains of *E. amylovora* was similar to that reported for the *Escherichia coli* K-phages

TABLE 2. Purification and concentration of *Erwinia amylovora* bacteriophages PEal(h) and PEa7

Steps in purification	Vol. (ml)	Recovery of PEal(h) (%)	Recovery of PEa7 (%)
Crude lysate	1,000	100	100
After DNase and RNase	1,000	100	100
After PEG precipitation	140	56	9
After 10–40% sucrose gradient	60	26	0.3
After 22,000 rpm for 2 hr	6	7	0.2
After CsCl and dialysis	2	0.6	0.06

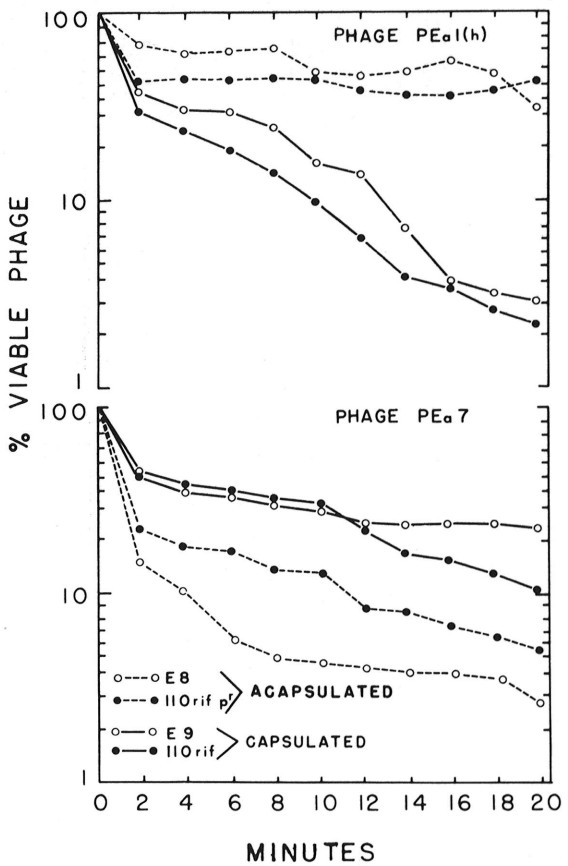

Fig. 5. Adsorption curves of *Erwinia amylovora* bacteriophages PEal(h) and PEa7 to encapsulated and nonencapsulated strains of *E. amylovora*.

(21,22) and several *Klebsiella* phages (13,16,17). This specificity has its cause in their adsorption site, which was located on the bacterial capsule. Although up to 65% of PEal(h) adsorbed to the nonencapsulated strains of *E. amylovora,* no increase in titer occurred. This adsorption may result from the presence of capsular material not detected by the India ink stain (3). Alternatively, infection may have been blocked after adsorption site recognition, as was hypothesized for *Klebsiella* phage 7 (22). Phage PEa7 was adsorbed more readily to nonencapsulated than to encapsulated strains of *E. amylovora,* suggesting that the receptor for PEa7 is located on the cell wall beneath the capsule. These data support Billing's observations that sensitivity for different phage types is related to the presence of a bacterial capsule (6).

The lower recovery of PEa7 after purification may be related to the more fragile tail structure of PEa7, which is probably necessary for adsorption site recognition. Another possibility is that the combination of salts and precipitating agents was not appropriate.

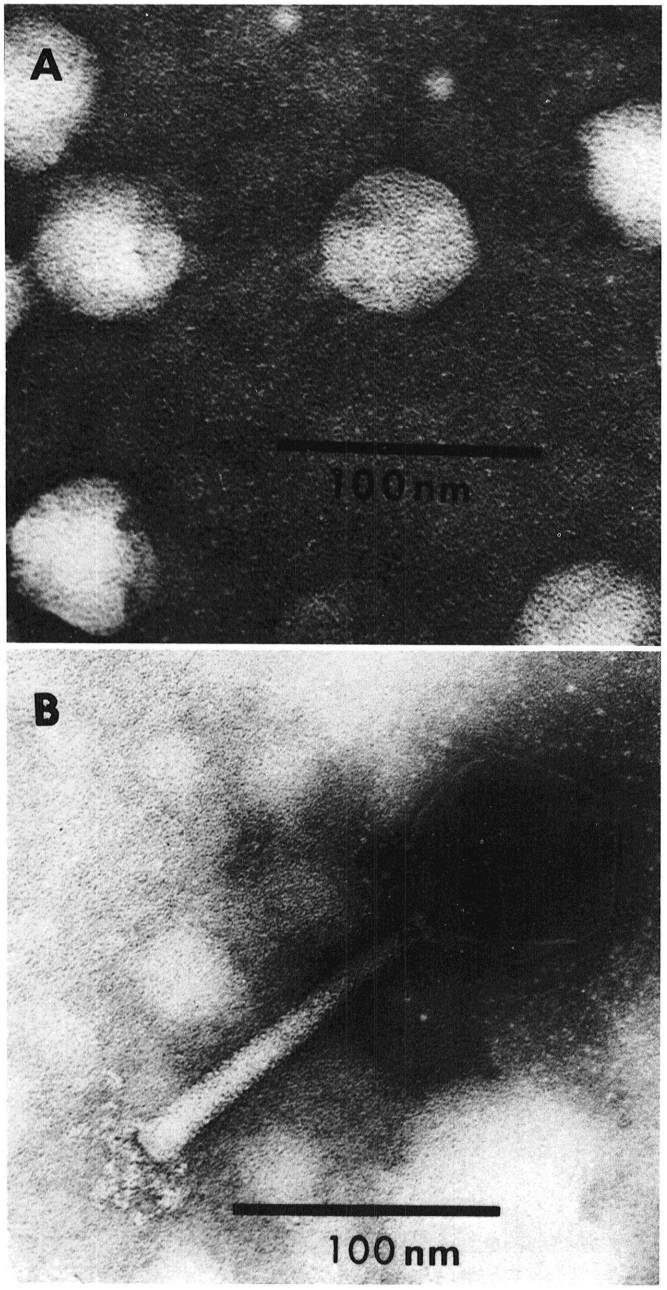

Fig. 6. Electron micrographs of *Erwinia amylovora* bacteriophages. **A,** Phage PEal(h), a polyhedral phage with a spikelike tail; **B,** Phage PEa7 has an octahedral head and a rigid, striated tail with fibral appendages.

The more than 1,000-fold decrease in pfu/ml of PEal(h), when incubated with *E. amylovora* 110 rif at 30 C, was not caused by thermal inactivation since the phage tolerated heating for 10 min at 45 C. Some phages infect their bacterial hosts but are then unable to multiply because a necessary protein is either not made or is nonfunctional at the temperature at which the bacterial host grows normally (20). This condition may exist for phages PEal(h), PEal(nh), and PEal2(h). The temperature sensitivity of the nonhalo-producing phage PEal(nh) adds evidence that PEal(h) and PEal(nh) are similar.

Calculation of the velocity constant, K, of phage antisera neutralization is valid only over a limited range of inactivation, usually 90–99% of the phage, and is not valid outside this range (1). The K values for PEal(h) antiserum with phage PEa7 and PEa7 antiserum with phages PEal(h) and PEal(nh) thus cannot be calculated. For comparison, estimates have been made that result in higher K values, suggesting greater serologic relatedness than exists.

Antiserum to PEal(h) neutralized its heterologous phage PEal(nh) more rapidly than its homologous phage PEal(h) K = 597/min and K = 321 min, respectively. Antiserum prepared to other phages that produced halo plaques neutralized both the phage and the enzyme that caused the halo (2). Lysis of host cells by PEal(nh) did not result in a translucent halo as did lysis by PEal(h). The lower K value for PEal(h) than for PEal(nh) may have resulted from competition for antibody between the virion and the factor causing the halo.

Serologic typing experiments indicated that the 14 phage isolates could be placed in two groups that correlated with groups I and II plaque types.

Sedimentation coefficients of PEal(h) and PEal(nh) were similar to that of the T-odd coliphages, and PEa7 was similar to the T-even coliphages (10). PEal(h) and PEal(nh) also are morphologically similar to coliphage T3; PEa7 is similar to the noncontractile, tailed T-even phages (8).

Phages PEal(h) and PEal(nh), and PEa7 conform to Bradley's (8) groups C and B, respectively. Thus, they probably are double-stranded DNA phages as was *E. amylovora* phage S1 of Erskine (11).

These phages are of interest for several reasons: (i) they are the

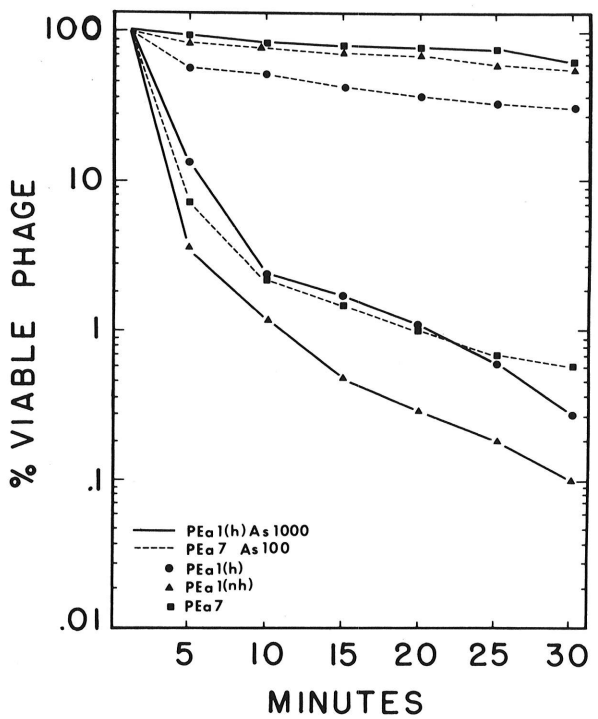

Fig. 7. Phage antisera neutralization curves for bacteriophages PEal(h), PEal(nh), and PEa7. Mean of two experiments.

first *E. amylovora* bacteriophages isolated from aerial parts of plants susceptible to *E. amylovora;* (ii) this is the first characterization of more than one strain of *E. amylovora* bacteriophage; (iii) lysis of the bacterial host by some of these phages results in an expanding, translucent halo surrounding the plaque, probably the result of enzymatic degradation of the bacterial capsule; and (iv) phage PEal(h) shows strong specificity for encapsulated strains of *E. amylovora,* suggesting that its attachment site is located on or in the bacterial capsule.

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