Isolation of Erwinia amylovora Bacteriophage from Aerial Parts of Apple Trees

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ABSTRACT

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Populations of *Erwinia amylovora* bacteriophage greater than 10^6 plaque-forming units (PFU) per gram of tissue were isolated without enrichment from diseased aerial parts of apple trees during the summer of 1975. Three phage isolates were selected from different geographical locations. Two types of plaques were produced; a clear-centered plaque with a spreading translucent halo and a smaller plaque without a halo. Thirty-five bacterial isolates, consisting of nine genera, 18 species, and 15 strains of *E. amylovora* were typed; the phages lysed only *E. amylovora*. The burst size of the three isolates was 40 to 50 PFU per cell. The phages could be stored at 4 C and −20 C but lost titer when stored at 24 C.

Additional key words: fire blight.

Studies of bacteriophages of plant pathogenic bacteria have dealt primarily with their use as a diagnostic tool (5) or in characterization of phage-bacteria interactions (3, 6, 11). Phages of phytopathogenic bacteria may be isolated from soil in the vicinity of the diseased plant (7) and often from the diseased tissue of the plant (11).

Erskine (8) isolated a phage from soil at the base of *Erwinia amylovora*-infected trees, but was unable to isolate it from infected or healthy aerial tissues. This phage (S1) lysed both *E. amylovora* and a yellow saprophytic bacterium which could also be lysogenized.

This report deals with the isolation and partial characterization of *E. amylovora* phages from diseased and symptomless plant tissues of aerial parts of apple trees during the summer of 1975.

MATERIALS AND METHODS

Erwinia amylovora isolation.—Diseased and symptomless aerial tissues of apple trees, *Malus sylvestris* Mill., at Michigan State University (MSU) and three growers' orchards in southwestern Michigan were sampled. No attempt was made to disinfest the tissues. Bacterial populations were quantified on a tissue weight basis unless stated otherwise. Tissues were washed in distilled water for 30 to 60 minutes. Serial dilutions ranging from 10^{-1} to 10^{-5} were made in 0.02 M potassium phosphate buffer, pH 6.8, and 0.1-ml samples were spread over the surface of a differential medium (13). Representative isolates were tested for pathogenicity using the seedling technique (12).

Bacteriophage isolation.—The tissues, washing procedures, and dilution series were the same as those described for the isolation of *E. amylovora*. The plating procedures were as outlined by Adams (1). Plaques, if present, were counted after 24 hours incubation at 24 C. Three phage isolates were chosen for further study. Phage PEa1 was isolated from blighted 'Jonathan' apple terminals from southwestern Michigan, PEa2 was isolated from soil at MSU, and PEa5 was isolated from a symptomless 'Jonathan' apple terminal at MSU. The phage isolates were purified by single-plaque isolation five times using *E. amylovora* isolate #110 from the MSU orchard as propagating host.

Phage characterization.—Phage lysates were prepared by scraping the top agar from plates with confluent lysis, centrifuged at 12,000 *g* for 10 minutes, and filtered through Millipore filters (pore size, 0.45 μm). The lysates were stored over a drop of chloroform in 3.54-g (2.0 dram) screw-cap vials covered with aluminum foil. Vials of each isolate were placed at 24, 4, and −20 C to determine longevity of storage.

The double-layer agar technique (1) in standard 100-mm diameter plastic petri plates was used to examine plaque morphology. The bottom layer consisted of 12 to 15 ml of 2.0% nutrient agar supplemented with 0.5% glucose; the top layer consisted of 2.5 ml of 0.7% nutrient agar, 0.5% glucose, and 0.5% yeast extract. The phage titers were adjusted to approximately 50 plaque-forming units (PFU) per plate. An 18- to 24-hour nutrient broth-glucose culture of *E. amylovora* isolate 110 was used as host. The plates were incubated at 27 C and examined over a 48-hour period.

The double-layer agar technique was used to type the host-range of the three phage isolates. One-tenth milliliter of a 24-hour nutrient broth-glucose culture of each bacterial isolate was added to the 2.5 ml of warm top agar. After the top layer had solidified, one loopful of each

phage isolate (titers ranged from 10^6 to 10^7 PFU/ml) was spotted on sections of the plates. The host-range typing was done twice at different times. Thirty-five bacterial isolates representing nine genera, 18 species, and 15 strains of *E. amylovora* were typed.

The one-step growth experiment for each of the three phage isolates was done in 0.8% nutrient broth, 0.5% glucose, 0.5% yeast extract at 24 C, using *E. amylovora* isolate 110 as the host. Phage were added to the bacteria at 1:10 ratio and allowed to adsorb for 10 minutes then diluted to approximately 10^3 bacteria per milliliter. Beginning at 20 minutes, samples were taken every 10 minutes through a 90-minute period. The one-step growth experiment was repeated three times for each phage isolate.

RESULTS AND DISCUSSION

During the monitoring of *E. amylovora* populations on 'Jonathan' apple trees at the MSU experimental orchard during the summer of 1975, *E. amylovora* phages were detected without enrichment. Phages could be isolated easily from three growers' orchards in southwestern Michigan during a fire blight epiphytotic. Phage was detected on all tissues sampled where *E. amylovora* was detected except on symptomless leaves (Table 1). Failure to detect phage on symptomless leaves may have been due to several factors: (i) the phage populations may have been below the detection levels, (ii) *E. amylovora* populations were lower on symptomless leaves than on other tissues, and (iii) there may be less protection on leaf surfaces from factors such as ultraviolet light, effect of moisture, etc., than on other tissues. It is not known whether the phages were located internally or externally to the plant surface, but they were present wherever *E. amylovora* was found extensively.

The three phage isolates could be stored for at least six months at 4 C and −20 C without significant loss of titer. Titers dropped rapidly when stored at 24 C.

Two distinct types of plaques were produced by the phage isolates (Fig. 1). The first type produced a clear

TABLE 2. Host range of *Erwinia amylovora* bacteriophages PEa1, PEa2, and PEa5

Bacterial isolate	Phage isolate PEa1	PEa2	PEa5
Erwinia amylovora			
Cal Ea1	hazy	hazy	hazy
Ill 68	+[a]	+	+
M S U 110	+	+	+
M S U 111	+	+	+
Traverse City 112	+	+	+
G. Rapids 113	+	+	+
G. Rapids 114	+	+	+
Paw Paw 115	+	+	+
Spinks Corners 116	+	+	+
Cal Ea5	+	+	+
Cal Ea38	+	+	+
M S U Mac 715	hazy	hazy	hazy
N.C. EACC512 120	+	+	+
N.C. EA518 121	+	+	+
Paw Paw 122	+	+	+
Agrobacterium tumefaciens UC3416	−[b]	−	−
A. tumefaciens UC 78	−	−	−
Corynebacterium fascians	−	−	−
C. flaccumfaciens	−	−	−
Enterobacter aerogenes	−	−	−
Erwinia atroseptica SR 8	−	−	−
E. carotovora SR 165	−	−	−
E. herbicola ZP-1	−	−	−
E. herbicola ZP-2	−	−	−
E. herbicola A-E	−	−	−
Escherichia coli	−	−	−
Pseudomonas aeruginosa	−	−	−
P. fluorescens	−	−	−
P. lachrymans	−	−	−
P. solanacearum	−	−	−
P. syringae	−	−	−
Rhizobium sp.	−	−	−
Serratia sp.	−	−	−
Xanthomonas juglandis	−	−	−
X. pruni PF-2	−	−	−

[a]Plus (+) indicates lysis.
[b]Minus (−) indicates no lysis.

TABLE 1. The occurrence of *Erwinia amylovora* and *E. amylovora* bacteriophages on aerial parts of Jonathan apple trees during the summer of 1975 at East Lansing, Michigan

Types and approximate amounts of tissues sampled	Samples containing *E. amylovora* and approximate concentration (CFU)[a] of *E. amylovora* per unit sampled	Samples containing bacteriophage and approximate concentration (PFU)[b] of bacteriophage per unit sampled
Symptomless terminals (~1.0 g/terminal)	Eight of 25 terminals 10^2 to 2×10^4 CFU/terminal	Four of 25 terminals 10^2 to 3×10^4 PFU/terminal
3- to 4-week-old infected terminals and leaves (~2 to 4 g/sample)	25 of 25 terminals 5×10^3 to 6×10^6 CFU/terminal	25 of 25 terminals 3×10^2 to 7×10^6 PFU/terminal
Newly formed cankers (~0.2 to 0.5 g/canker)	15 of 15 cankers $>10^6$ CFU/g	Nine of 15 cankers 10^2 to $>10^6$ PFU/g
Symptomless leaves Five samples, 10 leaves per sample	Four of five samples ~10^3 CFU/sample	0 of five samples None
Three blighted fruits (~0.5 g/fruit)	Three of three fruit ~10^6 CFU/g	Three of three fruit ~10^6 PFU/g

[a]CFU, colony-forming units.
[b]PFU, plaque-forming units.

center 4-5 mm in diameter with a distinct translucent, spreading halo occurring at 18 hours; the second type produced a smaller plaque 1-2 mm in diameter with an irregular margin and no halo. The halo may have resulted from lysogeny or the production of an enzyme capable of hydrolyzing the capsular polysaccharide (2, 4). The small, non-halo plaque form was easily separated from the larger, halo-forming plaques but the larger, halo-forming plaques could not be isolated readily from those produced by the non-halo plaque form.

Of the 35 bacterial isolates tested, only the *E. amylovora* isolates were lysed (Table 2). With two isolates, Ea1 from California and Mac 715 from MSU, hazy plaques were produced. These two bacterial isolates are different from the other isolates in that they did not produce copious amounts of slime on nutrient agar-glucose medium.

All three phage isolates had a similar one-step growth curve with latent periods of 30 to 40 minutes, a rise period occurring from 35 to 65 minutes from start of incubation, and a burst size of 40 to 50 PFU/cell (Fig. 2).

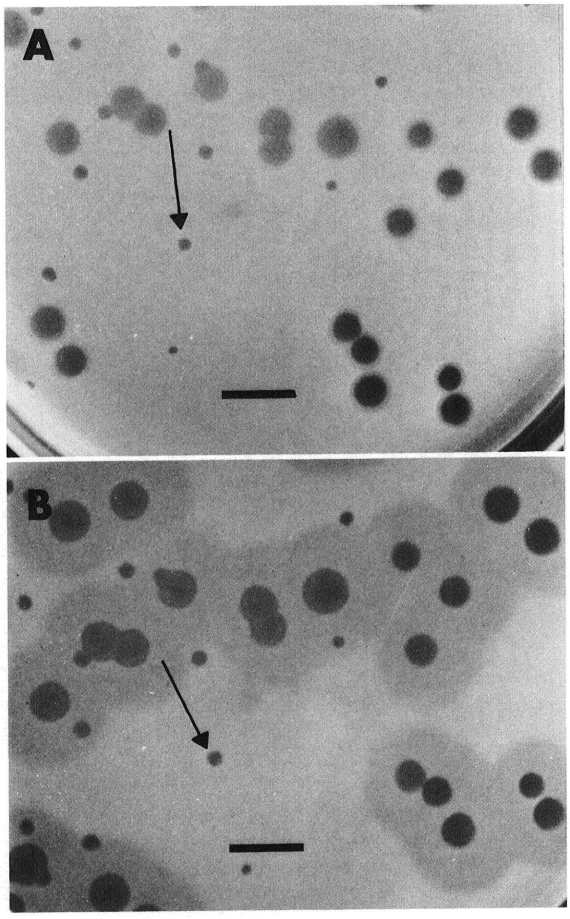

Fig. 1-(A, B). **A)** Plaque morphology of phage PEa1 (halo) and PEa1 (non-halo), arrow, after 18 hours incubation at 27 C with *Erwinia amylovora* isolate 110 on 2.5 ml of top-agar consisting of 0.7% nutrient agar, 0.5% glucose, and 0.5% yeast extract. **B)** Plaque morphology of phage PEa1 after 48 hours under the same conditions as in A. Note the extending, translucent halo of PEa1 (halo) while the plaque morphology of PEa1 (non-halo) has remained the same as at 18 hours (arrow). The scale bar equals 1.0 cm.

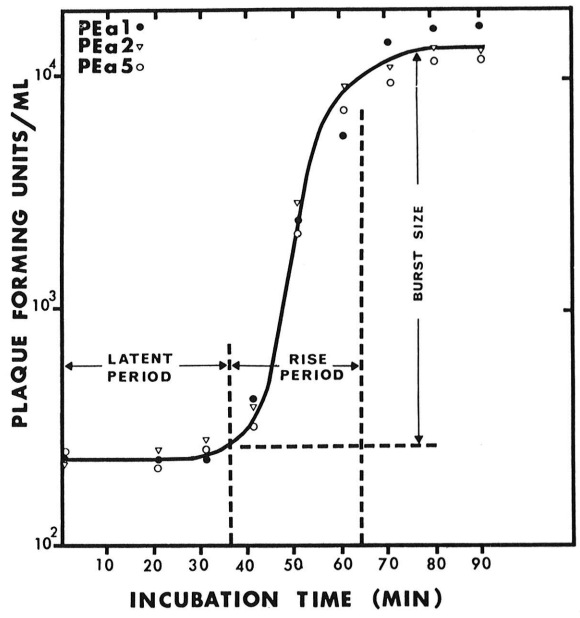

Fig. 2. One-step growth curve of phage isolates PEa1, PEa2, and PEa5 with *Erwinia amylovora* 110 as host.

Whether the phage plays a role in fire blight etiology is not known. Erskine investigated this possibility under laboratory conditions and hypothesized that the yellow, saprophytic bacterium may frequently be lysogenized and serve as a reservoir of phage which under appropriate conditions may affect the severity of fire blight (8). A major criticism of this hypothesis was the failure to isolate phage from aerial plant parts. Harrison and Gibbins (10) recently reported the isolation of a temperate phage from the yellow bacterium, *Erwinia herbicola,* isolate Y46, after treatment with mitomycin C; however, none of the *E. amylovora* isolates tested was sensitive to that phage.

One of the results of phage-bacteria interaction is the killing of the phage-sensitive bacteria and the selection of phage-resistant mutants. Preliminary data from our laboratory indicate that the virulence of *E. amylovora* was attenuated in phage-resistant bacteria. This was reported previously for *E. amylovora* (8) and *Pseudomonas morsprunorum* (9).

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