BACTERIOPHAGES OF *ERWINIA AMYLOVORA*: THEIR ISOLATION, DISTRIBUTION, CHARACTERIZATION, AND POSSIBLE INVOLVEMENT IN THE ETIOLOGY AND EPIDEMIOLOGY OF FIRE BLIGHT

By

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ABSTRACT

BACTERIOPHAGES OF *ERWINIA AMYLOVORA*: THEIR ISOLATION, DISTRIBUTION, CHARACTERIZATION, AND POSSIBLE INVOLVEMENT IN THE ETIOLOGY AND EPIDEMIOLOGY OF FIRE BLIGHT

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Studies were conducted exploring the involvement of *Erwinia amylovora* bacteriophages in the etiology and epidemiology of fire blight. Phages were isolated from aerial structures of apple in association with *E. amylovora*.

Phages and *E. amylovora* were monitored and isolated in a Michigan State University (MSU) orchard during 1975 and 1976 and in 10 commercial, south-western-Michigan apple orchards in 1976. Fourteen phage strains isolated from nine apple orchards were used in the characterization studies. These were divided into two major groups. Two phage types from group I and one phage type from group II were characterized using chemical, serological, ultra-centrifugal, and electron microscopic methods. Phage and *E. amylovora* interactions were studied in liquid and solid media and in apple seedlings.

David F. Ritchie

Phage and *E. amylovora* populations reached 10^6 plaque-forming-units (pfu)/g of fresh plant tissue and 10^8 colony-forming-units (cfu)/g of fresh plant tissue, respectively. Maximum phage and *E. amylovora* populations coincided with maximum fire blight severity. Isolation of phage was seasonal, while *E. amylovora* was isolated from diseased tissue throughout the year.

Two morphological and serological groups of phages were isolated. Group I phages produced two plaque types: PEa1(h) plaques were 2-3 mm in diameter surrounded by an expanding, translucent halo; while PEa1(nh) plaques were 1-2 mm in diameter without the halo. Group II phages represented by PEa7, produced 0.5-1.0 mm diameter plaques with an expanding, translucent halo. PEa1(h) was polyhedral, ca. 60 nm in diameter with a spike-like tail structure; PEa7 had an octahedral head, ca. 75 nm in diameter with a rigid, striated tail, ca. 135 nm long. Phages PEa1(h) and PEa1(nh) had a buoyant density of 1.53 g/cc and a sedimentation coefficient of 566 S. The buoyant density of PEa7 was 1.44 g/cc with a sedimentation coefficient of 1037 S. The growth cycle of PEa1(h) was 70 min yielding ca 50 pfu/productive cell, while PEa7 was 135 min with ca. 8 pfu/productive cell. PEa1(h) and PEa1(nh) required 10 min at 55 C for

complete thermal inactivation; 10 min at 65 C was required for PEa7. Host range for both phage groups was limited to *E. amylovora* and several *Erwinia herbicola* strains. Phage PEal(h) adsorbed more rapidly to capsulated than to acapsulated strains of *E. amylovora* while PEa7 adsorbed more rapidly to acapsulated strains of *E. amylovora*.

Growth of PEal(h) with *E. amylovora* 110 rif^r resulted in the synthesis of a capsular degrading factor (CDF). The CDF activity was inhibited by PEal(h) antiserum but not by PEa7 nor *E. amylovora* 110 rif^r antisera. The CDF affected only capsulated strains of *E. amylovora* and several strains of *E. herbicola*. Its activity was completely destroyed by heating for 10 min at 85 C. The degradation of the capsule was responsible for the expanding, translucent halo surrounding the plaque; alteration of the bacterial growth from a mucoid, white characteristic to non-mucoid, vitreous growth; high frequencies of PEal(h)-resistance; a delay in symptom development; and an increased sensitivity to streptomycin sulfate.

The phages produced their greatest effect on *E. amylovora* indirectly through the CDF degradation of the bacterial capsule. Phage and *E. amylovora* monitoring and laboratory data suggested that phage may have an effect on the epidemiology of fire blight.

This dissertation is dedicated to my parents

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GENERAL INTRODUCTION AND LITERATURE REVIEW

Fire blight, proven to be caused by *Erwinia amylovora* (Burrill) Winslow, *et al.*, almost 100 years ago (4,10) remains an erratic but serious disease on pome fruits. In recent years new methods of apple culture have utilized dwarf rootstocks resulting in high density plantings of old and new fire blight-susceptible varieties. These factors have increased the importance of fire blight. The erratic and usually devastating occurrence of fire blight cannot be fully explained and has normally been attributed to optimum climatic conditions and host factors. Even though these factors are important, the effects of other factors on the genetic potential of *E. amylovora* have received little research attention. With the discovery of *E. amylovora* bacteriophages on aerial structures of apple trees in 1975 (42) and awareness of Erskine's hypothesis (17) that phage may be involved in the epidemiology of fire blight, it became apparent that further investigations were warranted.

The major objective of this study was to explore the possible role which phage may have upon the etiology and epidemiology of the fire blight bacterium. This was done in three ways: 1) monitoring the distribution and populations of phages on the aerial structures of apple, 2) characterization of these phages, and 3) determination of the effects of these phages on *E. amylovora*.

Two excellent reviews on the history, epidemiology, and control of fire blight have recently been written (5,45). A voluminous amount of literature has been published starting in 1794 with Denning's (13) description of the disease, followed by a steady flow of research reports beginning with Burrill in 1877 (10). However, fire blight is still very difficult to control, continues to spread (5,45,51), and remains a constant concern in many countries where pome fruits are grown. In the United States, fire blight has at different times and places threatened the survival of the pome fruit industry (5). It continues to limit pear production and is becoming increasingly important to apple culture in the northeastern United States (7), especially where the growth of certain susceptible varieties is desirable (1,2).

The literature contains numerous unsubstantiated claims, contradictory reports, and working hypotheses which, with time and repeated citations, have become accepted as facts (45). The usual taxonomic inconsistencies of *E. amylovora* have been reported (8,15,33) as well as variations in virulence (3,45, 47) and morphology (3,28,53) among strains, including the instability of colony types and virulence when continuously transferred (3,25).

Factors contributing to pathogenicity are not understood. Host cell wall degradation does not appear to be involved (39,46) while a bacterial produced toxin has been implicated (22,26,39).

Much fire blight research has been descriptive and the study of possible factors which may affect disease development have been mostly climatic (9,32, 35,40). Efforts have been made to study the effect of coinhabitors, particularly *Erwinia herbicola* (Lohnis) Dye, on the survival of *E. amylovora* and disease development, but results have been variable or inconclusive (19,21,38,41,43,49).

There is little doubt that climatic conditions are important for disease development. Even so, in certain years and locations, blight infections are insignificant (45); furthermore, it has been

observed in California that seldom more than 100 flower strikes occurred per tree during an epiphytotic even though every flower was infested with *E. amylovora* and climatic conditions appeared optimal for infection (34). The physiological condition of the host plant is important for fire blight development; any factor which stimulates the development of succulent tissue enhances fire blight susceptibility (24,30,36,48). Host defense mechanisms have been examined but their role in pathogenesis, particularly under field conditions, has not been defined (11,21,44). Little is known concerning genetic variability in *E. amylovora* and the factors which may affect it in nature and how this may relate to fire blight epidemiology (45).

Viruses which specifically infect bacteria, bacteriophages, are capable of altering the genetic expression of their bacterial host via two major mechanisms: transduction (55) and lysogenic conversion (6). Bacteriophages of plant pathogenic bacteria are common (37,52) and often have received attention in and of themselves because of unusual characteristics (52) or have been used in bacterial identification and detection (37). Attempts to use phages for disease control have met with only limited success (12,37,52). Phages are involved in

the pathogenicity of several animal bacteria (16,23, 27) as well as in the induction of extensive phenotypical alterations in their bacterial hosts (29,31, 50). The effect of phage and their possible role in the virulence of several phytopathogenic bacteria has been reported (14,20,54).

Even though *E. amylovora* phages have been reported since the 1950's (8,37), only one strain obtained from soil has been characterized (17). Attempts to isolate phage from aerial structures of host plants have either been negative (17) or not reported prior to 1975 (42). Two papers have implicated the possible involvement of phage in the epidemiology of fire blight (17,18).

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PART I

ISOLATION, DISTRIBUTION, AND POPULATIONS OF *ERWINIA AMYLOVORA* BACTERIOPHAGES ON AERIAL STRUCTURES OF APPLE

INTRODUCTION

Prior to 1975 only one reported attempt to isolate *Erwinia amylovora* bacteriophages from aerial structures of fire blight-susceptible plants had been published, and it was negative (4). Up to this time, no information existed on phage populations, distribution, and their association with *E. amylovora* on aerial plant structures. With the isolation of *E. amylovora* phages from these structures in 1975, it seemed important to study the distribution, relative populations, and the association of phage with *E. amylovora* on apple. The objective of this section is to report the isolation of *E. amylovora* bacteriophages from aerial structures of apple and to describe the distribution and populations of these phages and *E. amylovora* on such structures in several Michigan orchards.

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Isolation of Erwinia amylovora Bacteriophage from Aerial Parts of Apple Trees

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ABSTRACT

RITCHIE, D. F., and E. J. KLOS. 1977. Isolation of Erwinia amylovora bacteriophage from aerial parts of apple trees. Phytopathology 67: 101-104.

Populations of *Erwinia amylovora* bacteriophage greater than 10^4 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 $>10^4$ CFU/g	Nine of 15 cankers 10^2 to $>10^4$ 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

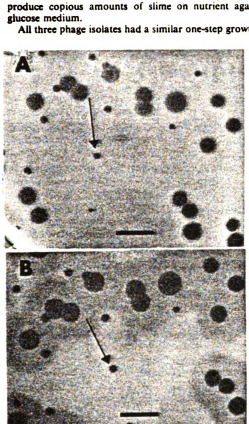

Fig. 1-(A, B). A) Plaque morphology of phage PEa1 (halo) and PEa1 (non-halo), arrow, after 18 hours incubation at 27 C with *Erwinia amylovora* isolate 110 on 2.5 ml of top-agar consisting of 0.7% nutrient agar, 0.5% glucose, and 0.5% yeast extract. B) Plaque morphology of phage PEa1 after 48 hours under 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.

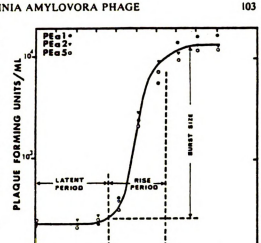

Fig. 2. One-step growth curve of phage isolates PEa1, PEa2, and PEa5 with *Erwinia amylovora* 110 as host.

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).

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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MATERIALS AND METHODS

Monitoring of *Erwinia amylovora* and its bacteriophages. The type of apple tissue selected for monitoring was dependent on the time of year; during winter, populations in cankers were monitored, while during the growing season populations in association with current year's tissue were checked. Tissue during the growing season was collected by slipping a plastic bag over the tissue and breaking it off thus reducing the possibility of cross contamination between samples. Tissue fresh weight was determined, a known quantity of distilled water added, and the tissue was washed in the bags for 1-2 hr. Samples from cankers were collected by removal of sections with a sterile scalpel. These sections were dissected into approximately 1.0 mm long pieces, fresh weight was determined, and tissues were washed for 1-2 hr in 10 ml of distilled water. Assays for phage were done using the agar overlay method (1) with *E. amylovora* 110 as host and a differential medium for *E. amylovora* (8). The number of plaque-forming-units (pfu) and colony-forming-units (cfu) are

reported as per gram fresh weight of tissue.

Monitoring of populations in a MSU orchard. *Erwinia amylovora* and its phage populations were monitored on Jonathan apple trees from June 1975 to December 1976. Symptomless tissues as well as blighted leaves and stems, blighted fruits, newly formed cankers, and overwintering cankers were sampled.

Monitoring of populations in ten growers' orchards. Ten apple orchards in southwestern Michigan were monitored in 1976 with samples taken in June, July, August, and October. Samples consisted of approximately 30 g of blighted leaves and terminals taken randomly throughout the orchard and combined into a composite sample of each orchard.

The effect of desiccation on phage PEa1(h). Leaf discs of 1.0 cm diameter were cut from healthy, mature apple leaves (*Malus sylvestris* cv. 'Jonathan') using a sterile corkborer. Discs were individually inoculated on the upper surface with 0.1 ml of phage PEa1(h) from a chloroformed, filtered (Millipore 0.22 µm pore size) lysate; inoculum contained ca. 10^9 pfu/ml in 0.02 M potassium phosphate buffer, pH 6.8. One set of discs was placed on a wire screen suspended over a pan of water in a clear plastic bag to maintain 95-100% relative humidity (RH). A second set was placed in a pan without water and left

uncovered in ambient RH of 15-25% measured with a recording hygrothermograph. The pans remained on the laboratory bench where temperatures ranged from 21-23 C. Three discs from each set were randomly chosen over a 28-day period and individually washed in 0.02 M potassium phosphate buffer, pH 6.8. Washings were diluted and assayed for phage using *E. amylovora* 110 as host.

RESULTS

Populations in a MSU orchard. Table 3 summarizes the monitoring results in a MSU orchard from June 1975 to December 1976. There was extensive variation in phage and *E. amylovora* populations among the samples; however, phage were easily detectable during certain periods (Figure 3). These periods coincided with fire blight epiphytotics during which time *E. amylovora* populations were maximal and widely distributed on blighted as well as symptomless apple tissues. During 1975, phages were detected from June through August but not during the winter of 1975-76. In 1976, phages were not detected until July, coinciding with fire blight outbreaks and were detectable through October but not in December. Even though phage populations were variable the average population for six sampling periods was approximately 10-fold greater in 1975 (2.3×10^5 pfu/g of tissue) than in 1976 (1.2×10^4 pfu/g of tissue). The percent of samples in which both *E. amylovora* and phage were detected was greater in 1975 (66.5%) than in 1976 (52.2%). In 1975, fire blight was severe from mid-May

Table 3. *Erwinia amylovora* and *E. amylovora* bacteriophage populations in a Michigan State University orchard at East Lansing during 1975 and 1976

Sample date	Type of tissue sampled	Number of individual samples	CFU[a]/g of tissue		PFU[b]/g of tissue		% of samples[c] containing CFU & PFU
			Mean	Range	Mean	Range	
6/23/75	Symptomless terminals[d]	25	1×10^4	$0 - 2 \times 10^5$	2×10^3	$0 - 3 \times 10^4$	16
6/24/75	Blighted terminals[d]	25	1×10^6	2×10^3-2×10^6	5×10^5	1×10^4-2×10^6	100
7/2/75	Blighted terminals	30	2×10^4	$0 - 5 \times 10^4$	4×10^5	$0 - 1 \times 10^6$	73
7/7/75	Newly formed cankers[e]	15	4×10^7	2×10^7-7×10^7	2×10^5	$0 - 7 \times 10^5$	60
7/30/75	Blighted terminals	5	1×10^4	$0 - 3 \times 10^4$	3×10^4	$0 - 6 \times 10^4$	60
8/5/75	Blighted terminals	10	1×10^6	1×10^5-8×10^6	3×10^5	$0 - 2 \times 10^6$	90
8/20/75	Cankers[f]	10	5×10^5	$0 - 4 \times 10^6$	n.d.[g]	n.d.	0
9/18/75	Cankers	10	4×10^5	$0 - 3 \times 10^6$	n.d.	n.d.	0
10/5/75	Cankers	10	4×10^4	$0 - 3 \times 10^5$	n.d.	n.d.	0
11/8/75	Cankers	10	1×10^4	$0 - 1 \times 10^5$	n.d.	n.d.	0
2/28/76	Cankers	10	5×10^4	$0 - 4 \times 10^5$	n.d.	n.d.	0
5/21/76	Cankers	10	8×10^4	$0 - 3 \times 10^5$	n.d.	n.d.	0

Table 3 (continued).

5/27/76	Cankers	4	2×10^5	$0 - 5 \times 10^5$	n.d.	n.d.	0
6/1/76	Symptomless terminals	10	n.d.	n.d.	n.d.	n.d.	0
6/12/76	Symptomless terminals	10	n.d.	n.d.	n.d.	n.d.	0
7/12/76	Blighted terminals	5	2×10^7	1×10^5-4×10^7	5×10^4	$0 - 2 \times 10^5$	60
8/2/76	Blighted terminals	10	5×10^3	9×10^1-2×10^4	1×10^4	2×10^2-6×10^4	100
8/12/76	Blighted terminals	3	5×10^4	3×10^4-6×10^4	4×10^4	$0 - 1 \times 10^2$	33
8/24/76	Blighted terminals	5	8×10^5	5×10^5-1×10^6	1×10^1	$0 - 4 \times 10^1$	40
9/1/76	Cankers	5	1×10^6	$0 - 4 \times 10^6$	4×10^3	$0 - 2 \times 10^4$	60
10/21/76	Cankers	10	7×10^4	$0 - 3 \times 10^5$	1×10^3	$0 - 1 \times 10^4$	20
12/17/76	Cankers	10	3×10^6	$0 - 2 \times 10^7$	n.d.	n.d.	0

[a] CFU = colony-forming-units of *Erwinia amylovora*.

[b] PFU = plaque-forming-units of *Erwinia amylovora* bacteriophage.

[c] The percent of the total samples in which both *Erwinia amylovora* and *E. amulovora* bacteriophages were detected.

Table 3 (continued).

[d]Symptomless and blighted terminals consisted of the distal 25 cm of the current year's growth composed of 6 - 10 leaves and the stem.

[e]Newly formed cankers were formed on wood 2 years or older as the result of infections which had occurred on the current year's growth such as terminals and fruit spurs. These cankers differed from overwintering cankers in the presence of indefinite margins, continual advancement into healthy wood, and the abundance of bacterial exudate.

[f]Classical, overwintering cankers; they exhibited little or no advancement into healthy tissues, no bacterial exudate, and were essentially dormant from September to April.

[g]n.d. = not detected.

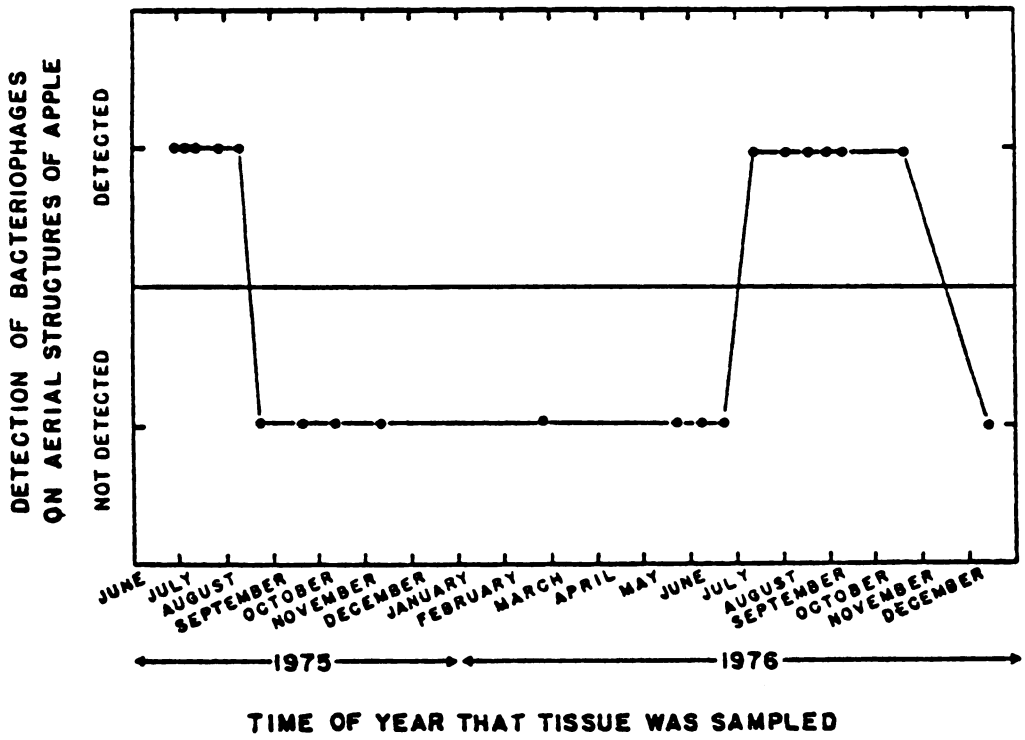

Figure 3. Seasonal detection of *Erwinia amylovora* bacteriophages in association with aerial structures of apple in a Michigan State University orchard at East Lansing. Note that Y-axis is qualitative not quantitative.

to mid-June; all of the Jonathan trees exhibited at least 50-100 strikes/tree. During 1976, fire blight was not observed until late June; some trees showed no strikes while the most severely infected trees showed fewer than 25 strikes/tree. It was the exception to detect phage in overwintering cankers; only in September and October of 1976 were they detected associated with such structures (Table 3).

Populations in ten growers' orchards. During the June monitoring period phage were detected in 8 of 9 orchards and in 7 of 9 orchards during July (Table 4). In August phage were detected in only 1 of 10 orchards and 2 of 10 orchards in October (Table 4). Phages were readily detected as long as *E. amylovora* was present but only occasionally detected when *E. amylovora* was non-detectable or at low levels. No new strikes were observed in the orchards after the 18 June monitoring period.

Effect of desiccation on infectivity of phage PEa1(h). Phage PEa1(h) was extremely sensitive to desiccation, exhibiting a 10^5-fold decline in titer within 24 hr at 15-25% RH (Table 5). The titer then declined to non-detectability after 16 days. Phage titers on leaf discs maintained at 95-100% RH remained relatively stable for 16 days then declined to ca. 1×10^1 pfu/leaf disc by the fourth week (Table 5).

Table 4. *Erwinia amylovora* and *E. amylovora* bacteriophage populations in ten growers' orchards in southwestern Michigan.

Orchard Number	Sample Dates								
	6/18/76			7/26/76		8/20/76		10/20/76	
	CFU[a]/g	PFU[b]/g	Fire blight[c] severity	CFU/g	PFU/g	CFU/g	PFU/g	CFU/g	PFU/g
1	1×10^7	6×10^3	0 - 25	4×10^7	n.d.	n.d.	n.d.	n.d.	n.d.
2	1×10^8	3×10^3	ca. 50	3×10^6	5×10^5	n.d.	n.d.	n.d.	n.d.
3	2×10^8	1×10^4	ca. 100	2×10^7	n.d.	n.d.	n.d.	n.d.	n.d.
4	1×10^8	$1 z 10^6$	0 - 100	$2 z 10^6$	3×10^4	n.d.	n.d.	n.d.	n.d.
5	7×10^7	2×10^3	0 - 50	3×10^6	4×10^4	n.d.	n.d.	n.d.	n.d.
6	8×10^4	n.d.[d]	0 - 25	n.s.[e]	n.s.	n.d.	n.d.	n.d.	n.d.
7	7×10^7	1×10^4	ca. 100	2×10^6	1×10^6	n.d.	n.d.	n.d.	1×10^4
8	4×10^7	1×10^3	10 - 100	2×10^7	7×10^5	n.d.	6×10^2	n.d.	n.d.
9	n.s.	n.s.	0 - 50	5×10^6	2×10^5	n.d.	n.d.	3×10^4	2×10^3

Table 4 (continued).

10	4×10^7	1×10^3	10 - 100	5×10^6	8×10^3	n.d.	n.d.	n.d.	n.d.

[a] CFU = colony-forming-units of *Erwinia amylovora*.

[b] PFU = plaque-forming-units of *E. amylovora* bacteriophages.

[c] The number of blight strikes per tree in the orchard. Zero indicates that not every tree had fire blight strikes. No new strikes were observed after the initial count was made on 6/18/76.

[d] n.d. = not detected.

[e] n.s. = not sampled.

Table 5. Effect of desiccation on the survival of *Erwinia amylovora* bacteriophage PEa1(h) on apple leaf discs.

Sample Period (Days)	Number of[a] PFU/disc	
	95 - 100% R.H.	15 - 25% R.H.
0[b]	5×10^8	5×10^8
1	$1.1 \times 10^8 \pm 2.1 \times 10^6$	$3.5 \times 10^3 \pm 4.5 \times 10^3$
6	$1.2 \times 10^8 \pm 4.9 \times 10^6$	$1.2 \times 10^3 \pm 9.1 \times 10^2$
10	$1.6 \times 10^8 \pm 4.4 \times 10^7$	$2.3 \times 10^2 \pm 1.1 \times 10^2$
16	$1.1 \times 10^8 \pm 5.3 \times 10^7$	0
21	$7.6 \times 10^5 \pm 1.0 \times 10^5$	0
28	$2.0 \times 10^1 \pm 1.0 \times 10^1$	0

[a] PFU = plaque-forming-units.

[b] Samples taken 4 hr after inoculation.

DISCUSSION

Erwinia amylovora bacteriophages were commonly found on aerial structures of apple in orchards infested by *E. amylovora*. Not only were phages detected in an MSU orchard during consecutive summers of 1975 and 1976, but also in 10 orchards in southwestern Michigan during the summer of 1976. During fire blight epiphytotics, phages were detected in association with all types of blighted tissue and occasionally in association with symptomless tissue as well. The more severe (number of strikes) fire blight was in an orchard the easier it was to detect phage. As the incidences of fire blight strikes decreased and the blighted tissue desiccated the ability to detect phage decreased.

As obligate parasites bacteriophages are entirely dependent on their bacterial host for multiplication, and are therefore affected by the metabolic condition of the bacterial host (1). In nature *E. amylovora* should grow optimally when climatic conditions are favorable, coupled with an abundance of susceptible

host tissue. Such conditions are usually present in Michigan orchards from mid-May through June. During this period the most rapid development of fire blight epiphytotics occur, resulting in a sufficient population of *E. amylovora* to support phage growth.

Even though phages cannot multiply apart from their bacterial host they can survive in the absence of their host; the length of time they remain infectious is dependent on numerous factors (1). The *E. amylovora* phages were able to tolerate numerous physical conditions, e.g. high and low temperatures, survival in absence of their host (Section II of dissertation), but were very sensitive to desiccation (Table 5). Similar drying conditions are common in blighted tissue one to two months following infection by *E. amylovora*. During the absence of susceptible plant tissue and favorable climatic conditions *E. amylovora* remained detectable (Table 3), but probably existed in an hypobiotic state (5) not conducive for phage growth. Thus, drying of blighted tissues and reduction in the presence of a highly metabolically active bacterial host were probably instrumental in the seasonal detectability of the phages.

This seasonal detectability of *E. amylovora* phages may explain why they were previously unreported

from aerial structures of plants susceptible to *E. amylovora*. Numerous studies involving the isolation of *E. amylovora* from blighted tissue have dealt with isolations from cankers during the winter and early spring (2,3,11); phages were not detected in cankers later than October. *E. amylovora* populations have been monitored primarily in symptomless rather than blighted tissue (6,9,10); phages were not commonly detected in association with blighted rather than symptomless tissue. Furthermore, dilutions are commonly used to obtain individual colonies (6) reducing the likelihood of detecting phage. Phage plaques were normally discernible for only 24-36 hr before becoming masked by phage-resistant *E. amylovora* and other phage insensitive bacteria.

At both the MSU orchard and the 10 southwestern Michigan orchards, fire blight epiphytotics were stationary or declining at the time of maximum recovery of *E. amylovora* phages. It would be tempting to suggest that this was the effect of phage. Phage does affect virulence of *E. amylovora* under laboratory conditions (Section III of dissertation); however, the decline in fire blight epiphytotics coincided closely with the stoppage of terminal-bud growth, thus reducing the amount of

susceptible tissue. Possibly a more quantitative monitoring of phages and the development of fire blight would provide a more definitive answer to the role of phage in fire blight development in Michigan orchards. Other factors such as nutritional condition and growth stage of the host plant, climatic conditions (high humidity, warm temperatures, rain, etc.), and insects are also involved in maximum development of fire blight epiphytotics. It has been suggested that saprophytic microorganisms can affect the development of fire blight (7), since these phages are parasites of *E. amylovora* it is possible that they may be one of the controlling factors in the development of fire blight.

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PART II

CHARACTERIZATION OF *ERWINIA AMYLOVORA* BACTERIOPHAGES

INTRODUCTION

Erwinia amylovora bacteriophages have been isolated from soil since the 1950's (14); however, only one phage strain, obtained from soil, has been partially characterized (10). In 1975 and 1976, *E. amylovora* phages were isolated from aerial structures of apple in several Michigan orchards. Some of the biological, chemical, and physical properties of these bacteriophages are presented in this section.

MATERIALS AND METHODS

Media and cultural conditions. Bacteria were grown on 2.0% (w/v) nutrient agar supplemented with 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) with 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, composing the bottom and top layers respectively.

The 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 (NGBYE medium). All liquid cultures, unless stated otherwise, were incubated at 22-23 C on a reciprocal shaker (80 oscillations/min, stroke length 4.0 cm). *Erwinia amylovora* strain 110 rifampin resistant (110 rif^r) was used for phage propagation and assay. Assay cultures were grown overnight in 125-ml flasks containing 25 ml of NBGYE. Prior to experiments, 5.0 ml of inoculum were added to 25 ml of fresh NBGYE and incubated 1-2 hr. Phage assay plates were incubated at 27 C and scored

after 12-36 hr. All bacteria and phage dilutions were made in sterile 0.02 M potassium phosphate buffer (PPB), pH 6.8.

Phage isolation. Bacteriophages were isolated from aerial structures of apple as described (17). Phages were purified by single-plaque isolation 3-5 times. High titer lysates for storage and preliminary characterization were obtained from plates having confluent lysis by adding 5.0 ml of PPB, scraping off the soft agar, adding chloroform to 1.0% (v/v), centrifuging for 10 min at 12,100 x g in a Sorvall RC2-B centrifuge, and filtering the supernatant (Millipore 0.22 µm pore size). Filtrates were stored over a drop of chloroform at 4 C.

Host range and plaque morphology. Host range was determined as described (17). Plaque morphology was examined using the agar overlay method (1). Phages were diluted so that approximately 50 plaques occurred per plate. The plates were incubated at 27 C and examined during a 12-48 hr period.

Phage growth and purification. *Erwinia amylovora* 110 rif^r was grown overnight in 25 ml of NBGYE, then 10 ml were added to 1000 ml of NBGYE in a 2-liter flask and grown to an optical density (O.D.) A_{525} of 0.06 - 0.10 (ca. 10^8 colony-forming-units [cfu]/ml). The appropriate amount of phage solution was added to give

a final multiplicity-of-infection (m.o.i.) of 0.1-1.0. The culture was incubated with shaking for 12 hr, chloroform was added to 1.0% (v/v), and shaken an additional 5-10 min. One µg/ml of bovine pancreatic DNase and 1.0 µg/ml of beef pancreatic RNase (U.S. Biochem. Corp., Cleveland, OH) were added in the presence of 10^{-3} M $MgSO_4 \cdot 7H_2O$ and shaken for 1 hr at 23 C. Bacterial debris was removed by centrifugation at 12,100 x g for 10 min (Sorvall RC2-B) and the supernatant decanted and stored.

Phages were precipitated with 10% (w/v) polyethylene glycol 6000 (PEG, J.T. Baker Chemical Co., Phillipsburg, New Jersey) in the prsence of 0.5 M NaCl with magnetic stirring for 60 min at 4 C (23). The phages were pelleted by centrifugation at 12,100 x g for 20 min and the pellets resuspended in 5.0 ml of PPB. The phage solution was then subjected to a cycle of low speed (10 min at 3,020 x g in conical tubes) — high speed centrifugation (2 hr at 22K using a rotor SW30 in a Beckman Model L Ultracentrifuge). Following high speed centrifugation, supernatants were decanted and the pellets resuspended overnight in 5.0 ml of PPB at 4 C then centrifuged for 10 min at 3,020 x g in conical tubes. The partially purified phage solution was then stored at 4 C over a drop of chloroform.

Further purification was obtained by layering 2.0 ml of phage solution on liner 10-40% sucrose gradients (4, 7, 7, 7 ml), in 0.1 M potassium phosphate buffer pH 7.8, and centrifuged for 1 hr at 22 K in a Beckman SW 25.1 rotor. Phages were collected by withdrawing the band with a needle or in 1.0 ml fractions with an ISCO Model D Density Gradient Fractionator scanned with an ISCO Model UA-4 Absorbance Monitor at 254 nm. Fractions were diluted with 9.0 ml of PPB, assayed, and the fractions containing maximum phage infectivity centrifuged for 2 hr at 22K and the pellets resuspended in 2.0 ml of PPB. When greater purity was required, isopynic centrifugation was performed in 50% (w/v) cesium chloride (CsCl) for 18-24 hr at 37.5K using an SW50.1 rotor.

Phage thermal inactivation and optimum growth temperature. One ml aliquots (ca. 10^7 plaque-forming-units [pfu]) of PEa1(h), PEa1(nh), and PEa7 suspended in PPB were added to thin-walled glass tubes (outside diameter 1.5 cm). The tubes were immersed in a water bath preheated and maintained (± 1.0 C) at 10 degree intervals of temperature ranging from 25-65 C. After 10 min, the tubes were immediately placed in an ice bath preceeding assay for viable phage. Each phage isolate was tested twice with two replications per test.

Optimum temperatures for infectivity and growth of phages PEa1(h) and PEa7 were determined over a range of 0-30 C. A 12 hr culture of *E. amylovora* 110 rif^r was diluted 1:10 in fresh NBGYE resulting in 7 x 10^7 cfu/ml. One ml aliquots were pipetted into tubes (3 tubes for each phage at each temperature) and placed in the incubators. After 30 min of acclimation, 0.1 ml of PEa1(h) (12 x 10^7 pfu/ml) and PEa7 (20 x 10^7 pfu/ml) were added to the tubes and incubated with shaking at 30-min intervals. Following 3 hr of incubation, a drop of chloroform was added to each tube and the tubes were stored at 10 C prior to assay.

One-step growth and adsorption experiments. One-step growth experiments were performed as described for phage PEa1 (17). The adsorption rates were determined using the chloroform method (1). One ml of phage suspension was added to 9.0 ml of NBGYE containing a known number of cfu/ml and the number of plaque-forming-centers was determined. One-tenth ml samples were diluted into 4.5 ml of NBGYE containing 0.5 ml of chloroform at 2-min intervals over a 20-min period.

Electron microscopy. A drop of sucrose-gradient-purified phages PEa1(h), PEa1(nh), and PEa7 suspended in PPB at titers of 2 x 10^{10} pfu/ml, 3 x 10^{10} pfu/ml and 1 x 10^{10} pfu/ml, respectively, was placed on

parlodion, carbon-coated, copper grids (mesh 300). After 3 min the drop was removed with filter paper and a drop of 2.0% (w/v) ammonium molydate was placed on the grid for 1 min. The excess was removed with filter paper, the grids dried and examined in a Philips 300 transmission electron microscope operating at 60K volts.

Serology. Bacteriophages PEa1(h) and PEa7 were purified through two cycles of 10-40% linear sucrose gradients, suspended in PPB, and stored at 4 C. The titers were 7×10^{10} pfu/ml and 6×10^{8} pfu/ml for PEa1(h) and PEa7, respectively. Female, New Zealand white rabbits (3.2-3.6 kg) were injected at 7-day intervals with 2 ml of a 1:1 emulsified mixture of phage and Freund's complete adjuvant (Difco). The first two injections were given intramuscularly into the thighs while the third was given subcutaneously in the dorsal region. Weekly ear-bleedings (25 ml) were initiated two weeks after the final injection. Processed sera were stored frozen at -20 C.

Antisera titers were determined by using 2-fold dilutions in PPB containing 10^{-3} M NaCl. Homologous phages were added to a final titer of ca. 10^{7} pfu/ml, allowed to react for 10 min at 23 C then viable pfu/ml assayed. A normal serum control was similarly treated. The reciprocal of the dilution at which no significant

decline in pfu could be detected was considered antiserum titer. Neutralization-rate experiments (1) were done with phages PEa1(h), PEa1(nh), and PEa7 using hololologous and heterologous antisera. The remaining 11 phage strains were typed by diluting each to ca. 10^7 pfu/ml and mixing with PEa1(h) antiserum (titer 100) and PEa7 antiserum (titer 40) for 10 min then assaying for viable pfu/ml.

Sedimentation coefficients. Sedimentation coefficients for phages PEa1(h), PEa1(nh), and PEa7 were determined by comparison to tobacco mosaic virus (TMV) essentially as outlined by Brakke (7). Ten-40% sucrose gradients (4, 7, 7, 7 ml), in 0.1 M potassium phosphate buffer pH 7.8, were allowed to become linear by setting at 4 C for 18 hr. One ml (O.D. A260 = 0.7) of virus suspension (in PPB) was layered on the gradients and centrifuged at 20K at 4 C in an SW25.1 rotor using a Beckman Model L Ultracentrifuge. Measurements, to ± 0.5 mm, were made at 20-min intervals over a 140-min period by measuring from the meniscus of the gradient to the top of the virus band. TMV and PEa1(h) were run twice while PEa1(nh) and PEa7 were run once. Sedimentation distance versus time was plotted and sedimentation rates calculated for the viruses while moving from 7.5 mm to 17.5 mm in the gradients. Sedimentation coefficients were calculated

based on the sedimentation coefficient of TMV as 187S(12).

Buoyant density. Phages PEa1(h), PEa1(nh), and PEa7 purified in sucrose gradients, were centrifuged to equilibrium using an SW50.1 rotor in a Beckman Model L2-65B Ultracentrifuge. Four-tenths ml of phage (2×10^{10} pfu/ml, 6×10^{10} pfu/ml, and 2×10^{10} pfu/ml, respectively) were layered on 4.5 ml of 40% (w/v) CsCl in distilled water. After 25 hr of centrifugation at 37.5K at 4 C, 10-drop fractions were collected from the bottom of the tubes. The density of each fraction at 23 C was determined by direct weighing (8) of 10 µl quantities using preweighed 10 µl disposable pipets. Fractions were dialysed for 12 hr against 0.1 M NaCl at 4 C, brought to 1.0 ml volume, assayed, and absorbance read at 260 nm. In the first experiment phages PEa1(h) and PEa1(nh) were found in the first fraction, therefore the experiment was repeated using 60% (w/v) CsCl. The results for phage PEa7 were from the first experiment while the results for PEa1(h) and PEa1(nh) were from the second experiment.

RESULTS

Isolation, plaque morphology, and host range. Fourteen phage strains from eight Michigan orchards were chosen because of their source of isolation and plaque morphology (Table 1). The most common type of phage plaque was 2-3 mm in diameter surrounded by an expanding, translucent halo (Figure 1-A). Times of initial halo development and rate of halo expansion varied among the phage-bacterial strain combinations. The second plaque type was 1-2 mm in diameter and lacked a halo (Figure 1-B); it was never isolated from plant material but was commonly detected in halo type lysates. It was impossible to propagate the halo producing types in broth without the production of non-halo producing types even when the halo type phage had been single-plaqued five times and sucrose gradient purified (Table 2). Non-halo producing phage were not found in halo plaques; however, when phage from halo plaques were incubated with 110rif^{r} in NBGYE both halo and non-halo plaques were detected (Table 3). The third plaque type, produced by phages PEa7 and PEa15,

Table 1. Bacteriophage strains, plaque morphology, source, and date of isolation from Michigan orchards

Isolate	Plaque Morphology		Sources and Date of Isolation	
	Plaque diameter (mm)	Expanding halo	Blighted leaves & terminals	Other
PEa1(h)	2 - 3	yes	Paw Paw, 6/17/75	
PEa1(nh)	1 - 2	no		Derived from PEa1(h)
PEa2(h)	2 - 3	yes		Soil, MSU, 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	

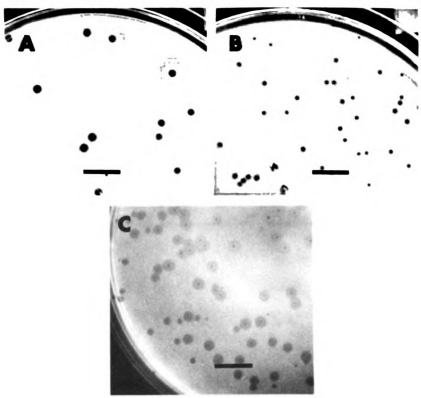

Figure 1 (A to C). Plaque morphologies of bacteriophages after 60 hr of incubation at 23 C with *Erwinia amylovora* 110 rif^r as host. Phages were plated using 2.5 ml of 0.7% nutrient agar, 0.5% glucose, and 0.25% yeast extract for the top layer; the bottom layer consisted of 2.0% nutrient agar and 0.5% glucose. A) Phage PEa1(h). B) Phage PEa1(nh). C) Phage PEa7. Bar = 1.0 cm.

Table 2. Propagation of group I bacteriophages and the development of halo and non-halo plaques.

Plaque Isolate	Plaque-Forming-Units/ml			
	Assay from plaque		After 24 h incubation in NBGYE	
	Halo	Non-halo	Halo	Non-halo
PEa1(h)	27×10^6	n.d.[a]	13×10^8	50×10^6
PEa1(nh)	n.d.	80×10^5	n.d.	14×10^8
PEa2(h)	53×10^6	n.d.	13×10^8	70×10^6
PEa2(nh)	n.d.	12×10^6	n.d.	12×10^8
PEa5(h)	70×10^6	n.d.	95×10^7	n.d.

[a] n.d. = not detected.

Table 3. Occurrence of halo and non-halo plaque-forming phages in halo plaques of phage PEa1(h).

Plaques	Plaque-Forming-Units/ml			
	Assay from plaques		After 24 h incubation in NBGYE	
	Halo	Non-halo	Halo	Non-halo
a	54×10^5	n.d.[a]	21×10^9	29×10^8
b	70×10^5	n.d.	19×10^9	18×10^8
c	63×10^5	n.d.	26×10^9	80×10^8
d	87×10^5	n.d.	22×10^9	75×10^8
e	68×10^5	n.d.	19×10^9	90×10^8

[a] n.d. = not detected.

was 0.5-1.0 mm in diameter with an expanding halo (Figure 1-C). At least 12 hr of incubation at 27 C were required before this plaque type could be observed, whereas the PEa1(h) plaque type was observed after 6 hr. Phages that produced plaques of the first and second types were placed in group I while phages producing plaques of the third type were placed in group II.

Host range for the 14 phage strains was essentially limited to strains of *E. amylovora* as was reported for PEa1 (17). Three strains (130, 150, and 151) of the closely related, yellow saprophyte *Erwinia herbicola* (Lohnis) Dye (13) were lysed by PEa7 but by PEa1(h) and PEa1(nh) only if spotted at titers of ca. 10^9 pfu/ml. Efficiencies of plating for PEa7 (compared to 110rif^r) were 0% for 130, 11.1% for 150, and 27.8% for 151. A weak area of lysis could be observed when a drop of 9 x 10^9 pfu/ml of PEa7 was spotted on strain 130 but not when lower titers were used. When group I phages were spotted on a mutant of 110rif^r, resistant to PEa1(h), the mutant was resistant to all group I phages but was lysed by group II phages.

Phage growth and purification. NBGYE lysates of PEa1(h) and PEa7 cleared only slightly even though 10^{10}-10^{11} pfu/ml were produced. The purification

scheme yielded better recovery of group I phages than of group II phages (Tables 4, 5). Centrifugation of phages PEa1(h), PEa1(nh), and PEa7 in linear 10-40% sucrose gradients for 1 hr at 22K resulted in a single, distinct band in the lower half of the gradient (Figure 2). Phages PEa1(h) and PEa1(nh) had maximum infectivity and absorbance in fraction 12, for PEa7 maximum infectivity and absorbance occurred in fraction 16. Electron microscopy showed the peaks at the top of the gradients to contain bacterial debris and empty virus particles. The ultraviolet adsorption spectra of 2-cycle, 10-40% sucrose-gradient purified preparations of phages PEa1(h) and PEa7 are shown in Figure 3.

Maximum infectivity and absorbance for PEa1(h) and PEa1(nh) were found in fraction 24 following 25 hr of isopynic centrifugation in 60% CsCl. The density of CsCl in this fraction was 1.53 g/cc (Figure 4). For phage PEa7 maximum infectivity and absorbance were found in fraction 6 following 25 hr of isopynic centrifugation in 40% (w/v) CsCl. The density of this fraction was 1.44 g/cc (Figure 4).

Thermal inactivation and optimum growth temperature. Phages PEa1(h) and PEa1(nh) were completely inactivated following 10 min at 55 C while PEa7 was completely inactivated after 10 min at 65 C (Figure 5).

Table 4. Purification and concentration of bacteriophage PEa1(h).

Steps in purification	Vol. (ml)	Titer pfu/ml	Total pfu	Recovery (%)
1- Crude lysate	1000	1.6×10^{10}	1.6×10^{13}	100
2- After DNase and RNase	1000	1.6×10^{10}	1.6×10^{13}	100
3- After PEG sedimentation	140	6.4×10^{10}	8.9×10^{12}	56
4- After 10-40% sucrose gradient	60	7.0×10^{10}	4.2×10^{12}	26
5- After 22K for 2 h	6	1.8×10^{11}	1.1×10^{12}	7
6- After CsCl and dyalysis	2	5.0×10^{10}	1.0×10^{11}	0.6

Table 5. Purification and concentration of bacteriophage PEa7.

Steps in purification	Vol. (ml)	Titer pfu/ml	Total pfu	Recovery (%)
1- Crude lysate	1000	1.6×10^{10}	1.6×10^{13}	100
2- After DNase and RNase	1000	1.6×10^{10}	1.6×10^{13}	100
3- After PEG sedimentation	140	1.1×10^{10}	1.4×10^{12}	9
4- After 10-40% sucrose gradient	60	7.0×10^{8}	4.2×10^{10}	0.3
5- After 2K for 2 h	6	6.0×10^{9}	3.6×10^{10}	0.2
6- After CsCl and dialysis	2	5.0×10^{9}	1.0×10^{10}	0.06

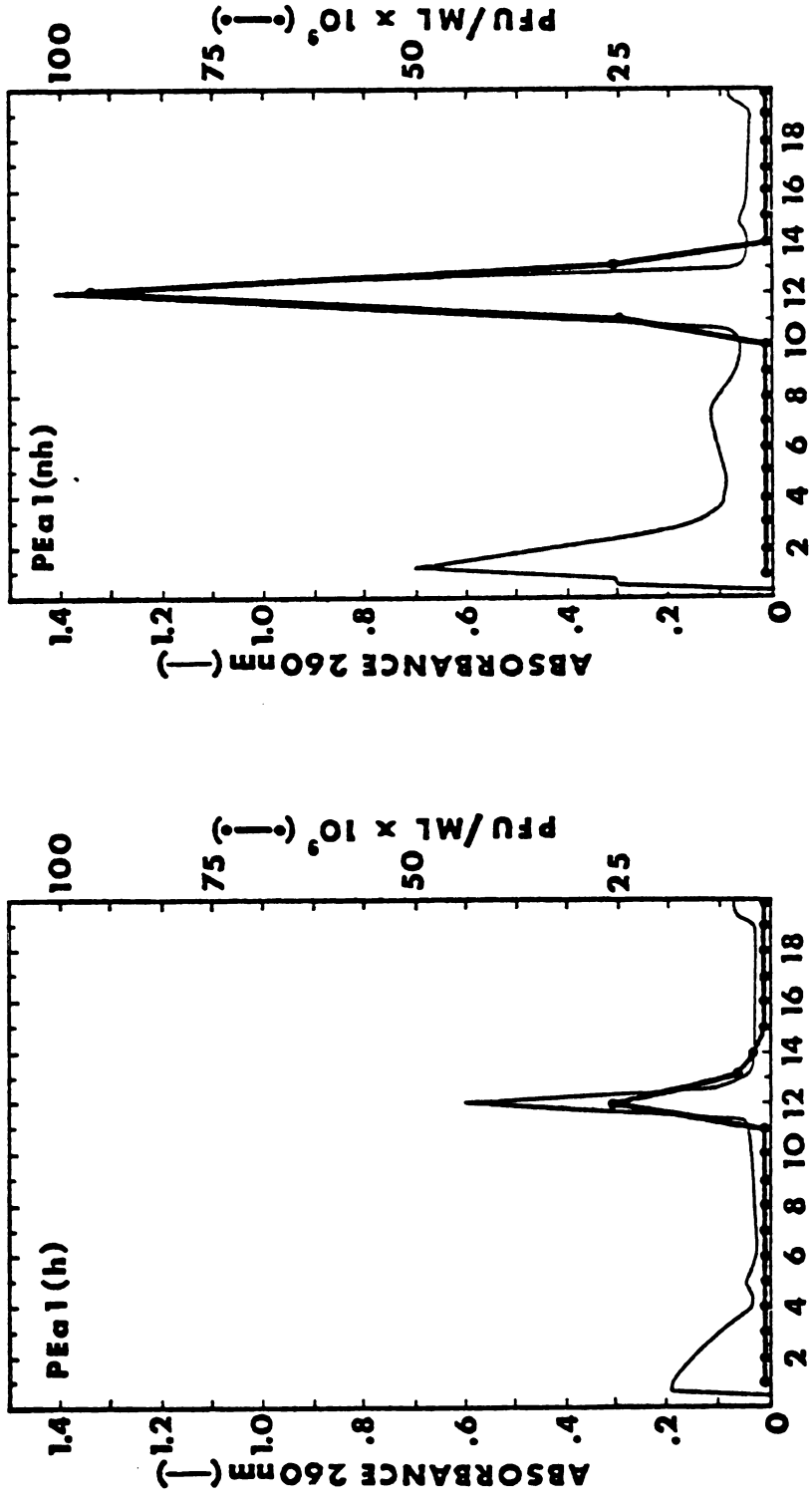

Figure 2. Sedimentation profiles of bacteriophages PEa1(h) and PEa1(nh) in 10 - 40% sucrose gradients.

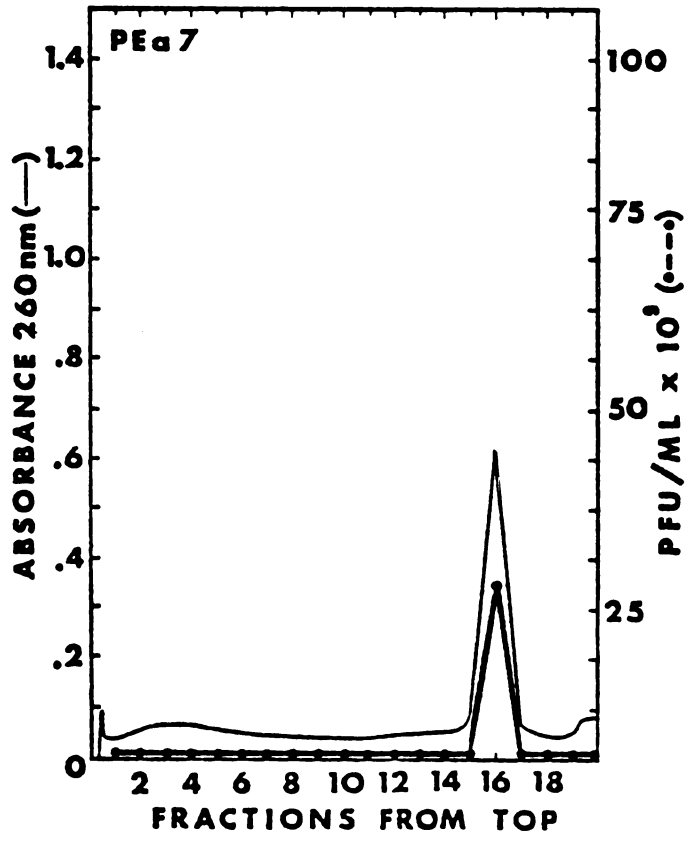

Figure 2 (cont'd). Sedimentation profile of bacteriophage PEa7 in 10 - 40% sucrose gradient.

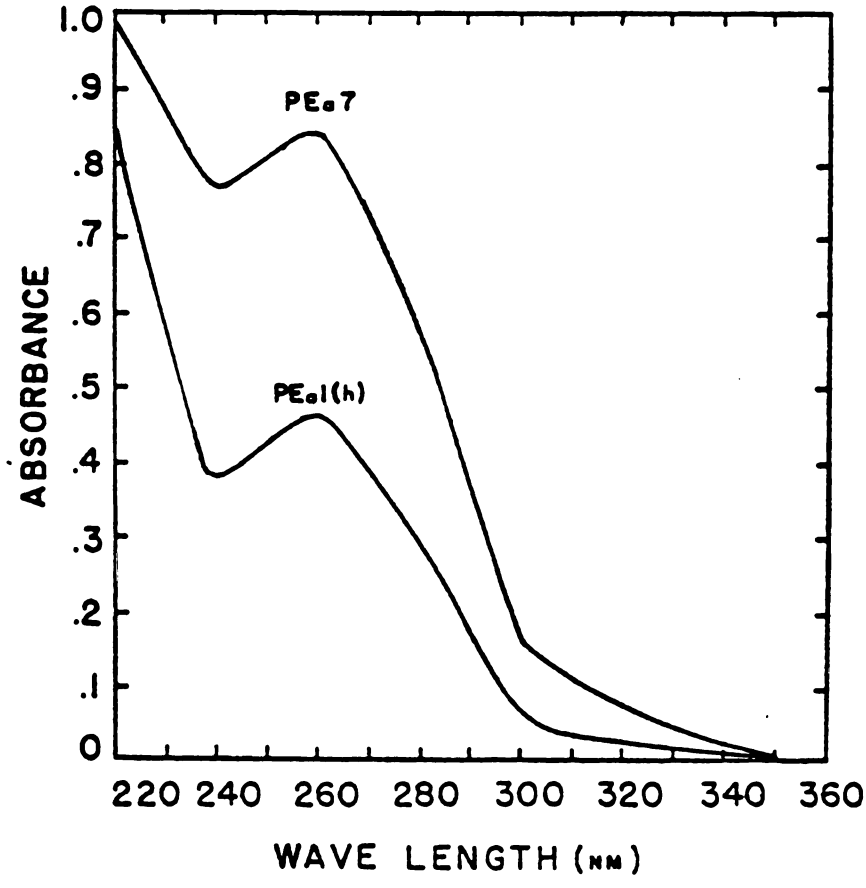

Figure 3. Ultraviolet adsorption spectra of 2-cycle, linear 10 - 40% sucrose gradient purified bacteriophages PEa1(h) and PEa7.

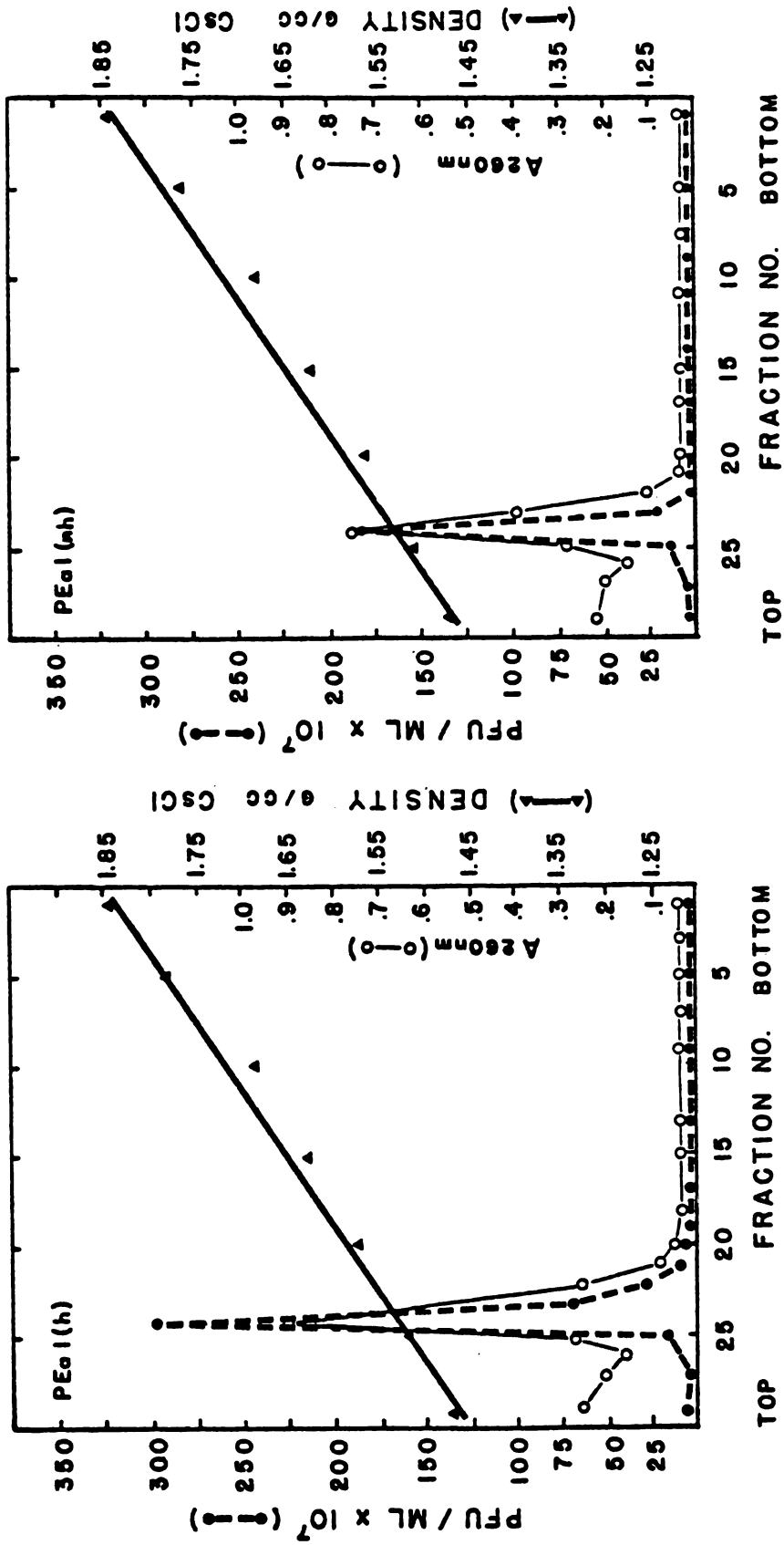

Figure 4. Isopynic centrifugation profiles and buoyant densities of bacteriophages PEa1(h) and PEa1(nh) in cesium chloride gradients.

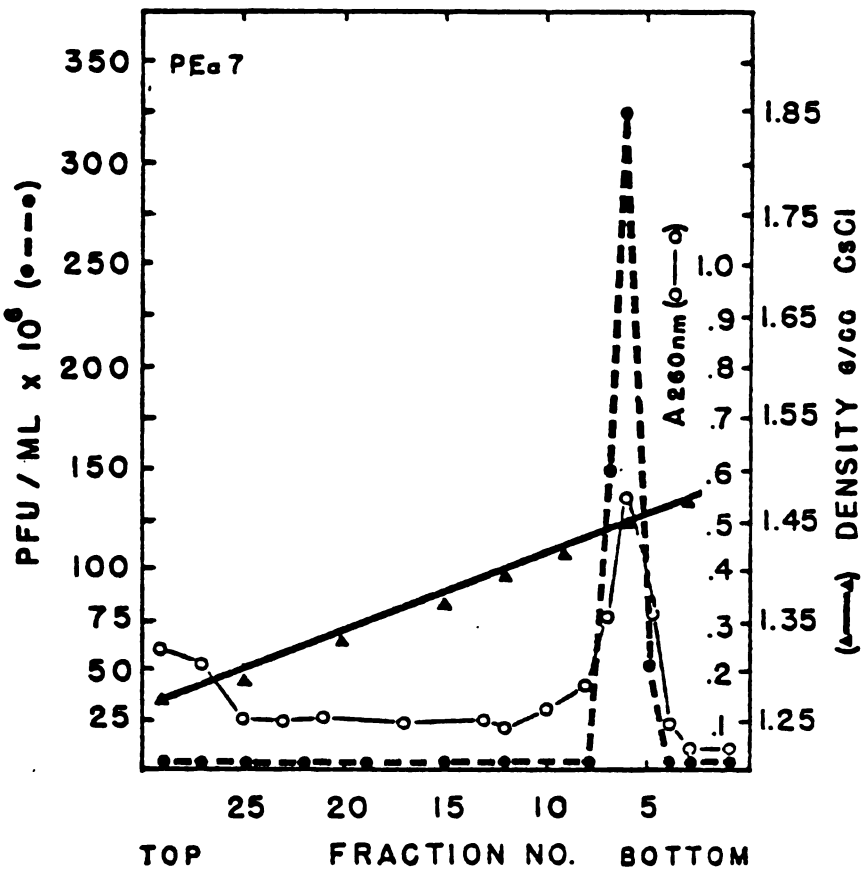

Figure 4 (cont'd). Isopynic centrifugation profile and buoyant density of bacteriophage PEa7 in cesium chloride gradient.

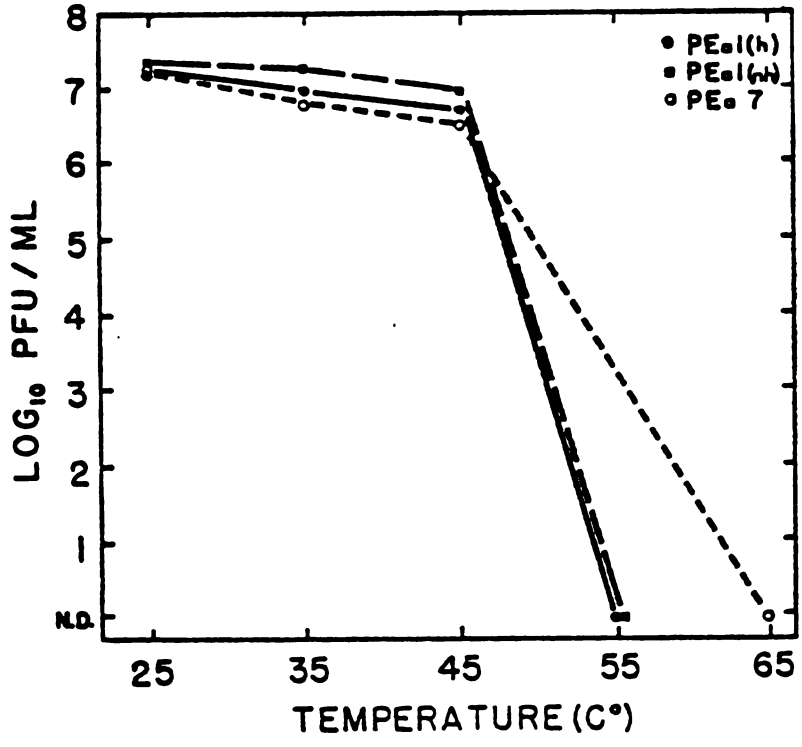

Figure 5. Thermal inactivation of bacteriophages PEa1(h), PEa1(nh), PEa7. N.D. = not detected.

PEa1(h) had an optimum growth temperature of 15-27 C yielding approximately a 100-fold increase in pfu after 3 hr of incubation (Figure 6). At temperatures of 10 C or less only 3-4 pfu per pfu inoculated were produced, while at 30 C more than a 10^3 decrease in pfu occurred. All the other group I phage strains except PEa1(nh) and PEa12 caused lysis when incubated at 30 C. Phage PEa7 grew optimally at 27 C yielding 9 pfu for each pfu inoculated (Figure 6). There was a net loss of pfu at temperatures of 15 C or less.

One-step growth and adsorption rates. PEa7 had a latent period of approximately 100 min, a rise period of 35 min, and an average burst size of 8 pfu per productive cell compared to a 40 min latent period and an average burst size of 50 pfu per productive cell for PEa1(h) (Figure 7).

Ninety percent of PEa1(h) adsorbed to *E. amylovora* 110rif^r within 10 min after 20 min were required to adsorb approximately 90% of PEa7 phages to the same bacterial strain (Figure 8). Contrasting results occurred when PEa1(h) and PEa7 were adsorbed to a PEa1(h)-resistant strain of 110rif^r, *E. amylovora* 110rif^{r}p^r (Figure 8). Similar results were also obtained using *E. amylovora* strains E8 and E9 (Figure 8). The adsorption rate constants, k, were calculated

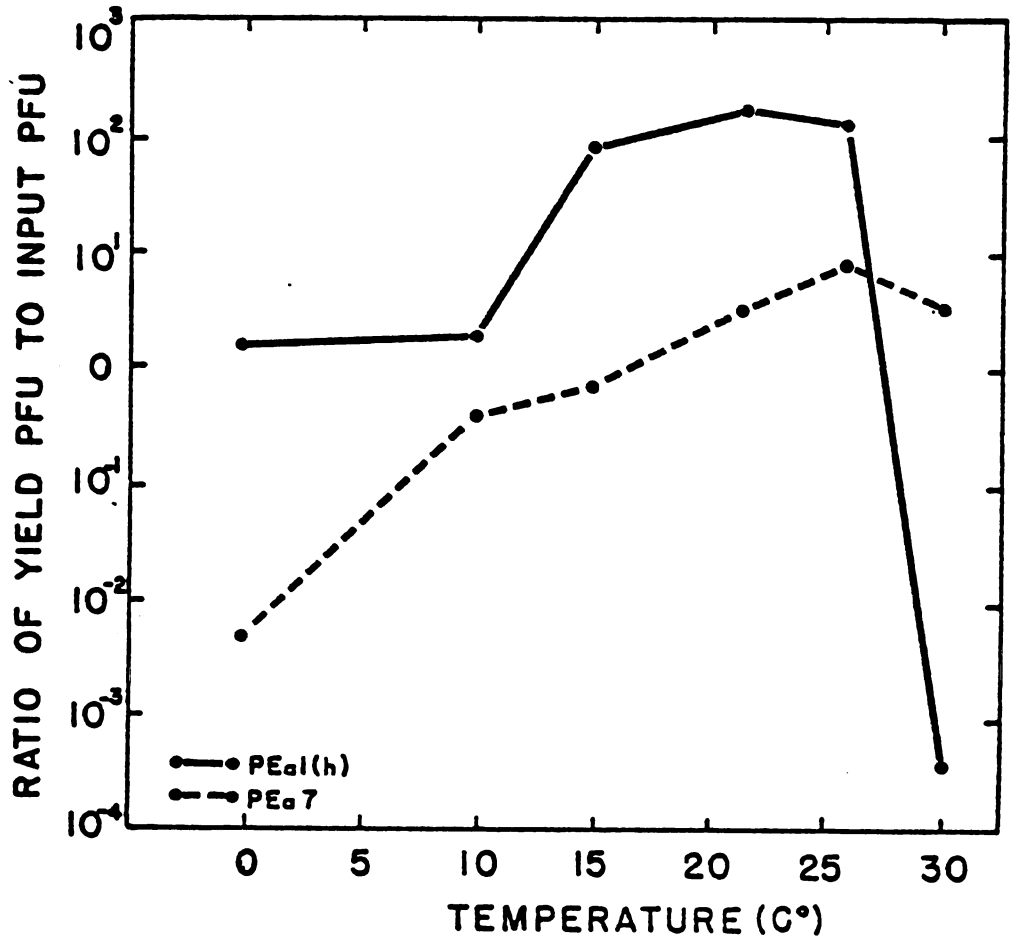

Figure 6. Optimum growth temperatures of bacteriophages PEa1(h) and PEa7.

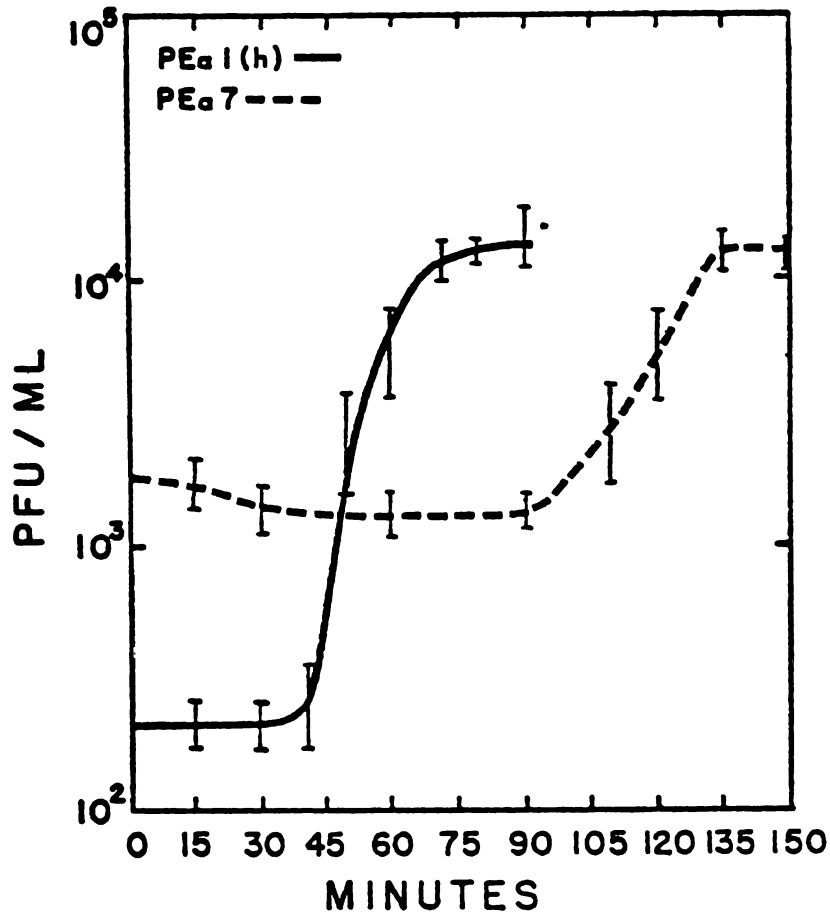

Figure 7. One-step growth curves for bacteriophages PEa1(h) and PEa7. Bars indicate range of the samples.

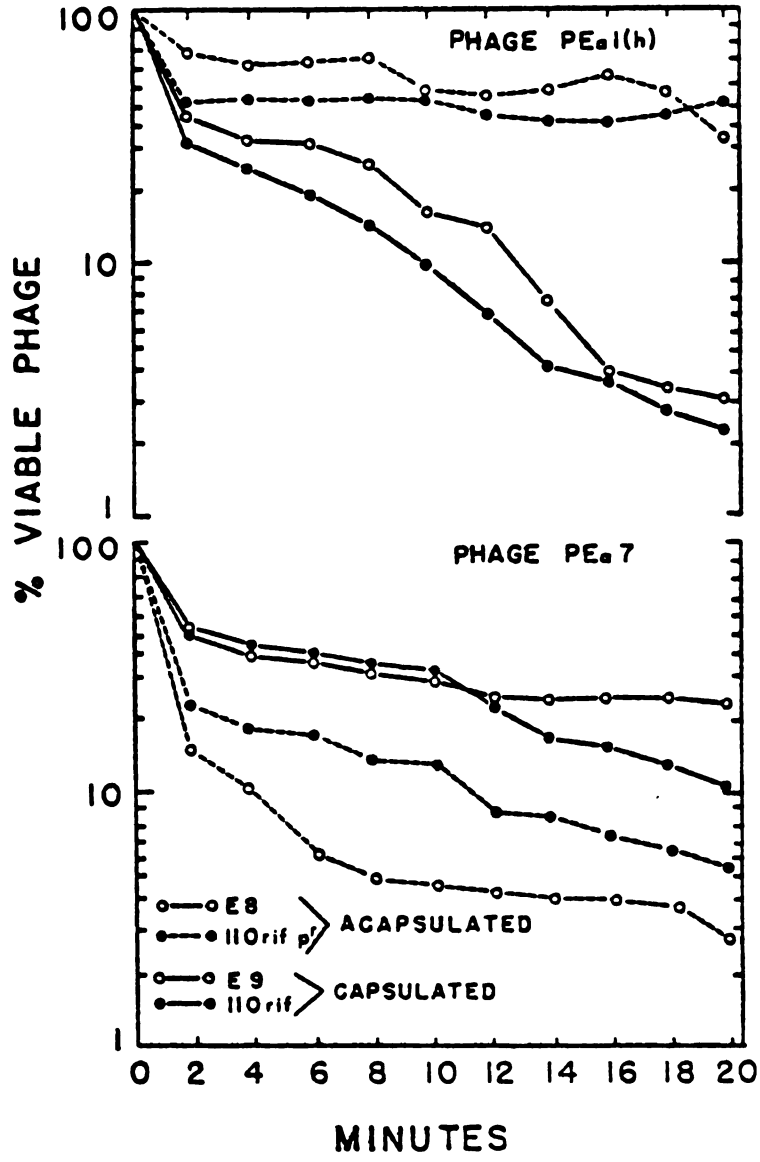

Figure 8. Adsorption curves of bacteriophages PEa1(h) and PEa7 to capsulated and acapsulated strains of *Erwinia amylovora*.

for PEa7 with different strains of *E. amylovora*, the results are summarized in Table 6. Phage PEa1(h) adsorbed rapidly to capsulated but not acapsulated strains of *E. amylovora*, while PEa7 adsorbed rapidly to acapsulated but slowly to capsulated strains (Figure 8 and Table 6).

Electron microscopy. Electron microscopy showed PEa1(h) to be polyhedral, probably iscoahedral, approximately 60 nm in diameter with a spike-like tail structure (Figure 9-A) thus placing it in Bradley's group C (6). Although not shown phage PEa1(nh) was morphologically indistinguishable from PEa1(h). PEa7 (Figure 9-B) had an octahedral head approximately 75nm in diameter with a rigid, non-contractile, striated tail approximately 135 nm long, thus fitting the criteria of Bradley's group B (6).

Serology. Serological neutralization results of phages PEa1(h), PEa1(nh), and PEa7 are shown in Figure 10. PEa1(h) antiserum, at a titer of 1000, neutralized 97% of its homologous phage within 10 min having a velocity constant K = 321 per min. Heterologous phages PEa1(nh) and PEa7 were 94% neutralized within 5 min, K = 597 per min, and 64% neutralized within 30 min, approximate K = 80 per min, respectively. PEa7 antiserum, titer 100, neutralized 93% of its

Table 6. Adsorption of phages PEa1(h) and PEa7 to capsulated and acapsulated strains of *Erwinia amylovora*.

Bacterial and Phage Strains			[b/] % Phage Adsorbed	[c/] Adsorption Constant (k) ml/min
E. amylovora	[a/] Capsulated	Phage		
110 rif^r	+	PEa1(h)	90 - 99	2.03×10^{-9}
110 $rif^r p^r$	-	PEa1(h)	50	2.04×10^{-10}
E 8	-	PEa1(h)	60	6.23×10^{-10}
E 9	+	PEa1(h)	90 - 99	1.97×10^{-9}
110 rif^r	+	PEa7	85 - 90	6.97×10^{-10}
110 rif^r	-	PEa7	90 - 99	1.72×10^{-9}
E 8	-	PEa7	90 - 99	2.47×10^{-9}
E 9	+	PEa7	77	2.97×10^{-10}

[a/] detected by the India ink procedure.

[b/] percent of adsorbed phages used to calculate the adsorption constant.

[c/] calculated at the time at which 90-99% of the phage had adsorbed or at the termination of the 20-min incubation period if 90-99% of the phage had not adsorbed during that time period.

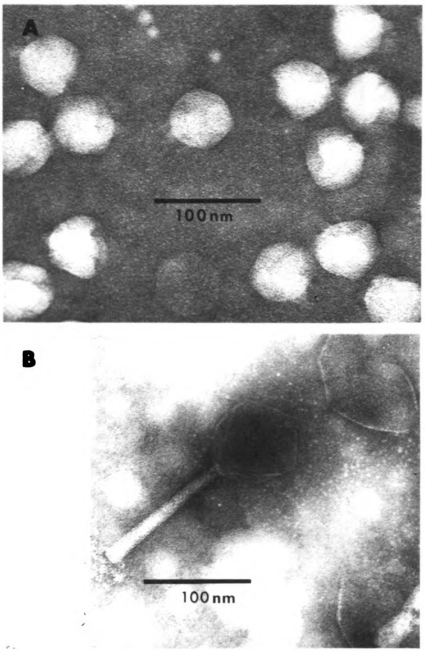

Figure 9 (A and B). Electron micrographs of *Erwinia amylovora* bacteriophages.
A) Bacteriophage PEa1(h), a polyhedral phage with a spike-like tail.
B) Bacteriophage PEa7 with an octahedral head and a rigid, striated tail.

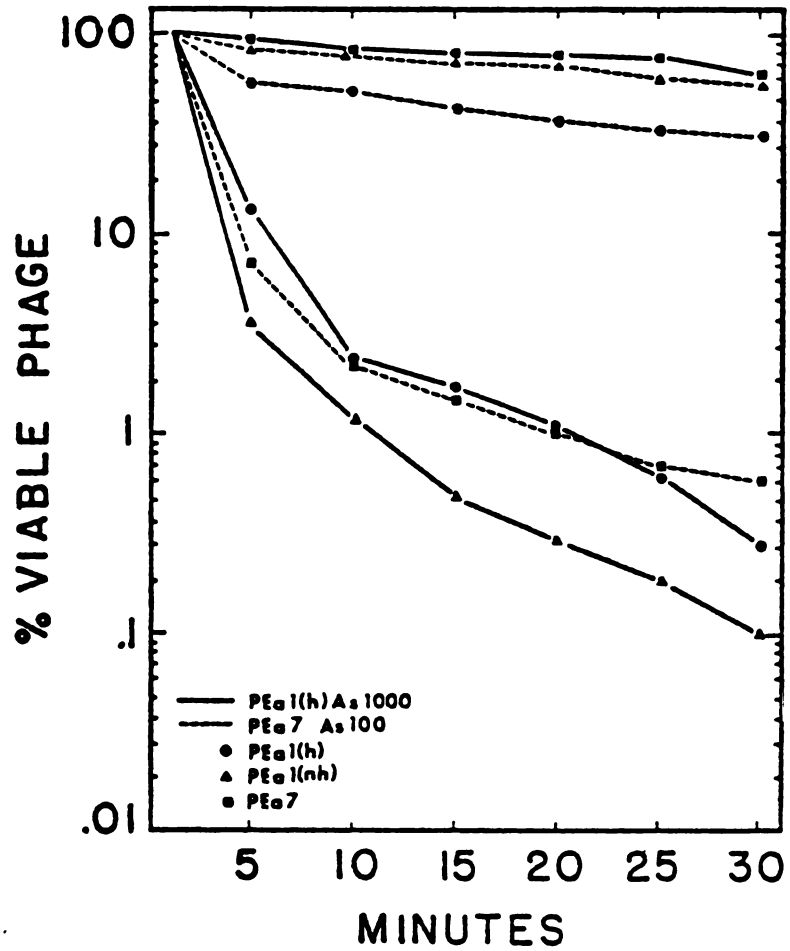

Figure 10. Antiphage sera neutralization curves for bacteriophages PEa1(h), PEa1(nh), PEa7.

homologous phage within 5 min, K = 56 per min. For heterologous phages PEa1(h) and PEa1(nh) 64% and 40%, respectively were neutralized after 30 min giving an approximate K = 8 per min for both. Serological typing of the 11 remaining strains resulted in the neutralization of all group I phages with PEa1(h) antiserum but not with PEa7 antiserum; PEa7 antiserum neutralized only group II phages (Table 7).

Sedimentation coefficients. Estimates of sedimentation coefficients for PEa1(h) and PEa1(nh) were 566S and 1037S for PEa7 (Figure 11, Table 8).

Table 7. Antiphage sera neutralization of the 14 *Erwinia amylovora* bacteriophage strains.

Phage Strains	Percent Viable Phage[a/]	
	PEal(h) antiserum	PEa7 antiserum
PEal(h)	1	98
PEal(nh)	1	97
PEa2(h)	1	99
PEa2(nh)	1	98
PEa3(h)	1	96
PEa4(h)	1	94
PEa5(h)	1	98
PEa6(h)	1	92
PEa7	97	2
PEa8(h)	1	88
PEal2(h)	1	86
PEal3(h)	1	99
PEal5	99	5
PEal6(h)	1	90

a/ Phage (ca. 10^7 pfu/ml) in 0.02 M potassium phosphate buffer plus 10^{-3} M NaCl, pH 6.8, were mixed with the antiserum and allowed to react at 23 C for 10 min, diluted, and plated with bacterial host 110 rif^r. The percent viable phage was determined by comparing the number of pfu propr to addition of the antiserum to the number of pfu following exposure to the antiserum. PEal(h) antiserum was used at a titer of 100 while PEa7 antiserum was used at a titer of 40.

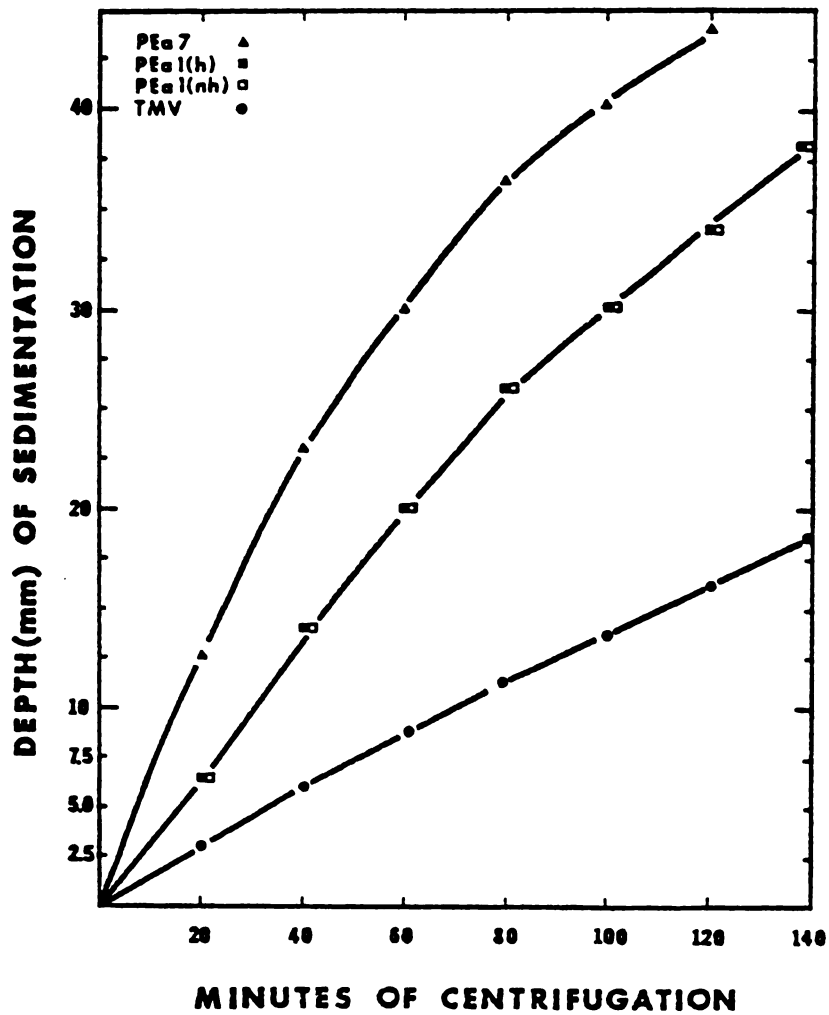

Figure 11. Sedimentation curves used to calculate the rate of sedimentation for sedimentation coefficient determinations.

Table 8. Sedimentation coefficient calculations for phages PEa1(h), PEa1(nh), and PEa7.

Virion	Time[a/] (min)	Rate of Sedimentation (mm/min)	Ratio of Phage Rate to TMV	$S_{20,w}$
TMV	88.7	0.1127	-----	187 S
PEa1(h)	29.3	0.3413	3.028	566 S
PEa1(nh)	29.3	0.3413	3.028	566 S
PEa7	16.0	0.6250	5.546	1037 S

[a/] Time for the virus bands to move from 7.5 mm to 17.5 mm in the gradients.

DISCUSSION

Based on the biological, chemical, and physical data these *E. amylovora* phages were placed in two groups. Group I was represented by PEa1(h) while group II was represented by PEa7. Except for plaque morphology, the data indicated that halo and non-halo producing phages of group I were identical. Several other phage-bacterial combinations result in the production of an expanding, translucent halo which was due to the enzymatic removal of the bacterial capsule (2,4,11,22). A similar phenomenon may cause the halo surrounding the plaque produced by the *E. amylovora* phages. Failure of PEa1(nh)-type phages to produce a halo may be due to nonsynthesis of the enzyme or a situation similar to a *Pseudomonas aeruginosa* phage-2 mutant, PDPI, in which the enzyme is produced but is not enzymatically active (4). The smaller plaque produced by PEa7 compared to that of PEa1(h) may be due to the longer latent period, smaller burst size, slower adsorption rate, and differences in phage morphology, all of which affect

plaque morphology (1).

Specificity of the group I phages for capsulated strains of *E. amylovora* was similar to that reproted for the *Escherichia coli* K phages (18) and *Klebsiella* phages (11,15,16). These phages were specific to capsulated strains because their attachment site was located on the bacterial capsule. Although up to 65% of PEa1(h) absorbed to the acapsulated strains of *E. amylovora* no increase in titer occurred. This adsorption may have been due to the presence of capsular material not detected by the India ink stain (3). A second and more likely explanation is that infection was blocked after capsule recognition. This was hypothesized to be the case with *Klebsiella* phage 7 (19). In comparison to PEa1(h), phage PEa7 attached more readily to acapsulated than capsulated strains of *E. amylovora* suggesting that the receptors for PEa7 are located on the cell wall beneath the capsule. These data would support and explain Billing's observations that sensitivity for different phage types was related to the presence of a bacterial capsule (5). As the data indicated, group I phages have their receptors on the bacterial capsule, thus this is the first substantiated report of K-phages infecting phytopathogenic bacteria and the presence of

such phages may be rather common in Enterobacteriaceae as suggested by Stirm (18).

The lower percentage of recovery during purification of PEa7 compared to PEa1(h) may be related to the more fragile tail structure of PEa7 which is probably necessary for attachment site recognition. It is also possible that during purification the capsular degrading factor was lost which may aid the phage particle in reaching its receptor (19,20). That reduced infectivity was the result of the physical disruption of the intact phage particles may be gained by measuring protein absorbance versus infectivity. PEa1(h) had an O.D. (280 nm) of 0.30 and an infectivity of 7×10^{10} pfu/ml with an A 260/280 ratio of 1.51. PEa7 had an O.D. (280 nm) of 0.63 but an infectivity of only 6×10^{8} pfu/ml with an A 260/280 ratio of 1.44 (Figure 3). This suggests that the protein content was greater for the PEa7 phage suspension than for PEa1(h) yet the infectivity of PEa7 was approximately 100-fold less. Significant amounts of bacterial protein were eliminated since the phages had passed through 2-cycles of sucrose gradient centrifugation; furthermore, after only 1-cycle no bacterial proteinaceous material such as flagella were observed in electron micrographs. Thus, PEa7 could be purified using the PEG method but

its infectivity was reduced.

Phage PEa1(h) had a wide temperature range for optimum growth compared to PEa7; however, when incubated at 30 C more than a 1000-fold decrease in pfu/ml occurred (Figure 6). This was not the result of physical thermal inactivation since the phage could tolerate heating for 10 min at 45 C without a significant drop in titer (Figure 5). Phages are known to adsorb to and inject their nucleic acid into their bacterial hosts. The phage is then unable to produce progeny because a protein necessary for its growth is nonfunctional at the higher temperature even though its bacterial host grows normally at that temperature (21). This situation may exist for phages PEa1(h), PEa1(nh), and PEa12(h). The temperature sensitivity of the non-halo form of PEa1(h), PEa1(nh), adds evidence that PEa1(h) and PEa1(nh) are very similar even though PEa1(nh) does not produce a halo-plaque. Temperatures produced a different effect on the growth of PEa7 than PEa1(h) (Figure 6). This indicated the the multiplication processes of these two phage types differed. The differences in their one-step growth (Figure 7) also supports this hypothesis.

Calculation of the velocity constant, K, of antiphage sera neutralization is valid only over a

limited range of inactivation, usually 90-99 percent of the phage, and cannot be validly calculated outside this range (1). Thus the K values for PEa1(h) antiserum with phage PEa7 and PEa7 antiserum with phages PEa1(h) and PEa1(nh) cannot be validly calculated; however, for purposes of comparison, estimates have been made. This results in higher K-values which suggest greater serological relatedness than actually exist.

Along with the other similarities between PEa1(h) and PEa1(nh) was the ability of PEa1(h) antiserum to neutralize homologous phage, PEa1(h), K was 321 per minute; however, for heterologous phage, PEa1(nh), it was 597 per minute. Antiserum prepared to other phages that produced halo plaques was shown to also neutralize both the phage and the enzyme that caused the halo (2). Lysis of host cells by PEa1(nh) did not result in detectable amounts of capsule degrading factor as did lysis by PEa1(h) (Section III of dissertation). The lower K for the homologous phage, PEa1(h), than for heterologous phage, PEa1(nh), may have resulted from competition between the virion and the capsule degrading factor for antibody. The data from the serological typing experiments (Table 7) indicated that the 14 phage strains could be placed in two groups which correlated with group I and group

II plaque types.

The sedimentation coefficients of PEa1(h) and PEa1(nh) were similar to those of the T-odd phages while that of PEa7 was similar to the T-even phages (9). This is also true for phage morphology; PEa1(h) and PEa1(nh) are morphologically similar to coliphage T3 while PEa7 is similar to the tailed T-even phages (6).

Group I phages were placed in Bradley's morphological group C, while group II phages were placed in Group B; the phages in groups B and C contain double-stranded DNA (6). Thus, these *E. amylovora* phages are probably double-stranded DNA phages as was the *E. amylovora* phage, S1, characterized by Erskine (10).

These phages are of interest for several reasons: 1) they are the first *E. amylovora* bacteriophages isolated from aerial structures of plants susceptible to *E. amylovora*, 2) this is the first extensive characterization of more than one strain of *E. amylovora* bacteriophage, 3) the phages produce an expanding, translucent halo surrounding the true plaque, and 4) the group I phages are specific for capsulated strains of *E. amylovora*.

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PART III

INTERACTION OF *ERWINIA AMYLOVORA* WITH ITS BACTERIOPHAGE

INTRODUCTION

Bacteriophages of phytopathogenic bacteria have been known since the 1920's (11,36). Three reviews dealing with these phages have been written since 1963 (11,36,48). A number of these phages have been extensively characterized, and several show unusual morphologies or biochemical properties (29,30,33,47, 48).

Because of the impressive destruction of bacteria by phage in the laboratory, the temptation has always been to test the therapeutic effect of phages. Yet, no substantiated evidence exists where a pathogen causing an epidemic disease has been controlled by a virulent phage under natural conditions. Several attempts to control phytopathogenic bacteria through the use of phages have been unsuccessful (11,36,48).

It was thought that the influence of phage on a bacterial population was limited to the elimination of the sensitive cells by virulent phages. Phage have now been shown to control or influence the virulence as well as control some of the other phenotypic

characteristics of their bacterial host via lysogenic conversion (6,14,20,21,27,32,46) or transduction (53). Many of these systems have been extensively studied; however, there are few cases where similar situations with phytopathogenic bacteria and their phages have been reported and extensively studied (12,15,16,17,52).

When strains of *Erwinia amylovora* were mixed with phage of the PEa1(h) type, the following phenomena occurred: development of a spreading, translucent halo surrounding the true plaque; alteration of colony morphology; high frequency of phage resistant survivors which easily reverted to phage sensitivity; and a delay in symptom development.

This section deals with the description and explanation of these phenomena and the possible involvement of phage in fire blight epidemiology.

MATERIALS AND METHODS

Media. For the growth of bacteria on solid medium 2.0% nutrient agar (Difco) with 0.5% glucose, pH 6.5, was used (NAG medium). Broth medium was composed of 0.8% nutrient broth (Difco), 0.5% glucose, and 0.25% yeast extract, pH 6.5 (NBGYE medium). Where the agar overlay method (1) was used the bottom layer consisted of 12-15 ml of NAG and the top layer consisted of 2.5 ml of 0.7% nutrient agar, 0.5% glucose, and 0.25% yeast extract, pH 6.5. The growth of *E. amylovora* in inoculated apple seedlings was monitored with TTN medium (42) and NAG to which 50 µg/ml of rifampin (NAG/rif) had been added after autoclaving. All dilutions, resuspensions, and inoculations of phages and bacteria were done in 0.02 M potassium phosphate buffer, pH 6.8 (PPB).

Selection of *Erwinia amylovora* mutants. Spontaneous mutants resistant to the antibiotic, rifampin (49) were selected from nine pure, pathogenic strains of *E. amylovora*. Bacteria from three to five colonies of each strain were streaked on NAG plates. After 24 hr

of incubation at 27 C, 2.0 ml of sterile PPB were added to each plate and the bacteria resuspended with a sterile, glass rod to make a slurry containing 10^{10}-10^{11} colony-forming-units (cfu)/ml. One-tenth ml of this slurry was plated on NAG rif plates and incubated at 27 C for 48 hr. Mutation frequencies were approximately 1 x 10^{-9}. Two of the fastest growing colonies were selected for each strain, resuspended in PPB, streaked on NAG, incubated for 48 hr, and representative colonies tested for rifampin resistance (rif^r) on NAG rif.

Phage resistant mutants (p^r) of the *E. amylovora* rif^r strains were selected to phage PEa1(h). One ml (ca. 3 x 10^8 cfu) of a 12 hr old broth culture of *E. amylovora* was added to 1.0 ml (ca. 4 x 10^8 pfu) of phage. Five minutes were allowed for phage adsorption, dilutions of 10^{-2}, 10^{-4}, and 10^{-6} were made, and 0.1 ml of each dilution was spread over the surface of NAG plates over which had previously been spread 0.1 ml (ca 1 x 10^9 pfu/ml) of a chloroform, filter-sterilized phage suspension. Following 48 hr incubation at 23 C, 3-5 colonies from each strain were resuspended in PPB and streaked on NAG. Cultures were tested for phage and rifampin resistance and stored on nutrient agar at 4 C.

Expanding, translucent halo. Plaque and halo development were studied using the agar overlay method (1). To test the number of phage-*E. amylovora* strain combinations that resulted in halo formation, 13 phage strains and 20 *E. amylovora* strains were used. One-tenth ml of ca. 2×10^9 cfu/ml of each bacterial strain was added to top agar and layered over the bottom layer, after solidification a loopful of phage suspension (ca. 10^4 pfu) was spotted on the plates and incubated at 27 C for 36 hr.

Effect of temperature on the development and expansion of the halo. *Erwinia amylovora* 110 rif^r and phage PEa1(h) were plated such that ca. 25 plaques per plate developed. The plates were incubated at 27 C for 6 hr, at which time plaques were first visible, the plates were then transferred to incubators set at 0, 10, 15, 23, 27, and 35 C air temperatures. The diameters of five plaques, including the halo, per plate were measured to ± 0.1 mm at specified time intervals. The effect of temperature on the expansion of established haloes was tested in a similar manner; however, haloes were allowed to develop for 18 hr at 27 C prior to transfer to the desired temperatures.

Diffusion of the phage from the plaques. Three, 24 hr old plaques produced by phages PEa1(h) and

PEa1(nh) were sampled for pfu. The points of sterile toothpicks were inserted into the true plaque and 2-3 mm outside of the plaque prior to halo development then dropped into 1.0 ml of PPB and assayed for phage. Plates were incubated an additional two days during which time the halo extended through the previously sampled areas. Samples were taken again from these areas and outside of the halo as described.

Altered colony morphology and sensitivity to phage. Bacterial colony morphology was studied using NAG plates. Phage sensitivity was determined by the agar overlay technique; test bacteria were added to the top agar. After solidification diluted phage solutions were spotted on the agar surface. The plates were incubated at 27 C for 24 hr and observed for lysis. The ability of phage to increase in titer in a bacterial culture was also used as a test for phage sensitivity.

Stability of phage resistance and p^r colony characteristics. Ten single-colony subcultures of *E. amylovora* 110 rif^r were selected. These cultures were incubated for 14 hr in NBGYE then diluted and plated on NAG and NAG over which 0.1 ml of a sterile solution of phage PEa1(h) had been spread. Plates were incubated for 48 hr at 23 C. The number of colonies which grew on NAG and NAG + PEa1(h) were

recorded. Three colonies from four of the NAG + PEa1(h) plates were transferred to NBGYE and incubated on a reciprocal shaker for 12 hr then plated on NAG for p^r and wild type (w.t.) colonies as well as tested for phage sensitivity.

Effect of temperature on the development of p^r colony characteristics. One-tenth ml (2×10^9 pfu/ml) of PEa1(h) was spread over the surface of NAG plates. One-tenth ml of a 10^{-6} dilution of an 18 hr NBGYE culture of *E. amylovora* 110 rif^r were spread over the surface and the plates incubated for a minimum of 48 hr at 10, 15, 23, 27, 30 and 35 C.

Interaction of PEa1(h) and *Erwinia amylovora* 110 rif^r in broth and the development of p^r colony characteristics. One ml of an 18 hr NBGYE culture of *E. amylovora* 110 rif^r was added to 125-ml flasks of 25 ml of fresh NBGYE and incubated at 1 hr. Phage PEa1(h) was then added to the 110 rif^r cultures at multiplicities-of-infection (m.o.i.) of 0.1, 1.0, 10.0, and 100.0. The cultures were incubated an additional 1 hr, diluted, plated on NAG, incubated at 23 C for 48 hr, and the number of p^r and w.t. colonies counted.

Effect of PEa1(h) antiserum on the development of p^r colony characteristics. One-tenth ml of PEa1(h) antiserum, diluted 1:10, was spread over the surface

of NAG plates, phage PEa1(h) and *E. amylovora* were then spread over this surface. The plates were incubated for 48 hr at 23 C at which time p^r and w.t. colonies were counted.

Agar diffusible substance. A test similar to that described by Rakienten *et al.* (40) was used to determine whether a colony morphology alteration could be caused by an agar diffusible substance. One loopful of a NBGYE culture of 110 rif^r was spotted in the center of an NAG plate, approximately 10. cm from this spot and surrounding it were placed four additional spots of 110 rif^r. On the center spot was placed a loopful of phage PEa1(h) suspension (ca. 10^8 pfu/ml) and the plate incubated at 23 C and observed over a 10-day period for changes in colony morphology.

Effect of phage lysates on mature lawns of *Erwinia amylovora*. Phage require a metabolically active host for multiplication (1) thus once the bacterial lawn of the agar overlay is more than 18-24 hr old plaques will not develop nor continue to expand. If plaque-like zones do occur or plaques continue to spread, then some factor other than phage multiplication is involved. To test for such effects produced by lysates of phages PEa1(h), PEa1(nh), and PEa7, mature lawns (lawns 2 days or older) of *E. amylovora* 110 rif^r were used. A spot test similar to that

described by Adams and Park (2) was used to test for these effects and to determine the titer of such a substance.

Effect of phage lysates on the *Erwinia amylovora* capsule. One-ml aliquots of a 48 hr old NBGYE culture of 110 rif^r were pipetted into sterile test tubes, India ink stains were (5) made, and immediately examined under phase contrast for the presence of a capsule. One ml quantities of chloroform-sterilized lysates of phages PEa1(h), PEa1(nh), PEa7, and *E. amylovora* 110 rif^r were added to the NBGYE cultures of 110 rif^r. India ink stains were made and examined at 5-min intervals. This method made it possible to directly observe the loss of bacterial capsules and to estimate the rate of capsule degradation.

Serological relationship between p^r and wild type *Erwinia amylovora*. Antiserum to *E. amylovora* 110 rif^r was prepared in a rabbit according to the method outlined by Allan and Kelman (3). Double diffusion tests with Ouchterlony plates (38) were done using NBGYE cultures of 110 rif^r and 110 $rif^r p^r$ which had been pelleted and resuspended in PPB to ca. 10^9 cfu/ml and steamed for 1 hr.

Mutation frequencies of phage resistance. The percent of *E. amylovora* cells resistant to phages

PEa1(h), PEa1(nh), and PEa7, after growth for 18 hr in NBGYE was determined for 20 *E. amylovora* strains. One-tenth ml of chloroform, filter-sterilized phage lysates (ca. 10^8 pfu/ml) were spread over the surface of NAG plates. Dilutions of 10^{-2}, 10^{-4}, and 10^{-6} of the *E. amylovora* strains were made and 0.1 ml aliquots spread over the surface of NAG + phage and NAG plates. After incubation for 48 hr at 23 C the number of colonies which grew on NAG and NAG + phage were counted and the percent of *E. amylovora* survivors calculated.

Fluctuation test. The fluctuation test developed by Luria and Delbruck (35) was used to determine whether the resistance to phage PEa1(h) was the result of a spontaneous mutation or an adaptation. Two strains of *E. amylovora*, 110 rif^r and 118 w.t., were tested with phage PEa1(h) as outlined by Braun (9).

Test for lysogeny. Temperate phages can often be detected by one of several methods: a low percentage may be spontaneously released, or inducing agents may be used such as ultraviolet light or the antibiotic mitomycin C (4,37). Several of the stable p^r strains of *E. amylovora* were tested for the production of phage using these methods.

Growth and effect of phage PEa1(h) on *Erwinia amylovora* 110 rif^r. Ten ml of an 18 hr old NBGYE

culture *E. amylovora* 100 rif^r (ca. 10^9 cfu/ml) were added to each of two flasks of 100 ml of fresh NBGYE and incubated an additional 30 min. Bacteria were assayed for the presence of a capsule using the India ink stain (5) and the number of cfu/ml determined by plating on NAG. To one of the flasks was added 1.0 ml of approximately 10^9 pfu/ml and the actual number of pfu/ml immediately assayed. At 10 min intervals, starting at 10 min after the addition of PEa1(h), the absorbance at 525 nm was read. The presence or absence of a bacterial capsule was determined, 1.0 ml of the culture was added to tubes containing 0.1 ml of chloroform and saved for capsule degrading factor assay, and the number of cfu and pfu/ml were determined by plating on NAG and using the agar overlay method, respectively. The presence of a capsule degrading factor was determined by the spot method (2) on mature lawns of 110 rif^r. Once the fractions containing the factor were determined these fractions were diluted 2-fold and the reciprocal of the greatest dilution exhibiting the activity was taken as the titer.

Symptom development by *Erwinia amylovora*. The effect of phage PEa1(h) on symptom development of *E. amylovora* was tested using open pollinated

Jonathan apple seedlings (41). The bacteria were grown for 18-24 hr on NAG, resuspended in PPB, standardized to 0.30 O.D. at 525 nm (ca. 10^8 cfu/ml), and diluted 1:10. A sterile, disposable syringe with a 25-gauge needle was used to wound and inoculate the seedlings. The needle was pushed through the hypocotyl, withdrawn, and a droplet of inoculum placed on the wound. Droplet size could be controlled by using the plunger; the bacterial concentration applied was ca. 10^6 cfu/droplet. Nine *E. amylovora* strains were used: wild types 105, 110, 112, 113, 115, 118, 121, 131, and 134 as well as rifampin and phage resistant mutants of these strains. Virulence was determined by the period of time required to produce a droplet of bacterial exudate at the point of inoculation. Several types of experiments were done. Seedlings were inoculated with wild type, rifampin resistant, and phage resistant strains of *E. amylovora*, and the rate of symptom development was monitored. *Erwinia amylovora* strains and phage PEa1(h) were mixed at an m.o.i. of 1.0, 10.0, and 100.0 and inoculated for 10, 15, or 30 minutes, then inoculated into the seedlings. Four to 12 seedlings per bacterial strain were used. A third experiment consisted of monitoring the symptom development and growth of three *E. amylovora* strains, 105, 110, and

134 plus their rifampin and phage resistant mutants. The seedlings were inoculated and the bacterial growth was monitored by removing a 1.0 cm section, centered on the point of inoculation, from each seedling. Each section was crushed in PPB with a sterile glass rod, diluted, and assayed on TTN or NAG rif plates. Each sample period consisted of three seedlings per bacterial strain.

Hypersensitive reaction in tobacco. Some plant pathogenic bacteria, including *E. amylovora*, induce a hypersensitive reaction in tobacco leaves (28). To determine whether the mutation to phage resistance affected the hypersensitive response by the tobacco leaf, seven wild type, rifampin resistant, and phage resistant strains were standardized to ca. 10^7 cfu/ml and infiltrated into tobacco leaves as described by Klement (28).

RESULTS

Expanding, translucent halo. One of the major characteristics of these *E. amylovora* phages, particularly the PEa1(h) type, was the development of a continually expanding, translucent halo (Figure 1). The true plaque did not increase in diameter after 18 hr but the translucent halo surrounding the plaque continued to expand. Halo formation was a common occurrence following the lysis of *E. amylovora* by most of the phages (Table 1). Exceptions were phage PEa1(nh) which did not produce a halo in combination with any of the *E. amylovora* strains and bacterial strains 104 and 119 which did not result in a halo with any of the phage strains.

Effect of temperature on the development and expansion of the halo. The halo surrounding the plaque produced by PEa1(h) failed to develop when incubated at 0 and 35 C (Figure 2-A). Once the halo was initiated it expanded most rapidly when incubated at 35 C (Figure 2-B). There was an increase in the rate of halo development and expansion as the incubation

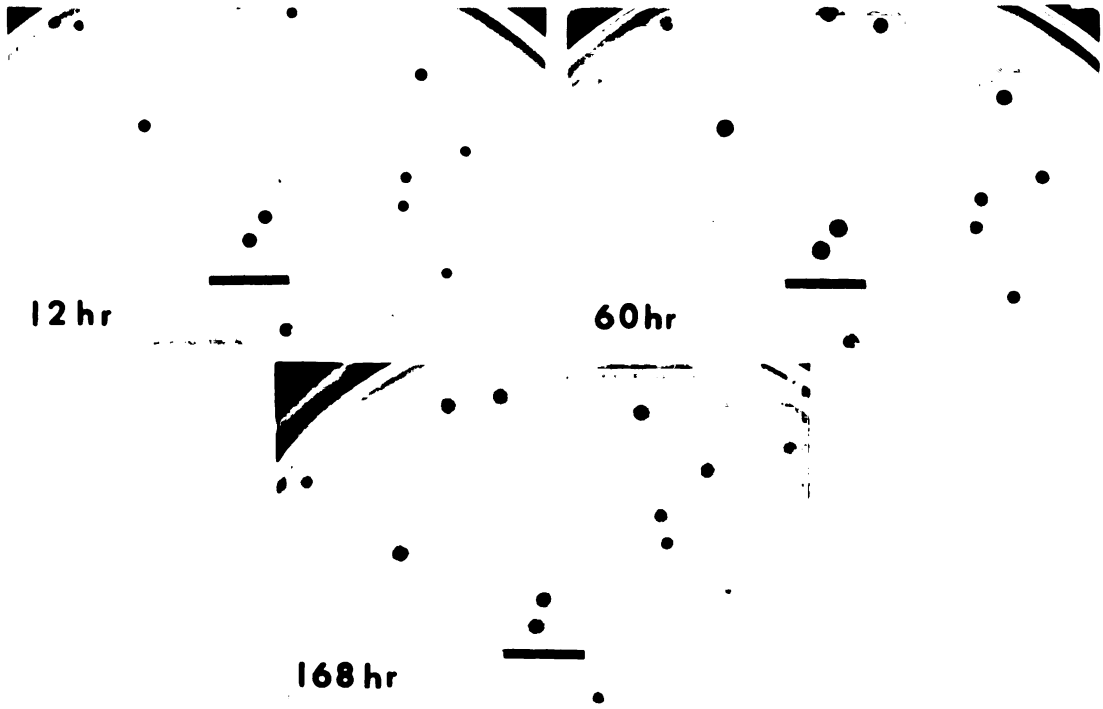

Figure 1. Expanding, translucent halo associated with the plaque formed by bacteriophage PEa1(h) grown on *Erwinia amylovora* 110 rif^{r}. Time is hours of incubation at 23 C. Bar = 1.0 cm.

Table 1. Phage - *Erwinia amylovora* combinations which resulted in halo formation.

E. amylovora strains	Phage strains (PEa)												
	1(h)	1(nh)	2	3	4	5	6	7	8	12	13	15	16
104	-	-	-	-	-	-	-	-	-	-	-	-	-
105	+	-	+	+	+	+	+	+	+	+	+	+	+
110	+	-	+	+	+	+	+	+	+	+	+	+	+
112	+	-	+	+	+	+	+	+	+	+	+	+	+
113	+	-	+	+	+	+	+	+	+	+	+	+	+
114	+	-	+	+	+	+	+	+	+	+	+	+	+
115	+	-	+	+	+	+	+	+	+	+	+	+	+
117	+	-	+	+	+	+	+	+	+	+	+	+	+
118	+	-	+	+	+	+	+	+	+	+	+	+	+
119	-	-	-	-	-	-	-	-	-	-	-	-	-
121	+	-	+	+	+	+	+	+	+	+	+	+	+
122	+	-	+	+	+	+	+	+	+	+	+	+	+
125	+	-	+	+	+	+	+	+	+	+	+	+	+
126	+	-	+	+	+	+	+	+	+	+	+	+	+
128	+	-	+	+	+	+	+	+	+	+	+	+	+
133	+	-	+	+	+	+	+	+	+	+	+	+	+
134	+	-	+	+	+	+	+	+	+	+	+	+	+
135	+	-	+	+	+	+	+	+	+	+	+	+	+
137	+	-	+	+	+	+	+	+	+	+	+	+	+
138	+	-	+	+	+	+	+	+	+	+	+	+	+

All the *E. amylovora* strains were lysed by the 13 phage strains but only the combinations marked with "+" resulted in halo formation.

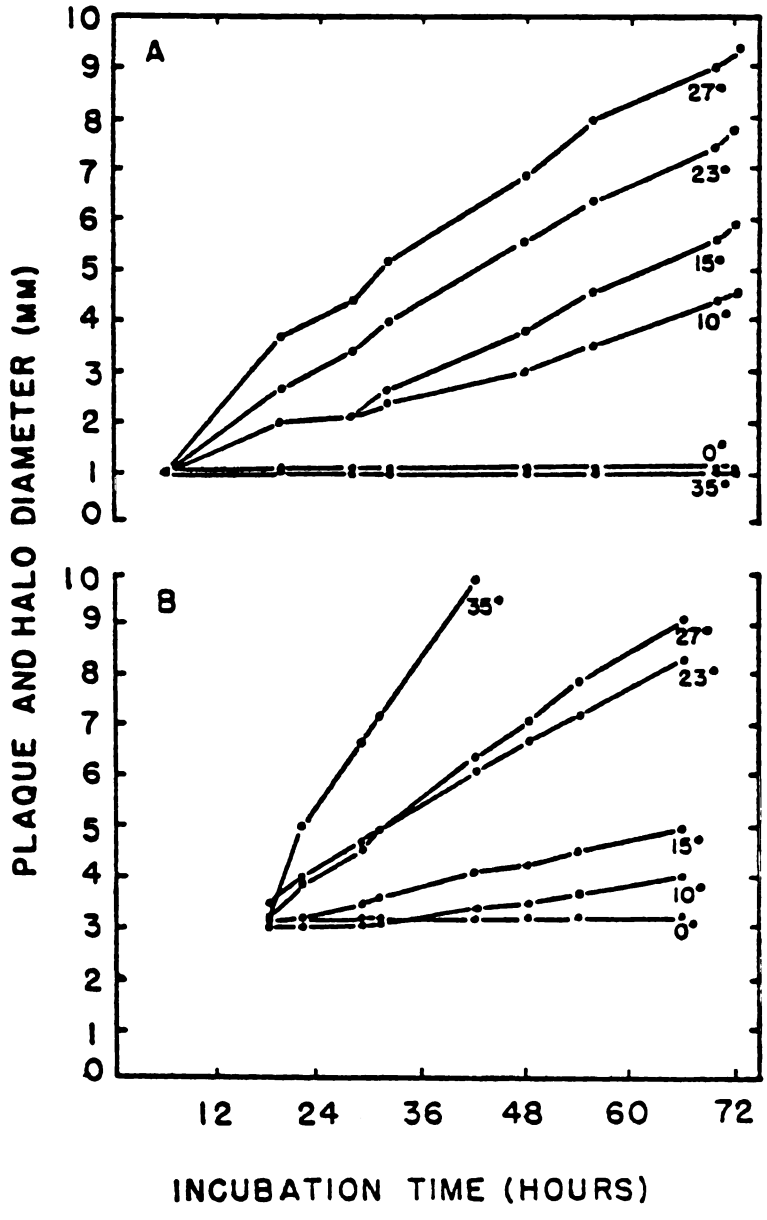

Figure 2(A and B). Effect of temperature on expansion of the translucent halo surrounding plaques produced by bacteriophage PEal(h). A) Effect on the establishment of the halo. B) Effect of temperature on expansion of the halo after its establishment.

temperatures were increased from 10 to 27 C.

Diffusion of the phage from the plaques. Phages of PEa1(h) diffused from the true plaque but not beyond the halo; their diffusion coincided with or followed the development of the expanding halo that surrounded the true plaque (Table 2). Phage PEa1(nh), which did not produce a halo, were not detected beyond the margins of the true plaque (Table 2).

Altered colony morphology and sensitivity to phage. When rifampin labeled (rif^r) *E. amylovora* was grown on NAG, convex, chalky white, mucoid colonies 2.0-2.5 mm in diameter developed (Figure 3-A). The rif^r colonies were indistinguishable from wild type *E. amylovora* except for their ability to grow on media containing rifampin. Colonies which developed on NAG in the presence of phage PEa1(h) were flat, vitreous, non-mucoid, and 1.0-1.5 mm in diameter (Figure 3-B). These p^r colonies were also resistant to rifampin.

When p^r type colonies were streaked with NAG, two different types of bacterial growth occurred. The growth which developed from the initial portion of the streak-process had p^r characteristics, but near the end of the streak-process wild type (w.t.) bacterial growth occurred. PEa1(h) phages could be detected in

Table 2. Diffusion of phages PEa1(h) and PEa1(nh) from the true plaque.

Plaque No.	Before Halo Development			
	PEa1(h)[a]		PEa1(nh)	
	Inside of plaque	2-3 mm outside of plaque	Inside of plaque	2-3 mm outside of plaque
1	+[b]	-	+	-
2	+	-	+	-
3	+	-	+	-

Plaque No.	After Halo Development				
	PEa1(h)			PEa1(nh)	
	Inside of plaque	Inside of plaque	2-3 mm outside of halo	Inside of plaque	2-3 mm outside of plaque
1	+	+	-	+	-
2	+	+	-	+	-
3	+	+	-	+	-

[a] Phage PEa1(h) produces a plaque having an expanding, translucent halo surrounding the true plaque while PEa1(nh) does not produce a halo.

[b] "+" indicates that phage was detected; "-" indicates that phage was not detected.

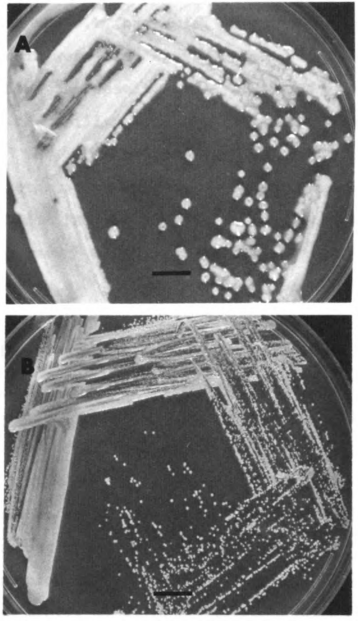

Figure 3 (A and B). Bacterial growth of *Erwinia amylovora* after 48 hr incubation at 23 C. A) Capsulated, mucoid, wild type growth. B) Bacteriophage PEa1(h)-resistant, acapsulated, non-mucoid. Bar = 1.0 cm.

the p^r type of bacterial growth but no phages could be detected in the w.t. bacterial portion of growth, the w.t. bacteria were also sensitive to PEa1(h) phages. Table 3 shows the results of an experiment to illustrate the instability of the p^r type *E. amylovora* colonies. The 10 subcultures of 110 rif^r were all of w.t. characteristics when grown on NAG, but when grown on NAG + PEa1(h) all of the colonies were of p^r type. The growth of p^r type colonies in NBGYE for 12 hr resulted in the failure to detect any p^r type colonies when plated on NAG, the colonies which developed were also phate PEa1(h) sensitive.

Factors contributing to the establishment of p^r type colonies. Growth of *E. amylovora* 110 rif^r at 10, 15, 23, and 27 C on NAG plates in the presence of phage PEa1(h) resulted in p^r type colonies; however, when incubated at 30 and 35 C, p^r type colonies failed to develop but retained w.t. characteristics (Table 4).

Incubation of *E. amylovora* 110 rif^r in NBGYE with PEa1(h) at m.o.i. of 0.1-100 failed to produce any p^r type colonies when plated on NAG; all of the colonies were of w.t. (Table 5). Incubation of 110 rif^r on NAG + PEa1(h) resulted in p^r type colonies.

Phage PEa1(h) antiserum also inhibited the development of p^r type colonies on NAG plates and in NBGYE medium (Table 6).

Table 3. Instability of bacteriophage PEa1(h)-resistance and altered colony morphology of *Erwinia amylovora* 110 rif^r.

Subcultures or 110 rif^r	cfu on NAG (10^8)		cfu on NAG + PEa1(h) (10^8)		Following 12 hr of culture in NBGYE broth. CFU on NAG (10^8).			
	w.t.	p^r	w.t.	p^r	Colonies[a] from NAG + PEa1(h)	w.t.	p^r	PEa1(h) sensitivity
a	50	0	0	23	a_1	29	0	+
b	50	0	0	28	a_2	49	0	+
c	60	0	0	30	a_3	21	0	+
d	63	0	0	26	b_1	58	0	+
e	22	0	0	14	b_2	46	0	+
f	17	0	0	13	b_3	56	0	+
g	49	0	0	21				
h	25	0	0	22	d_1	77	0	+
i	52	0	0	20	d_2	85	0	+
j	48	0	0	23	d_3	90	0	+
					f_1	49	0	+
					f_2	44	0	+
					f_3	31	0	+

cfu = colony-forming-units.

w.t. = wild type of mucoid *E. amylovora*, p^r = phage resistant and non-mucoid.

[a] Three p^r colonies that were selected from four 110 rif^r subcultures which were grown on NAG + Pea1(h).

Table 4. Effect of temperature on the development of the p^r type of colony.

Incubation Temperature (C)	NAG (cfu)		NAG + PEal(h) (cfu)	
	w.t.	p^r	w.t.	p^r
10	56	0	0	35
15	36	0	0	37
23	41	0	0	28
27	35	0	0	32
30	34	0	22	0
35	13	0	5	0

w.t. = wild type, phage PEal(h) sensitive, mucoid *Erwinia amylovora*.
p^r = phage PEal(h) resistant, non-mucoid *E. amylovora*.

Table 5. Results of the incubation of *Erwinia amylovora* 110 rif^r with bacteriophage PEal(h) in NBGYE and the development of p^r type colonies when plated on NAG.

M.O.I.[a/]	Colony type that developed on NAG	
	wild type (10^6 cfu/ml)	p^r (10^6 cfu/ml)
0.1	80	0
1	60	0
10	60	0
100	110	0
no phage	430	0
[b/]	0	330

[a/] M.O.I. = multiplicity of infection.
[b/] Phage were spread over the surface of NAG plates prior to plating 110 rif^r.

Table 6. Effect of bacteriophage PEal(h) antiserum on the development of p^r type colonies.

Treatment	Reaction on NAG (10^7 cfu/ml)		Reaction in NBGYE (10^7 cfu/ml)	
	w.t.	p^r	w.t.	p^r
110 rif^r alone	239	0	135	0
110 rif^r + PEal(h) antiserum	230	0	150	0
110 rif^r + PEal(h)	0	160	91	0
110 rif^r + PEal(h) antiserum + PEal(h)	225	0	80	0

w.t. = wild type, PEal(h) sensitive.

p^r = PEal(h) resistant.

Agar diffusible substance. Lysis of *E. amylovora* cells by phage PEa1(h) resulted in an agar diffusable substance which could move across bacterial-free areas of NAG and affected the appearance of *E. amylovora* growth (Figure 4).

Effect of phage lysates on *Erwinia amylovora*. Chloroform-sterilized lysates of phages PEa1(h) and PEa7 produced a substance that caused a depression or clearing in mature lawns of *E. amylovora* 110 rif^r. The substance had a titer of 64 in the PEa1(h) lysate, was non-detectable in PEa1(nh), a titer of 2 in the PEa7 lysates, and was not detected in chloroform-sterilized cultures of 110 rif^r or in cultures of 110 $rif^r p^r$ incubated with phage PEa1(h) (Table 7). None of the lysates had any detectable effect on mature lawns of 110 $rif^r p^r$.

Incubation of capsulated cells of *E. amylovora* 110 rif^r with a sterile lysate of *E. amylovora* 110 rif^r + PEa1(h) resulted in partial degradation of the capsule within 5 min and failure to detect the capsule after 10 min (Table 8). A lysate of PEa7 produced an effect on the *E. amylovora* capsule after 15 min of incubation and no capsule was detected after 30 min while PEa1(nh) and a chloroform-sterilized lysate of 110 rif^r culture had no detectable effect on the

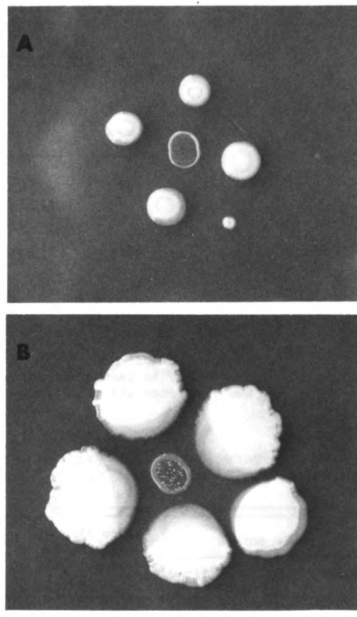

Figure 4 (A and B). Diffusion of capsular degrading factor across bacterial-free 2.0% nutrient agar and 0.5% glucose. A) Appearance of bacterial growth 24 hr after placement of bacteriophage PEa1(h) on the center colony. B) Appearance of bacterial growth 10 days later. Note vitreous appearance of inner portions of the colonies surrounding the phage-inoculated center colony.

Table 7. Ability of bacteriophage lysates to affect mature lawns of *Erwinia amylovora*.

Source of lysate[a/]	Titer[b/]	
	Lawn of 110 rif^r	Lawn of 110 $rif^r p^r$
110 rif^r alone	0	0
110 rif^r + PEa1(h)	64	0
110 rif^r + PEa1(nh)	0	0
110 rif^r + PEa7	2	0
110 $rif^r p^r$ + PEa1(h)	0	0

[a/] Lysates were composed of cultures grown for 24 hr then chlorform-sterilized.

[b/] The titer is the reciprocal of the greatest dilution producing a visible depression in the bacterial lawn.

Table 8. The ability of bacteriophage lysates to remove the capsule from *Erwinia amylovora* cells.

Minutes after addition of lysate	110 rif^r cells without the addition of lysate	Source of lysate[a/]			
		110 rif^r	PEa1(h)	PEa1(nh)	PEa7
0	+[b/]	+	+	+	+
5	+	+	±	+	+
10	+	+	-	+	+
15	+	+	-	+	±
30	+	+	-	+	-
60	+	+	-	+	-

[a/] Lysates were composed of cultures grown for 24 hr then chloroform-sterilized.

[b/] + indicates the detection of a capsule using the India ink stain, - indicates non-detection of a capsule.

E. amylovora capsule (Table 8).

Serological relationship between p^r and wild type *Erwinia amylovora*. The serological relationship between phage PEa1(h)-resistant, acapsulated, less virulent strain of *E. amylovora* 110 rif^r and phage PEa1(h)-sensitive, capsulated, virulent strain of *E. amylovora* 110 rif^r is shown in Figure 5. The capsulated (w.t.) *E. amylovora* had an extra band not produced by the acapsulated (p^r) *E. amylovora*.

Resistance of *Erwinia amylovora* to phage strains. Mutation frequencies of 20 *E. amylovora* strains to phages PEa1(h), PEa1(nh), and PEa7 were high (Table 9). With only a few exceptions, less than half the cells in a bacterial culture failed to survive exposure to phage.

The results of the fluctuation test with the selective agent, phage PEa1(h), and *E. amylovora* strains 110 rif^r and 118 w.t. are shown in Table 10. There was no significant difference in the number of PEa1(h)-resistant cfu among the individual cultures compared to the number of PEa1(h)-resistant cfu among the individual samples from the same culture.

All tests for temperate phages in the stable p^r strains of *E. amylovora* were negative.

Growth and effect of phage PEa1(h) on *Erwinia amylovora* 110 rif^r. The results of the experiment to

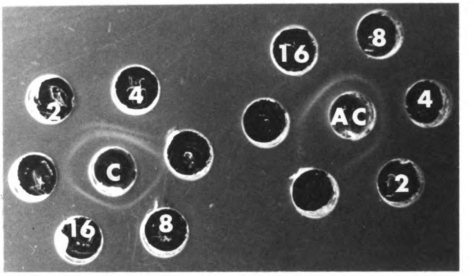

Figure 5. Agar gel serological tests with steamed suspensions of *Erwinia amylovora* strain 110 rif^r. Capsulated (C) and acapsulated (AC) suspensions were placed in the center wells while the outside wells were filled with dilutions of antiserum prepared to capsulated *E. amylovora* 110 rif^r. Numbers in outside wells represent the titer of the antiserum placed in the well.

Table 9. Mutation frequencies of *Erwinia amylovora* strains to bacteriophages.

E. amylovora	Percent *E. amylovora* survivors		
	Phage PEa1(h)	Phage PEa1(nh)	Phage PEa7
105 rif^r	28.9	26.9	62.8
110 rif^r	57.4	67.3	98.2
112 w.t.	59.0	n.d.[a/]	n.d.
113 w.t.	37.5	n.d.	n.d.
115 w.t.	47.7	64.9	82.4
116 w.t.	47.3	n.d.	n.d.
117 w.t.	22.7	26.7	58.7
118 w.t.	54.2	67.1	88.2
120 w.t.	0.014	n.d.	n.d.
121 w.t.	0.003	0.002	98.7
122 w.t.	18.2	n.d.	n.d.
126 w.t.	30.6	16.0	73.8
128 w.t.	37.3	n.d.	n.d.
131 w.t.	59.0	n.d.	n.d.
132 w.t.	79.0	n.d.	n.d.
133 w.t.	51.7	n.d.	n.d.
134 rif^r	0.005	0.001	90.0
135 w.t.	60.4	n.d.	n.d.
136 w.t.	78.5	95.1	95.5
138 w.t.	50.3	88.3	96.4

[a/] n.d. = not determined.

Table 10. Fluctuation test for *Erwinia amylovora* and resistance to bacteriophage PEa1(h).

Erwinia amylovora 110 rif^r

Samples from single culture		Samples from individual cultures	
Sample No.	No. resistant cfu (10^6)	Sample No.	No. resistant cfu (10^6)
1	29	1	25
2	29	2	23
3	32	3	29
4	45	4	42
5	40	5	18
		6	35
		7	19
		8	16
		9	22
		10	23
Average	35		25
Variance	10.3		6.5
Chi-square	5.89		23.20
P	.27		.005

Table 10 (continued).

Erwinia amylovora 118 w.t.

Samples from single culture		Samples from individual cultures	
Sample No.	No. resistant cfu (10^6)	Sample No.	No. resistant cfu (10^6)
1	53	1	45
2	46	2	45
3	50	3	48
4	52	4	44
5	49	5	52
		6	50
		7	51
		8	43
		9	49
		10	52
Average	50		48
Variance	1.5		1.2
Chi-square	.438		2.19
P	.96		.98

determine the growth and effect of PEa1(h) on *E. amylovora* 110 rif^r are shown in Figure 6. The 110 rif^r culture not inoculated with PEah(h) increased from 2.8 x 10^8 cfu/ml to 1 x 10^9 cfu/ml corresponding with an increase in O.D. from 0.3 to 0.8. Bacterial capsule was detected during the entire experiment, and no capsule degrading factor was detected. The culture inoculated with PEa1(h) showed and increase of 2.3 x 10^8 cfu/ml to 3.0 x 10^8 cfu/ml during the first 40 min followed by a sharp decrease to 4.0 x 10^7 cfu/ml during the next 10 min. Colony-forming-units remained stable for 10 more min, then increased logarithmically to 7.5 x 10^8 cfu/ml by the end of the experiment. The sharp decline in cfu corresponded to a 0.1 O.D. decrease, the loss of the bacterial capsule, an increase in the titer of the capsular degrading factor, and a sharp rise in the number of pfu from 6.5 x 10^7 pfu/ml to 10^{10} pfu/ml. Capsular degrading factor became maximal after 60 min at a titer of 32, and remained at this level. Optical density of the 110 rif^r plus PEa1(h) culture declined steadily as pfu increased and continued for 60 min then gradually increased. Even though the number of cfu in the 110 rif^r and 110 rif^r + PEa1(h) were very similar, by the termination of the experiment there was approximately a 0.5 O.D. difference between the 110 rif^r and the 110 rif^r + PEa1 (h)

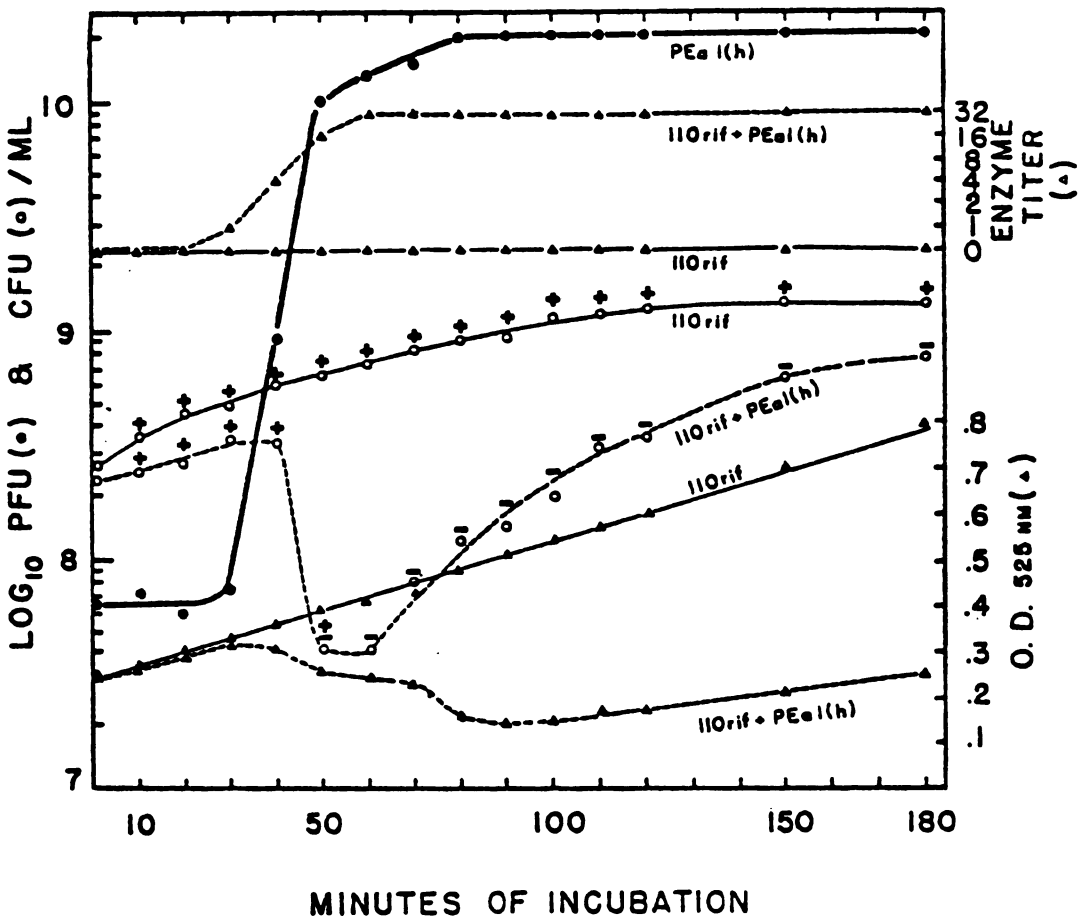

Figure 6. Growth of bacteriophage PEa1(h) and its effect on *Erwinia amylovora* 110 rif^r in NBGYE culture. The "+" or "-" along the CFU lines indicate the presence (+) or absence (-) of a bacterial capsule.

cultures.

Symptom development by *Erwinia amylovora*. *Erwinia amylovora* resistant to phage PEa1(h) exhibited a delay in the production of symptoms compared to wild type and rifampin resistant strains (Figure 7). Even though there was variability in rate of symptom development among the *E. amylovora* strains, the phage resistant strains consistently were slower in production of symptoms (Appendices C1, C2). Three *E. amylovora* strains resistant to phage PEa5(h) also exhibited a delay in symptom development (Appendix C3).

The inoculation of seedlings with a mixture of phage PEa1(h) and strains of *E. amylovora* resulted in a delay in symptom development (Figure 8). All strains mixed with PEa1(h) elicited delayed symptom development as compared to strains not mixed with PEa1(h) (Appendices C4-C6).

Wild type and rifampin resistant strains of *E. amylovora* 105, 110, and 134 grew exponentially and produced symptoms within 36 hr after inoculation into apple seedlings (Figure 9A-C). Populations of phage PEa1(h)-resistant strains of 105 and 110 grew similar to their wild type strains during the first 12-24 hr then remained relatively stable in the plant tissue (Figure 9A & B). After 120 hr none of the seedlings

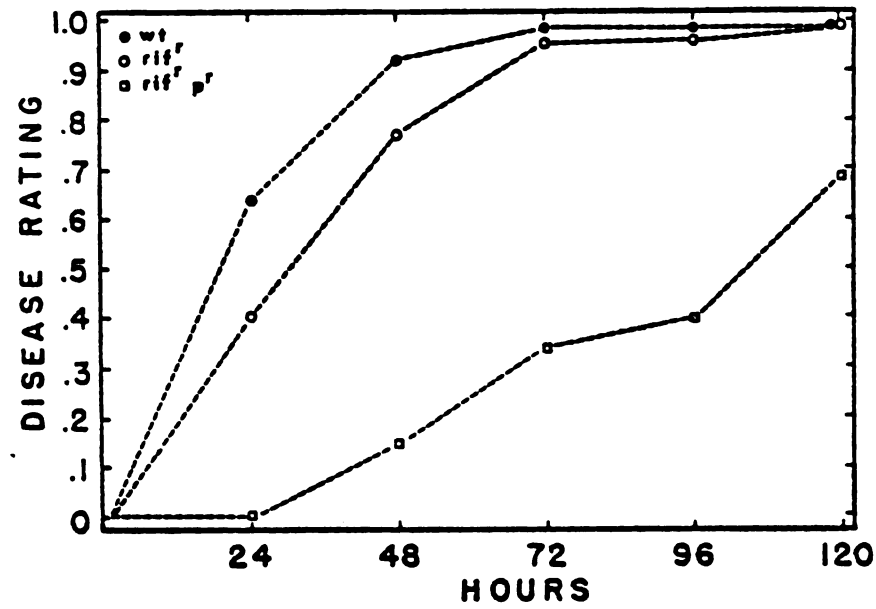

Figure 7. Rate of symptom development of wild type (w.t.), rifampin-resistant (rif^r), and bacteriophage PEal(h)-resistant ($rif^r p^r$) strains of *Erwinia amylovora*. Results are the means of eight different strains and two separate experiments.

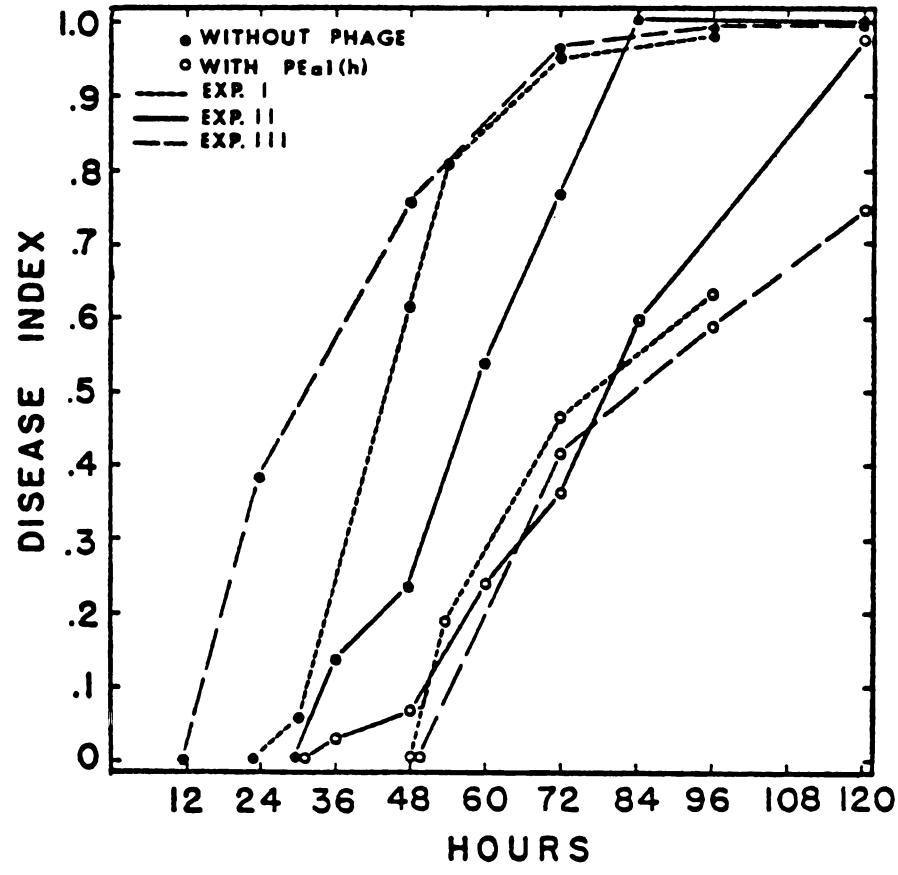

Figure 8. Rate of symptom development following inoculation of seedlings with *Erwinia amylovora* and a mixture of *E. amylovora* and bacteriophage PEa1(h).

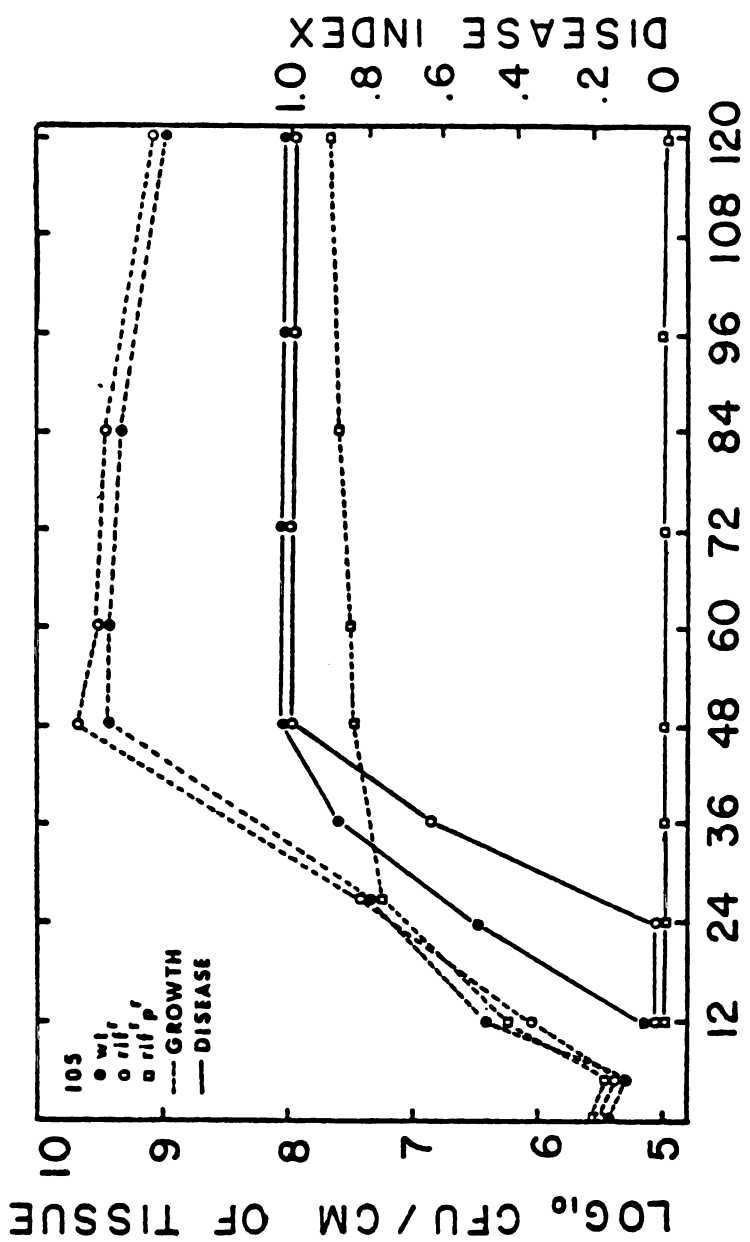

Figure 9-A. Growth and disease development of *Erwinia amylovora* 105 in apple seedlings. W.t. is capsulated, wild type, rif^r is wild type with rifampin resistance, and $rif^r p^r$ is rifampin resistant and phage PEa1(h) resistant.

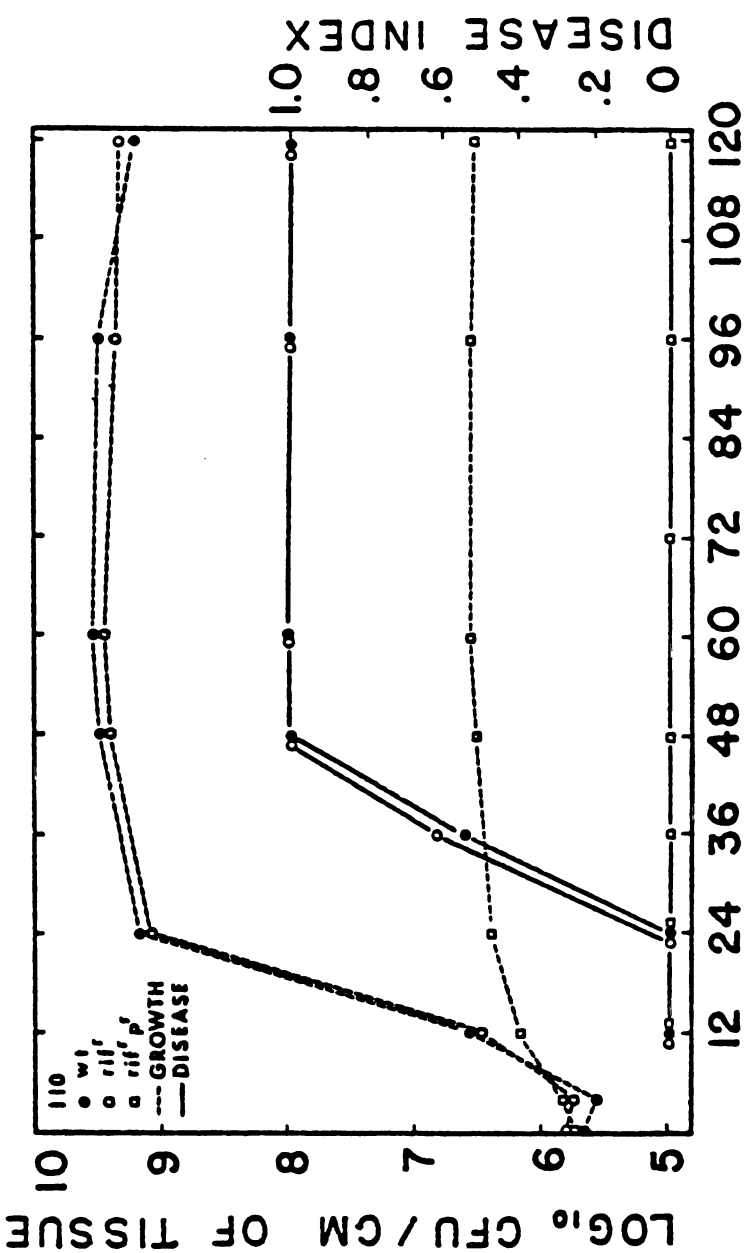

Figure 9-B. Growth and disease development of *Erwinia amylovora* 110 in apple seedlings. W.t. is capsulated, wild type, rif^r is wild type with rifampin resistance, and $rif^r p^r$ is rifampin resistant and phage PEa1(h) resistant.

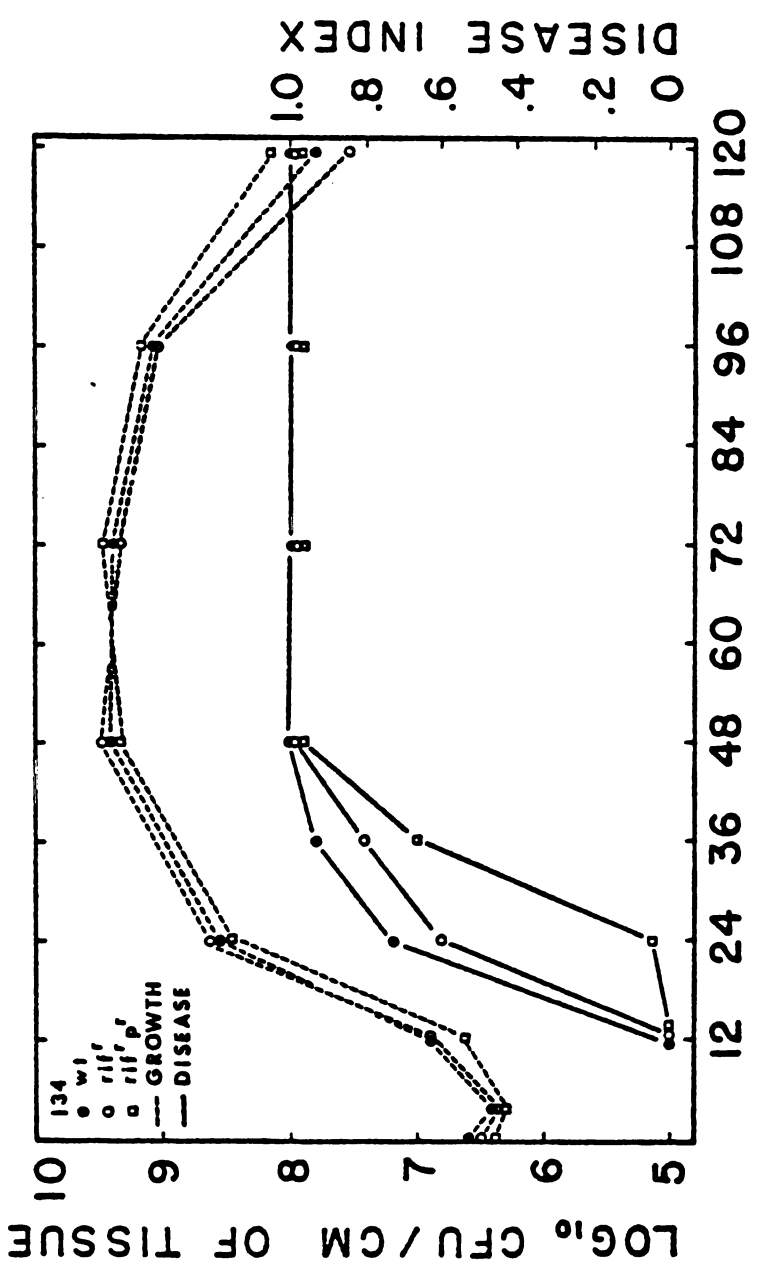

Figure 9-C. Growth and disease development of *Erwinia amylovora* 134 in apple seedlings. W.t. is capsulated, wild type, rif^r is wild type with rifampin resistance, and $rif^r pr$ is rifampin resistant and phage PEa1(h) resistant.

inoculated with the PEa1(h)-resistant strains exhibited symptoms. The PEa1(h)-resistant strain of 134 exhibited a growth curve similar to that of wild type 134 and all of the seedlings developed symptoms within 48 hr of inoculation (Figure 9C). Acapsulated strains 104 and 119 supported growth of PEa1(h) to a lesser extent then the capsulated 110 rif^r and E9 strains (Table 11). They showed no reduction in rate of symptom development compared to the capsulated and acapsulated strains of 110 rif^r (110 $rif^r p^r$) and E9(E8) (Table 12). Phage PEa7 could infect and grow on capsulated and acapsulated strains but approximately 100-fold less on acapsulated 104 and 119 then on 110 $rif^r p^r$ and E8 (Table 11).

Hypersensitive reaction in tobacco. *Erwinia amylovora* strains resistant to phage induced the typical hypersensitive response when infiltrated into tobacco leaves (Table 13).

Effect of phage PEa1(h) purification on the titer of *Erwinia amylovora* capsule degrading factor. The titer of the capsule degrading factor decreased as phage PEa1(h) was purified in sucrose and cesium chloride (CsCl) gradients (Table 14). The factor was still detectable after 24 hr of centrifugation in a 50% CsCl gradient.

Table 11. Ability of bacteriophages PEa1(h) and PEa7 to form plaques on several capsulated and acapsulated strains of *Erwinia amylovora*.

Erwinia amylovora strain	Presence of capsule	Bacteriophage PEa1(h) (plaque-forming-units/ml)	Bacteriophage PEa7 (plaque-forming-units/ml)
110 rif^r	+	8.2×10^7	5.5×10^7
110 $rif^r p^r$	-	0	2.0×10^7
E9	+	3.3×10^7	5.6×10^7
E8	-	0	4.2×10^7
119	-	2.4×10^5	4.0×10^5
104	-	1.0×10^6	9.7×10^5

Table 12. Symptom production by several capsulated and acapsulated strains of *Erwinia amylovora*.

Erwinia amylovora strain	Rate of symptom development[a/]									
	Capsulated					Acapsulated				
	[b/] 24	30	36	48	60	24	30	36	48	60
110 rif^r	.40	.80	.80	1.00	1.00					
110 $rif^r p^r$						.20	.20	.20	.40	1.00
E9	.40	1.00	1.00	1.00	1.00					
E8						.00	.20	.40	.60	.60
119						.75	.75	.80	.80	1.00
104						.40	.80	.80	.80	1.00

[a/] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate not present.

[b/] Hours after inoculation of seedlings. Bacteria (ca. 5×10^8 cfy/ml) were suspended in 0.02 M potassium phosphate buffer, pH 6.8, and 0.1 ml inoculated at point of injury.

Table 13. Ability of wild type (w.t.), rifampin-resistant (rif^r), and rif^r-PEal(h)-resistant ($rif^r p^r$) *Erwinia amylovora* strains to the hypersensitive reaction in tobacco leaves.

Erwinia amylovora strain	Reaction in tobacco		
	w.t.	rif^r	$rif^r p^r$
105	+[a/]	+	+
110	+	+	+
112	+	+	+
113	+	+	+
115	+	+	+
118	+	+	+
121	+	+	+

Sections of tobacco leaves were infiltrated with ca. 5×10^7 cfu/ml of bacterial suspension (0.02 M potassium phosphate buffer, pH 6.8).

[a/] + indicated hypersensitive response.

Table 14. Effect of bacteriophage PEa1(h) purification on the titer of the *Erwinia amylovora* capsule degrading factor.

Purification step[a/]	Titer at which clearing on mature lawns of *Erwinia amylovora* 110 rif^r occurs after 24 h at 23 C.
1. Chloroformed lysate centrifuged 10 min at 10,000 rpm	32
2. One-cycle of linear 10-40% sucrose-density-gradient centrifugation for 1 hr at 22,000 rpm	16
3. Two-cycles of linear 10-40% sucrose-density-gradient centrifugation for 1 hr at 22,000 rpm	4
4, Isopynic centrifugation in 50% (w/v) cesium chloride for 24 hr at 37,500 rpm	1

[a/] Detailed procedures given in Section II of dissertation under MATERIALS AND METHODS — Phage growth and purification.

DISCUSSION

Certain strains of bacteriophages which infect mucoid, capsulated strains of bacteria produce plaques surrounded by haloes or zones in which the bacteria are not lysed but the bacterial layer has become translucent and thinner than the surrounding normal bacterial growth (1,7,25,40). This expanding, translucent halo has been shown to be the result of phage-induced enzymes that hydrolyse the bacterial capsular expolysaccharides (7,45). A similar phenomenon occurs with many of the *E. amylovora* strains and most of its bacteriophage strains (Table 1). Evidence for such an enzyme-substrate reaction is also suggested by the direct relationship between the increase in temperature and increase in the rate of halo expansion (Figure 2-B).

Bacterial strains 104 and 119 did not have a detectable capsule whereas all the other strains were capsulated. Capsule degrading factor could not be detected in a PEa1(nh) + 110 rif^r lysate (chloroform-

sterilized) using the spot test (Table 7). Possibly, PEa1(nh) cannot direct the synthesis of or produce an active form of the factor. Such a non-halo mutant infecting *Pseudomonas aeruginosa* has been reported (7). Thus, for the production of an expanding, translucent halo the bacterial host must be capsulated and the infecting phage must direct the synthesis of an active form of the capsule degrading factor.

Synthesis of the factor was linked to phage multiplication. Phage PEa1(h) was temperature sensitive, being unable to multiply at 30 C or greater and slowly to 10 C (Figure 6, Section II). Halo formation also did not occur at 0 and 35 C (Figure 2-A). Capsule degrading factor was not detected in chloroform -sterilized *E. amylovora* cultures in which PEa1(h) had not multiplied (Table 7 and Figure 6).

Bacterial cells from colonies showing altered morphology (Figure 3) or grown in the presence of PEa1(h) (Figure 6) were acapsulated. Thus, phage PEa1(h) or a phage product (capsule degrading factor) was responsible for removal of the capsule resulting in the vitreous, non-mucoid colony. The capsule degrading factor prevented the build-up of capsule exopolysaccharides surrounding the bacterial cell but did not affect the ability of *E. amylovora* to multiply. This explains part of the O.D. difference

between 110 rif^r and 110 rif^r + PEa1(h) cultures, yet the acapsulated cells in 110 rif^r + PEa1(h) grew logarithmically once the capsule was removed (Figure 6).

The high mutation frequencies shown in Table 9 were not due to a spontaneous mutation since there was no significant difference in fluctuation among the individual cultures compared to the samples from the same culture (Table 10). The resistance to phage occurred following exposure to the phage thus the resistance was adaptational (35). Phage PEa1(h) adsorbed to capsulated strains of *E. amylovora* (Figure 8, Section II) suggesting that the adsorption site was located on the capsule. Removal of the capsule by capsule degrading factor would explain the high frequency and adaptational nature of resistance to PEa1(h). Removal from the factor such as streaking on NAG plates allows the formation of the capsule resulting in rapid reversion to wild type characteristics (Table 3). A similar situation was shown to occur with *Shigella dysenteriae* strain 136-R4 and bacteriophage T7 (32).

Incubation of capsulated *E. amylovora* and PEa1(h) at 30 C or greater (Table 4) or in the presence of PEa1(h) antiserum (Table 6) prevented the establishment of p^r type colonies. These two factors prevented the infection and growth of PEa1(h) (Section II). Thus, a

factor that prevented the infection and growth of PEa1(h) also prevented the establishment of p^r type colonies.

Failure to obtain p^r type colonies following incubation of capsulated *E. amylovora* and PEa1(h) in NBGYE (Table 5) may have been due to the dilution and plating process removing the bacteria from the factor and phage. Even though the cells were acapsulated in the NBGYE, when plated on NAG in the absence of the factor and phage they formed capsules resulting in wild type growth.

There was also a high frequency of resistance to PEa1(nh) but no capsule degrading factor was detected using the spot test (Table 7) nor was it observed that a PEa1(nh) lysate could remove the capsule (Table 8). Exposure of capsulated *E. amylovora* to PEa1(nh) on NAG resulted in vitreous, non-mucoid colonies indicating capsule removal. Possibly, infection by PEa1(nh) resulted in synthesis of a small, non-detectable (with the spot test) quantity of the factor requiring more than 60 min to remove a detectable amount of capsule.

The frequency of resistance to PEa7 was greater than for PEa1(h) or PEa1(nh) (Table 9). Phage PEa7, although able to adsorb to capsulated strains, adsorbed more rapidly to acapsulated strains (Figure 8, Section II) suggesting that its adsorption site lies

beneath the capsule. A possible explanation of the resistance to PEa7 may be its inability to penetrate the bacterial capsule or by the time penetration is accomplished the cell is unable to support phage growth. This hypothesis also may account for some of the PEa1(nh) resistance.

Capsule degrading factor is probably located in the spike-like tail structure of the phage as serologically shown for a similar phage (7). The data in Table 14 also indicate a close association of the factor with the PEa1(h) virion. One of the functions of this factor may be to allow the virion to penetrate the capsule to the cell wall where the nucleic acid can be injected into the bacterium. Such a tunneling effect was shown for *Klebsiella* phage 29 (8). Evidence that the factor enables the virion to diffuse more easily through capsulated bacterial masses is shown by the ability of phages to diffuse from the true plaque in association with the halo expansion (Table 2).

Many species of Gram-positive and -negative bacteria produce extracellular polysaccharides in the form of capsules and slime (45). For some pathogenic bacteria these non-toxic extracellular polysaccharides are important for the invasiveness of the bacteria by preventing phagocytosis (2,34,51). Extracellular

polysaccharides and glycopeptides are also produced by plant pathogenic bacteria. At least three species of *Corynebacterium* have been reported to produce glycopeptides (39), while *Xanthomonas phaseoli* (31), and *Pseudomonas solanacearum* (26) produce extracellular polysaccharides that may play a role in pathogenesis. All strains of *P. solanacearum* which failed to produce extracellular slime were avirulent (26,43). *Erwinia amylovora* also produces extracellular polysaccharide in the form of a capsule as shown by the India ink stain. A host-specific toxic polysaccharide from *E. amylovora*-infected apple tissue was reported (13,18,22). The exact involvement of this polysaccharide in the pathogenesis of fire blight is not completely understood (18,22,44). Even though there are many pathogenic bacteria which produce extracellular polysaccharides there are also many nonpathogens which produce these compounds.

The interaction between a capsulated, virulent strain of *E. amylovora* (E9) and an acapsulated, avirulent strain (E8) in apple tissue has been studied (22,23,24). It was reported that these two strains were serologically similar, produced typical *E. amylovora* colonies on Cross & Goodman and Miller & Schroth media, and induced a hypersensitive reaction in tobacco (24). Growth of E8 and E9 on NAG and TTN

media was very different; E9 produced mucoid, wild type growth while E8 produced non-mucoid, p^r type growth. A serological difference between wild type and p^r type bacteria was detected, indicating that resistance to PEa1(h) resulted in an antigenic change in *E. amylovora*. Goodman *et al.* (23,24) reported that E8 was localized and unable to multiply and become systemic while E9 could. This suggested that the capsule protected the bacterial cells from being agglutinated and bound by the plant cells. Such phenomena were described for *Rhizobium* (10) in the nodulation process and for *Agrobacterium* (50) in gall formation. Binding in these systems leads to a symbiotic relationship or gall formation; whereas, with *P. solanacearum* (43), *P. pisi* (19), and *E. amylovora* (23,24) binding leads to incompatibility resulting in avirulence for the pathogen.

Exposure of wild type *E. amylovora*, including E9, to phage PEa1(h) resulted in the induction and selection of bacteria phenotypically similar to E8. The reduction in symptom development exhibited by the p^r type bacteria may be the result of a mechanism similar to that described by E8 (23,24). Evidence for localization and reduction of bacterial multiplication of p^r type cells in host tissue was shown for strains 105 $rif^r p^r$ and 110 $rif^r p^r$ (Figure 9A & B). Use of the seedling technique (41) for virulence assays showed

that none of the p^r type bacteria including E8 were completely avirulent (Table 12). The tissue generally used has been fruits or potted trees (18,22,23,24,44), possibly the seedling stage may have been more susceptible.

The ability of strains 134 $rif^r p^r$, 104, and 119 (acapsulated strains) to multiply and produce symptoms similar to capsulated strains cannot be completely explained with the available data. Possibly, these strains lack a binding site for the plant cell to recognize or capsular polysaccharide is present but not detectable with the India ink stain and is covering the binding site.

Erskine also found that phage-resistant mutants of *E. amylovora* exhibited reduced virulence (15). He suggested that the mutation to phage resistance was associated with a change in cell wall permeability so that the enzymes and/or toxic substances responsible for virulence passed through the bacterial cell wall at a reduced rate. Results with strain 134 $rif^r p^r$ would contradict this hypothesis. Whatever the mechanism involved, the data consistently showed that *E. amylovora* resistant to or in the presence of PEa1(h) type phages produced a delay in symptoms.

PEa1(h) type phages and the capsule degrading factor may be useful in inducing and selecting

acapsulated, virulently reduced strains of *E. amylovora* similar to E8.

Erwinia amylovora and its phages were detected in close association on aerial apple structures (Section I). It is thus reasonable to assume that some of the effects observed in the laboratory could occur under natural conditions. In the presence of phage, *E. amylovora* was at least attenuated in symptom induction. Removal from phage resulted in capsulation and restoration of normal rate of symptom development which would correspond to outbreaks of fire blight. The existence of *E. amylovora* in the acapsulated state does not, at least in the laboratory, affect the ability of the bacteria to survive, except it exists more as an attenuated pathogen than a pathogen. The acapsulated strains were still capable of inducing the hypersensitive response in tobacco (Table 13), indicating that these strains have not been reduced to the level of a saprophyte or nonpathogen (28). It is possible that in the presence of phage and the capsule degrading factor *E. amylovora* may exist much like an epiphyte.

It is not to be assumed that moisture, temperature, and the physiological condition of the host plant are not important in the development of fire blight. Yet, microbial interactions and processes at the microscopic

or submicroscopic level may become very important when magnified to the macroscopic level.

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PART IV

SOME PROPERTIES OF THE BACTERIOPHAGE PEa1(h)-ASSOCIATED CAPSULE DEGRADING FACTOR

INTRODUCTION

Synthesis of bacteriophage directed enzymes occurs soon after the insertion of the phage nucleic acid into the bacterial cell. The majority of these enzymes are primarily involved in the synthesis of various structural components of the progeny phage (5). Other enzymes also may be synthesized which become associated with the mature phage particle; among these enzymes are the depolymerases that act on the bacterial capsule and slime layers (6). Phage-associated capsule degrading enzymes have been shown for phages infecting *Klebsiella* (11,14), *Escherichia coli* (17), *Aztobacter* (8), *Pseudomonas* (3), and bacteria in a few other genera (18). The exact role of these enzymes is not known, but it has been postulated that they assist the phage in reaching its underlying bacterial cell wall receptor, aid in the release of phage from infected cells, and are involved in the specific molecular interaction required for phage adsorption (5,13,15). A capsule degrading factor (CDF) was found associated with phage PEa1(h) following infection of *Erwinia amylovora* (Section III).

This section describes some of the properties of a crude preparation of this CDF and its effect on *E. amylovora* sensitivity to streptomycin.

MATERIALS AND METHODS

Source of capsule degrading factor. *Erwinia amylovora* strain 110 rifampin resistant (rif^r) grown in 0.8% (w/v) nutrient broth, 0.5% (w/v) glucose, and 0.25% (w/v) yeast extract (NBGYE), was inoculated with approximately 5 x 10^8 plaque-forming-units (pfu)/ml of PEa1(h) during the exponential growth stage (ca. 5 x 10^8 colony-forming-units [cfu]/ml). The culture was incubated at 23 C on a reciprocal shaker (80 oscillations/min) for 12 hr, sterilized by adding chloroform to 1.0% (v/v), and after 30 min centrifuged at 12,100 g for 10 min to remove bacterial debris. The supernatant represented the crude preparation of the CDF which was stored over chloroform at 4 C.

Capsule degrading factor. The presence and titer of the CDF was assayed by the spot method (2). The agar overlay method (1) was used to obtain the CDF substrate. Mature lawns of *E. amylovora* 110 rif^r were obtained in incubation of the agar overlay plates at 27 C for 48 hr, the plates were stored at 4 C until required.

Effect of capsule degrading factor on different bacteria strains. Twenty-five strains of bacteria, including 11 *E. amylovora* strains were grown in 25 ml of NBGYE for 18-24 hr. One-tenth ml of the culture was added to molten top-agar and layered over the bottom layer, after solidification a loopful of the crude CDF was spotted on the surface and the plates incubated at 27 C. After 24 hr, lysis or no lysis of the bacterial lawn was recorded and the plates incubated an additional 24 hr at 23 C. Following this 48 hr incubation, a loopful of the crude CDF was again spotted on the mature lawns. The presence or absence of a translucent zone or depression in the bacterial lawn was recorded after 12 hr at 23 C.

Thermal inactivation of the capsule degrading factor. Five-tenths ml of the crude CDF were pipetted into thin-walled glass tubes (1.5 cm diameter) and placed in a preheated water bath for 10 min at temperatures of 25-95 C ± 1 C. After 10 min, the tubes were immediately placed in an ice bath. Two-fold dilutions were made in 0.02 M potassium phosphate buffer, pH 6.8 (PPB) and a loopful of each dilution spotted on 2-week old lawns of *E. amylovora* 110 rif^r.

Effect of antisera on capsule degrading factor activity. Bacteriophage PEa1(h) and PEa7 antisera were prepared as described in Section II while

E. amylovora antiserum preparation is described in Section III. The antisera were diluted 1:50 in PPB, 0.5 ml was mixed with 0.5 ml of the crude CDF, and mixtures were incubated at 23 C for 10 min. Two-fold dilutions were made in PPB and assayed for CDF activity.

Effect of crude capsule degrading factor on *Erwinia amylovora* sensitivity to streptomycin sulfate. Two types of experiments were done to determine the effect of using the phage PEa1(h)-associated CDF for removal of the *E. amylovora* capsule and its sensitivity to streptomycin sulfate (streptomycin sulfate 740 mg/g, Chas. Pfizer and Co., Inc., New York, NY). The first experiment used a disc assay procedure with 12.7 mm diameter antibiotic discs (VWR Scientific, S and S No. 740E). The antibiotic discs were saturated with different concentrations of streptomycin sulfate dissolved in PPB. These discs were placed on the surface of the top-agar which had been seeded with 0.1 ml of an 18 hr old NBGYE culture of 110 rif^r (ca. 10^9 cfu/ml) or 110 rif^r plus 0.1 ml of the CDF. After 24 hr incubation at 27 C, the inhibition zones were measured to ± 1.0 mm.

A second experiment was designed to determine if loss of the bacterial capsule would cause an increase in the rate of streptomycin uptake and thus a more

rapid killing of the cells. One ml (ca. 10^9 cfu/ml) of an 18 hr old NBGYE culture of 110 rif^r was added to two flasks of 25 ml each of fresh NBGYE. To one flask was added 1.0 ml of the crude CDF preparation and the flasks incubated 4 hr with shaking. Ten ml of the cultures were added to four, sterile 125-ml flasks, two flasks for the 110 rif^r culture and two flasks for the 110 rif^r plus CDF. To one flask in each set was added 10 ml of 100 µg/ml of streptomycin sulfate in PPB, thus giving a final concentration of 50 µg/ml, to the two remaining flasks were added 10 ml of PPB. The number of cfu/ml in the non-streptomycin inoculated flasks was determined by dilution and plating on 2.0% (w/v) nutrient agar plus 0.5% (w/v) glucose and taken as the cfu at time zero. In experiment I, samples were taken over a 120-min period while in experiment II samples were taken over a 60-min period.

RESULTS

Source of capsule degrading factor. The method used to obtain the crude factor preparation yielded factor having a titer of 32-64 assayed with the spot test. The enzyme remained active when stored at 4 C.

Effect of the capsule degrading factor on different bacterial strains. Phage PEa1(h) plus 110 rif^r lysate and PEa1(h) plus 110 $rif^r p^r$ lysate could lyse all the *E. amylovora* strains except 110 $rif^r p^r$. The two *E. herbicola* strains also showed areas of lysis but none of the other bacterial strains were affected (Table 1). Capsule polysaccharide degradation was shown only with the PEa1(h) plus 110 rif^r lysate and affected only the *E. amylovora* and *E. herbicola* strains but not *E. amylovora* 110 $rif^r p^r$ nor any of the other strains (Table 1). The supernatant from 110 $rif^r p^r$ showed no lytic or CDF activity.

Thermal inactivation. The CDF could be heated for 10 min at 75 C without a decrease in titer; however, heating for 10 min at 85 C caused complete loss of CDF activity (Table 2).

Table 1. Effect of capsule degrading factor on different bacterial strains.

Bacterial Strain	[a] Lytic Effect			Capsule Degrading Effect		
	[b] 110 rif^r	[c] PEal(h) + 110 rif^r	[d] PEal(h) + 110 $rif^r p^r$	110 rif^r	PEal(h) + 110 rif^r	PEal(h) + 110 $rif^r p^r$
Erwinia amylovora						
105 rif^r	[e] -	+	+	[f] -	+	-
110 rif^r	-	+	+	-	+	-
110 $rif^r p^r$	-	-	-	-	-	-
112 w.t.	-	+	+	-	+	-
115 w.t.	-	+	+	-	+	-
118 w.t.	-	+	+	-	+	-
121 w.t.	-	+	+	-	+	-
125 w.t.	-	+	+	-	+	-
126 w.t.	-	+	+	-	+	-
131 w.t.	-	+	+	-	+	-
138 w.t.	-	+	+	-	+	-
Erwinia carotovora var. *atroseptica*						
108	-	-	-	-	-	-
Erwinia chrysanthemi						
140	-	-	-	-	-	-

Table 1 (continued).

E. chrysanthemi (continued)						
141	-	-	-	-	-	-
Erwinia herbicola						
130	-	+	+	-	+	-
150	-	+	+	-	+	-
Pseudomonas syringae						
203	-	-	-	-	-	-
Pseudomonas solanacearum						
215	-	-	-	-	-	-
Pseudomonas tomato						
216	-	-	-	-	-	-
Pseudomonas fluorescens						
214	-	-	-	-	-	-
Xanthomonas pruni						
303	-	-	-	-	-	-
Xanthomonas pelargonii						
305	-	-	-	-	-	-
Agrobacterium tumefaciens						
401	-	-	-	-	-	-
Corynebacterium fasciens						
501	-	-	-	-	-	-
Corynebacterium michiganense						
502	-	-	-	-	-	-

Table 1 (continued).

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a/ Lytic effect was the ability of the lysate to produce a clear area on the young, growing bacterial lawn, whereas capsule degrading effect was the ability to produce a depression or translucent zone on bacterial lawns 48 hr or older.

b/ 110 rif^r was a 12 hr old culture which was chloroform-sterilized and the bacterial debris pelleted by centrifugation.

c/ PEa1(h) + 110 rif^r was a 12 hr old culture of phage PEa1(h) grown on 110 rif^r then chloroform-sterilized and the bacterial debris pelleted by centrifugation. The supernatant contained a capsule degrading factor which could be detected at a 1:64 dilution.

d/ PEa1(h) + 110 $rif^r p^r$ was a 12 hr old culture of phage PEa1(h) incubated with 110 $rif^r p^r$ [*E. amylovora* strain resistant to PEa1(h)] chloroform-sterilized and bacterial debris pelleted by centrifugation. Phage PEa1(h) could be detected in the supernatant but no capsule degrading factor could be detected.

e/ "-" indicates no area of bacterial lysis while "+" indicates area of bacterial lysis.

f/ "-" indicates no detectable capsule degradation while "+" indicates a depression or translucent zone in the mature bacterial lawn indicating the presence of capsule degrading factor.

Table 2. Thermal inactivation of bacteriophage PEa1(h)-associated capsule degrading factor.

Temperature of heating for 10 min	[a] Titer of factor
25	64
45	64
55	64
65	64
75	64
85	0
95	0

[a] Reciprocal of the greatest dilution at which a depression could be detected in mature lawns of *Erwinia amylovora* 110 rif^r after 24 hr of incubation at 23 C.

Table 3. Effect of bacteriophage and *Erwinia amylovora* antisera on the activity of phage PEa1(h)-associated capsule degrading factor.

Titer of Factor								
Erwinia amylovora antiserum (As)			PEa1(h) antiserum (As)			PEa7 antiserum (As)		
Normal serum	Before As	After Ss	Normal serum	Before As	After As	Normal serum	Before As	After As
[a] 32/64	32/64	32/64	32/64	32/64	none	32/64	32/64	32/64

[a] Reciprocal of the greatest dilution producing a translucent zone/depression on the mature lawns of *E. amylovora* 110 rif^r after 12 hr at 23 C.

Effect of antisera on capsule degrading factor activity. Phage PEa1(h) antiserum completely inhibited the CDF activity of the PEa1(h)-associated CDF (Table 3). Antisera to *E. amylovora* 110 rif^r, phage PEa7, and normal sera had no effect on the CDF activity (Table 3).

Effect of capsule degrading factor on *Erwinia amylovora* 110 rif^r sensitivity to streptomycin sulfate. Larger zones of inhibition were produced on plates to which the PEa1(h)-associated CDF was added (Table 4). Streptomycin at 25 µg/ml produced larger zones of inhibition on 110 rif^r plus the CDF than did 100 µg/ml on 110 rif^r alone (Table 4).

The addition of 50 µg/ml of streptomycin to 110 rif^r plus CDF culture showed no viable bacteria within 30 min, while the culture without the CDF showed no viable bacteria within 60 min (Table 5, Exp. I). In experiment II (Table 5) no cfu were detected within 15 min in the 110 rif^r CDF streptomycin culture while 30 min was required without the CDF. In both experiments I and II, incubation with the PEa1(h)-associated CDF alone did not reduce the viability of the number of *E. amylovora* cells.

Table 4. Results of disc assay of streptomycin sulfate on *Erwinia amylovora* 110 rif^r and *E. amylovora* 110 rif^r - associated capsule degrading factor (CDF).

Streptomycin sulfate concentration (µg/ml)	Diameter of zones (mm)	
	110 rif^r	110 rif^r + CDF
0	0	0
25	15.0	19.0
50	16.0	20.0
100	18.0	22.0

a/ Mean of two replications; no measureable variation between the two replications.

Table 5. Effect of bacteriophage PEa1(h) - associated capsule degrading factor (CDF) on the uptake of streptomycin sulfate by *Erwinia amylovora* 110 rif^r.

Experiment I

Time after addition of streptomycin (min)	Number of colony-forming-units/ml			
	110 rif^r alone (10^7)	110 rif^r plus 50 µg/ml of streptomycin	110 rif^r plus PEa1(h) - associated CDF (10^7)	110 rif^r plus PEa1(h) - associated CDF plus 50 µg/ml of streptomycin
0	17	17 x 10^7	20	20 x 10^7
5	15	10 x 10^7	18	10 x 10^7
15	16	26 x 10^6	20	43 x 10^5
30	11	1 x 10^5	19	[a] n.d.
60	31	n.d.	25	n.d.
120	58	n.d.	23	n.d.

[a] n.d. = not detected.

Table 5 (continued).

Experiment II

Time after addition of streptomycin (min)	Number of colony-forming units/ml			
	110 rif^r alone (10^7)	110 rif^r plus 50 µg/ml of streptomycin	110 rif^r plus PEa1(h) - associated CDF (10^7)	110 rif^r plus PEa1(h) - associated CDF plus 50 µg/ml of streptomycin
0	31	31×10^7	32	32×10^7
5	19	30×10^7	20	33×10^6
10	59	15×10^6	20	[a] n.d.
15	45	75×10^5	18	n.d.
20	22	40×10^3	26	n.d.
30	25	n.d.	35	n.d.
40	20	n.d.	24	n.d.
50	21	n.d.	25	n.d.
60	25	n.d.	23	n.d.

[a] n.d. = not detected.

DISCUSSION

Relatively few enzymes that hydrolyse the bacterial polysaccharide capsule material and slime layer have been isolated or characterized (18). One of the best sources of such enzymes has been certain phage-infected bacteria (3,8,11,14,17,18). Bacterial specificity of these enzymes has ranged from strain specificity (1,11) to hydrolysis of the extracellular polysaccharides of bacteria in different genera (18). The CDF produced following the lysis of *E. amylovora* 110 rif^r by phage PEa1(h) was quite specific for *E. amylovora* capsular polysaccharide. Exceptions were the *E. herbicola* strains. Failure to detect CDF in the 110 $rif^r p^r$ + PEa1(h) combination was the result of 110 $rif^r p^r$ resistance to PEa1(h). Lysis of the young lawns by this combination was the result of the unadsorbed phage.

Capsule depolymerases have been useful in fractionating bacterial capsular polysaccharides, thus making it possible to study their structure (18). With the implication of extracellular polysaccharides in the pathogenesis of some phytopathogenic bacteria (12,16),

including *E. amylovora* (9,10), PEa1(h)-associated CDF may be useful in not only better understanding the capsular composition but also the pathogenesis of *E. amylovora*.

The differential in the thermal inactivation of phage PEa1(h) (Section II) and PEa1(h)-associated CDF suggested a method of phage separation for the CDF similar to that described by Adams and Park (2). A preliminary experiment indicated that 10 min of 75 C did not eliminate the PEa1(h) infectivity. This may have been the result of the high phage titer (ca. 10^{10} pfu/ml). The CDF could probably be purified and concentrated using the scheme outlined for the depolymerase of *Pseudomonas aeruginosa* phage 2 (4).

The specific inhibition of the CDF by phage PEa1(h) antiserum suggested that the CDF was of PEa1(h) origin and not a factor occurring in uninfected *E. amylovora*. Phage PEa7 also produced a capsular degrading factor as indicated by the translucent halo surrounding the plaque (Section II). PEa7 antiserum did not inhibit the PEa1(h)-associated CDF indicating the two factors were different even though both were synthesized in the same bacterial strain. This also supports the hypothesis that synthesis of the factor was under control of the phage genome.

The larger zone of inhibition produced by streptomycin on the *E. amylovora* 110 rif^r plus CDF plates could have resulted from better diffusion of the anitbiotic. The CDF may have enhanced the diffusion by degradation of the bacterial extracellular polysaccharide.

The more rapid killing of the 110 rif^r cells incubated with streptomycin plus the CDF than with streptomycin alone indicated a more rapid uptake by the acapsulated cells. The capsule may serve as a protective barrier against the rapid uptake of some chemicals. This also suggested the CDF, if active under orchard environments, might be useful to make *E. amylovora* more sensitive to chemotherapeutic agents.

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APPENDICES

APPENDIX A

Table A1. Bacterial strains and their source

Laboratory I.D. Number	
400	*Agrobacterium tumefaciens*. U.S., Upjohn Company.
401	*A. tumefaciens*. UC 78, Upjohn Company.
10	*Bacillus cereus* var. *mycoides*. Pfizer Company.
501	*Corynebacterium fasciens*. ATCC 13000.
500	*C. flaccumfaciens*. Source unknown.
502	*C. michiganense*. Tomato fruit, Michigan, 1976.
12	*Enterobacter aerogenes*. MSU Dept. of Microbiology, 1975.
104	*Erwinia amylovora*. Ea1, Pear blossoms, California, 1974.
105	*E. amylovora*. III. 68, Illinois, apple.
110	*E. amylovora*. Jonathan canker, MSU, February 1975.
111	*E. amylovora*. Bartlett canker, MSU, February 1975.
112	*E. amylovora*. Greening apple canker. Old Mission, Michigan, March 1976.
113	*E. amylovora*. Bartlett canker, Tome, Grand Rapids, Michigan, February 1975.
114	*E. amylovora*. Idared canker, Clayton, Grand Rapids, Michigan, February 1975.
115	*E. amylovora*. Jonathan canker, Carpenter, Paw Paw, Michigan, February 1975.
116	*E. amylovora*. Bartlett canker, Spinks Corners, Michigan, March 1975.
117	*E. amylovora*. Ea5, Pear blossom, California, 1974, streptomycin resistant.
118	*E. amylovora*. Ea38, Pear blossom, California, 1974, streptomycin resistant.
119	*E. amylovora*. Mac715, McIntosh terminal, MSU.
120	*E. amylovora*. EACC512, apple, N. Carolina, 1975.
121	*E. amylovora*. EA518, apple, N. Carolina, 1975.
122	*E. amylovora*. Apple canker, Webb orchard, Paw Paw, Michigan, March 1976.

Table A1 (continued).

123	*E. amylovora.*	Jonathan blighted terminal, MSU, May 1976.
124	*E. amylovora.*	Blighted Jonathan leaves, Carpenter, Paw Paw, Michigan, June 1976.
125	*E. amylovora.*	Blighted apple terminal, McLean, Hart, Michigan, 1976.
126	*E. amylovora.*	Bbl, Blackberry cane, Illinois, 1976.
127	*E. amylovora.*	Jonathan terminal, MSU, June 1976.
128	*E. amylovora.*	E9, Missouri, Goodman, virulent, capsulated.
129	*E. amylovora.*	E8, Missouri, Goodman, avirulent, acapsulated.
131	*E. amylovora.*	#1, Blighted Jonathan terminal, R.Baiers, Keeler, Michigan, June 1976.
132	*E. amylovora.*	#2, Blighted Jonathan terminal, Herman, Watervliet, Michigan, June 1976.
133	*E. amylovora.*	#3, Blighted Jonathan terminal, Aschroft, Lawrence, Michigan, June 1976.
134	*E. amylovora.*	#4, Blighted Jonathan terminal, Webb, Paw Paw, Michigan, June 1976.
135	*E. amylovora.*	#5, Blighted Greening terminal, Baiers, Keeler, Michigan, June 1976.
136	*E. amylovora.*	#6, Blighted Jonathan terminal, Hasell, Keeler, Michigan, June 1976.
137	*E. amylovora.*	#7, Blighted crabapple terminal, Mankey, Berrien Springs, Michigan, June 1976.
138	*E. amylovora.*	#8, Blighted Jonathan terminal, Mitchum, Hartford, Michigan, June 1976.
108	*E. carotovora*	var. *atroseptica*. SR8, Wisconsin, potato, 1969, Kelman.
106	*E. carotovora*	var. *carotovora*. SR165, Minnesota potato, 1973, Kelman.
140	*E. chrysanthemi*.	Corn, Berrien Springs, Michigan, 1977.
141	*E. chrysanthemi*.	Corn, Three Rivers, Michigan, 1977.
100	*E. herbicola.*	ZP-1, Dye, New Zealand.
101	*E. herbicola.*	ZP-2, Dye, New Zealand.
103	*E. herbicola.*	A-E, from apricot, MSU.
130	*E. herbicola.*	#7, Jonathan canker, 1976.

Table A1 (continued).

150	*E. herbicola*. Bartlett canker, MSU, 1977.
151	*E. herbicola*. Jonathan canker, MSU, 1977.
11	*Escherichia coli*. MSU, Microbiology Dept., 1975.
206	*Pseudomonas aeruginosa*. MSU, Microbiology Dept., 1975.
205	*P. fluorescens*. MSU, Microbiology Dept., 1975.
214	*P. fluorescens*. Pf-1, from pigweed, 1977.
209	*P. lachrymans*. Williams, Wisconsin, 1976.
200	*P. morsprunorum*. C28, cherry stem canker, England, 1960.
210	*P. solanacearum*. Tobacco, Phillipines, Sequeira, 1967.
215	*P. solanacearum*. Tomato, Georgia, 1977.
203	*P. syringae*. DH24, cherry canker, Michigan, 1976.
216	*P. tomato*. Pt-1, tomato, southwest Michigan, 1977.
13	*Rhizobium* sp. Source unknown.
14	*Serratia* sp. Contaminate in tissue culture, 1975.
301	*Xanthomonas campestris*. Cabbage, Michigan, 1974.
307	*X. campaestris*. PHW42, cabbage, Wisconsin, 1977.
302	*X. juglandis*. Diseased walnut, California, 1975.
303	*X. pruni*. PF-2, Michigan.
304	*X. vesicatoria*. Pepper plant from Georgia, 1976.

APPENDIX B

Table B1. Optimum concentration of polyethylene glycol (PEG), 6000 average molecular weight, for the precipitation of bacteriophages PEa1(h) and PEa7.

Percent PEG (w/v)	Total plaque-forming-units of PEa1(h)	
	Supernatant	Pellet
0	13×10^{11}	—
5	12×10^{11}	44×10^{9}
10	26×10^{10}	68×10^{10}
15	60×10^{9}	58×10^{10}
20	92×10^{7}	48×10^{10}

Percent PEG (w/v)	Total plaque-forming-units of PEa7	
	Supernatant	Pellet
0	50×10^{10}	—
5	32×10^{9}	36×10^{9}
10	96×10^{8}	22×10^{10}
15	11×10^{8}	17×10^{10}
20	27×10^{8}	16×10^{10}

Forty ml of crude phage lysate, chloroform-sterilized and the bacterial debris pelleted by centrifugation for 10 min at 12,000 g, were used per concentration of PEG tested. The appropriate concentration of PEG plus 0.5 M NaCl was added to the 40 ml of lysate and stirred for 1.5 hr at 4 C. The solutions were centrifuged at 12,100 g for 20 min, the supernatants decanted, and 2 ml of 0.02 M potassium phosphate buffer, pH 6.8, used to resuspend the pellets by setting at 4 C overnight.

APPENDIX C

Table C1. Symptom development by bacteriophage PEa1(h)-sensitive (wild type and rif^r) *Erwinia amylovora* and PEa1(h)-resistant *E. amylovora* (p^r).

Erwinia amylovora strain	[a] Rate of symptom development														
	Wild type					Rifampin resistant (rif^r)					Rif^r and PEa1(h)-resistant (p^r)				
	[b] 24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
105	[c] 1.00	1.00	1.00	1.00	1.00	.75	1.00	1.00	1.00	1.00	.00	.75	1.00	1.00	1.00
110	1.00	1.00	1.00	1.00	1.00	.75	.75	1.00	1.00	1.00	.00	.25	1.00	1.00	1.00
112	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.00	.25	.75	.75	1.00
113	1.00	1.00	1.00	1.00	1.00	.75	.75	1.00	1.00	1.00	.00	.25	.25	.75	1.00
115	1.00	1.00	1.00	1.00	1.00	.75	1.00	1.00	1.00	1.00	.00	1.00	1.00	1.00	1.00
118	.75	.75	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	.00	.00	.50	.50	1.00
121	.75	.75	1.00	1.00	1.00	.50	.75	1.00	1.00	1.00	.00	.00	.25	.25	1.00
Mean	.93	.93	1.00	1.00	1.00	.79	.89	1.00	1.00	1.00	.00	136	.68	.75	1.00

[a] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate not present.

[b] Hours after inoculation of seedlings with bacterial suspension of ca. 5×10^7 colony-forming-units.

[c] Mean of four replications.

APPENDIX C

Table C2. Symptom development by bacteriophage PEa1(h)-sensitive (wild type and rif^r) *Erwinia amylovora* and PEa1(h)-resistant *E. amylovora* (p^r).

Erwinia amylovora strain	[a] Rate of symptom development									
	Rifampin resistant (rif^r)					Rif^r and PEa1(h)-resistant (p^r)				
	[b] 24	48	72	96	120	24	48	72	96	120
105	[c] .00	.60	1.00	1.00	1.00	.00	.00	.00	.00	.00
110	.00	.80	.80	.80	1.00	.00	.00	.00	.00	.00
121	.00	.60	1.00	1.00	1.00	.00	.00	.60	.80	1.00
134	.00	1.00	1.00	1.00	1.00	.00	.60	.80	1.00	1.00
Mean	.00	.75	.95	.95	1.00	.00	.15	.35	.45	.50

[a] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate absent.

[b] Hours after inoculation of seedlings with bacterial suspension of ca. 5×10^6 colony-forming-units.

[c] Mean of five replications.

APPENDIX C

Table C3. Symptom development by bacteriophage PEa5(h)-sensitive (wild type) *Erwinia amylovora* and PEa5(h)-resistant *E. amylovora* (p^r).

Erwinia amylovora strain	[a] Rate of symptom development									
	Wild type					PEa5(h)-resistant (p^r)				
	[b] 24	48	72	96	120	24	48	72	96	120
105	[c] 1.00	1.00	1.00	1.00	1.00	.00	.00	.00	.00	.33
115	.00	.67	1.00	1.00	1.00	.00	.00	.00	.00	.33
121	.00	.00	.67	1.00	1.00	.00	.00	.00	.33	1.00
Mean	.33	.56	.89	1.00	1.00	.00	.00	.00	.11	.55

[a] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate not present.

[b] Hours after inoculation of seedlings with bacterial suspension of ca. 5×10^6 colony-forming-units.

[c] Mean of three replications.

APPENDIX C

Table C4. Simultaneous inoculation of apple seedlings with a mixture of *Erwinia amylovora* and bacteriophage PEa1(h) and the effect on symptom development.

Erwinia amylovora strain	[a] Rate of symptom development											
	E. amylovora without phage						*E. amylovora* with phage					
	[b] 36	48	60	72	84	120	36	48	60	72	84	120
[c] 105 rif^r	[e] .00	.00	.25	.46	1.00	1.00	.00	.00	.25	.33	.92	1.00
110 rif^r	.25	.33	.67	.83	1.00	1.00	.00	.00	.33	.50	.75	1.00
[d] 118 w.t.	.08	.33	.83	1.00	1.00	1.00	.00	.25	.33	.75	1.00	1.00
121 rif^r	.17	.25	.50	.83	1.00	1.00	.00	.00	.08	.08	.08	.83
134 rif^r	.17	.33	.42	.67	1.00	1.00	.08	.08	.17	.17	.25	1.00
Mean	.13	.25	.53	.76	1.00	1.00	.02	.07	.23	.37	.60	.97

[a] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate not present.

[b] Hours after inoculation of seedlings. Bacteria (5 x 10^8 cfu/ml) and phage PEa1(h) (5 x 10^8 pfu/ml) were mixed 1:1 in 0.02 M potassium phosphate buffer, pH 6.8, and allowed to incubate for 15 min, the seedlings were then inoculated with 0.01 - 0.05 ml of this suspension.

[c] rif^r = resistant to the antibiotic rifampin.
[d] w.t. = wild type.
[e] Mean of twelve replications.

APPENDIX C

Table C5. Simultaneous inoculations of apple seedlings with a mixture of *Erwinia amylovora* and bacteriophage PEa1(h) and the effect on symptom development.

Erwinia amylovora strain	[a] Rate of symptom development									
	E. amylovora without phage					*E. amylovora* with phage				
	[b] 30	48	54	72	96	30	48	54	72	96
[c] 105 rif^r	[e] .00	.25	1.00	1.00	1.00	.00	.00	.25	.25	.75
110 rif^r	.00	.75	1.00	1.00	1.00	.00	.00	.00	1.00	1.00
[d] 112 w.t.	.25	1.00	1.00	1.00	1.00	.00	.00	.25	.50	.75
113 w.t.	.00	.75	.75	.75	.75	.00	.00	.25	.75	.75
115 w.t.	.00	.75	1.00	1.00	1.00	.00	.00	.25	.75	.75
118 w.t.	.00	.25	.25	1.00	1.00	.00	.00	.50	.50	.50
121 rif^r	.00	.25	.50	.75	1.00	.00	.00	.00	.00	.00
134 rif^r	.25	1.00	1.00	1.00	1.00	.00	.00	.00	.00	.50
Mean	.06	.63	.81	.94	.97	.00	.00	.19	.47	.63

[a] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate not present.

[b] Hours after inoculation of seedlings. Bacteria (5×10^8 cfu/ml) and phage PEa1(h) (5×10^9 pfu/ml) were mixed 1:1 in 0.02 M potassium phosphate buffer, pH 6.8, and allowed to incubate for 30 min, the seedlings were then inoculated with 0.01 - 0.05 ml of this suspension.

Table C5 (continued).

[c/] rif^r = resistance to the antibiotic rifampin.

[d/] w.t. = wild type.

[e/] Mean of four replications.

APPENDIX C

Table C6. Simultaneous inoculation of apple seedlings with a mixture of *Erwinia amylovora* and bacteriophage PEa1(h) and the effect on symptom development.

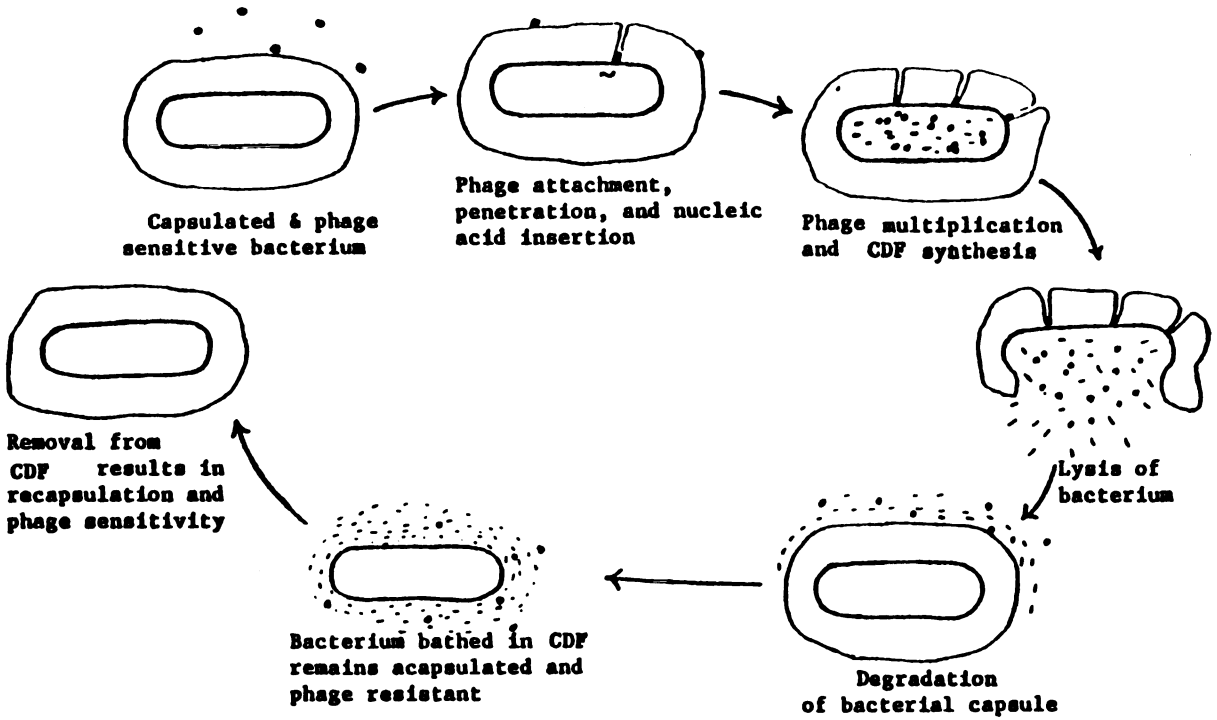
Erwinia amylovora	[a] Rate of symptom development									
	E. amylovora without phage					*E. amylovora* with phage				
[b]	24	48	72	96	120	24	48	72	96	120
[c] 105 rif^r [d]	.25	1.00	1.00	1.00	1.00	.00	.00	.00	.50	1.00
110 rif^r	.50	.75	.75	.75	1.00	.00	.00	.50	.75	1.00
121 rif^r	.25	.25	1.00	1.00	1.00	.00	.00	.00	.00	.00
131 rif^r	.50	1.00	1.00	1.00	1.00	.00	.00	1.00	1.00	1.00
Mean	.38	.75	.94	.94	1.00	.00	.00	.38	.56	.75

[a] Initial symptom was the presence of a droplet of bacterial exudate at the point of inoculation. 1.00 = exudate present; .00 = exudate not present.

[b] Hours after inoculation of seedlings. Bacteria (5×10^8 cfu/ml) and phage PEa1(h) (2×10^{10} pfu/ml) were mixed 1:1 in 0.02 M potassium phosphate buffer, pH 6.8, and allowed to incubate for 10 min, the seedlings were then inoculated with 0.01 - 0.05 ml of this suspension.

[c] rif^r = resistance to the antibiotic rifampin.

[d] Mean of four replications.



APPENDIX D

Figure D1. Diagrammatic representation of the interaction between bacteriophage PEa1(h) and capsulated *Erwinia amylovora*. CDF = capsulated degrading factor.