Comparative Relationships between Two Xanthomonas pruni Bacteriophages and Their Bacterial Host

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ABSTRACT

The comparative relationships between two virulent *Xanthomonas pruni* bacteriophages (Xp3-A, Xp3-I) that produce morphologically distinct plaques and their bacterial host are described. Adsorption rate constants are 1.1×10^{-9} and 1.8×10^{-9} ml/min for Xp3-A and Xp3-I, respectively. For Xp3-A, the latent period is 30-45 min; the eclipse period is between 15-30 min; and the burst size is 42-49 plaque-forming units (PFU). All phage particles are completed and released rapidly, reaching a maximum about 25 min after end of the adsorption period. For Xp3-I, the latent period is 60-75 min, the eclipse period is between 15-30 min, and the burst size is 176-296 PFU. Infectious Xp3-I phage particles are released slowly from infected cells over a period of 120 min. The infectivity of both phages in nutrient broth containing $1\text{-}2 \times 10^{3}$ PFU/ml is stable up to 55 C for 10 min but decreases markedly between 60-70 C. The relative roles of these relationships in the development of bacterial spot of peach are discussed. Phytopathology 60:1385-1386.

Additional key words: Bacterial viruses, comparative properties.

Bacterial spot disease of peach can be reduced experimentally by applying *Xanthomonas pruni* bacteriophage to foliage prior to inoculation with *X. pruni* (1). To study the role of this interaction as a factor in the epidemiology of bacterial spot, it is necessary to understand the nature of the relationship between pruniphages and their bacterial host.

Several types of *X. pruni* bacteriophages have been differentiated on the basis of plaque morphology, host range, serology, lysogenicity, and some growth characteristics (2, 4, 10, 11). The detailed structure, chemical composition, and physiochemical properties have been described for only one *X. pruni* bacteriophage (8).

Comparative quantitative data on adsorption rates and general growth characteristics of different *X. pruni* bacteriophages are not available. This paper describes the relationships between a single isolate of *X. pruni* and two virulent phages that produce morphologically distinct plaques and the relative effects of these relationships on bacterial spot disease of peach.

MATERIALS AND METHODS.—*Bacteriophage isolates.*—A sample of *X. pruni* bacteriophage was initially obtained from soil by the enrichment technique (3). Two phage isolates, designated Xp3-A and Xp3-I, were purified by five serial, single-plaque transfers from two morphologically distinct types of plaques. Xp3-A produces large, clear plaques with a narrow halo. Plaques produced by Xp3-I are small, irregular-shaped, and turbid. The plaques produced by Xp3-A and Xp3-I are morphologically similar to those produced by the virulent phage Xp4 and phage Xp8 released by a lysogenic strain of *X. pruni,* respectively (4).

Both phage isolates were propagated on a culture of *X. pruni,* and lysates were obtained as previously described (1). Plaque count assays were made by the single agar layer method (12). The relative efficiency of plating *X. pruni* bacteriophages by this method ranged between 0.91 to 1.29 in preliminary tests.

Irreversible adsorption, one step, and intracellular growth.—Irreversible adsorption was measured by the chloroform technique of Fredericq (7). Phage (plaque-forming units, PFU) was added to bacteria (colony-forming units, CFU) at an input ratio of 0.01 to 0.1 in nutrient broth containing 0.001 M $MgSO_4 \cdot 7H_2O$ and incubated at 27 C. One-step growth experiments were performed as described by Ellis & Delbruck (5). The input multiplicity of phage to bacteria was about 0.1. Phage was allowed to adsorb for 20 min at 27 C (90-95% adsorption) before the cultures were diluted into growth tubes and assayed. Intracellular phage growth was determined in cultures used for the one-step growth experiments according to the method of Sechaud & Kellenberger (13).

RESULTS.—*Phage growth characteristics.*—Lysis of phage-infected *X. pruni* cells was followed by measuring the change in absorbance at 620 nm of the cultures (Fig. 1). Phage was added to the cultures at input multiplicities of 1 to 22 so that most of the cells were infected. After no change in absorbance from 0 to 60 min, there was a marked decrease in absorbance during the next 210 min in the culture containing Xp3-A-infected cells, indicating almost complete lysis of the culture. No lysis was detected in cultures infected with Xp3-I, although the increase in absorbance was markedly lower in the culture with cells infected at a multiplicity of 22. Final titers were usually 10^{9} to 10^{10} PFU/ml and 10^{10} to 10^{11} PFU/ml in cultures of Xp3-A- and Xp3-I-infected *X. pruni,* respectively.

Adsorption.—Adsorption of Xp3-A and Xp3-I to *X. pruni* at 27 C in nutrient broth containing 0.001 M $MgSO_4 \cdot 7H_2O$ is shown in Fig. 2. Rapid adsorption occurs during the first 6-8 min. In this experiment, 64 and 88% of the PFU of Xp3-A and Xp3-I, respectively, were adsorbed initially. Further adsorption continues at a decreased rate until more than 90% of the PFU of both phages are adsorbed by 20 min. Values for adsorption rate constants, calculated from the initial slopes, are 1.1×10^{-9} and 1.8×10^{-9} ml/min for Xp3-A and Xp3-I, respectively. These values are similar to the maximum adsorption rate constant for several *X. phaseoli* bacteriophages of 10^{-9} ml/min

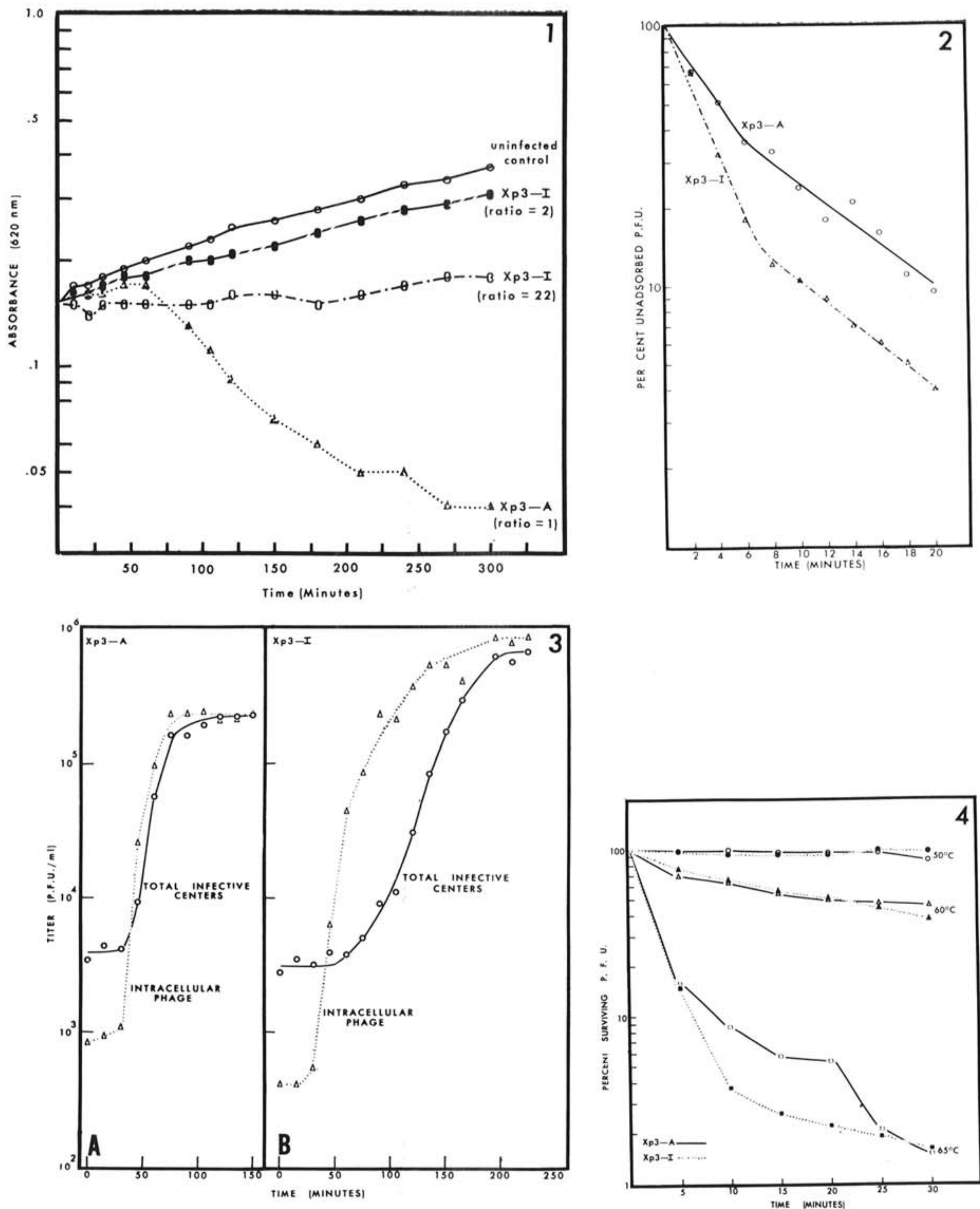

Fig. 1-4. 1) Lysis of *Xanthomonas pruni* infected with Xp3-A and Xp3-I. Cells were grown in nutrient broth at 27 C to a density of 1-6 × 10^8 colony-forming units/ml and infected with phage at the input multiplicities indicated. **2)** Adsorption of Xp3-A and Xp3-I to *Xanthomonas pruni*. Cells were grown in nutrient broth at 27 C to a density of about 2 × 10^8 colony-forming units/ml and infected at a multiplicity of 0.05 to 0.1. The medium contained 0.8% nutrient broth supplemented with 0.2% glucose, 0.5% NaCl, and 0.001 M $MgSO_4 \cdot 7H_2O$. **3)** One step and intracellular growth of Xp3-A (A) and Xp3-I (B). Phage (plaque-forming units) was added to cultures containing 2-3 × 10^8 colony-forming units/ml at an input multiplicity of about 0.1. After adsorption at 27 C for 20 min in the presence of 0.001 M $MgSO_4 \cdot 7H_2O$, cultures were diluted into fresh nutrient broth (0 min) and assayed for plaque-forming units at 15-min intervals. **4)** Rates of thermal inactivation of Xp3-A and Xp3-I in nutrient broth at 50, 60, and 65 C. One-ml samples containing 1-2 × 10^3 plaque-forming units/ml were heated for 10 min and rapidly cooled in an ice bath before being assayed for surviving plaque-forming units. Average values from three experiments.

given for phage Pg 60 (14). Adsorption rate constant values ranged from 0.2×10^{-9} to 1.2×10^{-9} ml/min for Xp3-A and from 1.3×10^{-9} to 3.3×10^{-9} ml/min for Xp3-I.

One-step and intracellular growth.—Typical virus growth curves are presented in Fig. 3. Phage was added to $2\text{-}3 \times 10^{8}$ bacteria (CFU)/ml in nutrient broth with 0.001 M $MgSO_4 \cdot 7H_2O$ at an input multiplicity of about 0.1. After 20-min adsorption at 27 C (more than 90% adsorption), the cultures were diluted into nutrient broth (0 min), incubated at 27 C, and assayed for infective centers at 15 min intervals.

The latent periods for Xp3-A and Xp3-I were 30-45 and 60-75 min, respectively. Infectious Xp3-A phage is released rapidly for the next 45 min, reaching a max about 75 min after the end of the adsorption period. Infectious virus is released more slowly from Xp3-I-infected cells from about 60 to 190 min.

The total eclipse phase of phage growth may not be evident, since phage antiserum was not used and there was probably some residual external phage in the growth tubes (6). But an eclipse period is suggested because the intracellular phage titer at the end of the adsorption period was about 4- to 8-fold lower than that of the total infectious center titer. Thus, intracellular phage starts to increase about 15-30 min after the end of the adsorption period in both Xp3-A- and Xp3-I-infected cells. Intracellular phage is equal to the total infectious center titer about 75 and 135 min after the end of the adsorption period for Xp3-A and Xp3-I, respectively. Therefore, all the phage appears to be released from Xp3-A-infected cells immediately after being completed, whereas newly synthesized phage is released slowly from Xp3-I-infected cells. The average burst sizes ranged from 42-49 for Xp3-A and 176-296 for Xp3-I in three experiments.

Thermal stability.—The thermal inactivation of Xp3-A and Xp3-I in nutrient broth was determined by heating 1-ml samples containing 1 to 2×10^{3} PFU/ml in a water bath for 10 min, followed by rapid cooling in an ice bath. Free phage particles in nutrient broth were not inactivated by 10-min exposures to temp from 30 to 55 C. Plaque formation decreases sharply between 55 and 60 C. In three experiments, Xp3-I was slightly less stable above 55 C than Xp3-A, and was completely inactivated when exposed to 70 C for 10 min. The kinetics of thermal inactivation of Xp3-A and Xp3-I at 50, 60 and 65 C are shown in Fig. 4. The rates of inactivation of both Xp3-A and Xp3-I at 50 C and 60 C are similar. At 65 C, Xp3-I is inactivated initially at a greater rate than Xp3-A.

Relationship to bacterial spot.—The relative efficacies of these two *X. pruni* phage types to reduce the severity of bacterial spot disease of peach foliage were compared. Phage-containing lysates and phage-infected *X. pruni* cells were applied to the foliage and allowed to dry. Infected *X. pruni* cells were obtained by adding phage to bacteria at ratios of 16 for Xp3-A and 57 for Xp3-I, allowing adsorption to proceed for 20 min at 27 C and collecting the cells by centrifugation. The foliage was inoculated with *X. pruni* cells from an overnight culture in nutrient broth collected by centrifugation at approx 5,000 *g* for 15 min, resuspended in 0.85% NaCl, and adjusted to a concn of approx 2×10^{8} CFU/ml. In order to assess the relative degree of protection provided by phage-containing lysates or phage-infected bacteria, a suspension of calcium oxytetracycline containing 100 ppm active oxytetracycline (Terramycin) was used as a standard.

In general, phage-containing lysates were slightly more effective than phage-infected *X. pruni* cells in reducing bacterial spot (Table 1). Greater disease reduction occurred on leaves treated with Xp3-I-infected cells than on leaves treated with Xp3-A-infected cells. But a greater degree of protection against *X. pruni* infection was obtained when the foliage was treated with the Xp3-A-containing lysate than with the Xp3-I-containing lysate. The concn of PFU in the Xp3-A-containing lysate was more than 10-fold less than that in the Xp3-I-containing lysate. Neither the use of phage-containing lysates or phage-infected *X. pruni* afforded as much protection of foliage against *X. pruni* infection as did the Terramycin treatments.

DISCUSSION.—The purpose of the present study is to compare the relationships between two *X. pruni* phages, distinguished on the basis of plaque morphology, and their host. This information is necessary to study the possible effect of this relationship to bacterial spot disease of peach.

Infective particles of Xp3-A are released immediately after being synthesized and lead to visible lysis of infected cultures. In contrast, newly synthesized Xp3-I particles are released slowly after being produced in vivo. Both Xp3-A and Xp3-I adsorb rapidly to *X. pruni* cells in the first 6-8 min. But it is not known why adsorption continues at a decreased rate after the initial adsorption rate. This phenomenon may be related to the availability of receptor sites.

It is not known yet whether the protection of peach foliage against *X. pruni* infection is the result of phage on the leaf surface or within intercellular spaces at the presumed sites of infection. Experiments are in progress to determine if phage-infected *X. pruni* can be introduced into the intercellular spaces of peach leaves where large numbers of phage particles can be released. There is no evidence that *X. pruni* cells in the environment of the intercellular spaces of the host are resistant to phage infection or that phage cannot replicate within such cells. The infectivity of free particles of the phage isolates used in these studies was stable up to 55 C in nutrient broth; however, temp may affect *X. pruni* phage multiplication (9).

Xp3-I-infected *X. pruni* cells were only slightly more effective in reducing bacterial spot of peach foliage than Xp3-A. This difference could be due to the larger numbers of phage particles released by Xp3-I-infected cells on the leaf surfaces or within the intercellular spaces. At the same time, however, the time between subsequent adsorption and release of progeny phage particles (about 80 min for Xp3-I) may be sufficient for *X. pruni* cells to escape phage attack and initiate infection. In contrast, phage exhibiting a growth pattern like that of Xp3-A could rapidly infect most of the invading bacterial cells and release progeny

TABLE 1. Effect of Xp3-A and Xp3-I pruniphages on bacterial spot disease (*Xanthomonas pruni*) of peach foliage

Treatment[a]	No. leaves	Avg % infected leaves/plant	Avg disease severity grade/infected leaf[d]	Disease index/plant	% Disease reduction
X. pruni—control	87	76.5	2.0	153.0	
Xp3-A-infected *X. pruni*—control	93	30.7	1.1	33.8	
Xp3-A-infected *X. pruni*	106	60.6	1.7	103.0	32
Xp3-A-containing crude lysate[c]	111	46.3	1.3	60.2	61
Terramycin (100 ppm)	106	8.9	0.8	7.1	95
X. pruni—control	117	59.1	2.2	130.0	
Xp3-I-infected *X. pruni*—control[b]	122	7.7	1.1	8.5	
Xp3-I-infected *X. pruni*	112	54.8	1.3	71.2	45
Xp3-I-containing crude lysate[c]	91	53.8	1.3	69.9	46
Terramycin (100 ppm)		4.6	0.7	3.2	98

[a] Foliage was inoculated with *X. pruni* at a concn of about 2×10^8 colony-forming units/ml after phage-containing lysates or phage-infected *X. pruni* cells were sprayed on foliage and allowed to dry. Four plants were included in each treatment.

[b] Ratios of phage (plaque-forming units) to bacteria (colony forming units) were 16 and 57 in the case of Xp3-A and Xp3-I, respectively. After adsorption for 20 min, the infected cells were collected by centrifugation at approx 5,000 *g* for 10 min, and the pellets covered with cold nutrient broth, kept in an ice bath, and resuspended just prior to spraying on the foliage.

[c] Titers were 8.6×10^9 and 1.0×10^{11} plaque-forming units/ml for lysates containing Xp3-A and Xp3-I, respectively.

[d] Disease rating per affected leaf based on a logarithmic scale: 0 = no bacterial spot disease; 1 = 0-3%; 2 = 3-6%; 3 = 6-12%; 4 = 12-25%; 6 = more than 50% of leaf surface exhibiting symptoms.

phage to check establishment of *X. pruni* infection until larger numbers of phage particles are released from Xp3-I-infected cells over a longer period of time. This may partially explain the somewhat higher degree of protection (85% disease reduction) observed earlier, when cell-free lysates containing *X. pruni* phage particles that produced a variety of morphologically distinct plaque types were used (1). The relative effects of the local environment on the leaf surfaces and within the intercellular spaces on the adsorption multiplication of Xp3-A and Xp3-I within *X. pruni* cells remains to be determined.

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