Relationships of Xanthomonas pruni Bacteriophages to Bacterial Spot Disease in Prunus

E. L. Civerolo

Research Plant Pathologist; Fruit Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Agricultural Research Center, Beltsville, Maryland 20705.

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ABSTRACT

Xanthomonas pruni phage (Xp3-A) plates with approximately the same relative efficiency of plating (EOP) on seven susceptible *X. pruni* isolates from peach, apricot, plum, and sweet cherry. Relative EOP of phage Xp3-I on the same isolates was more variable, but consistently lower on the heterologous *X. pruni* hosts than on the homologous host. There is no apparent direct relationship between virulence of the seven phage-sensitive *X. pruni* isolates on 'Sunhigh' peach seedlings and the relative EOP of phages Xp3-A and Xp3-I on these isolates. Extensive lysis of peach, apricot, and plum isolates of *X. pruni* by phage Xp3-A occurred at 20 and 27 C but not at 35 C. Limited lysis of a sweet cherry isolate of *X. pruni*, which produces turbid plaques when incubated at 27 C with phage Xp3-A, also occurred at 20 and 27 C but not at 35 C. Leaf infection, as measured by disease symptoms, was significantly less on Sunhigh peach seedling foliage pre-treated with phage and when phage was mixed with *X. pruni* inoculum. *X. pruni* phages may have an epidemiological role in bacterial spot disease development.

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Additional key words: Prunus persica, peach bacterial disease, phage-bacterium interaction.

Bacterial spot disease of stone fruits is caused by *Xanthomonas pruni*. Severe defoliation resulting in weakened trees, small fruit, and fruit spotting characterizes this disease (11, 23, 24). *X. pruni* overwinters in spring cankers resulting from twig infections in the previous fall (11, 23, 24). Several environmental factors influencing the occurrence and development of bacterial spot of peach have been described (11, 15, 23, 24).

Effects of biological interrelationships between individual components of natural epiphytic ecosystems on bacterial plant diseases are not fully known (7, 9, 16, 22, 26). Although bacteriophages may be important factors in the ecology of their host bacteria (1, 20), the epidemiological role of phages of phytopathogenic bacteria in plant disease development is less understood. The role of the lytic effect of bacterial viruses in certain bacterial plant diseases has been primarily of experimental interest only (5, 17, 19, 25, 29, 32).

Ubiquitous distribution and occurrence of specific phages in bacteria-infected plant tissues, irrespective of disease severity, suggest that, in nature, phages may check multiplication of sensitive phytopathogenic bacteria (20, 29). Plum and cherry isolates of *Pseudomonas mors-prunorum* were physiologically and biochemically indistinguishable, but exhibited some degree of pathogenic adaptation to their original stone fruit hosts (8, 9). Since these isolates could be differentiated by phage-typing, this pathogenic adaptation suggested either some form of association between phage sensitivity and specific virulence factors in *P. mors-prunorum* or a different history of phage infection (8, 9). Phage sensitivity of *P. syringae* and *P. savastanoi* was also associated with plant host origin, but there was no association between phage sensitivity patterns and plant host origin with isolates of *P. coronofaciens, P. pisi*, and *P. tomato* (3). There was no relationship between host specificity, pathogenicity, and lysogeny among several isolates of *P. syringae* (2).

Prior treatment of peach foliage with *X. pruni* phages may protect leaves, at least to a limited extent, against subsequent infection by *X. pruni* (4, 5, 6). However, the effect of the interactions between *X. pruni* phages and their bacterial hosts in nature on plant disease development is not clearly understood. This report describes some relationships of *X. pruni* phage-host interactions to bacterial spot disease development.

MATERIALS AND METHODS.–*X. pruni* was isolated from several naturally infected stone fruit hosts, including peach, apricot, plum, and sweet cherry. Single colony selections of all *X. pruni* isolates were maintained on nutrient agar supplemented with glucose (4, 6). Bacteriophages and their hosts were propagated in 0.8% nutrient broth containing 0.2% glucose and 0.5% NaCl (NGSB). Isolation, purification, propagation, and assay of pruniphages Xp3-A and Xp3-I have been described (4). A mixture of pruniphages for therapeutic use was isolated from diseased leaves or from soil collected from beneath naturally infected apricot trees. Soil and leaf samples were incubated in NGSB separately at room temperature for 24-48 hr. Alternatively, different *X. pruni* isolates were added to additional soil and leaf samples and incubated similarly. After centrifugation at 10,000 *g* for 20 min, the supernatant fractions from the enriched and nonenriched mixtures were combined and sterilized by passage through 0.45 μ (average pore size) membrane filters. These preparations contained several infectious units that formed morphologically distinct types of plaques and were propagated without fruther purification in peach and apricot isolates of *X. pruni*. Inoculation of peach seedling foliage and evaluation of bacterial spot disease development have been described (4, 6).

Lysis of *X. pruni* cells by phage isolate Xp3-A was followed by monitoring changes in optical density of inoculated cultures at 620 nm. Cultures were inoculated at multiplicities of 10 to 20

plaque-forming units (p.f.u.)/colony-forming unit (c.f.u.) so that the majority of cells were infected. Cells in log phase of growth were diluted into fresh NGSB and incubated at the appropriate temperature for 5 minutes before addition of phage. Inoculated cultures were incubated at 20, 27, and 35 C with rotary shaking (150 rpm).

In determining relative efficiency of plating, the number of plaques produced on the homologous *X. pruni* isolate was considered the standard. The homologous host was an isolate of *X. pruni* from naturally infected 'Coronet' peach tree and was used originally to isolate phage from soil by the enrichment technique (10).

Virulence of all *X. pruni* isolates was compared on 'Sunhigh' peach seedling foliage by atomizing bacterial suspensions onto the lower surfaces of the leaves. Each *X. pruni* isolate was grown for 18-24 hr in NSGB at 27 C in a water bath with rotary shaking (150 rpm). Cells collected by centrifugation at 5,000 *g* for 10 min were resuspended in sterile demineralized water and adjusted turbidimetrically to contain ca. 2×10^8 c.f.u./ml. After inoculation, plants were held in a mist chamber for 5 days before being returned to normal greenhouse conditions (4, 6). The disease index (4, 5) of inoculated foliage was taken as a measure of the relative virulence of each *X. pruni* isolate, using the Coronet peach isolate of *X. pruni* as a standard.

RESULTS.–*Relative virulence of X. pruni.*–All eight *X. pruni* isolates in this study were highly virulent on Sunhigh peach seedlings, suggesting that the general growth characteristics of *X. pruni* in peach leaves were similar in both the homologous and heterologous bacterium-host combinations. The apricot, sweet cherry, and plum isolates were slightly more virulent on the heterologous peach host plant than were three peach isolates, including the standard isolate. The two most virulent cultures were isolated from a naturally infected 'Suwanee' peach tree.

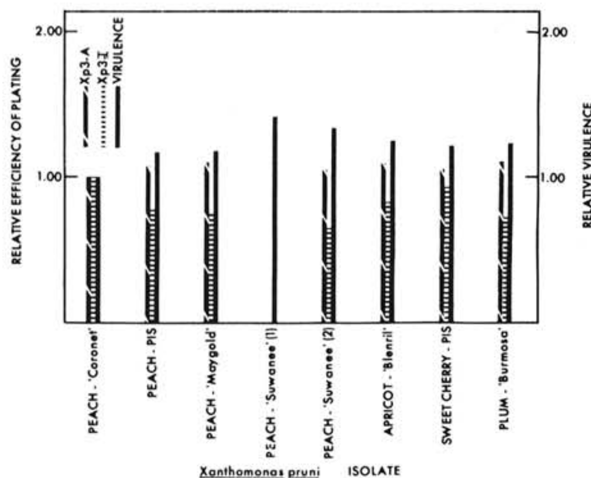

Fig. 1. Relationship of relative virulence of eight *Xanthomonas pruni* on 'Sunhigh' peach seedling foliage to relative efficiencies of plating of phages Xp3-A and Xp3-I on these isolates. Values are averages of four experiments. Relative virulence was estimated by comparing the disease index on foliage of Sunhigh peach seedlings after inoculation with *X. pruni* at concentrations of $1\text{-}3 \times 10^8$ colony-forming units/ml. In each experiment, four seedlings were inoculated with each isolate.

Relative EOP and plaque morphology.–Phage Xp3-A plated with a slightly higher relative EOP on the heterologous *X. pruni* isolates than on the homologous bacterial host (Fig. 1). Phage Xp3-I consistently plated with a lower relative EOP on the same heterologous *X. pruni* isolates than on the homologous host (Fig. 1). One *X. pruni* peach isolate, Suwanee-1, did not form plaques when plated with either of these two phages and it may be resistant or immune to infection by both phages (Fig. 1). There was no apparent direct relationship between the relative virulence of these *X. pruni* isolates and the relative EOP of Xp3-A and Xp3-I on these isolates (Fig. 1). However, plaque size and morphology which are characteristic features of the specific phage-host interactions may be epidemiologically significant (27, 30, 31). Xp3-A produced large clear plaques with a narrow halo when plated on the peach-Coronet, peach-PIS, and apricot-Blenril isolates; large plaques with a narrow halo and a small central clear area surrounded by a relatively wide turbid zone of bacterial growth on peach-Maygold, peach-Suwanee 2, and plum-Burmosa isolates; and large turbid plaques on the sweet cherry-PIS isolate. The peach-Coronet and apricot-Blenril isolates were completely susceptible to Xp3-A phage, since plaques remain clear. However, with prolonged incubation, minute colonies appeared within plaques produced by Xp3-A on the peach-PIS isolate, indicating the presence of a small proportion of resistant cells in these cultures. Plaques produced by Xp3-I on the susceptible hosts are usually less than 1 mm in diam and rounded to irregular with various degrees of opacity.

Effect of phage on bacterial spot disease.–The effect of a mixture of *X. pruni* phage on bacterial spot disease development on Sunhigh peach seedling foliage is presented in Table 1. Preinoculation treatment of foliage with crude lysates of the phage mixture resulted in 6-8% fewer infected leaves, decrease of 0.5-1.6 in the average leaf infection rating per infected leaf, and 17-31% reduction of disease index as compared to the water-treated controls. When phage was mixed with *X. pruni* immediately before inoculation, disease reduction was 79-92%, reflecting 51-54% decrease of infected leaves and 1.8-3.4 decrease in the average leaf infection rating per infected leaf compared to control leaves inoculated with *X. pruni* alone. These results are similar to those reported previously (4, 6). Preinoculation treatment with the phage-*X. pruni* mixture did not protect the foliage against subsequent *X. pruni* infection any more than preinoculation treatment of the foliage with phage alone.

Effect of phage treatment on leaf infection is shown in Fig. 2. On seedlings pretreated with phage before *X. pruni* inoculation, 70% of the leaves had

TABLE 1. Effect of *Xanthomonas pruni* bacteriophage on bacterial spot disease development on 'Sunhigh' peach seedling foliage

Expt.	Treatment[a] Preinoculation	Inoculum	Total number of leaves	Leaves infected (%)	Avg leaf infection[b] rating/infected leaf	Disease[c] index
1	Water	*X. pruni*	114	96.0	6.0	576.0
	Phage	*X. pruni*	120	90.3	4.4	397.3
	None	*X. pruni*	116	64.8	3.0	194.4
	None	Phage + *X. pruni*	140	13.7	1.2	16.4
2	Water	*X. pruni*	110	93.6	5.5	514.8
	Phage	*X. pruni*	116	85.3	5.0	426.5
	Water	Phage + *X. pruni*	100	48.0	2.1	100.8
	Phage + *X. pruni*	Water	105	28.6	1.4	40.0
	Phage + *X. pruni*	*X. pruni*	95	96.8	4.5	435.6

[a] Each treatment included three Sunhigh peach seedling. *X. pruni* inocula contained 2×10^8 c.f.u./ml, and crude lysates of a mixture of phages contained 4×10^9 p.f.u./ml. A mass isolate of phage was obtained from soil collected beneath a bacterial spot diseased peach tree and enriched with an apricot isolate of *X. pruni*. Large quantities of crude lysates were prepared by propagating the mass phage isolate in the same *X. pruni* isolate. *X. pruni* inocula and phage preparations used singly were appropriately diluted with dimineralized water to adjust concentrations of c.f.u. and p.f.u. to those in *X. pruni* and phage mixtures. Preinoculation treatment consisted of spraying the lower surfaces of the foliage at 30 p.s.i. with water, phage, or phage-*X. pruni* mixture until run-off. After allowing to dry for 1-2 hr, foliage was inoculated as indicated, by spraying as before. All plants were covered with polyethylene bags for 5 days in the greenhouse. Bacterial spot disease development was evaluated 14-21 days after inoculation.

[b] Leaf infection rating per infected leaf based on a logarithmic scale: 0 = no bacterial spot disease symptoms; 1 = 0-3%; 2 = 3-6%; 3 = 6-12%; 4 = 12-25%; 5 = 25-50%; 6 = 50-75%; 7 = 75-87%; 8 = 87-94%; 9 = 94-97%; 10 = 97-100%; 11 = 100% of leaf surface exhibiting symptoms.

[c] Disease index = % leaves affected × average leaf infection rating/infected leaf.

leaf infection ratings of 0-5, i.e., much less than 50% of the leaf surfaces exhibited symptoms (Fig. 2-B). In contrast, 58% of the water-treated control leaves had infection ratings of 6-10, indicating that more than 50% of the leaf surfaces exhibited bacterial spot symptoms (Fig. 2-A). On phage-treated plants, 33% of the leaves had infection ratings of 0-3; whereas only 12% of the leaves on the control plants had the same low infection ratings. When phage was mixed with *X. pruni* at the time of inoculation (ca. 20 p.f.u./c.f.u.), no leaf had an infection rating greater than 5 and 72% of the inoculated leaves did not show any bacterial spot symptoms (Fig. 2-D). In contrast, leaves inoculated with *X. pruni* alone had moderate to severe levels of infection, i.e., 54% had ratings of 4-9 (Fig. 2-C). The possible epidemiological significance of the degree of leaf infection may be that subsequent defoliation of infected plants pretreated with phage may not be as severe as on untreated plants.

Temperature effects.—Growth of four *X. pruni* single-colony isolates for 5 hr at 20, 27, and 35 C was determined in preliminary experiments. The maximum level and rate of increase in turbidity of 20-ml cultures occurred at 27 C. At 35 C a rapid initial increase in turbidity for 30-175 min was followed by an increase that continued at a lower rate than at 27 C. At 20 C there was no change in turbidity initially for 45 min, then a continual increase at approximately the same rate as at 27 C.

Lysis of 16- to 18-hr-old cultures of the four selected *X. pruni* isolates by phage Xp3-A was also followed at 20, 27, and 35 C by measuring the change in absorbance of infected cultures at 620 nm (Fig. 3).

At 20 C, a slight increase in absorbance from 0 to 90 min was followed by a rapid decrease for the next 240 to 270 min in the case of peach, apricot, and plum isolates indicating nearly complete lysis. There was a similar marked decrease in absorbance in the culture containing phage infected cells of the sweet cherry isolate. However, cultures of this *X. pruni* isolate are not extensively lysed. At 27 C a decrease in absorbance occurred approximately 60 min after addition of phage to the cultures. However, neither the rate nor the extent of lysis was as great at 27 as at 20 C, except in the case of the plum isolate. At 35 C, there was only a slight decrease in absorbance of inoculated cultures of all isolates from about 30 to 90 min after addition phage to the culture. After 90 min absorbance of phage-containing cultures continued to increase slowly, indicating that no lysis occurs at 35 C. Therefore, *X. pruni* may be more susceptible to phage infection at lower temperatures from 20-27 C than at higher temperatures. The slight decreases in turbidity of cultures initially at 35 C may have been due to lysis of susceptible cells that were grown at 27 C before being transferred to 35 C.

A significant proportion of the cells of the sweet cherry isolate of *X. pruni*, which forms turbid plaques at 27 C when plated with phage Xp3-A, may be lysogenic as suggested by plaque morphology. However, slight but marked decreases in absorbance at 20 and 27 C indicate some infection by Xp3-A resulting in lysis. Additionally, more cells of this

isolate may be lysed at 20 than at 27 C. No spontaneous lysis by conversion of a temperate phage in the lysogenic state to a virulent form occurred in noninoculated cultures. However, the nature of the resistant cells of this isolate is unknown.

DISCUSSION.—The *X. pruni*-phage relationships described herein have concerned lysis of susceptible *X. pruni* cells and its possible relationship to the ecological role of the phage-bacterial host interactions in plant disease development. However, other possibilities exist, such as the effects of lysogenization and transduction of genetic properties by temperate and virulent phages on *X. pruni* virulence. Neither phage in this study lyses all *X. pruni* isolates so far tested. It is not likely that a strictly species-specific phage exists which will lyse all or a majority of isolates of a single species despite species-specificity attributed to *X. pruni* bacteriophages (12, 30, 31).

Phage Xp3-A plates with approximately the same relative EOP on all susceptible *X. pruni* isolates used in this study. Relative EOP of phage Xp3-I on these same isolates was somewhat more variable, but consistently lower on the heterologous hosts than on the homologous host. Neither phage produces plaques when plated with the peach- Suwanee 1 isolate of *X. pruni* and attempts to induce a temperate phage in this isolate have failed. There is no apparent direct relationship between virulence of the seven phage-sensitive *X. pruni* isolates on Sunhigh peach seedlings and the relative EOP of phages Xp3-A and Xp3-I on these isolates. Virulence of *Xanthomonas oryzae* may be associated with colony type and phage susceptibility (18).

Although pathotypes (9) of *X. pruni* may exist, sensitivity of 18 *X. pruni* isolates to 12 virulent *Xanthomonas* phages was not related to the specific host plant habitats of the isolates (30, 31). On the other hand, certain phage sensitivity patterns of about 500 *Xanthomonas* cultures did reflect a specific plant habitat, while other sensitivity patterns were common to isolates from various host plants (30, 31). No data are available on the relative virulence of *Xanthomonas* isolates from different host plants, but with identical phage sensitivity patterns.

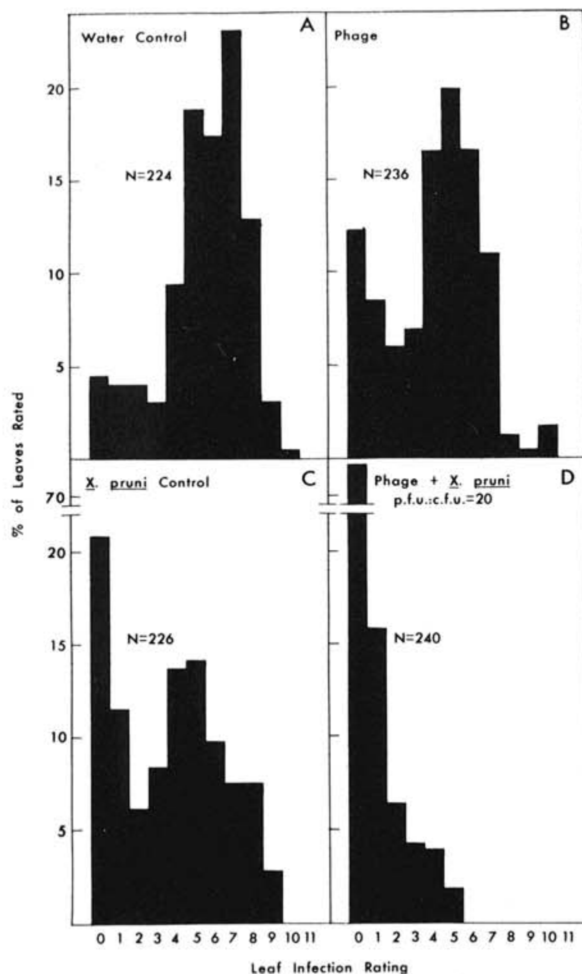

Fig. 2. Effect of *Xanthomonas pruni* bacteriophage on infection of 'Sunhigh' peach seedling foliage by *X. pruni*. Leaf infection rating per infected leaf based on a logarithmic scale. 0 = no bacterial spot symptoms; 1 = 0-3%; 2 = 3-6%; 3 = 6-12%; 4 = 12-25%; 5 = 25-50%; 6 = 50-75%; 7 = 75-87%; 8 = 87-94%; 9 = 94-97%; 10 = 97-100%; 11 = 100% of leaf surface exhibiting symptoms.

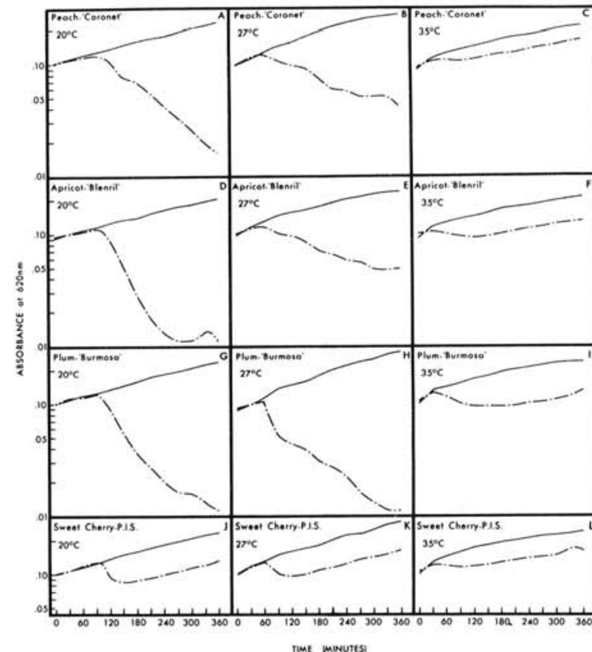

Fig. 3. Lysis of *Xanthomonas pruni* cells infected with phage Xp3-A at 20, 27, and 35 C. Cells grown in nutrient broth at 27 C were adjusted to a density of 1-3 × 10^8 colony-forming unit/ml and were incubated at the appropriate temperature for 5 min before adding phage at an input multiplicity of 1-10. Noninoculated cultures are represented by solid lines and inoculated cultures are represented by broken lines.

Despite plating with similar relative efficiencies on several *X. pruni* isolates, the plaques produced by Xp3-A on four selected isolates are morphologically distinct. This indicates differences in proportions of resistant cells or possibly conversion of different proportions of cells of each isolate into the lysogenic condition. Lysogeny occurs in *X. pruni* (13), but its relationship to virulence is unknown. There was no apparent direct relationship between virulent and temperate phages and virulence of *P. syringae* on peach seedlings (3).

Temperature affects the EOP of *X. phaseoli* phages on *X. phaseoli, P. phaseolicola* and *P. syringae* and possibly the ecological relationships between these phages and their bacterial hosts in nature (33). Thus, several *X. phaseoli* phages attack *X. phaseoli* (XP6022) over a wide temperature range from about 10 to 32 C, with optimal EOP for most phages at 24-28 C (33). Sensitivity of some *Pseudomonas* species to certain *X. phaseoli* phages occurs in a narrow temperature range from 20 to 30 C, with optimal EOP at 24 or 28 C on sensitive *P. phaseolicola* strains and 28 to 34 C on sensitive *P. syringae* strains.

Plaques produced by *X. pruni* bacteriophage were smaller at incubation temperatures above 25 C; whereas at 30 C, plaques were minute or irregular or not formed at all, depending on the phage (21, 27, 28). No plaque formation occurred when the incubation temperature was above 33 C (27). No plaques developed when a mixture of an isolate of *X. pruni* phage and *X. pruni* was incubated at temperatures below 24.2 C, and there was no significant difference between the number of plaques produced at 24.2, 27.0, and 29.7 C, although maximum number of plaques was produced at 27 C (21). From these observations, therefore, it might be concluded that there are definite limits to the temperature range within which plaque formation by pruniphages occurs, and within this range of 24 to 30 C plaque formation is a function of temperature (21).

Lysis of *X. pruni* cells by phage Xp3-A is also temperature-related. However, extensive lysis of *X. pruni* by phage Xp3-A at 20 C apparently contradicts the previous observation that no plaque formation occurred at incubation temperatures below 24 C (21). Absence of mass lysis may have been related to the specific phage isolate or phage-*X. pruni* combination studied. In any event, extensive lysis of several *X. pruni* isolates by phage Xp3-A at 20 C indicates that the temperature range in which infection and lysis of *X. pruni* can occur is wider than indicated by previous reports. This temperature range coincides with those temperatures which favor infection of stone fruit hosts by *X. pruni* under natural conditions if adequate moisture is available. Optimum EOP for pruniphages may occur at 27 C (21, 27). Growth of *X. pruni* in vitro for 6 hr at 35 C is indistinguishable from that at 27 C. However, growth of and infection by *X. pruni* at elevated temperatures under natural conditions may be unlikely. No information is available concerning the specific effect of temperature on the interaction between pruniphages and their host. Under natural conditions *X. pruni* may be more susceptible to phage infection and subsequent lysis at 20 C than at higher temperatures that favor infection.

Development of bacterial spot disease symptoms, reflected in the leaf infection rating, may be directly related to population levels of *X. pruni* within the intercellular spaces of inoculated leaves. The low level of infection of foliage treated with phage-*X. pruni* mixture before inoculation presumably resulted from *X. pruni* cells in the mixture that escaped or were resistant to phage infection. Adsorption of phage to *X. pruni* cells may be more efficient in a mixture before inoculation than when the components are introduced separately into intercellular spaces. The protective effect of phage against *X. pruni* cells on the leaf surfaces or within the intercellular spaces is presumably due to specific lysis of susceptible cells. However, other effects which might be related to virulence, such as lysogeny or transduction, can not be discounted. When peach seedling foliage is inoculated by spraying a bacterial suspension containing approximately 10^8 c.f.u./ml on the lower surface of leaves, 10^1-10^2 *X. pruni* c.f.u./mm^2 are generally recovered immediately after inoculation. If leaves are thoroughly rinsed with water after inoculation and leaf surfaces sterilized with 20% chlorox before isolation, this value of 10^1-10^2 c.f.u/mm^2 presumably represents the population level of *X. pruni* in the intracellular spaces capable of causing infection under experimental conditions. This order of concentration may be close to actual inoculum doses in natural infections by *X. pruni*. Furthermore, the minimum effective dose for in vivo growth of homologous bacterial in compatible (natural) hosts may be very low (14). Therefore, at low bacterial population levels and under suitable environmental conditions, sufficient *X. pruni* phage present on leaf surfaces or in intercellular spaces may effectively protect foliage against severe infection by lysing susceptible *X. pruni* cells. Conceivably, slight to moderate leaf infection under natural conditions may not result in severe defoliation and subsequent weakening of affected trees. Furthermore, under certain environmental conditions, *X. pruni* may occur on surfaces of plant parts, such as leaves, in a resident phase without causing obvious disease symptoms. Recovery of *X. pruni* and pruniphages from apparently healthy apricot leaves in the field (*unpublished*) supports this suggestion. Phages could conceivably have a role in regulating populations of *X. pruni* in a resident phase.

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