RESEARCH PAPER

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Soil-based systemic delivery and phyllosphere in vivo propagation of bacteriophages

Two possible strategies for improving bacteriophage persistence for plant disease control

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Soil-based root applications and attenuated bacterial strains were evaluated as means to enhance bacteriophage persistence on plants for bacterial disease control. In addition, the systemic nature of phage applied to tomato roots was also evaluated. Several experiments were conducted applying either single phages or phage mixtures specific for *Ralstonia solanacearum*, *Xanthomonas perforans* or *X. euvesicatoria* to soil surrounding tomato plants and measuring the persistence and translocation of the phages over time. In general, all phages persisted in the roots of treated plants and were detected in stems and leaves; although phage level varied and persistence in stems and leaves was at a much lower level compared with persistence in roots. Bacterial wilt control was typically best if the phage or phage mixtures were applied to the soil surrounding tomatoes at the time of inoculation, less effective if applied 3 days before inoculation, and ineffective if applied 3 days after inoculation. The use of an attenuated *X. perforans* strain was also evaluated to improve the persistence of phage populations on tomato leaf surfaces. In greenhouse and field experiments, foliar applications of an attenuated mutant *X. perforans* 91-118:Δ*OPGH* strain prior to phage applications significantly improved phage persistence on tomato foliage compared with untreated tomato foliage. Both the soil-based bacteriophage delivery and the use of attenuated bacterial strains improved bacteriophage persistence on respective root and foliar tissues, with evidence of translocation with soil-based bacteriophage applications. Both strategies could lead to improved control of bacterial pathogens on plants.

Introduction

Bacterial-incited plant diseases account for significant production losses to agricultural crops. Disease control is a major challenge as a result of various factors including pathogen variation, ability to overcome plant genetic resistance, lack of effective bactericides as a result of strains developing tolerance, and the pathogen's ability to reach high populations in a relatively short period of time when conditions are favorable for disease development. Antibiotics and copper-based compounds have been the principal bactericides used for disease control. Copper has been the most widely used bactericide; however, copper resistance is present in many plant pathogenic bacteria.[1–7] Antibiotics have also been used as part of a management strategy for various bacterial diseases since the 1950s.[8–10] Streptomycin, an aminoglycoside antibiotic, was used extensively for control of bacterial diseases and as a result, streptomycin-resistant strains became prevalent, resulting in reduced disease control efficacy of bacterial spot of tomato and pepper[8] as well as fireblight of apple and pear.[11] An alternative to conventional bactericides has been to use systemic acquired resistance (SAR) inducing compounds also known as plant activators, which have provided a level of control against various bacterial diseases,[12–16] but may have negative physiological effects on plant growth and yield.[15,16]

Bacteriophages (phages) offer an alternative to conventional management strategies for controlling bacterial plant diseases.[17–28] Although many studies provided positive results using phage, phage therapy has not been considered a good strategy

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for controlling plant pathogenic bacteria because of its unreliability[29] and the narrow spectrum of activity intrinsic to phages.[30] Additionally, the plant environments in which phage are required to operate are less than ideal. Within the phyllosphere, UV exposure, intense visible light and desiccation are all factors that reduce phage viability and disease control efficacy.[31] In studies examining persistence in the phyllosphere, phages applied to tomato leaves during the early morning in late May or early June were unrecoverable 24 h after application.[32] Compared with the phyllosphere, the rhizosphere environment is less harsh, but the phages have significant obstacles including a relatively low diffusion rate through heterogeneous soil matrices that changes as a function of available free water, biofilms that can trap phages,[33] soil clay particles that can reversibly adsorb phages,[34] and low soil pH that can inactivate phages.[35] In natural environments, as a result of low rates of phage diffusion and high rates of phage inactivation, low numbers of viable phages are available to lyse target bacteria.[31] One additional factor needed for a high degree of success is that high populations of both phage and bacterium must exist in order to initiate a chain reaction of bacterial lysis.[31]

Although some success has been achieved with phage for controlling bacterial foliar plant diseases,[36] deployment of phages in agricultural systems is challenging given the need to maintain high phage populations on plant surfaces and the inability of phages to persist on leaf surfaces for extended periods of time,[32] as well as the inability to deliver phages at sufficient quantities to the appropriate sites. Balogh et al.[37] improved efficacy by applying phages in the evening to extend the time phages persisted on the leaf surface and by identifying several formulations that extended the persistence of phages on leaf surfaces. Obradovic et al.,[17] used these findings and demonstrated that phages effectively reduced the bacterial spot pathogen in three different field trials, providing better disease control than the standard bactericide treatment, copper-mancozeb. Another approach for maintaining high phage populations in the phyllosphere is to co-apply them with bacteria that are able to persist in the plant environment and that are sensitive to the phage. Thus if the bacterial populations are maintained at fairly high concentrations, they will serve as hosts for the phage and potentially maintain high phage populations. Svircev et al.,[38] controlled fire blight of pear by utilizing a strain of *P. agglomerans* for delivering and sustaining a mixture of four phages, which were able to lyse strains of both *P. agglomerans* and *E. amylovora,* the causal agent of fire blight. A similar strategy was used for controlling tobacco bacterial wilt, where phages were applied together with a phage-sensitive avirulent strain of the pathogen *Ralstonia solanacearum* to control the disease.[28] Using a similar approach, Balogh[39] determined in greenhouse experiments that phage persisted for extended periods of time on tomato foliage colonized by a mildly pathogenic strains of the bacterial spot of tomato pathogen, but not on non-colonized leaves.

A second challenge in using phage relates to delivery site and application timing. The phage must come in direct contact with the pathogen prior to the bacterium entering the host. Therefore delivery of the phage in close proximity to potential infection sites is necessary for disease control. *Ralstonia solanacearum*, a soil inhabitant and causal agent of bacterial wilt of tomato, infects roots and then proceeds to colonize the vascular system in the stems, eventually causing the plants to wilt and die. Several studies have demonstrated control of bacterial wilt using phages.[22,27,40] Timely delivery of phages to the root zone prior to infection to allow for the phages to interact with the pathogen will likely be a critical factor in disease control. A second possible scenario relates to the phages ability to be taken up by the roots and then translocated in the xylem vessels. Translocation of phage and related reduction of crown gall incidence and severity was previously reported.[41] Therefore control of bacterial wilt by using phages as therapeutants following infection by the bacterium may be possible.

In this study, we tested two strategies for enhancing the use of bacteriophages for bacterial disease control on plants. The objectives of this study were to: (1) address the systemic nature and persistence of soil-applied phage in tomato plants, (2) assess the effectiveness of a commercial phage mixture against *R. solanacearum* for the control of tomato bacterial wilt, and (3) evaluate the use of an attenuated *X. perforans* strain to improve the phage persistence on tomato leaf surfaces.

Results

Systemic movement of phages in tomato plants. Phage from a commercial phage mixture specific to *X. perforans* strain 97-2 remained at detectable levels in the absence of the host bacterium in tomato roots for more than 14 d after root application (**Fig. 1**). Phage were also detected in foliar plant tissues at levels as high as 10^6–10^7 PFU/g tissue in the upper leaves and stems 2 d after initial application. Phage reached concentrations of up to 10^5 PFU/g in root tissues on the 15th day of sampling, regardless if roots were initially damaged and left undamaged at initial phage application. Phage levels in upper leaves and upper stems plummeted below the limit of detection by the 7th day in plants with damaged roots and by the 15th day in plants with undamaged roots. By the 10th day, phage were still detectable between 10^2 and 10^4 PFU/g of lower stem and leaf tissues in plants whose roots were damaged and left undamaged at initial phage application (**Fig. 1**).

In the second set of experiments using a single phage strain ΦMI2, the concentration of phage particles detected in the roots 13 d after application only dropped one log unit compared with the initial phage concentration 4 h after initial application (**Fig. 2A**). Phages were continually detected in the first and second internode within the two-week period (**Fig. 2A and B**). Although the concentration was lower than in roots, phages were detected within 24 h following application to the soil, and remained viable in plant tissue in the absence of the host bacterium. Three days after application, phages were detected in the first and second leaf, followed by detection in the third and fourth internode two days later (**Fig. 2A**), but this distribution was not confirmed in the second repetition of this trial (**Fig. 2B**).

In the third set of experiments where ΦRS5, a phage associated with *R. solanacearum*, was tested for systemic movement in tomato plants after applying a suspension of phages to the soil.

Phage ΦRS5 was detected 24 and 48 h after application in all plant sections except the second leaf (**Fig. 3**). The concentration of ΦRS5 was highest in the roots and progressively lower as sampling progressed up the plant. Five days after application, the ΦRS5 was only detected in the roots.

Control of tomato bacterial wilt with phages. When phage was applied at various time points prior and following the application of *R. solanacearum* to the soil, the most effective wilt control was achieved in the treatments where the commercial RS5-specific phage mixture (ΦRS5mix) was applied immediately after inoculation (**Fig. 4A**). However, there was no effect on disease control when the single phage ΦRS5 was applied immediately after inoculation (**Fig. 4B**). Plants that were not treated with the ΦRS5mix started wilting 3–5 d after inoculation (smaller weaker plants wilted first). Different stages of plant wilt were observed mainly in plants that did not receive the commercial RS5-specific phage mixture. Both ΦRS5mix and ΦRS5treatments were less effective when applied 3 d before inoculation and ineffective when applied 3 d after inoculation.

Effect of OPG mutant on phage persistence in greenhouse conditions. In the greenhouse, phage persistence was consistently higher on leaflets from plants treated with attenuated mutants compared with leaflets that only received phage (**Fig. 5**). Although phage populations were below the limit of detection 7 d after the phage application on leaflets that did not receive an attenuated mutant, phages were still recovered from leaves that were pre-treated with the attenuated *Xanthomonas perforans* strains 91-118:Δ*opgH,* 91-118:Δ*gumD* and 91-118::Δ*opgH*Δ*gumD* even 10 d after the initial phage application. Calculated AUPPC values were statistically lower ($p = 0.0249$) in phage alone applications compared with phage treatments that included the attenuated mutants (**Table 1**).

Effect of OPG mutant application on phage persistence in field conditions. In summer 2011, plots were sampled over a 7 d period on three separate occasions (**Fig. 6A–C**). During the three sampling periods (May 23–29, June 6–12 and June 20–25), the trends in phage populations were quite similar (**Fig. 6A–C**). In the absence of the OPG mutant, phage populations on tomato leaves dropped to levels of ≤ 10 PFU/g by day 2, 4 and 2 after initial phage application during the respective sampling periods. The addition of OPG mutant, regardless of level, improved phage population levels beginning at day 1 for the first two sampling periods (**Fig. 6A and B**) and at day 2 for the third sampling period (**Fig. 6C**), and greatly extended phage persistence on leaf surfaces at detectable levels for at least 5, 3 and 5 d, respectively (**Fig. 6A–C**). In the 2011 fall season, only one sampling period (December 8–14) was done, with similar results that by day 4 phage populations on leaves treated with the OPG mutant were higher than those treated with phage alone (**Fig. 6D**). AUPPC analysis substantiated that the application of the attenuated OPG mutant (at both rates) statistically improved phage persistence over time compared with phage applied alone to leaf surfaces during the first two sampling periods in the summer of 2011 (**Table 2**). Only the OPG applied at 10^7 cfu/ml statistically improved phage levels over that of the phage only treatment over the third sampling period during the summer of 2011 based on AUPPC. While in the fall of 2011, phage populations with the addition of the OPG mutant at 10^7 or 10^8 cfu/ml resulted in only numerically higher AUPPC values compared with phage applied alone.

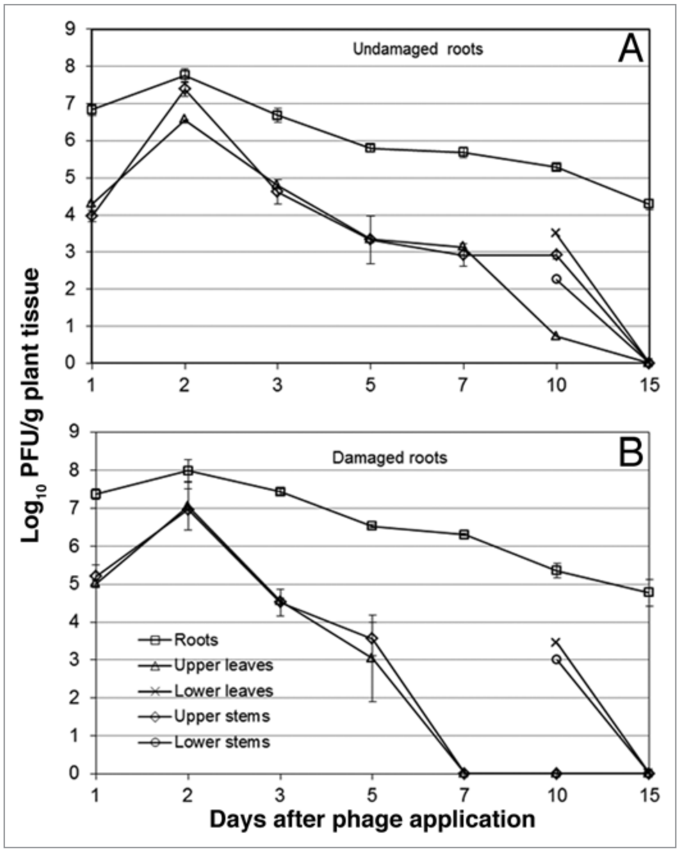

Figure 1. Systemic movement and persistence of *X. perforans* 97-2 specific phage mixture in tomato cultivar Bonny Best with undamaged (**A**) and damaged (**B**) roots. Four week-old plants were drenched with 30 ml of a commercial phage mixture provided by OmniLytics Inc. at a concentration of 10^8 PFU/ml. Control plants were treated with water. Destructive sampling was performed to evaluate phage presence in roots, upper leaves, and upper stems after 1, 2, 3, 5, 7, 10 and 15 d. Lower stems and lower leaves were evaluated after the 10th and 15th day only. Presented values are the average of two experiments.

Discussion

Translocation experiments using individual phage and commercial phage mixtures demonstrated that phage could move from the root zone to the lower foliar portions of the plant for short periods of time. We also noted that the phage could be maintained at high concentrations in the roots for at least 15 d, regardless if roots were damaged or left intact. Phage levels declined more rapidly in upper leaves and stems of tomato plants in which roots had been damaged and were detected for a week longer in plants where roots were not damaged. These results differed from those reported by Ward and Mahler,[42] who studied phage f2 uptake and translocation to distal tissues of soybean and corn

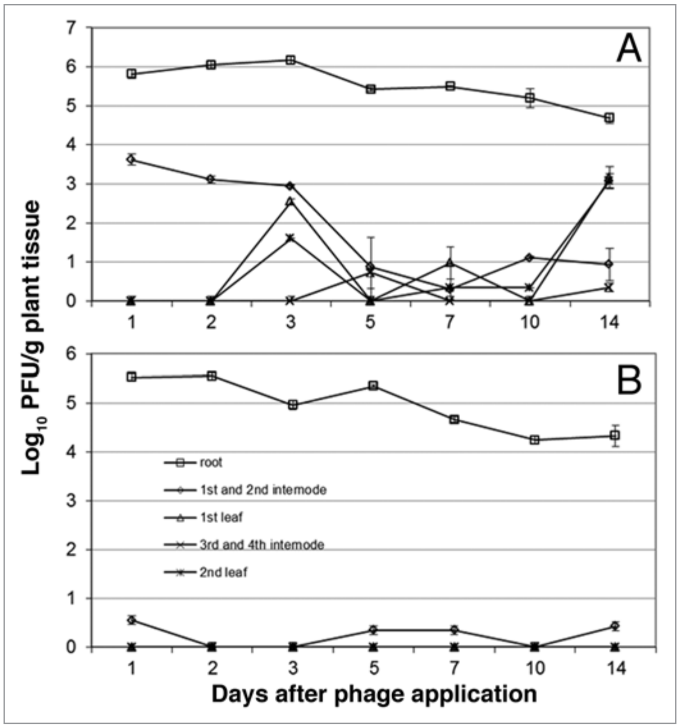

Figure 2. Systemic movement and persistence of *X. euvesicatoria* ΦMI2 in tomato cultivar Bonny Best. Four week-old plants were drenched with 30 ml of ΦMI2 suspension at a concentration of 3.7 × 10^8 PFU/ml in first experiment (**A**) and 1.7 × 10^8 PFU/ml in the second experiment (**B**). Control plants were treated with water. Destructive sampling was performed to evaluate phage presence in: roots, 1st and 2nd internode, 1st leaf, 3rd and 4th internode and 2nd leaf after 1, 2, 3, 5, 7, 10 and 14 d.

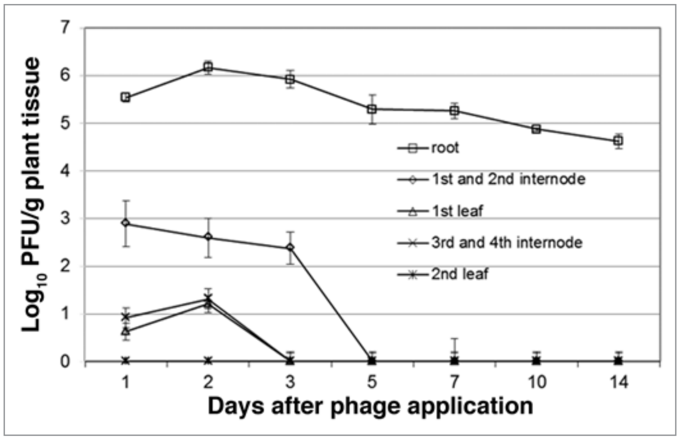

Figure 3. Systemic movement and persistence of *Ralstonia solanacearum* ΦRS5 in tomato cultivar Bonny Best. Four week-old plants were drenched with 30 ml of ΦRS5 suspension at a concentration of 1 × 10^8 PFU/ml. Control plants were treated with water. Destructive sampling was performed to evaluate phage presence in: roots, 1st and 2nd internode, 1st leaf, 3rd and 4th internode and 2nd leaf after 1, 2, 3, 5, 7, 10 and 14 d. Presented values are the average of two experiments.

grown in hydroponic solutions. They observed that uptake of phage f2, through the cut roots of corn and soybean plants, was consistently higher in the sampled upper tissues.

Reduction of the phage population below detectable levels in stems and foliage of the plants with damaged roots 5 d after treatment (**Fig. 1B**) may indicate reduced phage absorbing capacity in plants with injured roots. In the latter two experiments the level of phage uptake and the extent of systemic movement of phages were much lower (**Figs. 2 and 3**). One reason for this difference might be that in the latter experiments single phage strains were used as opposed to the first set of experiments where a commercial phage mixture was used; these differences may be due to differences in the phage virion properties. Our findings were similar to previous studies[42] that demonstrated that phage uptake, irrespective of root damage, will vary depending on the type of phage, plant species, plant size, plant age, and most likely with the kind of soil or media in which the plant is grown.

In our experiments using commercial phage mixtures, *X. perforans* 97-2 specific phage were recovered from roots 1 d after application at levels that did not differ significantly from the concentrations applied. However, in case of pure phage strains ΦMI2 and ΦRS5, the highest phage levels recovered from the roots occurred within 2–3 d after the initial application (**Figs. 2 and 3**), approximately two log units lower than the initial concentration. This could be either due to some differences in the phage properties or phage trapping by substrate particles. In all three sets of the phage translocation experiments, we observed similar trends regarding phage levels within the root system. The highest phage levels in roots typically occurred 2–3 d after initial soil application, regardless of the phage strain or root damage (**Figs. 1–3**). Our experiments also showed that phage could be initially recovered at higher levels from upper plant parts, which then rapidly declined from the 5th to the15th day. The decline may have been due to several factors, possibly plant defense responses or due to photosynthesis, since chlorophyll absorbs solar energy that might be detrimental to phage survival in the absence of a host bacterium.

In addition, the phage appeared to differ in their ability to persist in above ground tissues across experiments. The persistence of ΦRS5 inside stem and leaf tissue was limited to 3–5 d, whereas the *X. perforans* 97-2 specific phage mixture and *X. euvesicatoria* specific ΦMI2 were recovered from 7–15 d after application. However, these differences in phage persistence in above ground tissues might be due to the age of the plant at the time of phage application. Plants used to evaluate the *X. perforans* 97-2 specific phage mixture and *X. euvesicatoria* specific ΦMI2 were older than plants used to evaluate ΦRS5.

Regardless of whether individual phage or commercial phage mixtures were applied to the soil, phage persisted longer in tomato roots and reduced bacterial wilt severity (**Fig. 4**); although efficacy varied depending on the timing of phage treatments relative to the pathogen, *R. solanacearum*. Fujiwara et al.[22] recovered a large number of phages from the phage-treated roots of *R. solanacearum* inoculated and non-inoculated tomato plants for 4 mo after phage were applied. Phage titers recovered from *R. solanacearum* inoculated plants, were 10 times higher than non-inoculated plants, which would be expected considering that phage need bacterial cells to replicate. In the presence of a suitable bacterial host, it is expected that

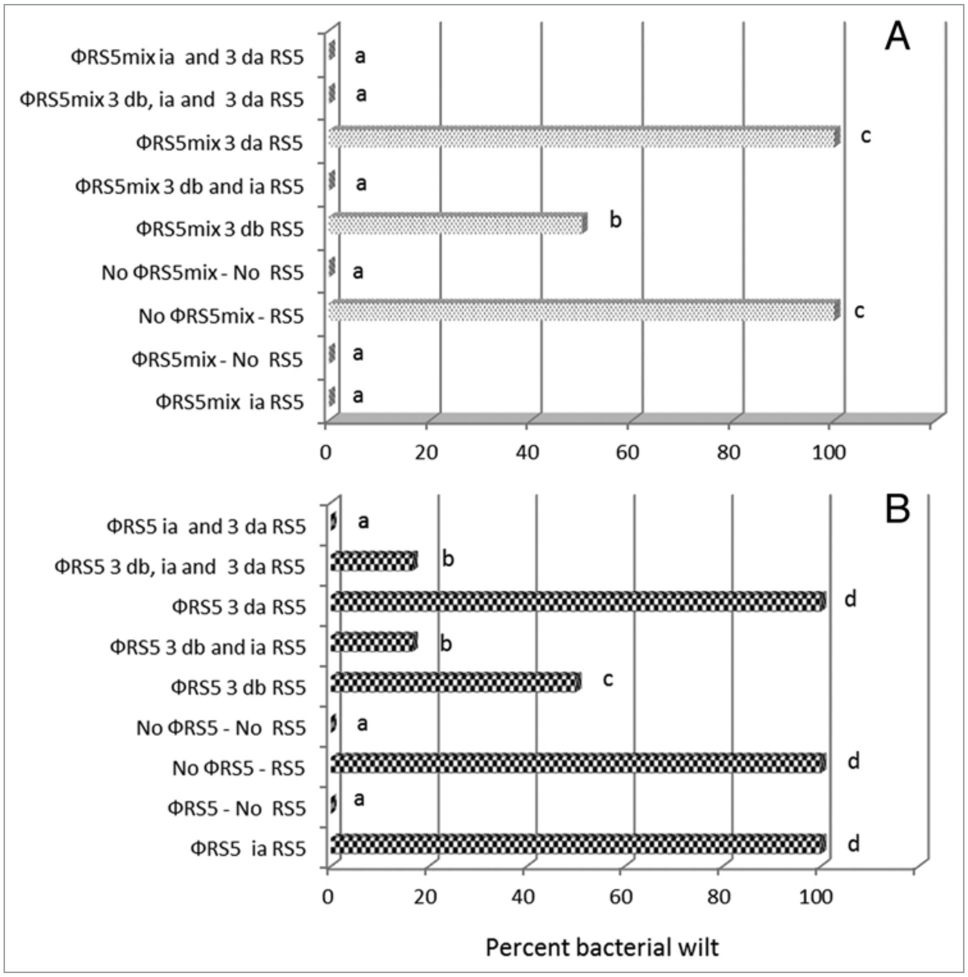

Figure 4. Control of tomato bacterial wilt with a commercial phage mixture or a purified phage against *Ralstonia solanacearum* strain RS5. (**A**) Four week-old tomato cv Solar Set was inoculated with *R. solanacearum* RS5 and treated with a 10^8 PFU/ml of a commercial phage mixture specific to *R. solanacearum* strain RS5 (ΦRS5mix*)* provided by OmniLytics, Inc. (**B**) Four week-old tomato cv Bonny Best was inoculated with RS5 and treated with a single phage strain (ΦRS5). Treatments from bottom to top were: (1) ΦRS5 mix immediately after inoculation (ΦRS5 mix, ia RS5), (2) ΦRS5 mix and non-inoculated (ΦRS5mix, No RS5), (3) Untreated-Inoculated (ΦRS5mix, No RS5), (4) Untreated, non-inoculated (ΦRS5mix, No RS5), (5) ΦRS5 mix 3 d before inoculation (ΦRS5mix, 3db RS5), (6) (ΦRS5mix, 3db and ia RS5), (7) ΦRS5 mix 3 d after inoculation (ΦRS5mix, 3da RS5), (8) (ΦRS5mix, 3db, ia and 3da RS5), and (9) (ΦRS5mix, ia and 3da RS5). Presented values are the average of two experiments. Means followed by the same letter are not significantly different based on Fisher's protected LSD method ($\alpha = 0.05$).

phage would persist longer in roots and confer further protection to plants from further bacterial infection unless the bacterium develops resistance to the phages.

Phage persistence in the phyllosphere is a limitation for the successful use of phages for control of foliar pathogens.[32] In greenhouse and field trials, phage persistence was dramatically improved with the prior application of a phage-sensitive, virulence-attenuated bacterial strain. This attenuated strain became established in the tomato phyllosphere and supported higher phage titers over a 7 d sampling period.

In this study we demonstrated two different approaches for applying bacteriophages which may prove useful for managing bacterial plant diseases. We demonstrated that some phages under certain conditions can be systemically translocated inside plants, and retain their viability there for days. In our present study, a commercial *X. perforans* 97-2 specific phage mixture reached the upper leaves of a tomato and maintained a 10^4 PFU/g leaf tissue concentration for 7 d, compared with a typical foliar application that would generally drop to undetectable levels within 1 or 2 d. Therefore, regular drench/drip applications could maintain a higher level of phage population in the tomato foliage, compared with what foliar sprays can provide. Of course, it is not known if phages present inside the leaves would have any way to contact foliar bacterial pathogens, and to a degree that would affect foliar disease development. It is also unknown whether the phage concentration achievable by root absorption is high enough to be effective for vascular pathogens, like *R. solanacearum*. Based on the importance of bacterial plant diseases and the need for effective control methods, further investigation on this topic would have merit. We also showed that phage populations could be maintained at significantly higher levels in the tomato phyllosphere in which an attenuated strain of its host had colonized. Although the attenuated strain resulted in visible disease on tomato leaves, it is plausible that other mutants can be identified that would colonize the phyllosphere without disease and serve as a suitable host for the phages.

Materials and Methods

Bacterial strains and phages. Bacterial strains used in these studies were stored at −80°C in sterile DI water with 30% glycerol and phages were stored at 4°C in dark. For all experiments, the strains used were grown on nutrient agar (NA) medium 0.8% (wt/V) (BBL, Becton Dickinson and Co.) at 28°C. The bacterial suspensions were prepared by using 24 h cultures grown on NA medium and suspensions were adjusted to $5–10^8$ cfu/ml ($A_{600} = 0.3$), and then were diluted appropriately.

Phage propagation. For field studies, phage-sensitive bacteria were grown in liquid Nutrient Broth (NB) (BBL, Becton Dickinson and Co.) or Luria-Bertani (LB) media shaking at 200 rpm at 28°C. After the addition of the phage and a 5 min incubation period on the bench top, the culture was shaken

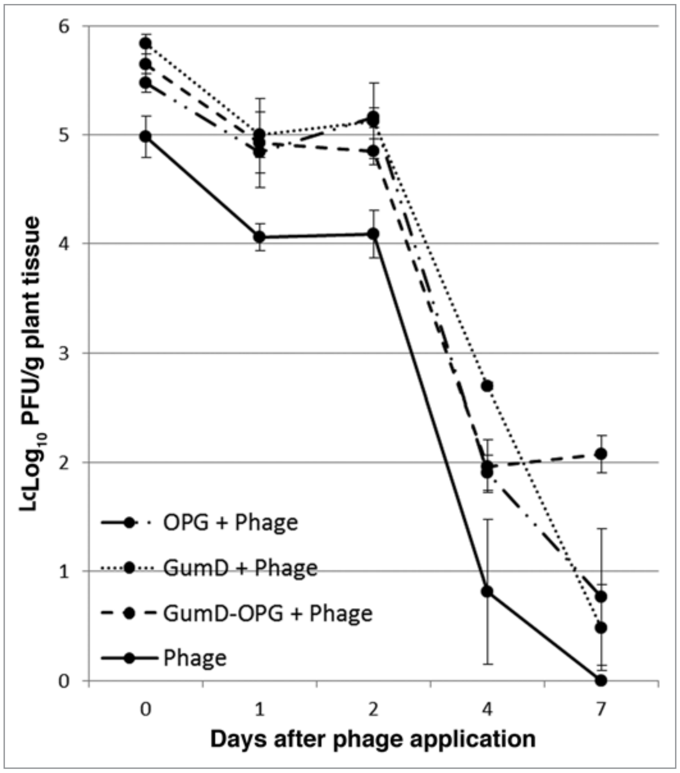

Figure 5. The effect of attenuated *Xanthomonas perforans* strains 91-118:Δ*opgH,* 91-118:Δ*gumD* and 91-118::Δ*opgH*Δ*gumD* on the persistence of phage Xv3-1 (Φ), specific to *X. perforans* 91-118, on tomato leaf surfaces over time. Three plants (3–4 weeks-old) were dipped in a 10^6 cfu/ml suspension of one of the *X. perforans* strains. Phage was applied at 5 × 10^8 PFU/mL 3 d later. Additional plants were treated with only Φ Xv3-1 as a control. A single leaflet from each plant was collected after phage application (0), 1, 2, 4 and 7 d later, and washed to enumerate phage levels, expressed as the number of plaque forming units (PFU) per gram of leaf tissue with standard error based on 3 replicate plants per a treatment.

Table 1. Effect of attenuated Xanthomonas perforans mutants on phage (Φ) persistence based on the area under phage population curve (AUPPC) on greenhouse grown tomato plants

Treatment[§]	AUPPC[ǁ]	
OPG + Φ	21.5	a[¶]
GumD + Φ	23.4	a
GumD-OPG + Φ	23.3	a
Phage	15.0	b
P_{TRT} =	0.0249	

[§] Three plants were dipped in a solution (10^6 cfu/ml) of attenuated *X. perforans* strains 91-118:Δ*opgH*, 91-118:Δ*gumD* and 91-118::Δ*opgH*Δ*gumD*. Phage Xv3-1 was applied at 5 × 10^8 PFU/mL 3 d later. Additional plants were treated with phage alone as a control. [ǁ] AUPPC was calculated using the formula: $\Sigma\ ([(x_i + x_{i-1}) / 2]\ (t_i - t_{i-1}))$ where x_i is the phage population (log PFU/ml) at each evaluation time and $(t_i - t_{i-1})$ is the time between evaluations. [¶] Means followed by the same letter are not significantly different based on Fisher's protected LSD method (α = 0.05).

at 150 rpm at 28°C for 16–18 h. Then the culture was sterilized, enumerated and stored at 4°C in the dark until use. This method yielded phage titers of approximately 10^{10} PFU/ml.[39]

Systemic movement of phage in tomato. For the first set of experiments, a proprietary mixture of phage (OmniLytics Inc.) active against *X. perforans* strain 97-2 were studied using tomato plants cv Bonny Best grown in 10-cm pots containing soilless medium. Plants were maintained in a greenhouse, watered daily, and fertilized every 14 d with a soluble 20-20-20 (N-P-K) fertilizer (0.4 g/pot; Peter's Fertilizer Products, W.R. Grace & Co.). The soil surrounding 4 week-old tomato plants was drenched with30 ml of the phage mixture (10^8 PFU/ml). Treatments consisted of (1) root-injured plants treated with phage, (2) non-injured plants treated with phage, and (3) non-injured and non-phage-treated control plants. Roots were injured in treatment 1 by stabbing the root system with a knife at four different locations in the pot close to the base of each plant. Each treatment consisted of 21 plants with three plants used for destructive sampling at each time point at days 1, 2, 3, 5, 7, 10 and 15 d after treatment. At each time point, the weights of washed roots, upper leaves, upper stems, lower leaves and lower stems (when plants had more than 3 leaves) were determined, before blending individual samples in 25 ml nutrient broth. Blended plant tissue was transferred to a 50 ml centrifuge tube and held for about 5 min at room temperature while plant material settled to the bottom of the tubes. One milliliter of supernatant was transferred to a 1.5 ml micro-centrifuge tube and 100 μl of chloroform was added. From this tube serial dilutions were made and plated with a bacterial suspension of *X. perforans* strain 97-2 for quantifying plaques after a 24 h incubation at 28°C as previously described.[37] The experiment was performed twice.

The next set of experiments was performed similarly, but used phage strain M12 active against *X. euvesicatoria* strain KFB189, which was isolated from the roots of field-grown pepper plants in Serbia. Treatments were similar to the previous study, except the injured root treatment was not included. Following the phage drench application (30 ml/plant), three treated and three non-treated control plants were sampled 1, 2, 3, 5, 7, 10 and 14 d after the initial phage application. The aerial portions of the plants were carefully collected to avoid contaminating the stem and foliage samples with the phage treated substrate. The substrate was thoroughly washed from the roots with tap water followed by removal of the free water from the plant surface by blotting with paper tissue. Plants were sectioned using a sterile scalpel on the following five sections: (1) root; (2) first and second internode; (3) first leaf; (4) third and fourth internode; and (5) second leaf. Phage was enumerated similar to the first set of experiments, except plant tissues were homogenized in sterile water (1 ml water per gram of tissue) using a mortar and pestle. The experiment with the pure phage strain MI2 was performed twice.

A third set of phage trials was performed to test the systemic nature of phages specific to *R. solanacearum*. These tomato experiments followed the same experimental procedure used in the previous MI2 phage trials, except phage strain RS5 (ΦRS5) compatible with *R. solanacearum* strain RS5 was used. The experiment was performed twice. Experimental data from all six trials were collected as the number of plaque forming units (PFU) per g of plant tissue and $\log_{10}$ transformed prior to calculating the mean value from the three replications and performing statistical analyses.

Control of tomato bacterial wilt with ΦRS5. In the first set of experiments, a 10^8 PFU/ml phage mixture specific to *Ralstonia solanacearum* strain RS5 (Φ*RS5mix*) provided by OmniLytics, Inc. was used. Inoculum of *R. solanacearum* strain RS5 was grown overnight on casamino acid peptone glucose broth in a shaker at 28°C. Inoculum concentration was determined with the aid of a spectrophotometer, and adjusted to 10^8 cfu/ml with the same broth. For this experiment, 4-week-old tomato plants cv Solar Set were transplanted to 10 cm pots containing plant growth medium and placed over individual saucers that were also used for watering to avoid cross contamination and maintain high moisture content. The experiment had nine treatments replicated six times and arranged on a greenhouse bench in a randomized complete block design. Bacterial inoculum (6 ml) was applied as a drench around the plant using a 10 ml pipet. Similarly, 5 ml of Φ*RS5mix* (MOI = 1) was applied according to the following treatments: (1) Φ*RS5mix* immediately after (*ia*) inoculation (Φ*RS5mix ia RS5*), (2) Φ*RS5mix* and non-inoculated (Φ*RS5mix*, *No RS5*), (3) untreated-inoculated (*No* Φ*RS5mix*, *RS5*), (4) untreated, non-inoculated (*No* Φ*RS5mix*, *No RS5*), (5) Φ*RS5mix* 3 d before (*3db*) inoculation (Φ*RS5mix 3db RS5*), (6) (Φ*RS5mix 3db* and *ia RS5*), (7) Φ*RS5mix* 3 d after (*3da*) inoculation (Φ*RS5mix 3da RS5*), (8) (Φ*RS5mix 3db, ia* and *3da RS5*) and (9) (Φ*RS5mix ia* and *3da RS5*).

For the second experiment, 4-week-old tomato plants cv Bonny Best were transplanted, moved to a growth chamber (16 h light/8 h dark; 26°C) and treated similarly as in previous experiment. In this experiment, treatments were applied 10 d after transplanting to give the roots time to heal and resume normal growth. Plants were similarly drenched with 6 ml *R. solanacearum* RS5 inoculum, but this time a single phage strain Φ*RS5* prepared as previously described at 10^8 PFU/ml (MOI = 1) was used instead of the OmmiLytics Φ*RS5mix*. To avoid cross contamination, six plants per treatment were placed in the same tray and the substrate was kept moist throughout the 14 d observation period by adding water to the trays. For both experiments, percent of wilted plants per treatment was evaluated after 14–21 d and each experiment was performed twice.

Role of attenuated strains of *X. perforans* in phage persistence in phyllosphere. *Greenhouse experiment.* Three- to 4-week-old tomato plants of cv Bonny Best grown in 10-cm pots were maintained in the greenhouse with temperatures ranging from 25–35°C. Plants were inoculated with *X. perforans* 91-118:Δ*opgH,* 91-118:Δ*gumD* or 91-118:Δ*opgH*Δ*gumD* strains[43,44] separately by dipping three plants each in the appropriate bacterial suspension adjusted to 10^6 cfu/mL and amended with 0.025% Silwet L-77 (Loveland Industries, Co.). Once disease symptoms were observed on inoculated plants, a phage suspension of 5 × 10^8 PFU/mL phage (MOI = 100) was sprayed once on all treatments. Phage suspensions used in greenhouse studies were a mixture (Agriphage from OmniLytics, Inc.) and phage stock Xv 3-1 propagated on *Xanthomonas perforans* 91-118:Δ*opgH* from phage stocks for field trials. The titer of the phage was determined over a 7 d period by sampling one leaflet from each of three plants and quantifying the phage concentrations as described above.

Field experiments. The field experiments were located at the University of Florida's Gulf Coast Research and Education Center (GCREC). Experiments were prepared along three plastic-mulched raised beds, 100 m in length on 1.5 m bed center spacing. Each group of 3 beds was separated by a 4.6 m ditch area. Individual plots consisted of three adjacent 6.4 m bed lengths with plants spaced every 46 cm, and included a 3.7 m non-planted buffer area between plots on the same beds to minimize inter-plot movement of phage and bacterial treatments. Treatments were replicated 4 times and arranged in a randomized complete block design. All treatments and measurements were made to the center bed of each plot, using plants in the outer beds to minimize inter-plot interference. Field experiments were conducted in the summer and fall of 2011 with tomato cultivar SecuriTY 28, and the *X. perforans* 91-118:Δ*opgH* attenuated mutant as the host strain for phage persistence studies. Either a 10^7 or 10^8 cfu/ml suspension of *X. perforans* 91-118:Δ*opgH* was prepared in 10 mM $MgSO_4$ and applied to tomato foliage in select plots before sunrise with a backpack sprayer. An enriched phage ΦXv 3-1 specific to *X. perforans* 91-118:Δ*opgH* in a 0.75% (wt/V) skim milk suspension was applied weekly in the evening to specific plots at 10^8 PFU/ml after the first *X. perforans* 91-118:Δ*opgH* applications were made (corresponding to an MOI of 0.1 and 1 for plots treated with 10^7 or 10^8 cfu/ml suspension of *X. perforans* 91-118:Δ*opgH*, respectively). Treatments included: (1) *X. perforans* 91-118:Δ*opgH* applied alone at 10^7 cfu/ml, (2) *X. perforans* 91-118:Δ*opgH* applied at 10^7 cfu/ml followed by phage, (3) *X. perforans* 91-118:Δ*opgH* applied at 10^8 cfu/ml followed by phage; (4) a phage alone control; and (5) a non-treated control. Initially, weekly applications of *X. perforans* 91-118:Δ*opgH* were made for the first 2 weeks, and then once every 2 weeks for the remainder of the summer trial and once every 3 weeks for the remainder of the fall trial.

Phage isolation from phyllosphere and quantification of phyllosphere populations. For detection of phage in the greenhouse and field studies, leaflets were sampled to monitor phage persistence on the leaf surface at days 0, 1, 2, 4, 7. For greenhouse studies, samples were also collected on day 10. For field trials, five leaflets were removed from the middle part of each plant to create a composite sample for each plot, while for greenhouse trials three leaflets were taken from the middle part of each plant. The samples were placed in a portable Styrofoam cooler and immediately carried to the laboratory and processed for phages as described above. The leaflets were placed in Erlenmeyer flasks containing 100 ml or 50 ml sterile DI water for field and greenhouse trials, respectively, and agitated for 15 min. One milliliter aliquots of the rinsate were transferred to 1.5 ml microcentrifuge tubes. To each tube 100 μL of chloroform was added. Tubes were incubated on a rotary shaker for 30 min. The chloroform was pelleted by centrifugation at 13,000 rpm speed for 15 min. The aqueous top phase was transferred into new centrifuge tubes. The tubes were centrifuged at 13,000 rpm for 15 min to remove cellular debris. The supernatant was used for enumeration of the phage titer after dilutions. For numeration of

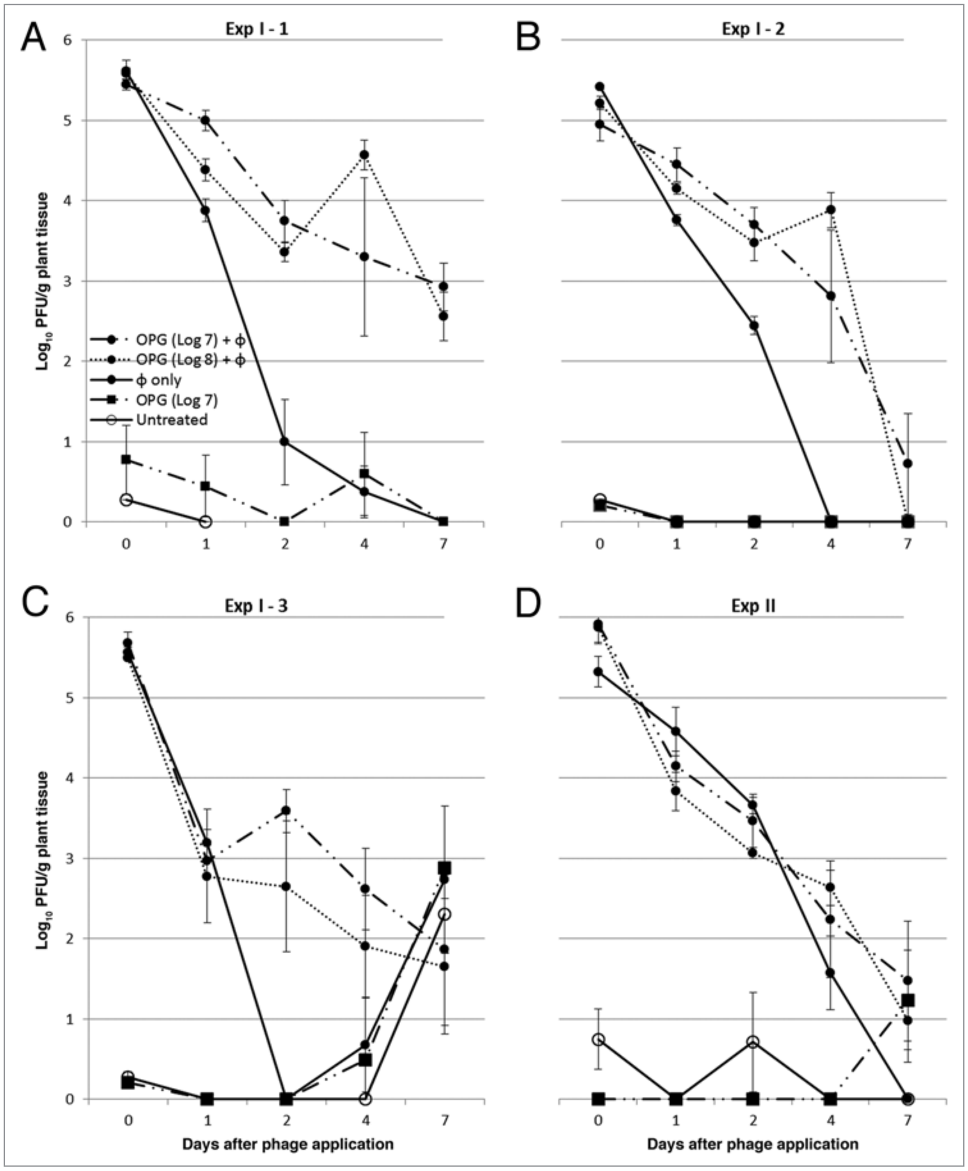

Figure 6 (See opposite page). The effect of an attenuated *Xanthomonas perforans* strains 91-118:Δ*opgH* (OPG) on the persistence of phage Xv3-1 (Φ) on tomato leaf surfaces over time. Three field trials were performed during the summer of 2011 (**A–C**) and a single field trial during the fall of 2011 (**D**). For each field trial, OPG was applied to tomato plants every 2 (**A–C**) or 3 (**D**) weeks as either a 10^7 or 10^8 cfu/ml bacterial suspension. Five random leaflets from each plot were collected after phage application (0), 1, 2, 4 and 7 d later, and washed after phage were applied to enumerate phage levels, expressed as the number of plaque forming units (PFU) per gram of leaf tissue with standard error based on 4 replicate plots per a treatment. Additional plots were treated with either phage or OPG, or left untreated as a control.

phage titer in greenhouse and field trials, soft nutrient agar yeast extract medium (NYA) [0.8% Nutrient Broth, 0.6% Bacto Agar and 0.2% Yeast Extract (Difco, Becton Dickinson and Co.)] was used. Bacterial cells from 24 h-old cultures were suspended in 2 ml $MgSO_4$ and 100 μL of the concentrated bacterial suspension was added in empty Petri dishes. Sixteen mililiters warm (48°C) NYA medium was poured into the plate. The dishes were gently swirled for even distribution of the bacteria. After the medium solidified, 10 μL dilutions of the phage suspension were spot inoculated. After the phage suspension dried, the plates were transferred to 28°C incubators and after 24 or 48 h the plaques were counted at the appropriate dilutions. The phage concentration was calculated from the plaque number and specific dilution and expressed as PFU/ml. Population data were log-transformed and standard errors were determined. The overall growth curve was determined by calculating the area under the population progress curve (AUPPC). The AUPPC is a modification of the area under the disease progress curve (AUDPC) which has been used to analyze population progress:[45] standardized AUPPC = $\Sigma\ ([(x_i + x_{i-1}) / 2]\ (t_i - t_{i-1}))$ where x_i is the phage population (log PFU/ml) at each evaluation time and $(t_i - t_{i-1})$ is the time in days between evaluations. The data were then subjected to an analysis of variance in SAS version 9.2 (SAS Institute, Inc.) using PROC GLIMMIX to assess the effect of treatments on AUPPC or phage populations over time. For the

Table 2. Effect of *Xanthomonas perforans OPG* mutant on phage (Φ) persistence on field grown tomato plants

Treatment[§]	Exp I-1		Exp I-2		Exp I-3		Exp II	
OPG (10^7) + Φ	25.8[‖]	a[¶]	20.5	a	20.4	a	20.0	a
OPG (10^8) + Φ	27.4	a	21.8	a	16.6	ab	19.3	a
Φ (Phage only)	9.0	b	10.0	b	11.7	bc	16.6	a
OPG (10^7)	2.2	c	0.0	c	5.5	cd	1.7	b
Untreated control	0.0	c	0.0	c	3.5	d	1.3	b
P_{TRT} =	< 0.0001		< 0.0001		0.0002		< 0.0001	

[§] Field plots were sprayed with a solution (10^7 or 10^8 cfu/ml) of attenuated *X. perforans* strains 91-118:Δ*opgH* (OPG) every two (Exp I-1, -2 and -3) or three (Exp II) weeks. Phage Xv3-1 was applied to foliage at 5 × 10^8 PFU/mL on May 23 (Exp I-1), June 6 (Exp I-2), June 20 (Exp I-3) and Dec 8 (Exp II). Additional plots were treated with either phage or OPG alone, or left untreated as controls. Five leaflets were collected from each plot immediately after phage application, and 1, 2, 4 and 7 d later to enumerate phage levels. [‖] Values indicate AUPPC, which was calculated using the formula: $\Sigma\ ([(x_i + x_{i-1}) / 2](t_i - t_{i-1}))$ where x_i is the phage population (log PFU/ml) at each evaluation time and $(t_i - t_{i-1})$ is the time between evaluations. [¶] Means followed by the same letter are not significantly different based on Fisher's protected LSD method (α = 0.05).

analyses of AUPPC data, block and the interaction of block × treatment were considered random effects in the model. Repeated measures were performed to examine phage populations over time in field and greenhouse trials, with block and the interaction of block × time fitted to a heterogeneous compound-symmetry covariance structure as a random effect in the analyses. Means separation were based on Fisher's protected LSD method (α = 0.05).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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