# Survey of Phages in *Xylella fastidiosa* Almond Leaf Scorch Strains

**Project No.:** **08-PATH10-Chen**

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**Objectives:**

To survey *Xylella fastidiosa* almond leaf scorch disease strains in the field for the presence of phages (viruses that infect bacteria) and estimate the occurrence frequency as an effort to understand the population dynamics of the bacterium under natural conditions.

**Interpretive Summary:**

*Xylella fastidiosa* causes almond leaf scorch disease (ALSD) in California. The bacterium is nutritionally fastidious and difficult to culture in artificial media. Studies on the biology of *X. fastidiosa* are highly challenging. As a result, our knowledge about the pathogen behavior and ALSD is limited. Previous observations have found that incidences of ALSD varied from year to year in the Central Valley of California. One reasonable explanation is related to the population changes of *X. fastidiosa*. Towards the understanding of the bacterial population dynamics, this project studied the *in planta* activities of the bacterial phages, or the bacterial viruses, and their impact on the bacterial population. Guided by the available genomic information, five phages of *X. fastidiosa* were indentified and their activities in almond orchard were monitored. Our data showed that there were strong phage activities in the *X. fastidiosa* population. The occurrence frequencies range from 0.21 to 0.84. A frequency of 0.16 was observed between the presence of bacterial phages and the absence of *X. fastidiosa* cells. This is tentatively interpreted as the reduction or killing power of phages on *X. fastidiosa*. Results from this study demonstrated that phage research in *X. fastidiosa* could provide clues to explain the fluctuation of ALSD incidences over different years and additional direction for future ALSD management strategies.

**Materials and Methods:**

Selection of phages

All of the phages in this study were prophages, i.e. they were identified from the bacterial genome sequences. Five phage sequences were selected for the field survey (**Table 1**). Phage 3-1 was originally identified in a Florida Pierce's disease (PD) strain (Chen et al., 2005a). Phages 346, M12#3, M23#4 and M23#5 were recently identified from ALSD strains isolated from California. With the phage sequences, polymerase chain reaction (PCR) primer were designed using Primer 3 program (Rozen and Skaletsky. 2000) by setting the annealing temperature at 60 ± 3 C. A PCR amplification from a primer set represented the occurrence of the corresponding phage in the ALSD sample.

**Table 1.** Phage primers, their nucleotide sequences and amplicon sizes in this study

| Phage | Forward primer | Reverse primer | Amplicon size |
|---|---|---|---|
| 3-1 | ATTGCGTCAGGCCAATGTAT | GATGCCAGGCACCAGTATTT | 300 bp |
| 346 | AAAGAAGACCAGCGATTTTT | GCAGGTACTTGAGGTAGTCG | 346 bp |
| M12#3 | GGAATAGCCTCCCATTAAGT | TGAAACATCGCTTGTACTTG | 954 bp |
| M23#4 | AGCAAAATAAGAAGCAATCG | TGAGGGGAAAACTTGTCTTA | 961 bp |
| M23#5 | CGATAAGGTGTCCACTGAAT | TTCATTGCCGATTCTTAAAT | 920 bp |

Sample collection

For detection of phages from different *X. fastidosa* strains, bacterial cultures maintained in the USDA-ARS facility at Parlier, California, were used. Strains of *X. fastidiosa* were grown in PW medium for 10-14 days. Bacterial cells were collected and suspended in sterile water. The cell suspensions were used as templates for PCR amplification.

For the field survey, almond leaves showing ALSD symptoms were collected from orchards in Kern County in October 2008 when ALSD symptoms are visible. Samples were immediately sent to the USDA laboratory at Parlier, California. Phages were detected directly in infected plant tissues. To prepare the plant tissue for PCR, the collected almond leaves were placed in a labeled paper envelope, freeze-dried in a freeze-drier following the published procedure (Chen et al., 2008). One dried petiole (about 2 cm long) was selected to represent the sample and pulverized with a Fast-prep machine (FP120, Qbiogene, Inc. Carlsbad, CA). The pulverized freeze-dried tissue was suspended in 500 µl of TE buffer and used for PCR after dilution.

The presence of a phage was determined by the amplification of an expected amplicon with the corresponding primer set. The presence of *X. fastidiosa* and its genotype were identified by multiplex PCR where primer set Teme150fc (5′ tctaccttat cgtgggggac 3′) and Teme454rg (5′ aacaactagg tattaaccaa ttgcc 3′) was specific to G-genotype and

primer set Dixon454fa (5′ccttttgttg gggaagaaaa 3′) and Dixon1261rg (5′ tagctcaccc tcgcgagatc 3′) was specific to A-genotype.

PCR procedure

PCR reaction (25 µl) was carried out using the TaKaRa Taq™ (Hot Start Version) kit (Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan). The reaction mixture contained: 2.5 µl of 10×DNA polymerase buffer, 2.5 µl of dNTPs (2.5 mM of each dNTP), 0.5 µl of each of the 10 µM forward and reverse primers, 1 µl of diluted petiole suspension, 0.2 µl of Taq DNA polymerase (5 U/µl) and 18.3 µl of H2O. The multiplex PCR procedure (Chen et al., 2005b) was used for *X. fastidiosa* and phage detection. Briefly, primers were used for PCR amplification in an MJ Research Tetrad II DNA engine with an initial denaturing at 96 °C for 10 min, followed by 30 cycles consisting of: denaturing at 96 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The amplification products were stored at 4 °C. The amplified DNAs were resolved through 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Transmission electron microscopy

A *X. fastidoisa* ALSD strain was cultured in 500 ml PW broth for 30 days. Bacterial cells were removed by centrifugation. The supernatants were then concentrated by high speed centrifugation at 155,000 g for 1.5–2 hours. The high speed centrifugation pellets were resuspended in 200–500 µl sterile distilled water. Five µl of phage suspension was prepared in a 400-mesh copper grid and stained in 2% uranyl acetate. The grid was air-dried and examined by TEM as described previously (Chen and Civerolo, 2008). Images were made with a Megaview III digital camera using analysis software.

Data analyses

The occurrence frequencies of phages were calculated based on the total number of samples collected after the exclusion of samples that were negative to both *X. fastidiosa* and phages. Comparisons were made among occurrence frequent between bacterial cells and phages. An association index was calculated based on the formula: 2xy/(x+y), where x is the number of positive detection of *X. fastidiosa*, y is the number of positive detection of a phage, and xy is the number of positive detection to both *X. fastidiosa* and a phage.

## Results and Discussion:

Phage 346 was recently identified from a *X. fastidiosa* ALSD strain. To test the host range of this phage, primers were designed and PCR was performed on 25 *X. fastidiosa* strains from almond, grape and oleander. Among them, 20 were from ALSD samples collected in California. With two exceptions, all almond strains and two grape strains were positive with phage 346. The two exceptional almond strains were weakly positive. Absence of phage 346 was found in the three oleander strains (**Figure 1**), suggesting host specificity of this phage.

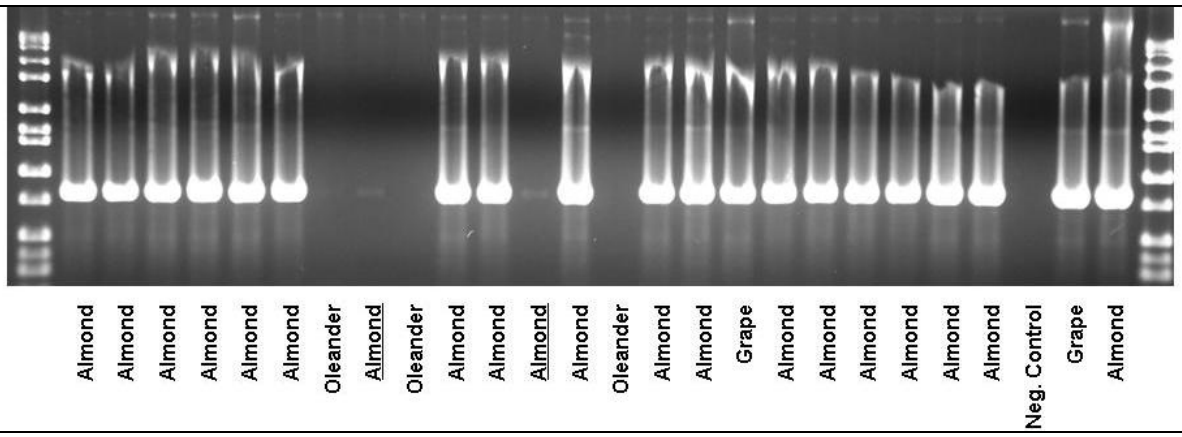



**Figure 1.** Detection of phage 346 from 25 Xylella fastidiosa strains isolated from California.

Results of *In Planta* detection of phage with phage 346 and *X. fastidiosa* are representatively shown in **Figure 2**. In most cases, when *X. fastidiosa* cells were detected, phages were detected. Our focus was, however, in the situation where phages were detected but not the bacterial cells. This may represent the reduction or killing power of phages on *X. fastidiosa.*

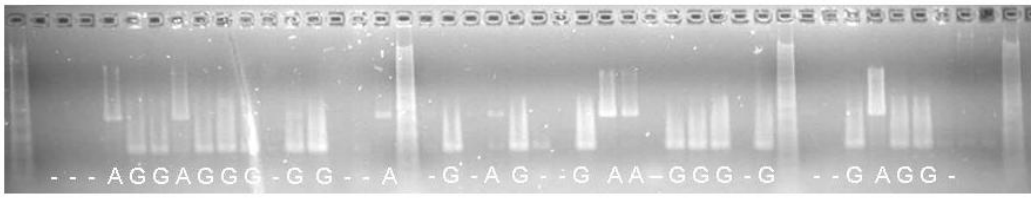
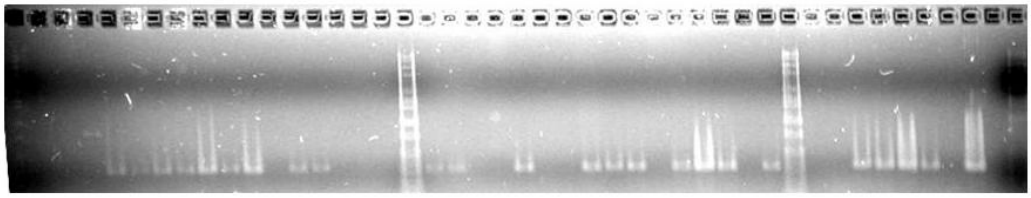
| | |
|---|---|
| X. fastidiosa cells |  |
| Phage 346 |  |

**Figure 2.** *In planta* detection of *Xylella fastidiosa* cells and phage 346.

A summary of detection of the five phages in 43 ALSD samples were shown in **Table 1.** Among the 7 samples with the absence of *X. fastidiosa*, the presence of phages was 1 for phage 3-1, 5 for phage 346, 2 for phage M12#3, 4 for phage M23#4, and 1 for phage M23#5. Considering the 43 samples together, a frequence of 0.16 was observed between the presence of bacterial phages and the absence of *X. fastidiosa* cells. Tentatively, this is regarded as the reduction power of tested phages on *X. fastidiosa*. Since *X. fastidiosa* harbors more phages, the overall reduction power remains unknown. A more comprehensive research will provide more accurate data in this regard. It is also unclear how these phages caused the reduction of *X. fastidosa* cells. This preliminary study provides additional clues towards the understanding the population dynamics of *X. fastidiosa* and for future disease management strategies.

**Table 1**. In planta detection *of Xylella fastidiosa* and its phages.

| | *X. fastidiosa* | | | Phage | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sample** | **G** | **A** | **Absence** | **3-1** | **346** | **M12 #3** | **M23 #4** | **M23 #5** |
| **371/25-3 E** | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| **371/25-3 J** | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| **371/25-3 C** | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| **9-1 19** | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| **9-1 20** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **370/29-3 4** | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| **R-370-29-3 5** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **371/25-3 D** | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| **370/29-3** | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| **R-370-29-3 3** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **371/25-3 L** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **371/25-3** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **9-1 11** | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| **9-1 14** | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| **9-1 13** | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| **370/29-3 3** | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| **370/29-3 2** | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| **R-370-29-4 2** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **9-1 21** | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| **371/25-3 G** | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **R-370-29-3 7** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **R-370-29-4** | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **371/25-3 A** | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| **371/25-3 I** | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| **371/25-3 H** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **9-1 2** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **9-1 23** | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| **371/25-3 Q** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **304/9-1 5** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **371/25-3 P** | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| **304/9-1 3** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **R-370-29-3 6** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **9-1 22** | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 |
| **9-1 10** | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 |
| **371/25-3 N** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **371/25-3 O** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **371/25-3 M** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **371/25-3 F** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| **371/25-3 B** | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| **R-37029-3 2** | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **9-1 18** | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| **R-370-29-3 1** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| **R-370-29-3 8** | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| | | | | | | | | |
| Total | 27 | 9 | 7 | 26 | 36 | 9 | 32 | 14 |
| Occurrence frequency | 0.63 | 0.21 | 0.16 | 0.60 | 0.84 | 0.21 | 0.74 | 0.33 |

We detected strong phage activities in the *X. fastidiosa* population. The occurrence frequencies range from 0.21 to 0.84. Phage 346 was the highest (0.84). It occurred in both A- and G-genotype strains. Phage M12#3 had the lowest occurring frequency (0.21). Interestingly, this phage was more associated with A-genotype strains with an association index of 0.67. On the other hand, phage 346 was G-genotype-associated with an association index of 0.76.

It has been highly challenging for phage research in *X. fastidiosa* to identify phage particles. In this study, the morphology of phage particles was examined. In addition to the previous observation of icosahedral morphology (Chen and Civerolo, 2008), short tails of some particles were observed (**Figure 3**), confirming that the phages are in the Family of Podoviridae.

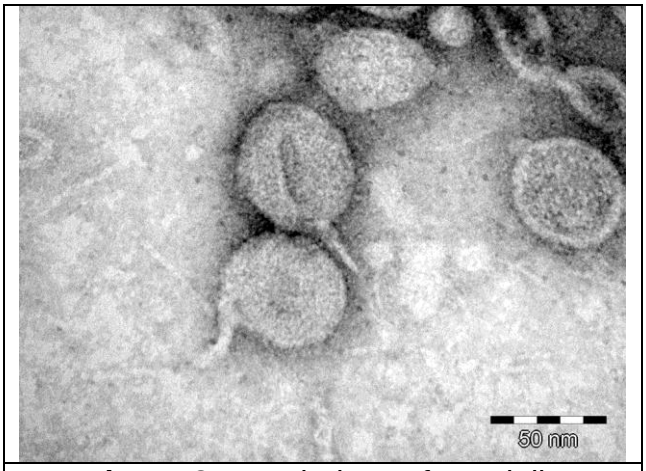



**Figure 3.** Morphology of a *Xylella fastidiosa* phage.

In summary, we have made a preliminary study on selected phages of *X. fastidiosa* ALSD strains in field and estimated their occurrence frequencies as an effort to understand the population dynamics of the bacterium and the disease it caused. Results from this study demonstrated that phage research in *X. fastidiosa* could provide clues to explain the fluctuation of ALSD incidences over different years and additional direction for future ALSD management strategies.

## References:

Chen, J., Civerolo, E.L., Jarret, R.L., Van Sluys, M.A., De Oliveira, M. C. 2005a. genetic discovery in Xylella fastidiosa through sequence analysis of selected randomly amplified polymorphic DNAs. 50:78-83

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Rozen, S. and H.J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. P. 365-386 In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ.

## Recent Publications:

A manuscript summarizing the results from this study is in preparation to submit to a refereed microbiology journal. An abstract has been submitted to the 2009 American Phytopathological Society Annual Meeting in Portland, OR.