BACTERIOPHAGE AND BACTERIOCINS OF *XYLELLA FASTIDIOSA*: POTENTIAL BIOCONTROL AGENTS

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ABSTRACT
The development of a bacteriophage (phage) and/or high molecular weight bacteriocin-based biocontrol strategy offers a novel approach for the control of Pierce's disease caused by *Xylella fastidiosa (Xf)*. We have isolated, propagated and characterized a functional *Xylella* phage, designated *Xf*as53. Phage morphology was examined by electron microscopy and was classified as belonging to the family *Podoviridae* with a head diameter of 55 nm and a short non-contractile tail having a diameter of 12 nm. The dsDNA genome of *Xf*as53 was determined to be 36,673 base pairs with a GC content of 57%. Bioinformatic analysis of the *Xf*as53 genome predicted a total of 46 protein coding genes.

INTRODUCTION
Pierce's disease of grapes, caused by the xylem-limited, fastidious bacterium *Xylella fastidiosa (Xf)*, is a limiting factor in the cultivation of high quality wine grapes. This disease is particularly damaging to Texas vineyards. The Texas Wine Marketing Institute in 2007 reported that the Texas wine industry currently consists of 2,900 bearing acres which produced 8,500 tons based on 2005 year end data (http://www.depts.ttu.edu/hs/texaswine/docs/TX_ECONOMIC_IMPACT_2007.pdf). The full economic benefit of Texas wine and grape industry is estimated at $997.3 million to the state's economy and provides over 8000 jobs. It is estimated that the disease has resulted in several millions of dollars in damage due to lost vines, replanting costs, and the closure of numerous productive vineyards. Pierce's disease has also become a major threat to the nation's most valuable wine producing regions in California, with the potential to have an economic impact in the billions of dollars. Other strains of the same bacterium cause disease in oleander, peach, plum, and several shade trees including sycamore and a number of oak species native to Texas. *Xf* has a wide host range and is transmitted by several species of sharpshooter leafhoppers.

The search for new ways to combat microbial pathogens is an ongoing process in both medicine and agriculture. The long-term goal of our project is to ultimately develop a phage and/or bacteriocin–based biocontrol agents to control *Xf,* the causal agent of Pierce's disease in grapes. The double-stranded DNA (dsDNA)-containing phages are very likely the most numerically abundant group of similar organisms in the biosphere (Hendrix et al. 1999). Treatment of a disease with phages, a practice also termed phage therapy, involves the use of bacterial viruses that can only attack specific bacteria to kill the targeted pathogenic microorganism. Despite a controversial legacy arising from the pre-DNA era of microbiology, there is growing interest in reconsidering therapy as an additional weapon against both human (Chibani-Chennoufi et al. 2004, Bruttin and Brussow 2005) and plant bacterial pathogenesis (Federal Register /Vol. 70, No. 248/Wednesday, December 28, 2005, Balogh et al. 2003, 2008). Phage therapy pre-dates antibiotics by decades, but was largely abandoned when chemical antimicrobials became readily available. Now, however, the emerging threat posed by antibiotic-resistant pathogens is spurring a resurgence of interest in phage as a potential therapy to cure or prevent infections, and as a tool to kill food-borne pathogens. A combination of six phages were recently approved by the FDA to be sprayed on ready-to-eat meat and poultry products, including sliced ham and turkey (Federal Register Vol. 71, No. 160, Friday, August 18, 2006). Multiple commercial efforts to develop phage therapeutics are underway (www. evergreen. edu/phage/companies.htm for a list). We submit that it is necessary to address experimentally the key scientific issues that are involved to establish practical phage and/or bacteriocin therapy for Pierce's disease.

The genomes of the *Xylella* strains have a high number of phage-related sequences dispersed in their chromosomes, constituting 7% of the CVC strain 9a5c genome and 9.02% of the Temecula strain genome (Simpson et al. 2000) suggesting that bacterial viruses have contributed to the evolution of *Xylella.* The 9a5c genome exhibits five potential prophage regions (Simpson et al. 2000, Canchaya, 2004) with a different GC content (57%) and several other phage-related genes dispersed throughout the sequence, which result in a high percentage of repeated fragments. These regions and genes are organized

differently in the Temecula strain genome. A total of eight clusters of phage-related genes have been identified in the Temecula strain, none of which is present in strain 9a5c genome (Moreira et al. 2005). A more recent analysis using the "Prophage Finder" program indicates four and three potential prophage clusters in the strain 9a5c and Temecula genomes, respectively (Bose and Barber 2006). Using manual annotation, we have identified three and four potential prophage clusters in the draft sequence contigs of the Dixon and Ann-1 strains, respectively. Presumptive phage particles associated with the Temecula strain grown in PW broth were recently observed by transmission electron microscopy (Chen and Civerolo 2008).

OBJECTIVES

1. To develop a method for the isolation and propagation of *Xylella* phage.
2. To isolate lysogenic and/or virulent phage.
3. To characterize the isolated phage.

RESULTS AND DISCUSSION

The formulation of a semi-solid medium that is conducive to even dispersal and confluent growth of *Xf*, a technique which is required for the efficient manipulation and study of phages and bacteriocins, has not been previously reported in the literature. We have established such techniques and developed an efficient plate assay for detection of phage and bacteriocins **(Figure 1)**. Using this novel method we were able to screen a 30 X 30 matrix using each isolate as an indicator to test supernatants of isolates grown in PW-M broth. California *Xf* isolates included in the study were Temecula, Ann1 and Dixon (Feil and Purcell 2001). Texas isolates included one each from American Sycamore (*Plantanus occidentalis)*, seacoast sumpweed (*Iva annua*), annual sunflower (*Helianthus annuus)*, redspike mexican hat (*Ratibida columnifera*), and western ragweed (*Ambrosia psilostachya*), black Spanish grape (*Vitis aestivalis* hybrid), mustang grape (*Vitis mustangensis*), as well three giant ragweed (*Ambrosia trifida var. texana*) isolates, two oleander (*Nerium olenander*), and 15 grape isolates (*Vitis vinifera*) isolated from different commercial varieties of grapevines grown in Texas.

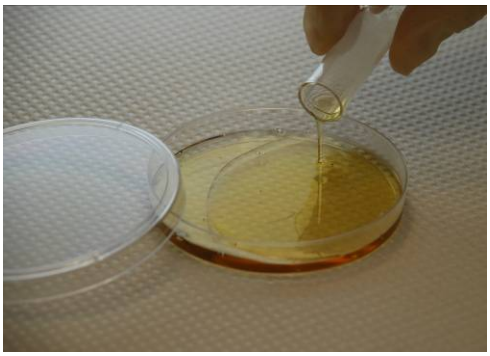

Figure 1. Plate overlay method for isolation and propagation of *Xylella* phage.

Using the overlay method, we were able to identify plaque production on plates seeded with hosts. Phage activity was indicated by plaque formation. Serial dilutions of supernatants indicating activity were plated using the overlay method in which the bacterial suspensions and phage were added and mixed before being applied. After incubation for five-seven days at 28 ºC, individual plaques were excised from the overlay, suspended in phage buffer and titered. This procedure was repeated twice to obtain a single clonal plaque isolate. High titer lysates (10^{10} PFU/ml) were prepared by harvesting overlays of plates exhibiting confluent lysis (**Figure 2A**). To perform further analysis, high titer lysates were purified using a CsCl gradient (**Figure 2B**).

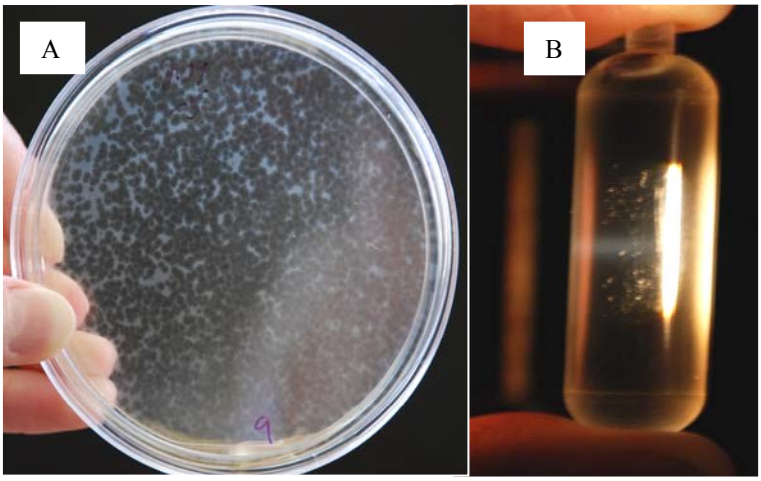

Figure 2. **A**. Dilution series of phage Xfas53 exhibiting confluent lysis of host. **B**. CsCl gradient showing phage band (red arrow).

Transmission electron microscopy of purified phage revealed that phage *Xf*as53 belonged to the family *Podoviridae* with a head diameter of 55 nm and a short non-contractile tail having a diameter of 12 nm **(Figure 3 A and B).**

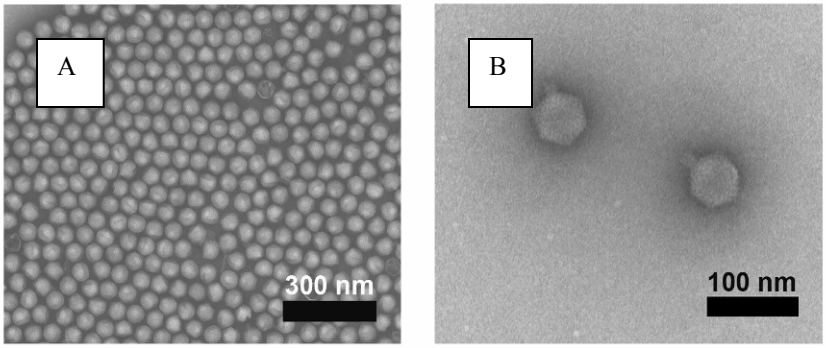

Figure 3A and B. Electron micrograph of CsCl purified phage Xfas53 stained with 2% (w/v) aqueous uranyl acetate.

The adsorption of *Xf*as53 was characterized by measuring the disappearance of free phage after mixing with the susceptible host cells. We observed 95% adsorption to susceptible host in 15 min at an MOI of 10^{-3}. Both sensitive and resistant *Xf* isolates from a variety plant hosts were identified in a host range study. Representative *Xf* isolates from a range of hosts or grape varieties that exhibited sensitivity to phage *Xf*as53 are listed in Table 1.

Table 1. Representative isolates, plant source and origin exhibiting sensitivity to phage *Xf*as53.

Plant source-variety	Isolate	Origin
Oleander	*Xf* 95	M. Black (TX)
Black Spanish Grape	*Xf* 39	M. Black (TX)
Mustang Grape	*Xf* 41	M. Black (TX)
Grape-Chambourcin	*Xf* 48	Apple/Torres(TX)
Grape-Sangiovese	*Xf* 76	Gonzalez/Enderle (TX)
Grape-Zinfandel	*Xf* 67	Apple/Torres(TX)
Grape-Zinfandel	*Xf* 78	Gonzalez/Enderle (TX)
Grape-Syrah	*Xf* 66	Gonzalez/Enderle (TX
Grape	Temecula	Temecula (CA)

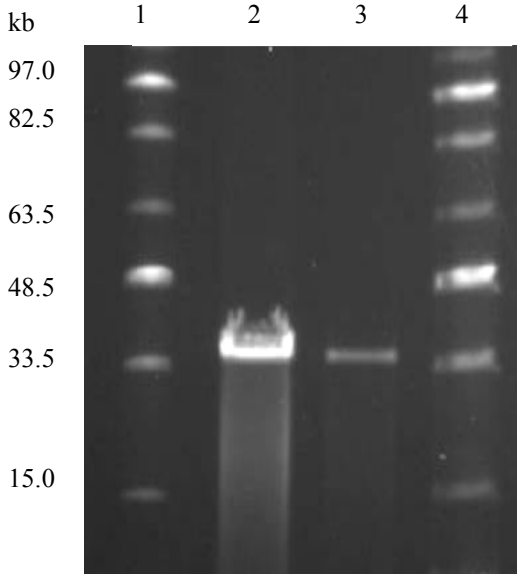

Figure 4. Ethidium bromide stained gel showing 0.5 µg (lane 2) and 0.05 µg (lane 3) of genomic DNA from phage Xfas53. MidRange PFGE Marker I (lanes 1 and 4). Marker sizes are indicated.

The genome size of phage *Xf*as53 was estimated to be approximately 36 kb by pulsed-field gel electrophoresis (**Figure 4**), and no genomic ladder, indicating end annealed multimers, was observed, suggesting that the phage uses pac-type rather than cos-type DNA packaging.

A random library of the phage DNA was sequenced with eight-fold coverage. The assembled reads resulted in the production of a single contig of 36,673 base pairs with a GC content of 57 %. A total of 46 protein coding genes were predicted. The genes are organized into two transcription units with the first 19 genes being transcribed from the reverse strand and all but three of the remaining 27 genes being transcribed from the forward strand **(Figure 5**). Genes at the divergent promoter region include some with similarity to proteins implicated in lysogenic control in other temperate phage. *Xf*as53 has a lysis cassette that includes a holin, endolysin, Rz, and Rz1 equivalent. Functional annotation of the 46 genes indicates these include genes for DNA replication and metabolism, lysogenic control, host cell lysis, and virion morphogenesis. Comparison of the *Xf*as53 encoded proteins to those from other phages indicated that the predicted structural proteins are most closely related to the Bpp-1 – like podophages as well as *Thalassomonas* phage BA3.

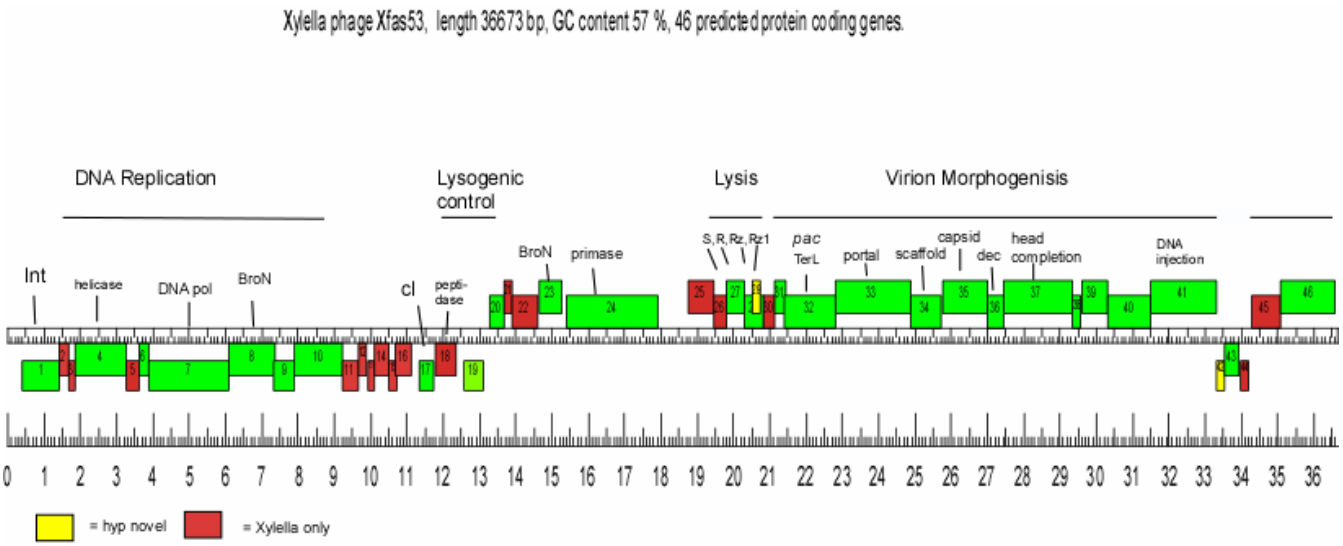

Figure 5. Map of phage *Xf*as53. Boxes are predicted genes drawn to scale and placed above or below the genome scale bar, based on transcription orientation. Gene numbers are listed in the boxes and homolog or functional assignments, where possible, are indicated. Yellow boxes indicate novel hypothetical proteins. Red boxes indicate proteins unique to *Xylella*. Green boxes indicate conserved hypothetical proteins.

CONCLUSIONS

We have isolated and characterized a functional lysogenic phage of *Xf*. This is a significant step forward in understanding the biology of *Xf* and its phages, which will allow us to study the phage-*Xylella* interaction and the potential use of phages as biocontrol agents. Our results increase the probability of success in identifying functional virulent phage and/or bacteriocins for the implementation of a control strategy that is not currently available against this economically important pathogen.

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