GENOMIC CHARACTERIZATION OF A LYSOGENIC PHAGE FROM *XYLELLA FASTIDIOSA*

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ABSTRACT
Xylella fastidiosa (Xf) is an important pathogen causing diseases on several economically important crops, such as grape and almond, in California. Despite recent intensive efforts to study this nutritionally fastidious pathogen, many biological traits of the bacterium, such as bacteriophages, remain unclear and deserve more research attention. We have consistently observed the presence of phage particles from supernatants of *Xf* culture. Further study on phage requires genome characterization. In this study, we conducted an experiment to induce the release of lysogenic phage particles from bacterial hosts in PD3 broth. Phage particles were enriched from culture supernatant. Examination by electron microscopy showed particles with morphology similar to those in Family Podovirideae. Strain Dixon showed a relatively higher phage titer and therefore was used for phage genome characterization. Bacterial chromosomal DNA in the phage preparation was removed by DNAse digestion. Phage DNA was amplified by PCR using random primers, cloned, and sequenced. A BLAST search identified a cloned sequence matching to a prophage region in the whole genome sequence of several *Xf* strains. This sequence also shares similarity (but was not identical) with a phage sequence from a Texas strain of *Xf*. Using the published whole genome sequences as a guide, we are trying to identify the whole phage genome. Several challenges need to be overcome: 1) Phage titer is still not sufficiently high to generate large quantity of phage DNA for analysis; 2) DNAse inhibitor are present in phage preparations; and 3) The genome sequence of strain Dixon is not enclosed and sequence quality remains to be evaluated.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) is a bacterium causing many plant diseases including grape Pierce's disease and almond leaf scorch disease in California. This research is to study a virus or bacteriophage that could infect the pathogenic bacterium. Current efforts are to identify the genetic make-up of the bacterial virus, which has received little studies by far. Five genes have been detected and more studies are underway. Our research goal is to learn more about this bacterial virus so that more information will be available for future use to control xylellae diseases.

INTRODUCTION
Xylella fastidiosa (Xf) is an important pathogen causing disease on several economically important crops, such as grape and almond, in California. Despite recent intensive efforts to study this nutritionally fastidious pathogen, many biological traits of the bacterium, such as bacteriophages, remain unclear and deserve more research attention. Bacteriophages play important roles in bacterial biology including nutrient cycling, horizontal gene transfer, environmental adaptation and pathogenicity. Characterization and understanding the bacteriophage could lead to the identification of critical genes or biological processes useful for disease control.

Typically, bacteriophages are observed and enumerated using a plaque assay. The host bacterial cells are exposed to some environmental stress such as heat, UV radiation or chemical compound such as mitomycin C. These stresses trigger the shift for bacteriophage from lysogenic to lytic phase and form phage particles. Bacterial cells are plated as a lawn and bacterial phage plaques can be observed as clearings in the lawn. However, *Xf* is nutritionally fastidious and grows very slowly on agar plates of the available media. Chen and Civerolo (2008) developed a technique to collect phage particles from supernatants of prolong broth culture. Phage particles were morphologically characterized and most of them belong to Family Podovirideae. Summer et al (2010) reported the use of a Texas strain of *Xf* that formed bacterial lawn to isolate a phage through plaque assay. Although we have not tested the Texas strain, our attempts to develop culture lawn with our *Xf* collection have not been successful.

OBJECTIVE
In this study, we continued the protocol of Chen and Civerolo (2008) to collect *Xf* phage particles and explored the use of whole genome sequence information to characterize a lysogenic phage from *Xf*. Strain Dixon was selected because it generated relatively high titer of phage particles under our experimental condition, and a shot-gun whole genome sequence for strain Dixon is also available for reference.

MATERIALS AND METHODS
Bacterial cultivation and phage isolation. Xf strain Dixon was maintained in PW (Periwinkle wilt) broth culture at room temperature on a shaker table. From a two week old culture, five ml were used to inoculate 250 ml of either PW broth or PD3 broth. The cultures were grown at 25 ºC for four weeks. Broth cultures were then centrifuged to pellet cells and

supernatant was retained. Supernatant was filtered through Centricon Plus-70 100K MWCO filters (Millipore, Carrigtwohill, Co Cork. Ireland). The presence of phage particles in the phage prep were confirmed by electron microscopy examination after negative stain.

For phage genomic analysis, 17µl of phage prep was treated with Baseline-ZERO DNase (Epicentre, Madison WI) at the concentration of 1 MBU for 0.5 hours at 37ºC. This step was to digest any remaining bacterial chromosomal DNA, leaving only phage DNA contained within the phage capsid. The removal of chromosomal DNA was confirmed by the lack of PCR amplification with primers (four Primers, namely) for the detection of *Xf* chromosomal DNA (**Table 1**).

Ampification and confirmation of phage DNA. Phage prep treated with DNase was then used for direct PCR. Phage DNA was amplified using random primers OPA02, OPA3, OPA09, OPA11 and OPA18 with the following PCR procedure; PCR master mix per reaction contained: 16.8 µl molecular biology grade water, 2.5 µl dNTPs, 2.5 µl 10x PCR buffer, 0.2 µl TaKaRa Taq (5U/µl, TaKaRa Taq™ Hot Start Version, TaKaRa BIO INC. Otsu, Shiga, Japan), 0.5 µl of each primer (5 µM) and 2 µl of phage prep. PCR was run on a PTC-200 Peltier Thermal Cycler (MJ Research). RAPD PCR program was set as 96 ºC denature one min, 35 ºC annealing two min, 72 ºC extension two min for each cycle and a total of 40 cycles with a final 72 º extension of six minutes. Amplified DNA was visualized after agarose gel electrophoresis and staining.

RAPD PCR amplicons were cloned in pGEM-T plasmid vector. Cloned plasmids were sequenced using BigDye Turminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin TX). Excess dye was removed using BigDyeXTerminator Purification kit (Applied Biosystems, Austin TX). Sequencing reaction was run on a 16 capillary 3130xl Genetic Analyzer (Applied Biosystems, Austin TX). DNA sequences were used as query for BLAST search against GenBank DNA sequence database that included seven whole genome sequences of *Xf* strains. Among them, the sequences of strain 9a5c, Temecula1, M12, M23 and GB514 were enclosed and the sequences of strain Ann-1 and Dixon were not enclosed.

Genomic characterization of phage genome. BLAST search with cloned RAPD identified a prophage region in the genome of Temecula1, GB514, M23 and 9a5c. Sequences in the open reading frame (ORF) up- and down- streaming this genomic locus were then used to design PCR primer sets using Primer 3 program (http://frodo.wi.mit.edu/primer3/). These primer sets were used for PCR amplifications in the attempt to identify more genes in the phage genome. Initial primers used were designed using the genome sequence of strain Temecula1 as a reference. These primers were not specific enough to amplify prophage genes of strain Dixon. Primers were then redesigned by finding the homologous sequence in Dixon chromosome using NCBI's BLAST analysis and Primer3 program. The following PCR procedure was used: PCR master mix included 16.8 µl molecular biology grade water, 2.5 µl dNTPs, 2.5 µl 10x PCR buffer, 0.2 µl TaKaRa Taq (TaKaRa Taq™ Hot Start Version, TaKaRa BIO INC. Otsu, Shiga, Japan) 0.5 µl of each primers and 2 µl phage prep. Setting for PCR program was: 96 ºC denature for one min., 55 ºC annealing for 30 sec, 72 ºC extension for 30 sec per cycle with a total of 30 cycles and a final extension of 72 ºC for onr min. Amplified DNA was visualized after agarose gel electrophoresis and staining.

RESULTS AND DISCUSSION

A total of 250 µl of phage preparation (phage prep) was obtained from 250 ml of bacterial culture. **Figure 1** shows phage particles from electron microscopy. Phage preps from PD3 broth showed more particles than those from PW broth culture. Previously, strain Dixon was considered not capable of growing on PD3 medium. In this study, strain Dixon was able to grow on PD3. However, initial inoculum was grown in PW medium and may have provided limited amount of essential nutrients. Nevertheless, strain Dixon was growing under stress. This explains why more phage particles were observed from PD3 medium, rather than PW medium. Morphology of phage particles is consistent with Podovirideae type phage particles (**Figure 1**).

Among the five RAPD primers, only OPA03 amplified a DNA band of ~1,200 bp from the phage prep (**Figure 2**). Sequence from this PCR amplicon matched with prophage regions in the whole chromosomal sequence of *Xf* strains GB514, M23, Temecula1, and 9a5c (**Figure 3**). Shorter sequence with high similarity were also found from strain M23 and phage Xfas53, isolated from a Texas strain of *Xf*. All these evidence indicates that the observed particles were indeed true phages. Interestingly, the amplicon from phage particles was slightly larger than the strain Dixon chromosomal DNA.

Since phage particles were induced from pure *Xf* culture, it is assumed that the phage genome was a direct excision of a prophage sequence from the bacterial genome. Both phage and prophage should share the same set of genes. Data obtained so far seem to support this assumption (**Table 2**). The 4Primer used in this study targets the 16S rDNA locus of the bacterial chromosome and does not amplify DNA from phage prep if chromosomal DNA was completely digested by DNase. This is, however, not always true in our experiment. This may be due to the incomplete removal of chromosomal DNA in some batches of the phage preps. Repeating PCR experiment helped to correct the problem. The whole genome sequence of *Xf* strain Dixon is currently available in public domain. Yet the genome has not been enclosed. The five ORFs tested in this study are located on contig 89. Using the whole genome sequence as a guide, we will continue to characterize the whole genome of the phage isolated from this bacterial strain.

CONCLUSIONS

This research provides genomic evidence, in addition to our previous morphological observation, that *Xf* strains harbor bacteriophage(s). One practical perspective of phage research is to review the mechanisms of phage-bacteria interaction, or how a phage lyses a bacterial cell. Phage research has a potential for disease control.

REFERENCES CITED

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Summer, E. J., Enderle, C. J., Ahern, S. J., Gill, J. J., Torres, C. P., Appel, D. N., Black, M. C., Young, R., and Gonzalez, C. F. 2010. Genomic and biological analysis of phage Xfas53 and related prophages of *Xylella fastidiosa.* Journal of Bacteriology 192:179–190

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Table 1. List of primers for phage genomic characterization from *Xf* strain Dixon.

Primer Name	Primer sequence	Size (bp)
Dix1091_564f	TAGCGATACCGCTCAGACCT	564
Dix1091_564r	GTGCACAGACATTGGATTGG	
Dix1092_572f	GCTGTTTTGCTTGACGTTGA	572
Dix1092_572r	TCCACCGTTGAGTATGACGA	
Dix1093_800f	GACGCGCTTTCTGTTACCTC	800
Dix1093_800r	ATTTTTGTCCGCAAAATTCG	
Dix1094_518f	GCAAAGTCGAAGCCCTACTG	518
Dix1094_518r	GAACGATCCGTAACCACCAC	
Dix1095_448f	TCCCAAACCTCGTTCATACC	448
Dix1095_448r	GCGACAGATTGAGGGGTAAA	
OPA 03	AGTCAGCCAC	variable

Table 2. Summary of phage genomic characterization from *Xf* strain Dixon .

Template	Primer	PCR			
		1	2	3	4
Phage prep	DIX1091	+	+	+	+
Dixon DNA	DIX1091	+	+	+	+
Phage prep	4Primer	+	+	-	-
Dixon DNA	4Primer	+	-	+	+
Phage prep	DIX1092	+	+	+	+
Dixon DNA	DIX1092	+	+	+	+
Phage prep	4Primer	-	-	-	+
Dixon DNA	4Primer	+	+	+	+
Phage prep	DIX1093	+	+	+	+
Dixon DNA	DIX1093	+	+	+	+
Phage prep	4Primer	-	-	-	-
Dixon DNA	4Primer	-	-	+	+
Phage prep	DIX1094	+	+	+	+
Dixon DNA	DIX1094	+	+	+	+
Phage prep	4Primer	-	-	-	-
Dixon DNA	4Primer	-	-	+	+
Phage prep	DIX1095	+	+	+	+
Dixon DNA	DIX1095	+	+	+	+
Phage prep	4Primer	-	-	-	+
Dixon DNA	4Primer	-	-	+	+

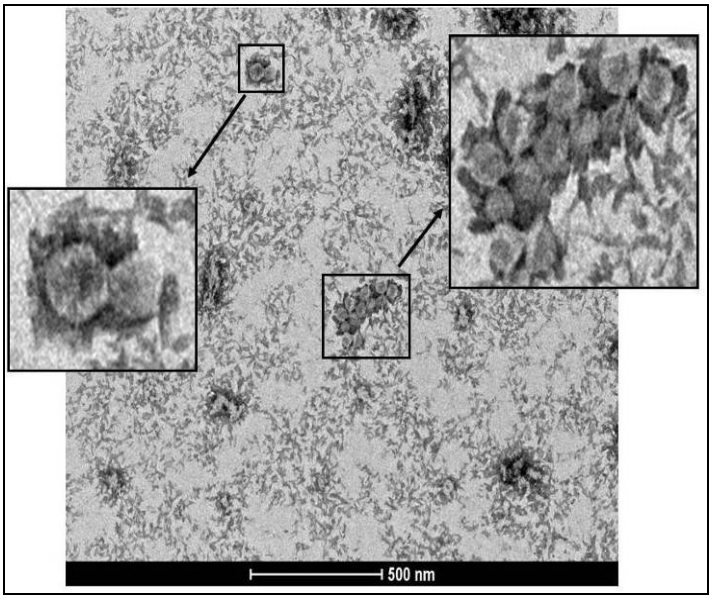

Figure 1. Electron micrograph of phage preparation from prolong (four weeks) culture of *Xf* strain Dixon.

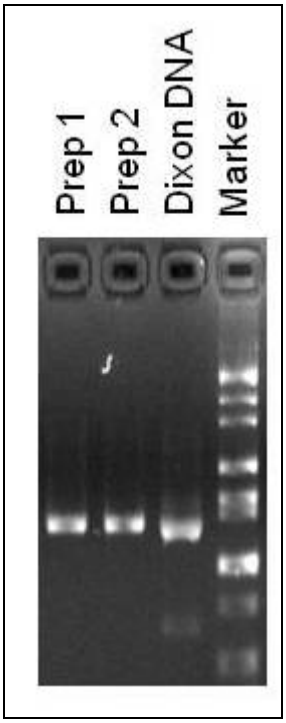

Figure 2. RAPD PCR from phage preparation of *Xf* strain Dixon.

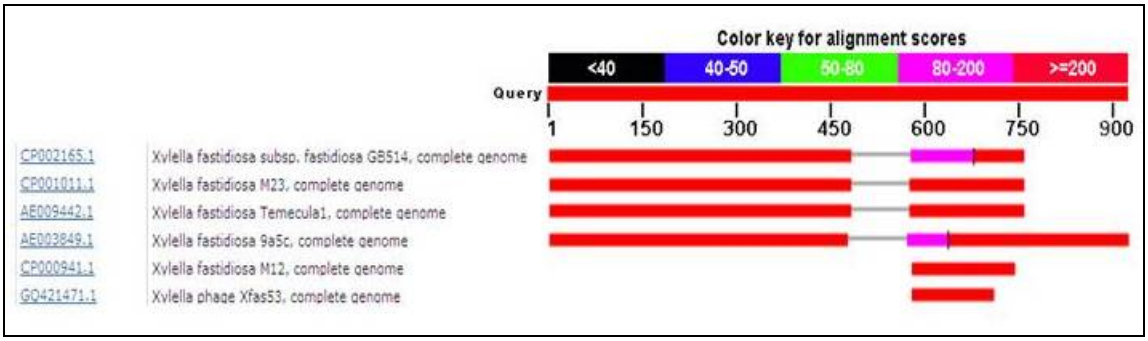

Figure 3. BLAST results of a phage sequence from *Xf* strain Dixon against GenBank DNA database.