Renewal Progress Report for CDFA Agreement Number 14-0136-SA.

Title of Project:
Defining the role of secreted virulence proteins LesA and PrtA in the pathobiology of *Xylella* and in the development of Pierce's disease

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Time period:
Five months (10/01/2014 to 02/28/2015)

Introduction
Xylella fastidiosa (*Xf*) is a fastidious, xylem-limited gamma-proteobacterium that causes several economically important diseases in many crops including grapevine, citrus, periwinkle, almond, oleander, and coffee (Davis et al. 1978, Chatterjee et al. 2008). In the field, *Xf* is vector-transmitted by various xylem sap-feeding sharpshooter insects (Purcell and Hopkins 1996, Redak et al. 2004). The *Xf* subspecies *fastidiosa* (*Xff*), as exemplified by the California strain Temecula 1, causes Pierce's disease (PD) in grapevine. The *Xf* life cycle and virulence mechanism are not entirely understood (Chatterjee et al. 2008). This research seeks to understand the pathobiology of *Xf* that leads to disease; specifically, the underlying mechanism that leads to leaf scorching symptoms. Understanding the underlying mechanism could help develop new strategies to control PD in grapevines in California. The secretion of virulence factors by pathogens is an important mechanism by which many plant diseases are triggered. Unlike closely related pathogens from genus *Xanthomonas*, *Xff* does not possess the type III secretion system (T3SS) (Van Sluys et al. 2002). However, *Xanthomonas* and *Xf* have in common a similar type II secretion system (T2SS) for a battery of important extracellular enzymes that are responsible for virulence (Ray et al. 2000). In *Xff*, genes have been identified that code for plant cell wall degrading enzymes (CWDEs) such as polygalacturonase, cellulase, lipase/esterase and several proteases (Simpson et al. 2000). These enzymes may aid *Xff* migration inside xylem vessels by degrading the pit membrane and also help release the carbohydrates necessary for bacterial survival. Cell wall degradation by CWDEs releases oligosaccharides as products, which can induce potent innate immune responses from plants. The plant defense responses include production of phytoalexins, fortification of cell walls through deposition of callose, oxidative burst, and induction of programmed cell death (Darvill and Albersheim 1984, Ryan and Farmer 1991, Braun and Rodrigues 1993). One T2SS secreted

protein, a polygalacturonase virulence factor encoded by pglA, lost pathogenicity when it was mutated and resulted in *Xf* that was unable to colonize grapevine (Roper et al. 2007). This confirmed an earlier finding that expression of a polygalacturonase inhibitory protein that blocked the action of pglA provided resistance to PD (Aguero et al. 2005).

List of objectives

Goal: Define the role that *Xylella*-secreted proteins LesA and PrtA play in the Pierce's disease phenotype of grapevine

Objectives:
Objective 1: Define the mechanism of action of LesA and PrtA gene products

> Activity 1: Express LesA, B, C and PrtA individually and examine their role in the virulence response of *Xf* cultures
> Activity 2: Perform metagenome analysis of xylem tissues infected by strains mutated for Les A, B, and C and PrtA
> Activity 3: Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xf* virulence

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Objective 1: Define the mechanism of action of LesA and PrtA gene products.

Our previous analysis revealed 24 secreted proteins in cultures of *Xf* Temecula 1. Of these, we have characterized two proteins, PrtA and LesA. A proteomic analysis of infected leaf tissues revealed five of the 24 secreted *Xf* proteins, the most abundant of which is LesA. To further characterize the role of these proteins, we used insertional mutagenesis of *Xf* cultures and expressed the respective proteins in *E. coli* to identify their function in PD.

Activity 1: Express LesA, B, C and PrtA individually and examine their role in the virulence response of *Xf* cultures.

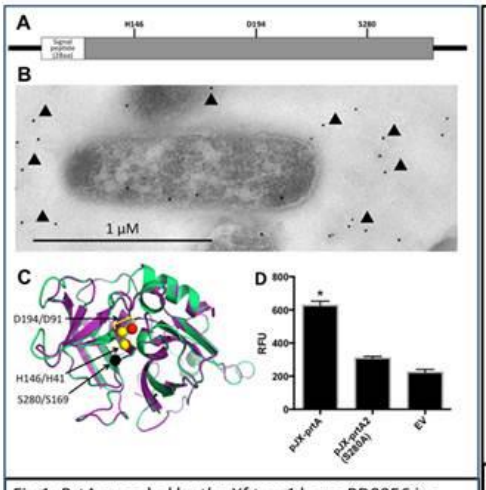

Fig 1: PrtA encoded by the Xf tem1 locus PD0956 is a secreted protein virulence factor with protease activity

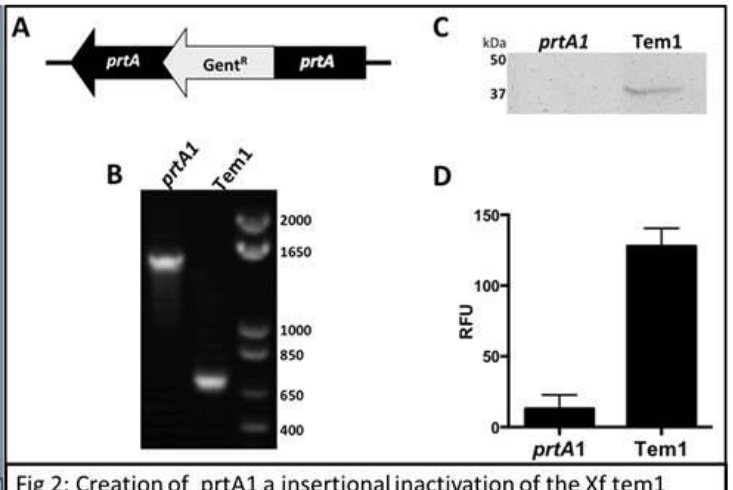

Fig 2: Creation of prtA1 a insertional inactivation of the Xf tem1 locus encoding PrtA.

The secreted protein PrtA is encoded for by the PD0956 locus at 1,159,634 to 1,160,638 bp in the *Xf* genome was previously annotated as an uncharacterized protein. It has a 28 amino acid signal peptide consistent with being secreted (Fig 1). Immunogold localization of PrtA in fixed

cells using antibodies against PrtA revealed that most of the protein was located outside the cell further confirming that it is secreted (Fig 1B). We analyzed the structure of PrtA, comparing it with proteins in the PDB database, and found a close structural similarity to an extracellular alkaline serine protease (PDB:3CP7A). Based on this structural prediction, we aligned the active site residues H146, D194 and S280 and created a threaded structure of PrtA showing the perfect alignment of active site residues (Fig 1C). prtA is highly conserved among *Xylella* strains, but not in Xanthomonas. To confirm if PrtA had protease activity, we expressed PrtA in *E. coli* and made a mutant version in which the S280 serine became an alanine residue, designated prtA2. We then compared the protease activity of *E. coli* extracts with extracts of strains expressing PrtA, PrtA2 and the empty vector (EV) using fluorescent labelled casein as the substrate. Extracts expressing PrtA had good protease activity, with lesser activity for PrtA2 and the empty vector (Fig 1D). We concluded that PD0956 encodes a protease designated PrtA. Using a cloned version of PD0956, we inserted gene encoding gentamycin resistance at the S280 position, electroporated it into *Xf* Tem1 cells, and selected for growth on gentamycin to identify a suitable homologous recombination event within the coding region of PD0956, designated prtA1 (Fig 2). We further confirmed an insertion disrupting the coding sequence by PCR comparison of wild type and prtA1 genes (Fig 2). PrtA1 displayed less protease activity was not detectable in secreted proteins from prtA1 cultures, but PrtA was detectable in wild type cultures (Fig 2). Growth characteristics of prtA1 cultures revealed that it was markedly more planktonic than wild type *Xf* Tem1 strains (Fig 3). Growth on plates showed less aggregation and when grown in flasks, a clear biofilm ring was formed by wild type but not prtA1 cultures. We used scanning EM to confirm that wild type cultures showed marked aggregation while prtA1 was exclusively planktonic. To investigate the role of prtA in the virulence response and PD, we infected grapevine plants as described (Dandekar et al. 2012), inserting ~10 million bacteria at the bottom of grapevines about 10 cm above the soil. Plants were scored at 10 weeks. Pierce's disease symptoms were clearly visible with prtA1 infections starting at six to eight weeks (Fig 4). A comparison of prtA1 and wild type colonies shows twitching motility at the margins of prtA1 colonies, confirming enhanced movement consistent with the observed enhanced virulence.

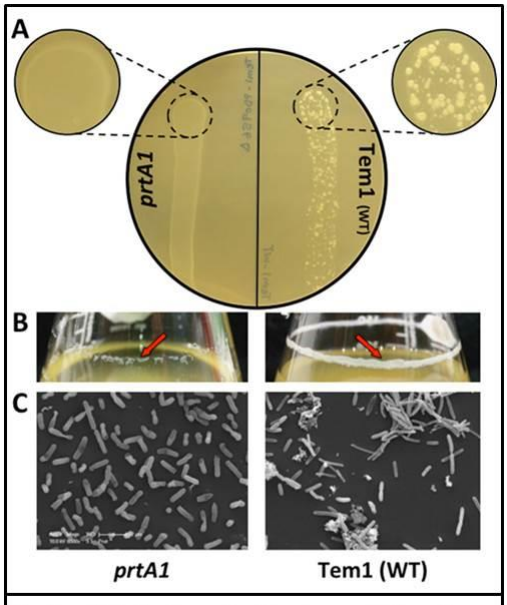

Fig 3: prtA1 displays planktonic growth in culture

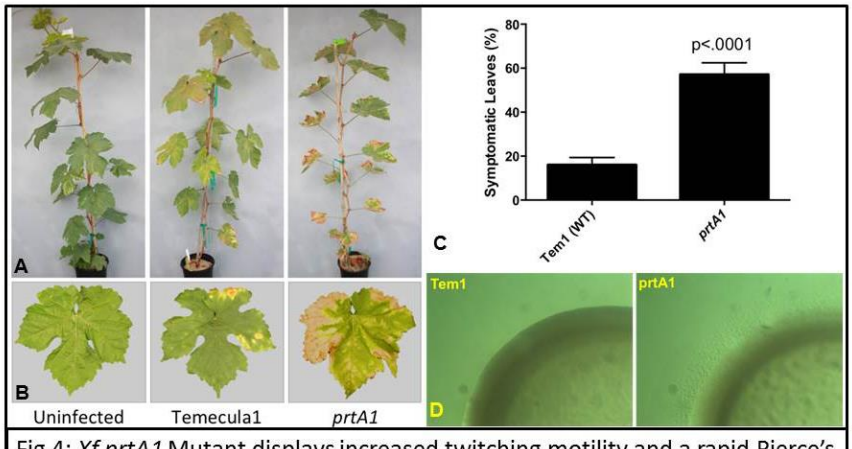

Fig 4: *Xf prtA1* Mutant displays increased twitching motility and a rapid Pierce's Disease phenotype earlier than WT *Xf* strains.

The secreted protein LesA is encoded for by the PD1703 locus present between 1,983,742 and 1,984,905 bp within the *Xf* genome; this gene was also previously annotated as an uncharacterized protein. It has a 35 amino acid signal peptide consistent with it being secreted (Fig 5). Immunogold localization of LesA in fixed cells using antibodies against LesA revealed that most of the protein was embedded within the secreted matrix surrounding *Xf* cells, confirming that LesA is a secreted protein. We compared the structure of lesA to proteins in the PDB database and found a close structural similarity to a *Xanthomonas oryzae* pv. oryzae (*Xoo*) LipA that has lipase and esterase activity (Aparna et al. 2009). *Xf* cultures grown on plates containing tributryn agar created zones of clearance indicating lipase activity. Based on this structural prediction, we aligned the active site residues S200, D360 and H402 of LesA with LipA from *Xoo*. We then threaded LesA with the known structure of LipA and there was a perfect match and alignment of active site residues (data not shown). Additionally, LesA is highly conserved among *Xylella* and *Xanthomonas* strains. To determine whether LesA had both lipase and esterase activities, we expressed LesA in *E. coli* and made a mutant version, LesA2, in which the S200 serine in the protein was substituted with an alanine residue. We then analyzed the lipase activity by growing the *E. coli* strains on agar containing tributryn and the esterase activity by assaying E. coli extracts for the ability to form 4-methyl umbelliferone (4MU) from 4-methyl umbelliferone butyrate. Esterase activity was clearly seen in strains expressing LesA and to a lesser extent in those expressing LesA2 and the empty vector (EV). *E.coli* expressing LesA showed zones of clearance but not those expressing LesA2. In addition, LesA protein was detected on western blots from cultures expressing both LesA and LesA2. We concluded that PD1703 encodes a lipase/esterase that we designated LesA. Using a cloned version of PD1703, we inserted a gene encoding kanamycin resistance at S200, electroporated into *Xf* Tem1 cells and selected for growth on plates containing kanamycin to identify suitable homologous recombination events that disrupted PD1703 within the coding region of LesA. We identified such an event and designated it LesA1. Among the 24 proteins secreted by *Xf* cultures, LesA was the most abundant, but we also identified LesB and LesC, proteins with strong homology to LesA. An alignment of the protein sequences revealed a conservation of the active site residues of Les A in LesB and C. Les B is encoded by PD1702, adjacent to the lesA coding region, but LesC is encoded by PD1211, at some distance away from lesA and B on the genome. Since lesA and B were located together, we created a double knock-out using kanamycin, strain lesA3B1. We expressed both LesB and LesC in *E.coli*. We confirmed by PCR comparison of wild type *Xf* with lesA1 and LesA3B1 that we had knocked out one and both genes (data not shown). LesA1 and

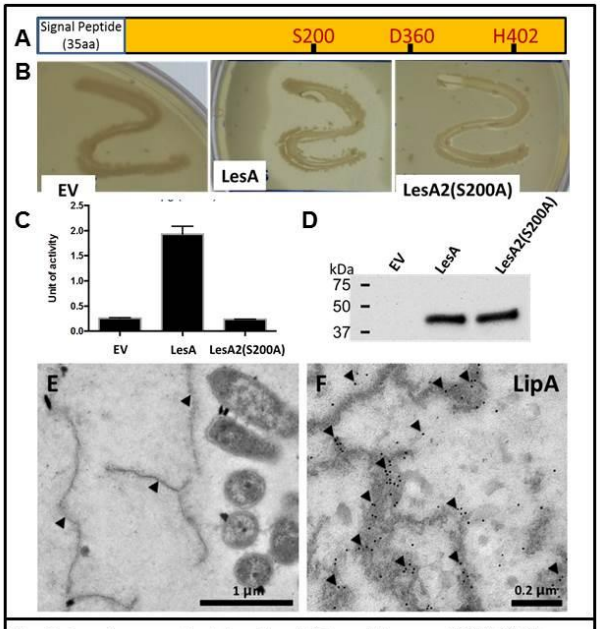

Fig 5: LesA encoded by the Xf tem1 locus PD1703 is a secreted virulence factor with lipase/esterase activity

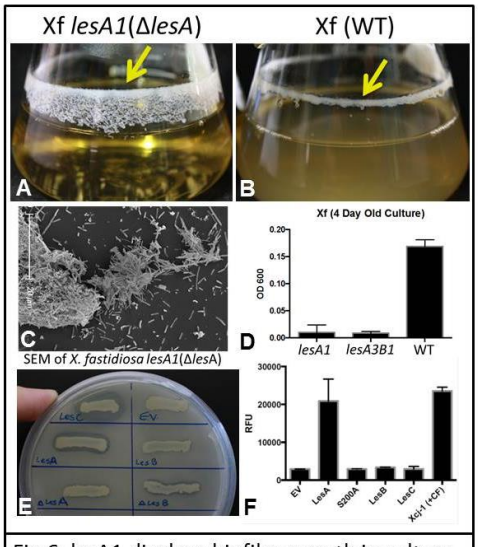

Fig 6: lesA1 displays biofilm growth in culture

LesA3B1 show less lipase and esterase activities than LesA and LesB (Fig 6). Additionally, there are some differences among the activities of LesA, B and C. Les A has both lipase and esterase activities, LesB has neither lipase nor esterase activity and LesC has lipase but no esterase activities directed to four-carbon substrates. LesA1 and lesA3B1 cultures were markedly in a biofilm state, in contrast to wild type *Xf* Tem1 strains. When grown in flasks, a clear biofilm ring was visible for the wild type, but a much larger and more profound ring of biofilm was visible for lesA1. We further confirmed this observation using scanning EM, where lesA1 showed marked aggregation of cells. To investigate the role of lesA in the virulence response and PD, we infected grapevine plants as described earlier (Dandekar et al. 2012) inserting ~10 million bacteria at the bottom of grapevines about 10 cm above the soil. Plants were scored at 10 weeks. Pierce's disease symptoms were clearly visible starting at 10 weeks for the wild type Tem1 strains, but neither the lesA1 nor the lesA3B1 strains showed symptoms (Fig 7). Infiltrating the LesA protein into grapevine leaves led to scorching, but infiltrating the LesA2 mutant protein that lacks both lipase and esterase activities did not. These results clearly show that the secreted LesA protein is related to leaf scorching and that the activity of the lipase/esterase is necessary for the PD phenotype.

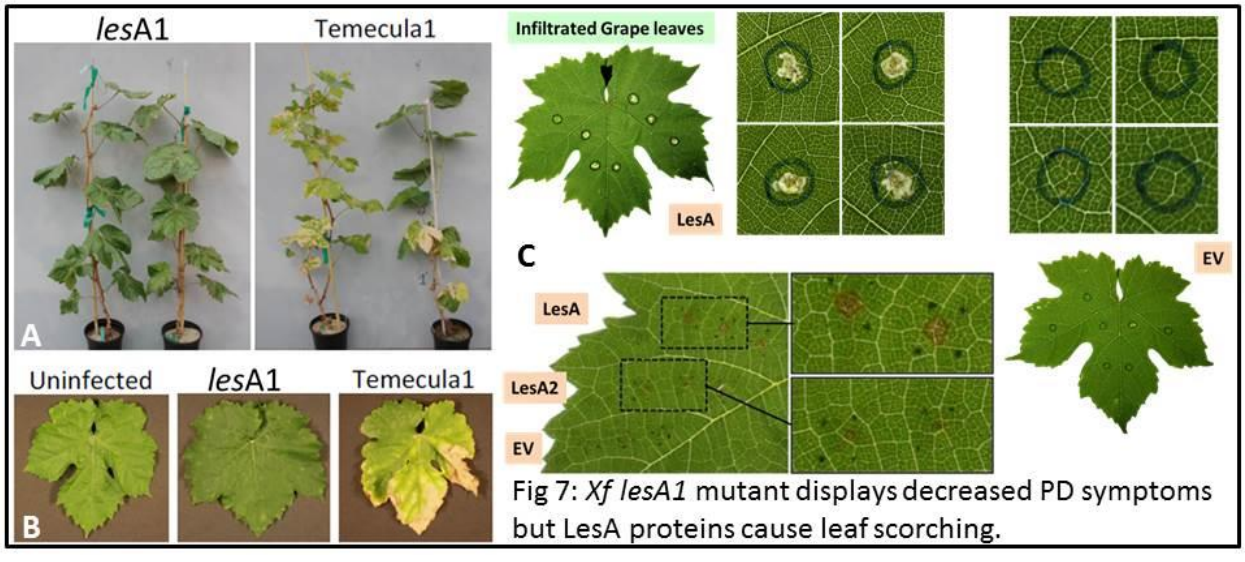

Fig 7: *Xf lesA1* mutant displays decreased PD symptoms but LesA proteins cause leaf scorching.

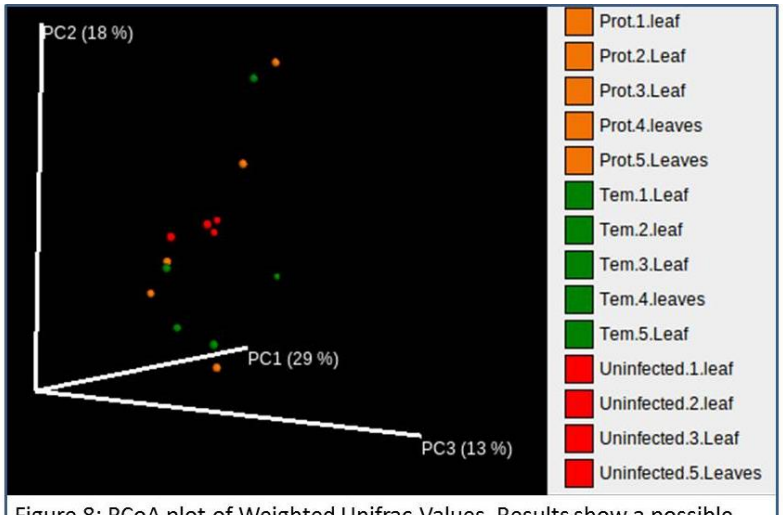

Figure 8: PCoA plot of Weighted Unifrac Values. Results show a possible correlation of *X.f. mt prtA* (Prot) and *X.f. Tem1* (WT) with possible clustering of uninfected samples.

<u>Activity 2: Perform metagenome analysis of xylem tissues infected by strains mutated for Les A, B, and C and PrtA</u>.

The secreted proteins could influence the grapevine microbiota and that interaction could influence the disease outcome. To investigate this possibility, we used mutants unable to make LesA (lesA1), LesAB (lesA2B3) and PrtA (prtA1). We conducted a preliminary survey of the alpha diversity of the resident microbiome of Thompson Seedless (TS) grapevines infected with wild type (Temecula 1) or prtA1 and that of an

uninfected control. TS grapevines were allowed to grow for 12 weeks and then five plants from each treatment were harvested. We collected from the 1st offshoot above the 10th node of each plant (the 2nd and 3rd petiole and leaf from this offshoot). Samples were placed on ice, brought to the lab, and frozen in liquid nitrogen. Samples were kept frozen and then ground into powder using Qiagen's grinding jar set and associated TissueLyser. DNA was extracted using the MoBio PowerPlant Pro DNA isolation kit. PCR and sequencing of the V4 region of the 16S rRNA gene using region-specific primers and PCR and sequencing were performed using standard protocols as agreed upon in the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/) using Illumina MiSeq (Caporaso et al. 2012).

The immediate problem emerging from the sequencing data was a high proportion of host chloroplast sequences that came from the extraction of the leaf samples. After removing chloroplast sequences, the sequencing depth was not sufficient for analysis. As such, the PCoa plot (Fig. 8) showed no large differences between samples. Additionally, due to sampling constraints our preliminary samples were obtained from new growth, far from the initial point of infection. Preliminary data demonstrate the low number of sequences after chloroplast removal and show the percentage of total sequences attributed to different microorganisms (Fig 9).

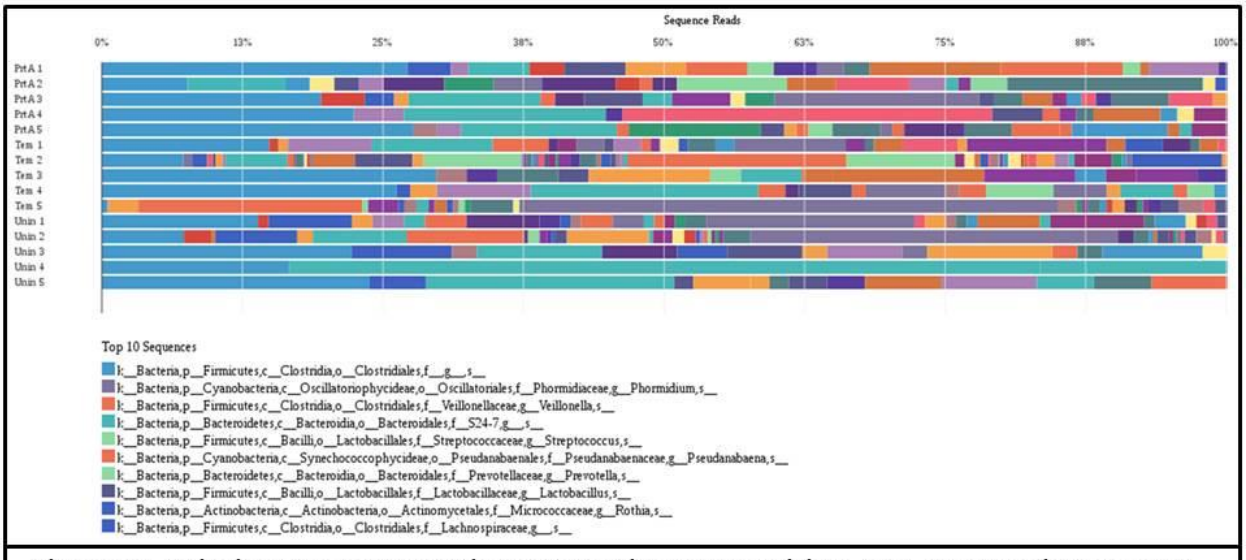

Figure 9: Relative 16S Taxonomic Community Composition. Top 10 constituents are identified to the Genus level where possible.

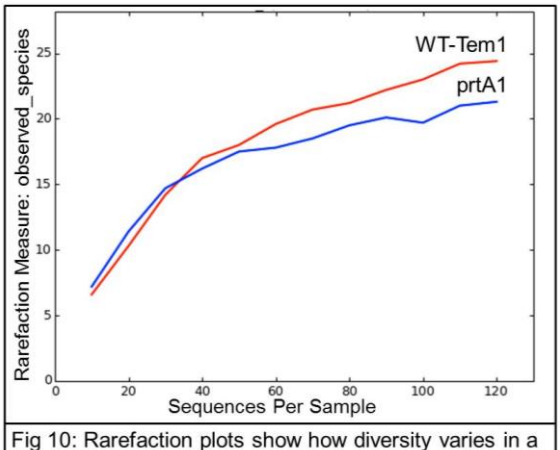

Fig 10: Rarefaction plots show how diversity varies in a sample with the number of sequences sampled.

To compensate for low sequencing depth due to the abundance of chloroplast sequences, we obtained PCR blockers that selectively inhibit amplification of chloroplast sequences (Orum 2000). Using PCR blockers, a rarefaction plot verified that the number of observed species was plateauing and thus that we sampled a majority of the 16S community (Fig. 10).

While an examination on the alpha diversity of the inside of TS grapevines continues, work has begun on an infection study to determine differences in the microbiome structure upon infection with *Xf*. For this infection study, we obtained non-leaf samples much closer to the inoculation point, as this is where symptoms are located and where

probable changes in the microbiota will first be evident. TS grapevines were grown 18 weeks and harvested at six different times. Six plants were sampled from each of five treatments: uninfected or infected with *Xf* Tem1 (WT), *Xf* lesA1, *Xf* lesA2B3 or *Xf* prtA1) at each time. From each plant, we obtained root tissue, stem tissue from two to three nodes above the infection point, and stem tissue between nodes 10 and 11. Samples were placed on ice, brought to the lab, washed with .04% Tween 20 and rinsed with ddH20 to minimize background surface microbiota. Samples were then frozen in liquid nitrogen and maintained at -80C until analyziz. Sample were ground into powder using Qiagen's grinding jar set and associated TissueLyser. Unfortunately, the previously used MoBio PowerPlant Pro DNA isolation kit and several other kits and protocols were insufficient to obtain high-quality DNA from these high-phenolic, woody samples. A kit was preferred to decrease possible microbiota background variability arising from user contamination during a phenol-chloroform DNA extraction. However, high-quality DNA was finally obtained using Qiagen's DNAeasy Plant Mini Kit with an additional 5% sodium metabisulfite in the lysis buffer. We are about to begin library prep of these DNA extractions for sequencing using Illumina MiSeq.

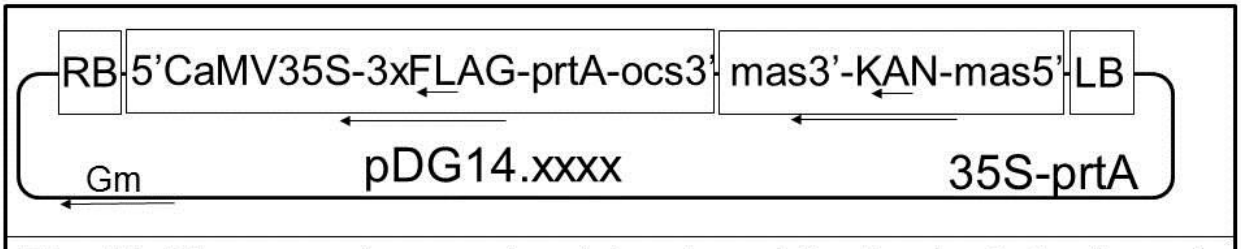

Fig. 11: Binary vector construct developed for *in planta* testing of anti-virulence activity of Xf prtA encoded protein in transgenic tobacco plants

Activity 3: Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xf* virulence.

To test the anti-virulence phenotype of PrtA, we cloned the prtA coding region into a binary vector under the CaMV35S promoter after codon optimization for expression in tobacco (Fig 11). The binary vector construct was introduced into a disarmed strain of *Agrobacterium* (EHA105) via electroporation to create a functional system for plant transformation. Currently, transgenic SR1 tobacco plants are being generated at the UC Davis Parson Transformation Facility. We have screened the initial batch and identified six that are positive for the presence of the kan genes and that express PrtA, as detected using an an-FLAG antibiody. We did not detect the protein using anti-PrtA antibody. The resulting six plants are currently being propagated to collect F1 seed that germinate on medium containing kanamycin. Kan-resistant transplants will be moved to the greenhouse and grown to six to eight leaves. We will infect fully expanded leaves with a virulent strain of *Xf* to evaluate whether PrtA has anti-virulence activity.

Publications produced and pending, and presentations made that relate to the funded project.

Dandekar, A.M., H. Gouran, R. Nascimento, H. Gillespie, L. Goulart, and S. Chakraborty. 2014. Defining the role of secreted virulence proteins LesA and PrtA in the pathobiology of *Xylella* and in the development of Pierce's disease. Proceedings of the Pierce's Disease Research Symposium held December 15-17, 2014, in Sacramento, California. pp. 50-56.

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California.

Our goal is to understand the virulence mechanisms of *Xf* that lead to leaf scorching symptoms observed in PD and to exploit this information to develop new strategies to control PD in

grapevines. Blockage of xylem elements and interference with water transport by *Xf* is regarded to be the main cause of PD symptom development. Analysis of *Xf* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA. We generated mutant *Xf* that are defective for each of these two genes that show alterations in disease phenotype: LesA1 is less virulent while PrtA1 is more virulent. LesA displays lipase/esterase activities and is the most abundant protein secreted by *Xf*, but is very similar to two additional, less abundant proteins: LesB and LesC. Expression of LesA, B and C individually in *E. coli* indicate that these proteins can induce scorching symptoms in grapevine and walnut leaves. These symptoms are related to the lipase/esterase activity present in these proteins. The PrtA protein has protease activity and Xf-prtA1 mutants are highly virulent, suggesting that this protein may somehow block disease. We are building vectors to test this protein for anti-virulence activity in transgenic SR1 tobacco plants. An understanding of how these two proteins work will provide new insights into this disease and provide new avenues of therapy.

Layperson summary of project accomplishments

Pierce's disease (PD) of grapevines is caused by the bacterium *Xylella fastidiosa* (*Xf*), a xylem-limited gamma-proteobacterium that is responsible for several economically important diseases in many plants. A characteristic symptom of PD is leaf scorching, with marginal regions of leaves developing chlorosis progressing to necrosis. Blockage of xylem elements and interference with water transport by *Xf* and its associated biofilm have been posited to be the main cause of PD symptom development. Analysis of *Xf* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA. We generated mutant *Xf* that are defective for each of these two genes, and which show alterations in adhesiveness/aggregation that are related to biofilm formation/dissipation or biofilm behavior, which in turn are related to disease symptom development. In this proposal, we will use these mutants and create others to analyze how these proteins affect biofilm development and the formation of disease symptoms like scorching. We need to better understand how this bacterium regulates pathogen behavior and how this behavior is related to pathogenicity. We will also study the impact of these genes on the social behavior of the microbiome in the xylem. An understanding of how these two proteins work will provide new insights into this disease and provide new avenues of therapy.

Status of funds.

All of the funds for the first year of this project are on track to be spent by June 30, 2015.

Summary and status of intellectual property associated with the project

We have made disclosures on LesA and PrtA and these are being evaluated by UCD Innovation Access. As additional insights are gained on the functioning of LesA and PrtA, additional disclosures will be made to secure the IP.

Literature cited

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