Interim 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:
Nine months (10/01/2014 to 06/30/2015)

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.

The secreted protein PrtA was previously annotated as an uncharacterized protein. We analyzed the structure of PrtA, comparing it with proteins in the PDB database, and found a close structural match to an extracellular alkaline serine protease. Based on this structural prediction, we were able to align the amino acid residues in the active site showing a perfect alignment of these active site residues. Also, prtA is highly conserved among various *Xylella* strains, but interestingly not among Xanthomonas strains. The protease activity of prtA was confirmed by expressing the encoded protein, PrtA in *E. coli* and we were able to demonstrate a lack/lesser activity in a mutant (prtA2) where one of the active site residues (S280 mutated to A280) was mutated as compared to the wild type PrtA enzyme using fluorescent labelled casein as the substrate. To investigate the function of this protein we created a functional knockout strain via homologous recombination where the genomic region encoding PrtA was disrupted via the insertion of a gene encoding resistance to the antibiotic gentamycin, this mutant strain was called prtA1. We confirmed via PCR analysis that the coding region of prtA was disrupted in the prtA1 mutant. Expectedly, the mutant strain displayed less protease activity and PrtA was not detectable in secreted proteins from prtA1 cultures, while PrtA was detectable in wild type cultures. Growth characteristics of prtA1 cultures revealed that it was markedly more planktonic than wild type *Xf* Tem1 strain. 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 whereas prtA1 appeared to be

exclusively planktonic. Since planktonic forms are more virulent we infected grapevine plants as described earlier (Dandekar et al. 2012) in the main stem, 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 much earlier to that observed with the wild type. 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.

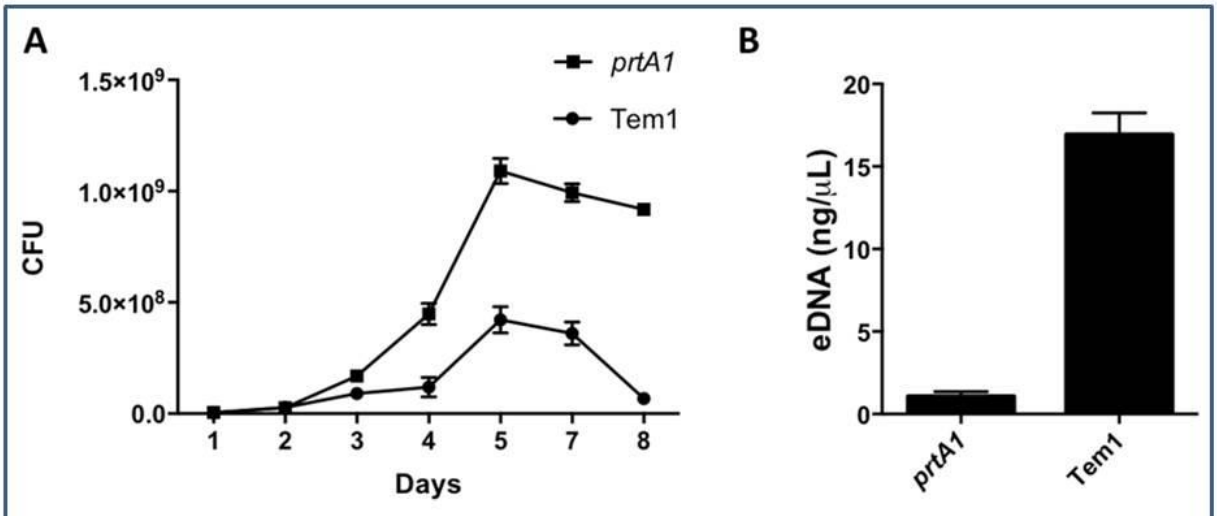


Fig 1: Cell lysis and eDNA quantification. (A) *X. fastidiosa prtA1* mutant and Tem1 wild type growth curves by plating on agar plate and counting number of colony-forming units (CFU) for 8 days. (B) eDNA quantification for *prtA1* mutant and Tem1 strains grown in liquid media for one week.

The secreted protein LesA was also previously annotated as an uncharacterized protein. It has a 35 amino acid secretion peptide consistent with it being secreted. 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). Lipase activity was confirmed by growing *Xf* cultures on plates containing tributryn, a triacylglyceride of butyrate, zones of clearance were clearly visable surrounding the colonies 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 the Xoo LipA and there was an excellent allignment of active site residues. Additionally, LesA was found to be highly conserved among both *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 activity by growing/harvesting *E. coli* strains they displayed both lipase activity evaluated on agar plates containing tributryn as well as esterase activity by assaying the E. coli extracts using the substrate 4-methyl umbelliferone butyrate and measuring the formation of 4-methyl umbelliferone (4MU) the product of the reaction. Esterase activity was clearly seen in strains expressing LesA and to a lesser extent in those expressing LesA2 or 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. To investigate the function of the LesA protein we created a functional knockout strain via homologous recombination where the genomic region encoding LesA was disrupted via the insertion of a gene encoding resistance to the antibiotic kanamycin, this mutant strain was called 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 located adjacent to the lesA on the Xf genome, but LesC is located at some distance away. Since lesA and B were located together, we created a double knock-out using kanamycin, this particular strain is designated 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. LesA1 and LesA3B1 show less lipase and esterase activities than LesA and LesB. 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 butyrate substrates. LesA1 and lesA3B1 cultures displayed a strong 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. 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.

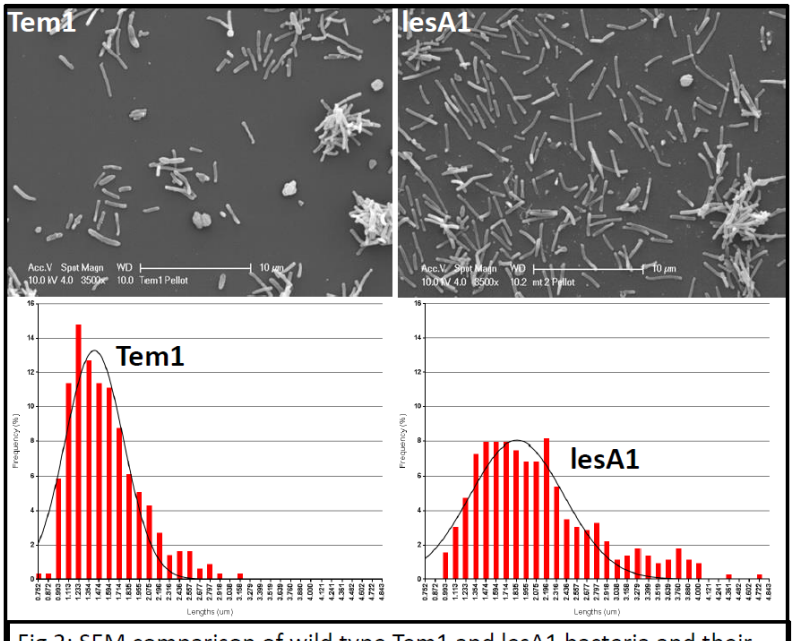
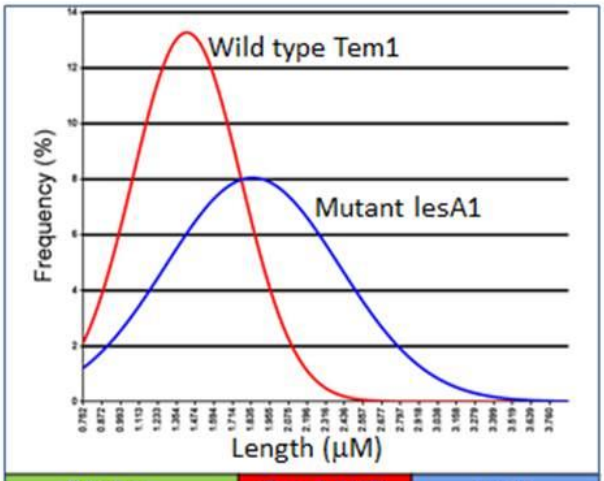


Fig 2: SEM comparison of wild type Tem1 and lesA1 bacteria and their length distribution.

Growth of the lesA1 in culture showed that a predominant proportion of the bacteria were in a biofilm state and when grown in a flask the culture displayed a strong ring of biofilm and the optical density (OD) of the culture was low compared to the wild type Tem1 strain. Shown in Fig 2 are scanning electron microscope (SEM) image panels on the top showing wild type and leaA1 cells grown in culture and on the bottom are the measurements of bacterial length of this population. The lesA1 strain that displayed a greater proportion in the biofilm state had a greater proportion of longer cells (Fig 2, 3). Expectedly, lesA1 was less pathogenic as compared to wild type Xylella. The biofilm and planktonic states have been shown to be regulated by quorum sensing behavior, regulated by DSF as C12 fatty acid molecule (Chatterjee et al., 2008). DSF synthesized by rpfF triggers biofilm formation and down regulates pathogenesis. Mutants in rpfF are more virulent than wild type Xylella. The DSF is sensed by a receptor rpfC that is part of a two-component regulatory system that senses DSF on the outside and triggers rpfG to mediate the response (Buttner and Bonas 2009). LesA is required for pathogenesis and so lesA1 that does not make LesA is a nonpathogenic biofilm in culture. In Fig 4 we measured the levels of LesA and there appears tto be a good correlation between the presence of lesA and pathogenesis, wild type Tem 1, prtA1 and rpfF that do not make biofilm and that are very

pathogenic make a lot of LesA, whereas mutants that make more of a biofilm like lesA1, lesA3B1 and rpfC express lower levels of LesA (Fig 4).



Strain:	Tem1 (WT)	lesA1
95% Confidence Interval Amplitude:	10.27 to 11.74	7.336 to 8.532
Mean Length:	1.646 to 1.730	1.825 to 1.959

Fig 3: Bacterial length measurements of wildtype and lesA1

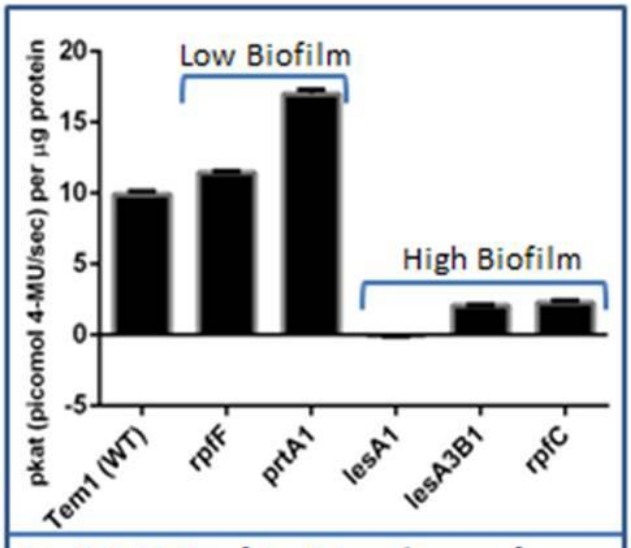


Fig 4: Activity of LesA in cultures of different mutants compared to wild type Tem1 cells

<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. Previously, we conducted a preliminary alpha diversity survey in which we took samples infected with *X. fastidiosa* unable to make PrtA (prtA1), wild type *Xf.* (Tem1), and uninfected tissue. 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).

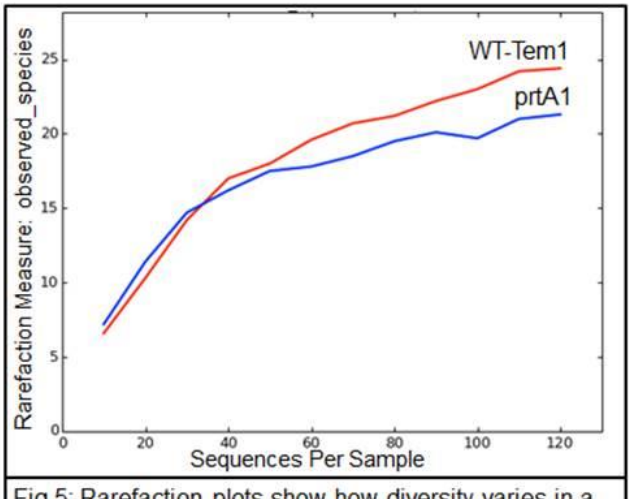


Fig 5: Rarefaction plots show how diversity varies in a sample with the number of sequences sampled.

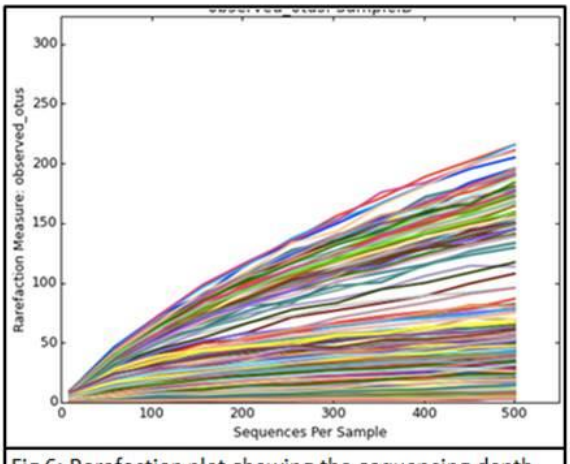


Fig 6: Rarefaction plot showing the sequencing depth of samples as it pertains to new microbial OTU discovery in the x.f. infection experiment.

The immediate problem which emerged from the preliminary sequencing data was the high proportion of host chloroplast sequences that came from the samples. Preliminary data demonstrated the low number of sequences after chloroplast removal. To compensate for low sequencing depth due to the abundance of chloroplast sequences, we obtained PCR blockers that selectively inhibited amplification of chloroplast sequences (Orum 2000). Using PCR blockers, a rarefaction plot verified that the number of observed species was plateauing indicating that we sampled a majority of the 16S community (Fig.5). We have since used the data and experience we obtained from our preliminary work to begin work on an infection study to determine differences in grapevine microbiome structure upon infection with *Xf*. A rarefaction plot of this new data shows a wide range in microbial diversity per sample (Fig. 6). For this infection study, we harvested non-leaf samples at a much closer distance to the inoculation point, as this was where symptoms were observed 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 (Wild Type), *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

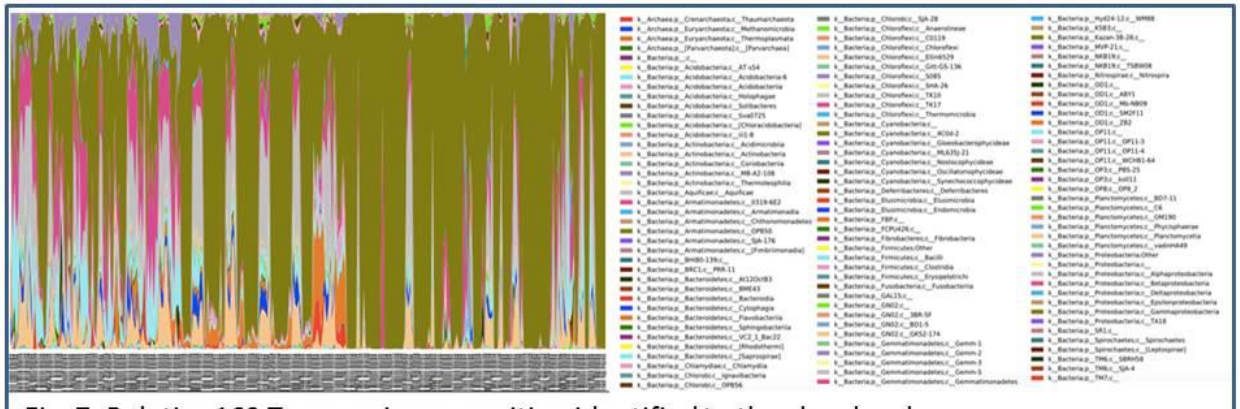


Fig. 7: Relative 16S Taxonomic composition identified to the class level.

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 analysis. 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. As a kit was preferred to decrease possible microbiota background variability arising from user contamination during a phenol-chloroform DNA extraction, a high-quality DNA was finally obtained using Qiagen's DNAeasy Plant Mini Kit with an additional 5% sodium metabisulfite in the lysis buffer. We made a library of these DNA extractions, and sequenced using Illumina MiSeq. Data was then demultiplexed and mitochondrial, singleton, and remaining chloroplast sequences were removed. Unique OTU's were defined as having no more than 97% similarity. We have begun our initial analysis of the sequencing data (Fig. 7), and begun comparing how diversity changes between tissues (Fig. 8). This will be followed by a diversity comparison between time points followed by a comparison of treatments at a single time point and then across time points.

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 9). 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 antibody. 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.

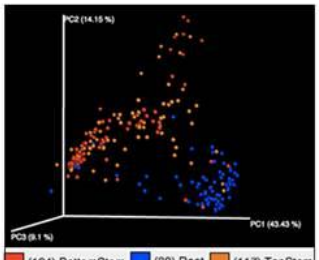


Fig. 8: PCoA plot of Weighted Unifrac Values. Initial data shows a clear difference between root tissue (blue) and stem (red and orange). Bottom Stem tissue does not appear to be highly differentiable from Top Stem tissue.

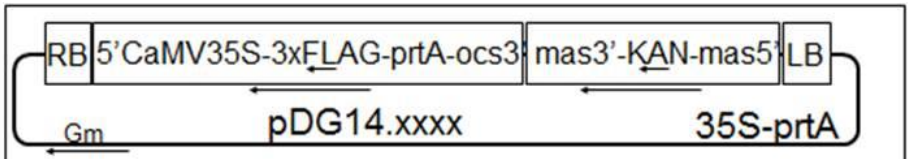


Fig. 9: Binary vector construct developed for *in planta* testing of anti-virulence activity of Xf prtA encoded protein in transgenic tobacco plants

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 were 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.

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