I. INTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 13-0096-SA

II. TITLE OF PROJECT. Identification of a new virulence factor required for Pierce's disease and its utility in development of a biological control

III. PRINCIPAL INVESTIGATOR, CO-INVESTIGATORS, AND COOPERATORS.

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IV. TIME PERIOD COVERED BY THE REPORT. April 2014-July 2014

V. INTRODUCTION.

X. fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium that induces Pierce's disease (PD) in grapevines (Chatterjee et al. 2008). *Xf* is transmitted to plants by insect vectors and once in the xylem, *Xf* is postulated to migrate, aggregate, and form biofilm that clogs the vessels leading to PD. We, and others, have studied key *Xf* genes and proteins involved in these steps (Guilhabert and Kirkpatrick 2005, Meng et al. 2005, Feil et al. 2007, Li et al. 2007, Shi et al. 2007, da Silva Neto et al. 2008, Cursino et al. 2009, Cursino et al. 2011) with the hope that better understanding of PD will lead to developing preventative strategies.

Recently we began to explore new virulence factors involved in PD development. We explored an *Xf* gene, *PD1311*, that is annotated as a putative peptide synthase (Altschul et al. 1990) or AMP-binding enzyme (Punta et al. 2012). The putative PD1311 protein contains the three motifs found in adenylate-forming enzymes (Fig. 1), also known as the ANL superfamily, which is composed of ACSs (acyl- and aryl-CoA synthetases), NRPS (nonribosomal peptide synthetase) adenylation domains, and Luciferases (Chang et al. 1997, Gulick 2009). *Xf* lacks luciferase activity and the size and domains of NRPS enzymes (Strieker et al. 2010), suggesting that PD1311 may be an ACS. The most studied bacterial ACS is the *Escherichia coli* FadD, which catalyzes exogenous long-chain fatty acyl-CoA from fatty acid, coenzyme A, and ATP

(Black et al. 1992). ACS metabolite intermediates are involved in β-oxidation and phospholipid biosynthesis, and ACS proteins are also implicated in cell signaling, protein transportation, and protein acylation (Korchak et al. 1994, Glick et al. 1987, Gordon et al. 1991). Importantly, ACSs are known to be involved in virulence factors, such as the *Xanthomonas campestris* ACS FadD homolog, RpfB, which appears to be involved in production of quorum-sensing molecule, DSF (diffusible signaling factor) (Barber et al. 1997). We deleted the *PD1311* gene and found that the resulting strain, Δ*PD1311*, is nearly non-pathogenic when inoculated in grapevines. These results indicate that the PD1311 protein is fundamental for PD development and therefore warrants further study.

motif I.....TSG[S/T]TGXPKG
(181)TSG S TGVPKG(190)

motif II.....YGXTE
(317)YGATE(321)

motif III.....YRTGD
(420)HRMGD(424)

Fig. 1. Sequence comparison of PD1311 to acyl-adenylate-forming enzyme superfamily signature motifs. For each motif, consensus sequence (bold) is shown above PD1311 (unbold) sequence with amino acid numbers in parenthesis. Motifs I/II are for ATP binding. Motif III is for substrate binding and catalysis at the invariant aspartate (Chang et al. 1997, Ingram-Smith et al. 2012).

Preliminary studies with the Δ*PD1311* strain suggest that beyond its role in disease, it blocks pathogenicity by the wild-type Temecula strain. Therefore we propose that it has potential as a biocontrol for PD. The weakly virulent *Xf* elderberry strain, EB92-1, has been studied as a PD biocontrol (Hopkins 2005, Hopkins 2012). Additional strategies involve naturally resistant rootstocks (Cousins and Goolsby 2011) and transgenic varieties (Dandekar 2012, Gilchrist and Lincoln 2012, Kirkpatrick 2012, Labavitch et al. 2012, Lindow 2012, Powell and Labavitch 2012) are being examined. However, continued research for PD controls is warranted. Given the essentially avirulent phenotype of the Δ*PD1311* strain, understanding how PD1311 orchestrates the disease response may also provide key insights into PD development.

VI. LIST OF OBJECTIVES.

The overall goal of this project is to understand how the PD1311 protein influences virulence, and test if the PD1311 mutant strain functions as a biocontrol for Pierce's disease. To examine these questions, we propose the following:

Objective 1. Characterize the *X. fastidiosa* Δ*PD1311* mutant.
- a. Complete *in vitro* behavioral assays critical for disease.
- b. Determine the role(s) of PD1311 in producing virulence factor(s).

Objective 2. Determine the effectiveness of Δ*PD1311* Temecula strain as a biological control of Pierce's disease.
- a. Determine conditions for biological control.
- b. Examine spread of Δ*PD1311* and wild-type strains simultaneously.

VII. DESCRIPTION OF ACTIVIES.

Objective 1. Characterize the *X. fastidiosa* Δ*PD1311* mutant.

1a. Complete *in vitro* behavioral assays critical for disease.

As reported previously, we deleted the *PD1311* gene and complemented the Δ*PD1311* strain as previously described (Matsumoto et al. 2009, Shi et al. 2009). The Δ*PD1311* strain grows in xylem sap and therefore is not a lethal mutation (Fig. 2). Since key processes leading to PD are motility, aggregation, and biofilm formation (Chatterjee et al. 2008), we examined these behaviors in the Δ*PD1311* strain. Such information will help us determine if PD1311 exerts its effects by common virulence methods or has more specialized function.

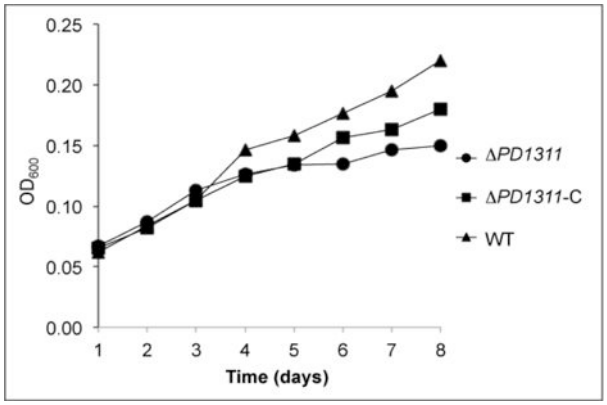

Fig. 2. Δ*PD1311* strain growth. Wild-type *Xf* (WT - triangle), mutant (Δ*PD1311* - circle), and complemented mutant (Δ*PD1311*-C – square) strains were grown for eight days in 100% *Vitis vinifera* cv. Chardonnay xylem sap and growth was determined by OD_{600} readings.

i) Motility:

We examined Δ*PD1311* movement by the *in vitro* fringe assay in which fringe around the bacterial colony directly correlates with type IV pilus twitching motility (Meng et al. 2005, Li et al. 2007). While the Δ*PD1311* strain is motile, it appears to be modestly affected (Fig. 3). **Given the potential reduced motility and that *in vivo Xf* migrates against the transpiration stream (Meng et al. 2005), we are creating chambers to assess the speed of cells in microfluidic chambers and will examine translocation in plants (Meng et al. 2005, de la Fuente et al. 2007a, de la Fuente et al. 2007b) [new results for the April 2014-July 2014 reporting period highlighted in bold].**

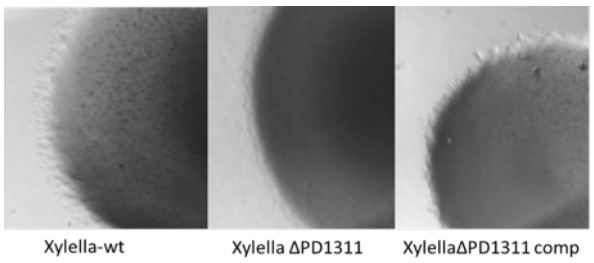

Fig. 3. Motility of Δ*PD1311* mutant strain. Colony fringes of wild-type (*Xylella*-wt), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311comp) strains were assayed on PW agar overlaid with cellophane. Colonies were assessed after five days of growth (Meng et al. 2005, Li et al. 2007). Colonies photographed at 90X magnification. Experiment was repeated three times.

ii) Aggregation and biofilm formation:

Cell aggregation is a critical step in biofilm formation, which is proposed to clog xylem vessels and prevent transport of nutrients and water resulting in PD (Chatterjee et al. 2008). We found that the Δ*PD1311* strain has decreased aggregation compared to the wild-type and complemented strains ($P<0.03$) (Fig. 4). In addition, the Δ*PD1311* strain produces less biofilm than wild-type *Xf* ($P<0.0001$) (Fig. 5). Decreased biofilm production generally correlates with decreased pathogenicity (Cursino et al. 2009, Shi et al. 2009, Cursino et al. 2011). However previously examined mutants that have reduced biofilm do not eliminate disease, unlike the Δ*PD1311* strain. The Δ*PD1311* strain result indicates that other factors besides altered biofilm production are involved in Δ*PD1311* significantly reduced virulence.

1b. Determine the role(s) of PD1311 in producing virulence factor(s).

Our studies suggest that PD1311 impacts *Xf* in ways beyond motility, aggregation, and motility. Many bacteria produce secondary metabolites that are critical to their pathogenic responses (Raaijmakers and Mazzola 2012). *Xf* creates quorum-sensing DSF, which coordinates motility, biofilm formation, and virulence (Chatterjee et al. 2008). *Xf* RpfB is a known ANL protein involved in the production of a subset of DSF products (Almeida et al. 2012). Since PD1311 may also be an ANL protein, we asked if it was involved in DSF production.

We streaked wild-type *Xf* and the Δ*PD1311* strain onto PW agar plates (Davis et al. 1981) for 8 days to allow production of DSF. The *Xanthamonas campestris campestris* (*Xcc*) indicator strain 8523 (kindly provided by Prof. Steven Lindow, U. Cal., Berkeley) was streaked perpendicular to either the wild-type or the Δ*PD1311* strain for 24 hours (Newman et al. 2004). A suspension was made of the *Xcc* strain 8523 cells and fluorescence was visualized using a confocal microscope. We found no changes in fluorescence by the Δ*PD1311* strain indicating that PD1311 is not involved in DSF production (data not shown).

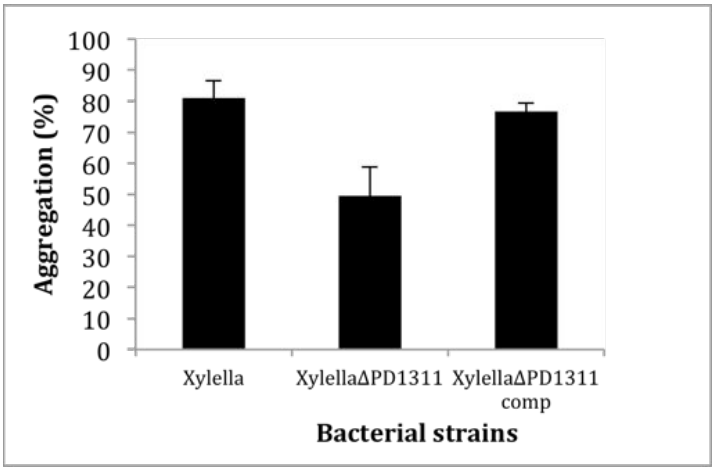

Fig. 4. Aggregation of *ΔPD1311* mutant strain. Aggregation of wild-type (*Xylella*), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311comp) strains were grown in test tubes for five days in 3 ml PD2 (Davis et al. 1980). After 5 days the OD_{540} was recorded (OD_T) and the bacteria resuspended before recording the OD (OD_S) again. The percent aggregation was calculated as $[(OD_T - OD_S)/ OD_T] \times 100$ (Burdman et al. 2000, Shi et al. 2007). The experiment was repeated three times.

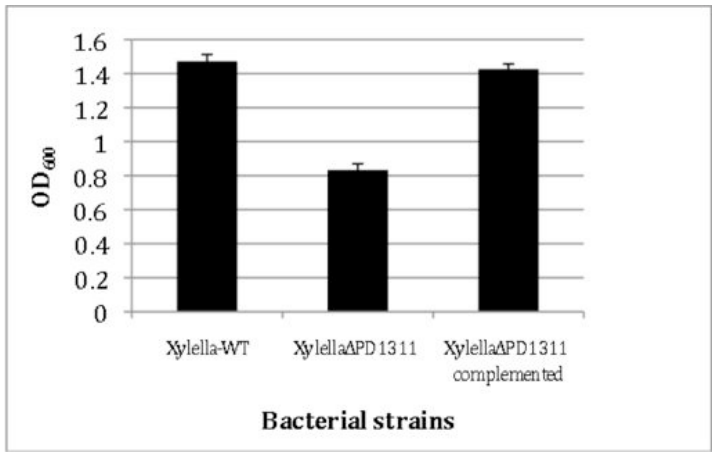

Fig. 5. Biofilm formation by *ΔPD1311* mutant strain. Quantification of biofilm formation in 96 well plates for wild-type (*Xylella*-WT), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311complemented) strains (Zaini et al. 2009). Experiment was repeated three times with 24 replicates each.

Objective 2. Determine the effectiveness of ΔPD1311 Temecula strain as a biological control of Pierce's disease.

We previously inoculated grapevines with the Δ*PD1311* strain and found the strain to be nearly avirulent (Fig. 6). As plants were started late in the season and the buffer control began to show signs of senescence around week 14, we do not believe the post-week 14 disease rating of *ΔPD1311* was true PD. **We have begun a repeat trials of the greenhouse experiments. While the experiment is only in week nine, trends support the past finding that Δ*PD1311* may not induce disease (Fig. 7). We will follow PD development until the wild-type strain has fully induced disease.**

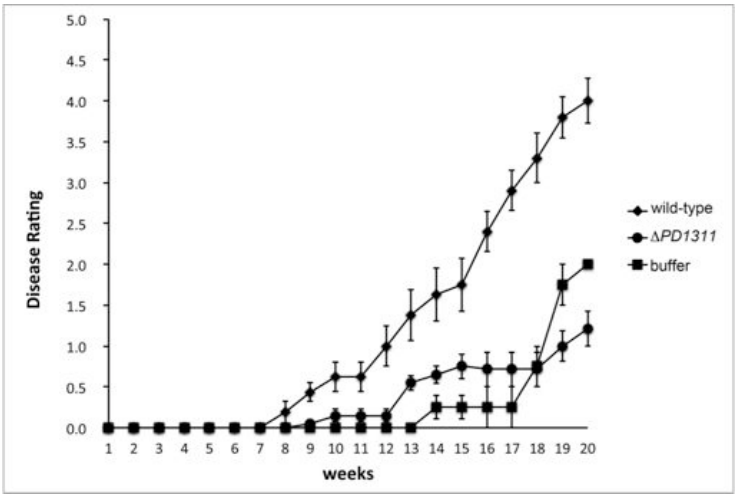

Fig. 6. Development of Pierce's disease trial 1. Grapevines were inoculated with wild-type *Xf* (diamond), *ΔPD1311* strain (circle), and buffer as a negative control (square). Symptoms were monitored on 10 plants for each treatment over a period of 20 weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Plants were started late in the season and the buffer control showed symptoms at week 14, suggesting that *ΔPD1311* symptoms may be due to senescence and not PD.

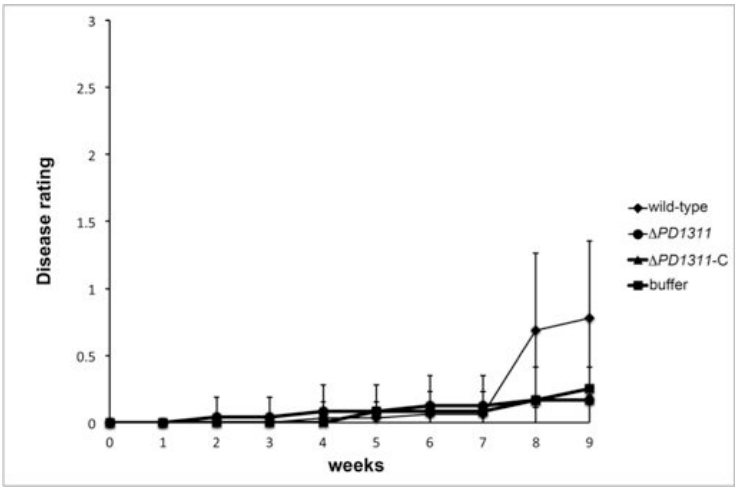

Fig. 7. Current study: development of Pierce's disease trial 2. Grapevines were inoculated with wild-type *Xf* (diamond), Δ*PD1311* strain (circle), Δ*PD1311* complement strain (Δ*PD1311*-C; triangle), and buffer (square). Symptoms have been monitored on 12 plants for each treatment for nine weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Disease monitoring will be continued for additional weeks. Trail 3 is also underway but it is too early for any PD symptoms to have developed.

We believe that the Δ*PD1311* strain may act as a biocontrol because we found that it reduced biofilm production by the wild-type *Xf* strain. For this study, we grew wild-type *Xf* cells constitutively expressing green fluorescent protein (wt-GFP) with either the Δ*PD1311* strain or wild-type cells. We previously used this strain (kindly provided by Prof. Steven Lindow, U. Cal., Berkeley) and found it to produce biofilm similar to wild-type *Xf* (data not shown). As stated above, wild-type cells produce more biofilm than the *ΔPD1311* strain (Fig. 5) so mixtures of wt-GFP*/ΔPD1311* should have equal or greater fluorescence than mixtures of wt-GFP/wt, if the strains did not impact each other. We found that the wt-GFP/ΔPD1311 mixture had less fluorescence than the wt-GFP/wt mixture (Fig. 8), indicating that the Δ*PD1311* strain impacts biofilm produced

by wt *Xf*.

2a. Determine conditions for biological control.

Given our findings that the Δ*PD1311* strain is nearly avirulent and impacts biofilm production by wild-type cells, we began greenhouse studies to determine if the Δ*PD1311* strain can be a viable biocontrol for PD. We inoculated *V. vinifera* cv. Cabernet franc vines per standard procedures (Cursino et al. 2011) and recorded disease development of PD using the five-scale assessment (Guilhabert & Kirkpatrick 2005). We have three different inoculation conditions: i) co-inoculated wild-type and Δ*PD1311* strains, ii) inoculation with the Δ*PD1311* strain followed two weeks later by wild-type *Xf* [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], and iii) inoculation of wild-type *Xf* followed two weeks later by the *ΔPD1311* strain. These experiments are only in their ninth week, however, early trends indicate that the mutant strain limits disease from wild-type *Xf* cells (Fig. 9).

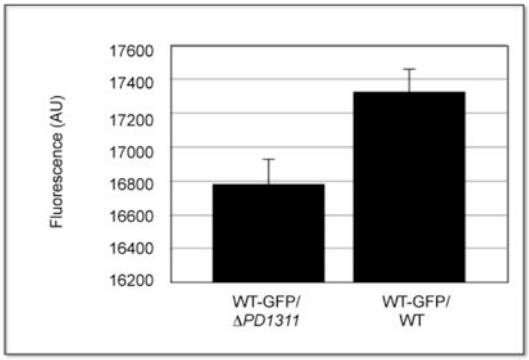

Fig. 8. The Δ*PD1311* strain impacts biofilm formation by wild-type cells. Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type *Xf* constitutively expressing green fluorescent protein (WT-GFP) and either wild-type *Xf* (WT) or the Δ*PD1311* strain (Δ*PD1311*). Experiment was performed with 24 replicates. Fluorescence in arbitrary units (AU).

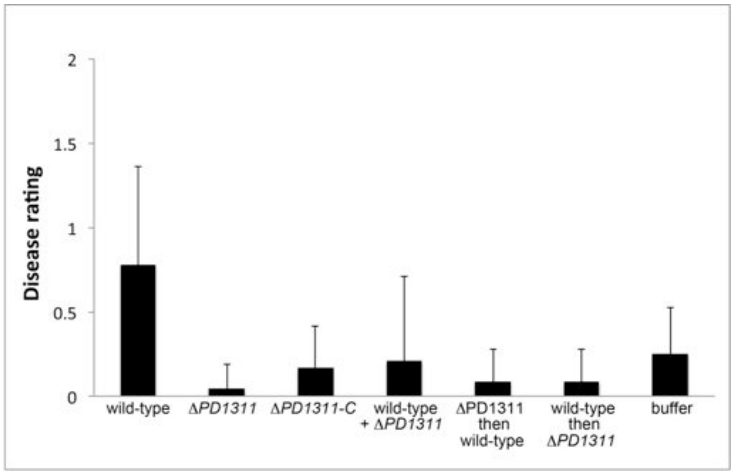

Fig. 9. Current study: the Δ*PD1311* strain impacts disease development by wild-type cells. Grapevines were inoculated with wild-type *Xf*, Δ*PD1311* strain, Δ*PD1311* complement strain (Δ*PD1311*-C), wild-type *Xf* co-inoculated with Δ*PD1311* strain (wild-type + Δ*PD1311*), Δ*PD1311* strain followed by wild-type *Xf* inoculation two weeks later (Δ*PD1311* then wild-type), wild-type *Xf* followed by Δ*PD1311* strain inoculation two weeks later (wild-type then Δ*PD1311*), or buffer. Symptoms monitored on 12 plants for each treatment. Bars represent findings on latest disease rating (week nine). Disease rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Disease monitoring will be continued for additional weeks. A repeat trial is also underway but it is too early for any PD symptoms to have developed.

2b. Examine spread of Δ*PD1311* and wild-type strains simultaneously.

We will perform these experiments upon completion of objective 2a. However, our initial trial *in planta* indicates that the Δ*PD1311* strain can be detected in plants (Table 1), suggesting that limited PD symptoms from Δ*PD1311* incoluation is not due to an inability of the Δ*PD1311* to survive in the grapevines.

VIII. SUMMARY OF ACCOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE.

Xf motility, aggregation, and biofilm formation are key steps in PD development (Chatterjee et al. 2008). Concerning objective 1a, we have shown that PD1311 plays a role in aggregation, biofilm formation, and potentially motility. **We are further refining our understanding of PD1311 and motility.** For objective 1b, we have found that PD1311 is not involved in DSF production. **In relation to objective 2, we are currently repeating greenhouse studies to confirm preliminary results that the *ΔPD1311* strain is nearly avirulent. While very preliminary, trends support earlier findings.** For objective 2a, we previously found that the *ΔPD1311* strain impacts biofilm production by wild-type *Xf*. **Therefore we are currently examining if *ΔPD1311* strain blocks PD induced by wild-type cells. Early results suggest that the mutant strain inhibits wild-type virulence *in planta*. Objective 2b will be performed once PD is more developed in the grapevines. Overall, this work will help further understanding of disease development and prevention.**

	Plant 1		Plant 2		Plant 3		Plant 4	
	up	down	up	down	up	down	up	down
WT	-	-	-	-	-	+	-	-
Δ*PD1311*	-	+	-	-	+	+	-	-

Table 1. Δ*PD1311* strain detected *in planta*. Five microliters of 10^9 CFU/mL of wild-type (WT) or mutant (Δ*PD1311*) *Xf* were inoculated into young grapevines in the 6-7th node counting from the top. The petioles directly above (up) and below (down) the inoculation point were sampled 10 days post-inoculation for PCR detection using *Xf* specific primers. + or – represents the presence or absence of the characteristic band.

IX. PUBLICATIONS PRODUCED AND PENDING, AND PRESENTATIONS MADE THAT RELATE TO THE FUNDED PROJECT.

Publications (Peer reviewed and Proceedings).

Burr, T.J., Mowery, P., Cursino, L., and K. Johnson. Identification of a new virulence factor required for Pierce's disease and its utility in development of a biological control. Proceedings of the Pierce's Disease Research Symposium 2013, pp. 41-47. Proceedings.

Mowery, P., T.J., Burr, Hoch, H.C., Cursino, L., Johnson, K., Galvani, C., Athiuwat, D., and Shi, X. Exploiting a chemosensory signal transduction system that controls twitching motility and virulence in *Xylella fastidiosa*. Proceedings of the Pierce's Disease Research Symposium 2012, pp. 59-64. Proceedings.

Cursino, L., Galvani, C.D., Athinuwat, D., Zaini, P.A., Li, Y., De La Fuente, L., Hoch, H.C., Burr, T.J., and P. Mowery. 2011. Identification of an Operon, Pil-Chp, that Controls Twitching Motility and Virulence in *Xylella fastidiosa*. *Mol. Plant Microbe Interact.* 24:1198-1206.

Mowery, P., T.J., Burr, Hoch, H.C., Cursino, L., Athiuwat, D., and Galvani, C. Exploiting a chemosensory signal transduction system that controls twitching motility and virulence in *Xylella fastidiosa*. Proceedings of the Pierce's Disease Research Symposium 2011, pp. 71-75. Proceedings.

Pending Publications.

Johnson, K.L., Burr, T.J., and Mowery, P. Affect of cell aggregation on development of targeted gene deletion clones in *Xylella fastidiosa*. Drafting for submission.

Cursino, L., Athinuwat, D., Patel, K., Galvani, C.D., Zaini, P.A., Li, Y., De La Fuente, L., Hoch, H.C., Burr, T.J., and Mowery, P. Characterization of the *Xylella fastidiosa chpY* gene and its role in the development of Pierce's disease. Drafting for submission.

Athinuwat, D., Johnson, K., Hao, L., Galvani, C.D., Cursino, L., Losito, E., Hoch, H.C., Burr, T.J. and Mowery, P. Analysis of the *Xylella fastidiosa* Pil-Chp operon genes and their relevance to Pierce's disease. Drafting for submission.

Presentations and Posters.

Burr, T.J. How *Xylella fastidiosa* Is Able to Move in Plants. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Presentation.

Johnson, K, Mowery, P., and Burr, T.J. Impact of aggregation on development of *Xylella fastidiosa* mutant clones. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Poster.

Mowery, P., Johnson, K.L., Cursino, L., and Burr, T.J. Identification of a new virulence factor required for Pierce's disease and its utility in development of a biological control. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Poster.

Johnson, K. Role of a thioredoxin family protein in *Xylella fastidiosa* virulence. APS-MSA, Austin, TX, 2013. Presentation.

Mowery, P., Johnson, K.L., Cursino, L., and Burr, T.J. *Xylella fastidiosa* virulence factor mutant strain as a potential biocontrol for Pierce's disease. APS-MSA, Austin, TX, 2013. Poster.

Mowery, P. "How does your vineyard grow? Understanding the grapevine pathogen, *Xylella fastidiosa*." Department of Biology. Ithaca College. Ithaca, NY, 2013. Presentation.

X. RESEARCH RELEVANCE STATEMENT.

Xylella fastidiosa is an important phytopathogen that infects a number of important crops including citrus, almonds, and coffee. The *X. fastidiosa* Temecula strain infects grapevines and induces Pierce's disease. We recently deleted the *X. fastidiosa PD1311* gene and found that the strain was no longer pathogenic. Based on sequence analysis, the PD1311 protein appears to encode an acyl CoA synthetase, which is a class of enzymes involved in many different processes including secondary metabolite production. Given the critical role of PD1311 in Pierce's disease development, we are exploring how it induces its phenotype. In addition, we are testing the *ΔPD1311* strain as a potential biocontrol for preventing Pierce's disease.

XI. LAY SUMMARY OF PROJECT ACCOMPLISHMENTS.

We discovered that deleting the *X. fastidiosa* Temecula gene, *PD1311*, results in a strain that does not induce Pierce's disease. This project will examine how PD1311 plays such a central role in disease. Given the importance of Pierce's disease, it is critical to understand how PD1311 exerts its effects. In addition, we will determine if the strain deleted for the *PD1311* gene can function as a biocontrol. Options for managing Pierce's disease are limited, which makes possible new biocontrols critically important. Together the results from these aims will expand our understanding of Pierce's disease and provide information in relation to preventing disease.

XII. STATUS OF FUNDS.

$54,421 of the funds have been spent.

XIII. SUMMARY AND STATUS OF INTELLECTUAL PROPERTY ASSOCIATED WITH THE PROJECT.

No intellectual property has resulted from research done under this grant.

XIV. LITERATURE CITED.

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