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. November 2013-March 2014

V. INTRODUCTION.

X. fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium that causes Pierce's disease (PD) of 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 *Xf* proteins and regulators 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 goal of better understanding PD in order to develop prevention strategies.

While the steps leading to blocked vessels appear to be key to disease, we wanted to explore if additional virulence factors facilitate symptoms. Of particular interest were genes with potential roles in secondary metabolite production. We explored a *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 has 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* does not have luciferase activity, nor the domains and size of NRPS megaenzymes (Strieker et al. 2010), suggesting that PD1311 is

potentially 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 discovered that deleting PD1311 results in a non-pathogenic strain when inoculated in grapevines, indicating that PD1311 is fundamental for Pierce's disease development.

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).

Given our findings with the *ΔPD1311* strain, we proposed it has potential as a biocontrol for PD. The weakly virulent *Xf* elderberry strain EB92-1 has been studied as a potential PD biocontrol (Hopkins 2005, Hopkins 2012). Other approaches include naturally resistant rootstocks (Cousins and Goolsby 2011) or transgenic varieties (Dandekar 2012, Gilchrist and Lincoln 2012, Kirkpatrick 2012, Labavitch et al. 2012, Lindow 2012, Powell and Labavitch 2012). However, continued research for PD controls is warranted. Given the avirulent phenotype of the *ΔPD1311* 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 ΔPD1311* 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 *ΔPD1311* Temecula strain as a biological control of Pierce's disease.
- a. Determine conditions for biological control.
- b. Examine spread of *ΔPD1311* and wild-type strains simultaneously.

VII. DESCRIPTION OF ACTIVIES.

Objective 1. Characterize the *X. fastidiosa ΔPD1311* mutant.

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

We deleted and complemented *PD1311* as previously described (Matsumoto et al. 2009, Shi et al. 2009). The Δ*PD1311* strain grows in xylem sap (**Fig. 2**), can be detected in plants (**Table 1**), and produces less aggregation and biofilm than wild-type cells. Key processes leading to PD are motility, aggregation, and biofilm formation (Chatterjee et al. 2008), and therefore we are examining these behaviors in the *ΔPD1311* strain. Such information will help us determine if PD1311 exerts its effects by common virulence methods or has more specialized function.

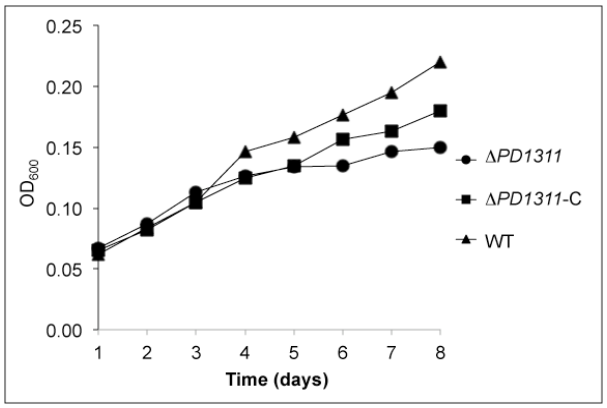

Fig. 2. Δ*PD1311* strain growth. Wild-type *Xf* (WT - triangle), mutant (Δ*PD1311* - circle), and complemented mutant (Δ*PD1311*-C – square) strains were grown for eight days in 100% *Vitis vinifera* cv. Chardonnay xylem sap and growth was determined by OD_{600} readings.

i) Motility:

Weakly virulent strains have been found that have low motility (Cursino et al. 2009). Our preliminary results show that *ΔPD1311* motility may be modestly affected, as examined by colony fringe assay (**Fig. 3**); fringe around the bacterial colony directly correlates with type IV pilus twitching motility (Meng et al. 2005, Li et al. 2007). Given the potential reduced motility and that in grapevines *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).

	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.

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). Our studies show that the *ΔPD1311* strain has decreased aggregation in comparison to wild-type and complement strains ($P<0.03$) (**Fig. 4**). We then examined biofilm production using a 96-well crystal violet assay (Zaini et al. 2009). In line with the aggregation findings, the *ΔPD1311* strain appears to produce 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 *ΔPD1311*. This result suggests that other factors besides altered biofilm production are involved in ΔPD1311 avirulence.

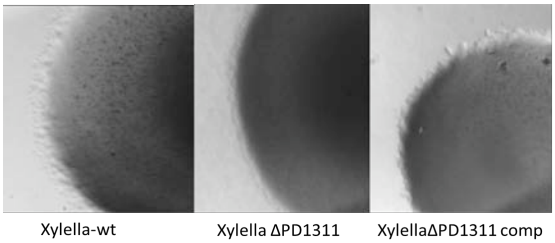

Fig. 3. Motility of *ΔPD1311* mutant strain. Colony fringes of wild-type (*Xylella*-wt), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311comp) strains were assayed on PW agar overlaid with cellophane. Colonies were assessed after five days of growth (Meng et al. 2005, Li et al. 2007).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

<u>1b. Determine the role(s) of PD1311 in producing virulence factor(s).</u>

Our preliminary results suggest that PD1311 affects virulence by methods beyond motility and biofilm formation, which are fundamental processes in PD (Chatterjee et al. 2008). Many bacteria produce secondary products that are critical to their pathogenic responses (Raaijmakers and Mazzola 2012). In terms of *Xf*, these molecules may be involved in pathogen regulation or secondary metabolite production. The *Xf* DSF is a quorum-sensing product that coordinates motility, biofilm formation, and virulence (Chatterjee et al. 2008). *Xf* is postulated to have multiple DSF products of which the known ANL protein, RpfB, appears only to be involved in the production of a subset (Almeida et al. 2012). Given that PD1311 appears to be an ANL member, it may also play a role in DSF production in conjunction with RpfB or in another DSF product. Determining if PD1311 is involved in DSF production will either place PD1311 within the growing class of *Xf* quorum-sensing associated molecules (Almeida et al. 2012) or direct our studies to other areas of ACS involvement.

To determine if PD1311 alters DSF production wild-type *Xf* and the *ΔPD1311* strain were streaked 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 *ΔPD1311* 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. Our findings showed no alterations in DSF production by the *ΔPD1311* strain suggesting that PD1311 is not involved in quorum sensing molecule production (data not shown).

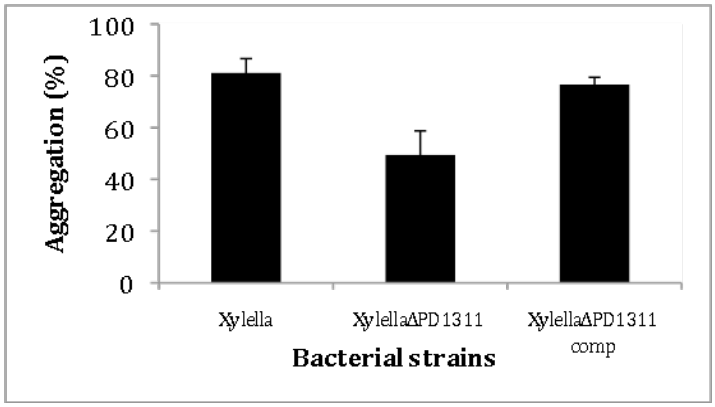

Fig. 4. Aggregation of *ΔPD1311* mutant strain. Aggregation of wild-type (*Xylella*), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311comp) strains were grown in test tubes for five days in 3 ml PD2 (Davis et al. 1980). After 5 days the OD_{540} was recorded (OD_T) and the bacteria 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.

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

When we test the *ΔPD1311* strain *in planta* we found that deleting it eliminated disease (**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. These results indicate that PD1311 product is critical for PD symptoms.

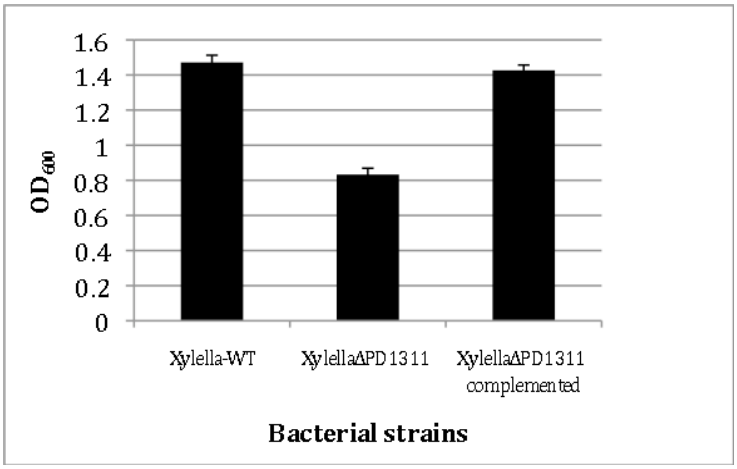

Fig. 5. Biofilm formation by *ΔPD1311* mutant strain. Quantification of biofilm formation in 96 well plates for wild-type (*Xylella*-WT), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311complemented) strains (Zaini et al. 2009). Experiment was repeated three times with 24 replicates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

We believe that the *ΔPD1311* strain may act as a biocontrol as it appears to have an impact on wild-type *Xf* cells in relationship to an important PD trait, biofilm formation. We have wild-type *Xf* cells constitutively expressing green fluorescent protein (wt-GFP) (kindly provided by Prof. Steven Lindow, U. Cal., Berkeley). We have used this strain before in a number of behavioral assays and found it to function like wild-type *Xf* (data not shown). To determine if *ΔPD1311* affected biofilm formation by wild-type strain Temecula *Xf*, half the cells were wt-GFP and the other half were either non-fluorescent wild-type (wt) or the *ΔPD1311* strain. 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 observed that the wt-GFP/ΔPD1311 mixture had <u>less</u> fluorescence than the wt-GFP/wt mixture (**Fig. 7**), suggesting that the *ΔPD1311* strain has an ability to reduce the virulence-associated biofilm produced by wt *Xf*.

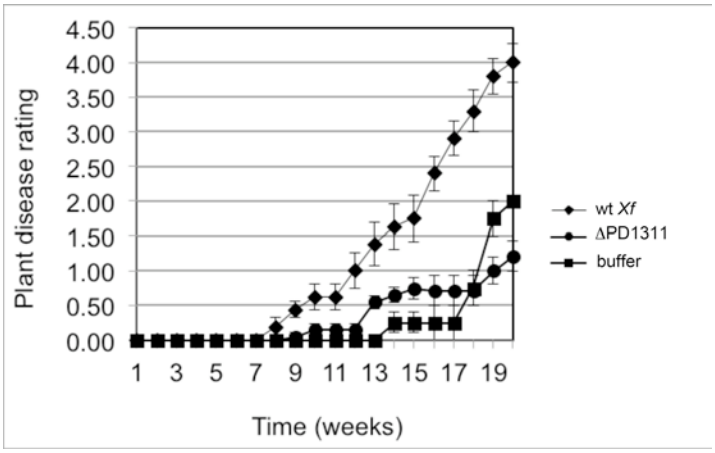

Fig. 6. Development of Pierce's disease. Grapevines were inoculated with wild-type *Xf* (diamond), *ΔPD1311* strain (circle), and buffer as a negative control (square). Symptoms were monitored on 10 plants for each treatment over a period of 20 weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 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.

<u>2a. Determine conditions for biological control.</u>

Given our findings that the *Xf* Δ*PD1311* Temecula strain does not cause PD and impacts wild-type biofilm production, we began pilot greenhouse studies to determine if the *Xf* Δ*PD1311* strain can be a viable biocontrol. 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 created three different inoculation conditions: i) wild-type *Xf* after a two week pre-treatment with the Δ*PD1311* strain [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], ii) wild-type and Δ*PD1311* strain co-inoculated, and iii) Δ*PD1311* strain after a two week pre-treatment with the wild-type strain. Our controls included vines inoculated with wild-type Temecula, the Δ*PD1311* strain, or buffer control (Hopkins 1984). At our facilitates a major greenhouse renovation was initiated last summer, and unfortunately, during the surging process all the plants grew very poorly not allowing a meaningful evaluation of PD symptoms. The construction is now complete and therefore we do not anticipate similar issues this year.

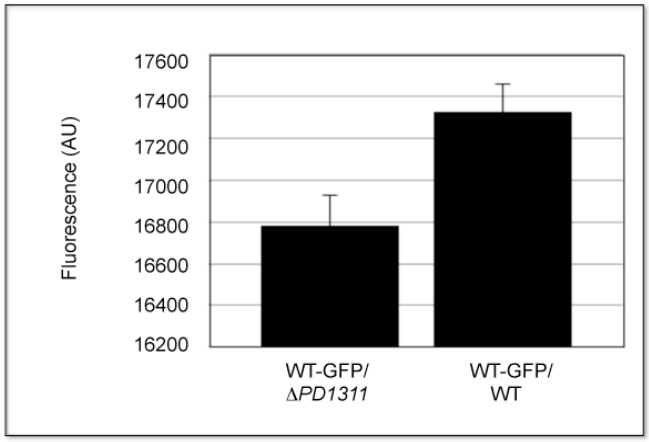

Fig. 7. The Δ*PD1311* strain impacts biofilm formation by wild-type cells. Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type *Xf* constitutively expressing green fluorescent protein (WT-GFP) and either wild-type *Xf* (WT) or the Δ*PD1311* strain (Δ*PD1311*). Experiment was performed with 24 replicates. Fluorescence in arbitrary units (AU).

2b. Examine spread of ΔPD1311 and wild-type strains simultaneously.

We will soon begin organizing the experiments in order to address objective 2b.

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 biofilm formation, aggregation, and maybe motility. We are further refining our understanding of PD1311 and motility. For objective 1b, we have found that PD1311 is not involved in quorum sensing but that the *ΔPD1311* strain appears to impact biofilm production by wild-type *Xf*. Therefore the mutant strain may alter wild-type-induced disease *in vivo*. In relation to objective 2a, we will complete greenhouse studies within the next year to determine if the *ΔPD1311* strain can block Pierce's disease development by wild-type *Xf*. For objective 2b, our preliminary results show that the mutant can compete with wild-type cells *in planta*. Overall, this work will help further understanding of disease development and prevention.

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, PD1311 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 PD1311 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.

$39,174 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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