

I. INTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 15-0138-SA

II. TITLE OF PROJECT. Development of a biological control for Pierce's disease

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

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IV. TIME PERIOD COVERED BY THE REPORT. October 2015-February 2016

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 genetic mechanisms 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, Cursino et al. 2015) with the goal of better understanding PD virulence and for development of prevention strategies.

We deleted the *Xf* PD1311 gene (Δ PD1311), a putative acyl-CoA synthetase (ACS), as we were interested in genes potentially involved in secondary metabolite production. ACSs catalyze long-chain fatty acyl-CoAs (Black et al. 1992) and are involved in numerous processes including pathogenicity (Barber et al. 1997). We found that PD1311 is a functional enzyme (data not shown), and that Δ PD1311 grows in PD2 and *Vitis vinifera* sap (**Fig.1**).

Motility, aggregation, and biofilm production are key behaviors of *Xf* that are associated with PD (Chatterjee et al. 2008). Δ PD1311 is reduced in type IV pili-mediated motility on PW plates

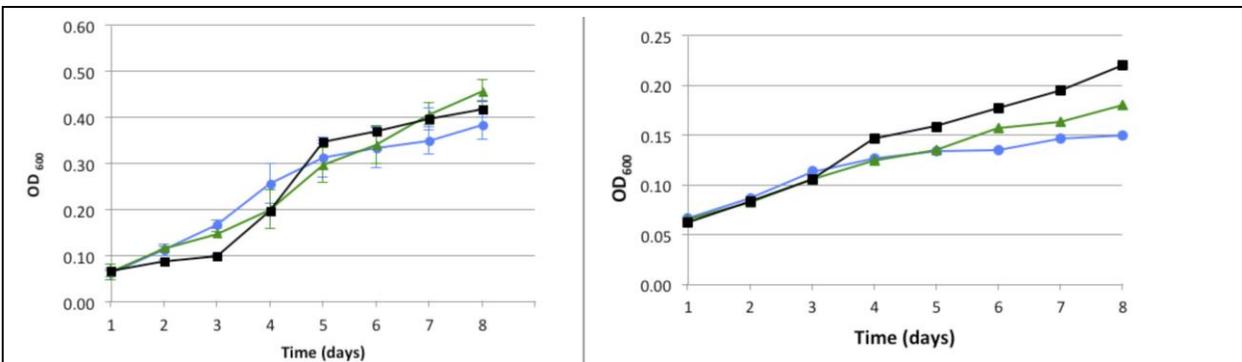


Fig. 1. ΔPD1311 strain growth curve. Wild-type *X. fastidiosa* (black square), ΔPD1311 mutant (blue circle), and complemented mutant (green triangle) strains were grown for eight days in PD2 (left) or 100% *Vitis vinifera* cv. Chardonnay xylem sap (right) and growth was determined by OD₆₀₀ readings.

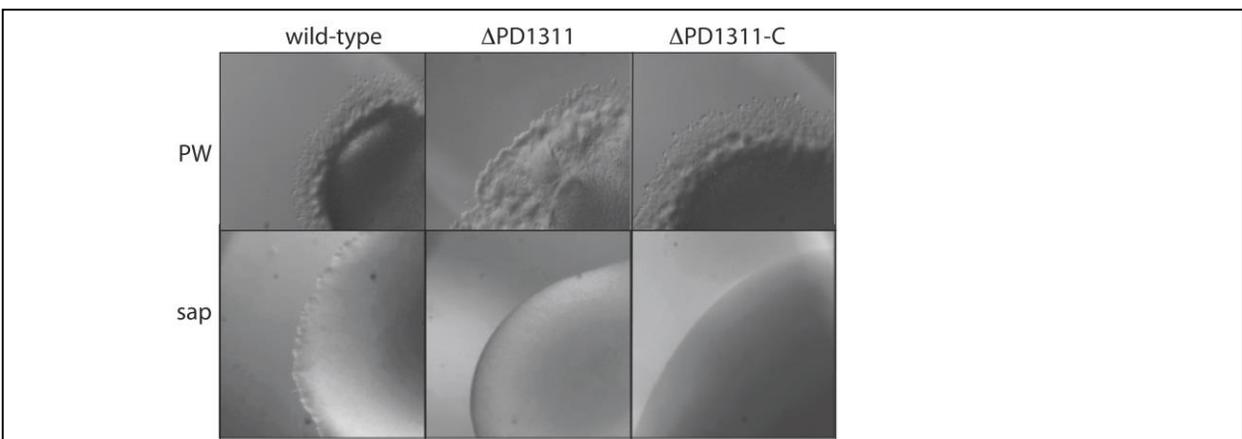


Fig. 2. Motility of ΔPD1311 mutant strain. Colony fringes of wild-type, ΔPD1311 mutant, or ΔPD1311 complement (ΔPD1311-C) strains were assayed on PW agar or 80% *V. vinifera* sap agar. Colonies were assessed after five days of growth (Meng et al. 2005, Li et al. 2007). Colonies photographed at 90X magnification. Experiment was repeated three times.

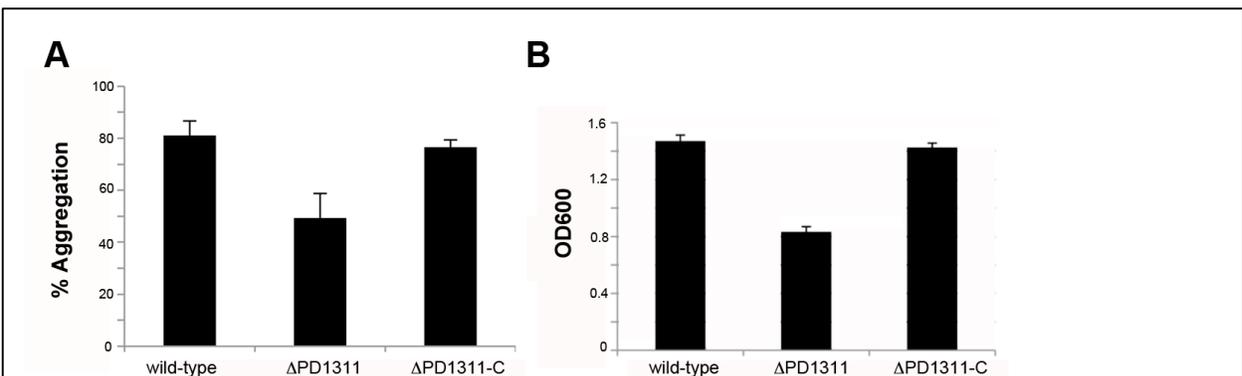


Fig. 3. Aggregation and biofilm formation by ΔPD1311 strain. A) Aggregation of wild-type, ΔPD1311 mutant, or ΔPD1311 complement (ΔPD1311-C) strains grown in test tubes for five days in 3 ml of PD2 (Burdman et al. 2000, Davis et al. 1980, Shi et al. 2007). The experiment was repeated three times. B) Quantification of biofilm formation in 96 well plates (Zaini et al. 2009). Experiment was repeated three times with 24 replicates each.

and is non-motile on sap agar (Fig. 2). In comparison to wild-type cells (Temecula 1), Δ PD1311 is reduced in aggregation and biofilm production (Fig. 3). We therefore hypothesized that Δ PD1311 is less virulent in plants, as mutants with similar phenotypes have been shown to have reduced or be avirulent (Cursino et al. 2009, Cursino et al. 2011, Guilhabert and Kirkpatrick 2005, Killiny et al. 2013). We found that Δ PD1311 was avirulent and showed no PD, even at twenty weeks post-inoculation (Fig. 4).

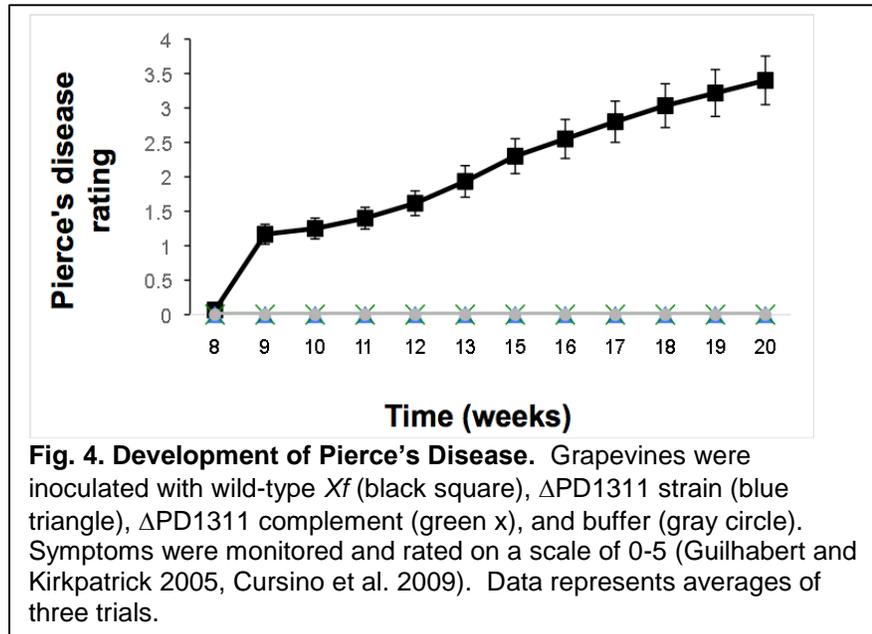


Fig. 4. Development of Pierce's Disease. Grapevines were inoculated with wild-type *Xf* (black square), Δ PD1311 strain (blue triangle), Δ PD1311 complement (green x), and buffer (gray circle). Symptoms were monitored and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Data represents averages of three trials.

The weakly virulent *Xf* elderberry strain EB92-1 has been studied as a potential PD biological control (Hopkins 2005, Hopkins 2012). Other approaches towards controlling PD include resistant rootstocks (Cousins and Goolsby 2011) and transgenic vines (Dandekar 2014, Gilchrist et al. 2014, Gilchrist and Lincoln 2014, Kirkpatrick 2014, Lindow 2014, Powell and Labavitch 2014). Continued research of PD controls is warranted. We had preliminary results that Δ PD1311 lowers the incidence of wild-type-induced PD (data not shown). Given the avirulent phenotype of Δ PD1311 and its ability to limit wild-type induced PD, this strain provides new potential for a commercialized biological control

VI. LIST OF OBJECTIVES.

The overall goal is to optimize Δ PD1311 as a biological control for PD and to understand the mechanisms of disease inhibition that will facilitate commercialization.

Objective 1. Examine aspects of Δ PD1311 Temecula strain as a biological control of PD.

- a. Optimize application timing and conditions for the Δ PD1311 strain.
- b. Determine if over-wintered Δ PD1311 inoculated plants maintain PD resistance.
- c. Explore leafhopper transmission of the Δ PD1311 strain.
- d. Develop clean deletion strain of Δ PD1311 that would be suitable commercialization.

Objective 2. Determine the function of the PD1311 protein and the mechanism by which Δ PD1311 acts as a biological control.

- a. Elucidate the role of PD1311 protein.
- b. Examine impact of the Δ PD1311 strain on wild-type *Xf* *in vitro* and *in planta*.

VII. DESCRIPTION OF ACTIVITIES.

Objective 1. Examine aspects of Δ PD1311 Temecula strain as a biological control of PD.

Objective 1a. Optimize application timing and conditions for the Δ PD1311 strain.

To examine if the *Xf* Δ PD1311 Temecula 1 strain could act as a potential biocontrol, we inoculated *V. vinifera* cv. Cabernet Sauvignon vines per standard procedures (Cursino et al. 2011) and recorded development of PD using the five-scale assessment (Guilhabert and Kirkpatrick 2005). We created three different inoculation conditions: i) wild-type *Xf* after a two week pre-treatment with Δ PD1311 [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], ii) wild-type and Δ PD1311 co-inoculated, and iii) controls (wild-type-only, Δ PD1311-only, buffer). We previously found that inoculating Δ PD1311 after a two week pre-treatment with the wild-type strain did not limit PD (data not shown). Our controls included vines inoculated with wild-type Temecula 1, Δ PD1311, or buffer (Hopkins 1984). We found that pre-treatment with Δ PD1311 inhibits PD, while co-inoculation does not alter disease development (**Fig. 5**).

Objective 1b. Determine if overwintered Δ PD1311 inoculated plants maintain PD resistance.

In 2014 we had *V. vinifera* plants infected with wild-type *Xf* or Δ PD1311 two weeks prior to wild-type *Xf*. These vines were cut back and placed in nursery storage for the 2015 winter. The plants were then grown in the greenhouse in Spring 2015 to follow potential PD development. Preliminary results showed that wild-type *Xf* could overwinter and cause PD in the following year. Plants treated with Δ PD1311 followed by wild-type *Xf* did not show symptoms either year and ELISA did not detect *Xf* (Temecula 1 or Δ PD1311) in year 2 (**Table 1**). This data suggests that Δ PD1311 protection may last overwintering. However, we have not explored whether biocontrol treatment in year 1 would protect against a fresh wild-type inoculation in year 2. If found, this result would indicate that the Δ PD1311 biocontrol may have

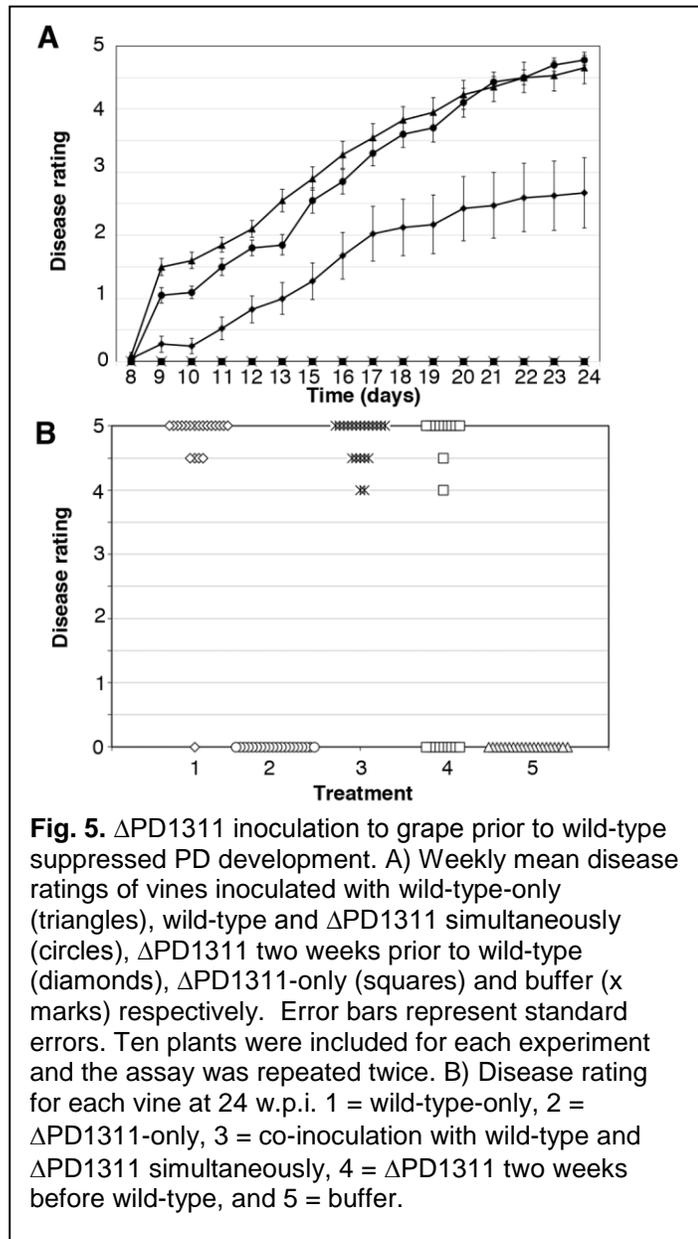


Fig. 5. Δ PD1311 inoculation to grape prior to wild-type suppressed PD development. A) Weekly mean disease ratings of vines inoculated with wild-type-only (triangles), wild-type and Δ PD1311 simultaneously (circles), Δ PD1311 two weeks prior to wild-type (diamonds), Δ PD1311-only (squares) and buffer (x marks) respectively. Error bars represent standard errors. Ten plants were included for each experiment and the assay was repeated twice. B) Disease rating for each vine at 24 w.p.i. 1 = wild-type-only, 2 = Δ PD1311-only, 3 = co-inoculation with wild-type and Δ PD1311 simultaneously, 4 = Δ PD1311 two weeks before wild-type, and 5 = buffer.

long-lasting protection in the field. If symptoms do develop in year 2 in the Δ PD1311-treated plants, this result will indicate that reapplication of the biocontrol will be necessary to maintain PD suppression.

Objective 1c. Explore leafhopper transmission of the Δ PD1311 strain.

Xylem-sap feeding leafhopper vectors transmit *Xf* from plant to plant (Chatterjee et al. 2008). The bacterium utilizes adhesins, such as FimA, HxfA, and HxfB, to attach and form biofilms on insect foreguts, which then becomes a source of inoculum for further disease spread (Killiny and Almeida 2009, Killiny et al. 2010).

Table 1. *Xf* ELISA results overwintered plants.^a

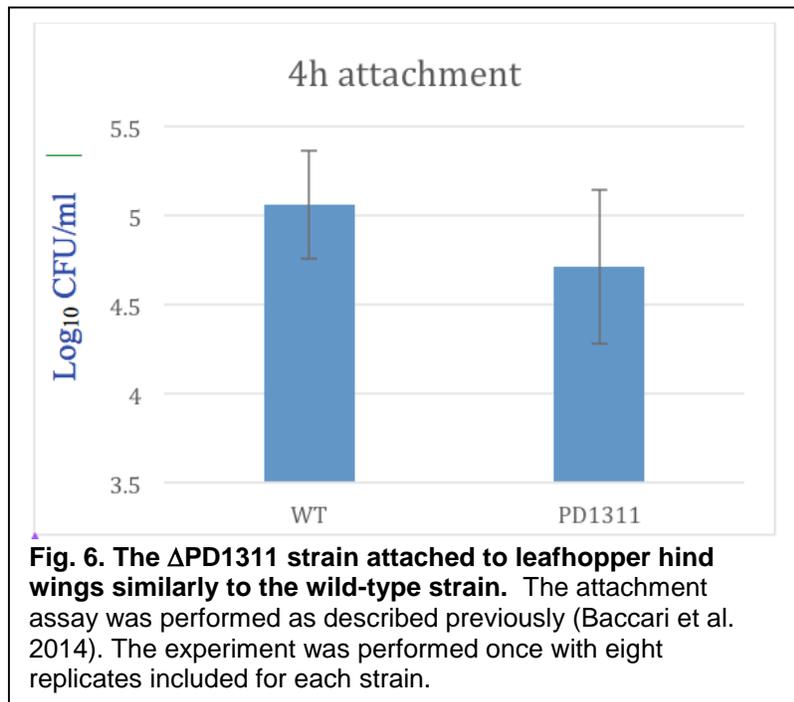
Treatment Year 1 ^b	Symptoms Year 1 ^c	Symptoms Year 2	0cm ^d	30cm ^d	150cm ^{dcm}
WT	+	+	+/1 ^e	+/1	+/1
		-	-/3	-/3	-/3
Δ PD1311 then WT	-	-	-/2	-/2	-/2

^a Plants overwintered in cold storage between year 1 and 2.
^b Plants were given no further inoculations in year 2.
^c “+” = PD symptoms; “-” = no PD symptoms.
^d Sample distance up from inoculation point in year 2.
^e “+” or “-” indicated positive or negative for *Xf*, respectively / “number” is the number of plants tested by ELISA in year 2.

Thus interaction with insects is a known key step for *Xf* to accomplish its life cycle. For development of Δ PD1311 as a commercially viable biological control agent and for future field studies, it will be necessary to understand its insect transmissibility. Because Δ PD1311 has reduced aggregation and biofilm (Fig. 3), we hypothesize that Δ PD1311 is altered in its ability to be insect vectored. As an initial assay, we want to examine the adhesion of the mutant strain to the hindwing of the leafhopper vector, as this assay has been found to mimic adhesion to the foregut region owing to similar chitinous nature of the cuticles (Killiny et al. 2010). We have preliminary data to show that Δ PD1311 attached to insect wings at a level similarly to the wild-type strain (Fig. 6). We will repeat the experiments for further verification.

Objective 1d. Develop clean deletion strain of Δ PD1311 that would be suitable commercialization.

Δ PD1311 was created via site-specific recombination of a kanamycin cassette into the *Xf* chromosome (Matsumoto et al. 2009, Shi et al. 2009). For commercial viability, the antibiotic marker needs to be removed from the strain. Unlabeled *Agrobacterium tumefaciens* mutants have been created (Merritt et al. 2007), which will be the first approach we attempt. This work will begin soon.



Objective 2. Determine the function of the PD1311 protein and the mechanism by which Δ PD1311 acts as a biological control.

The *Xf* PD1311 gene has motifs suggesting it encodes an ACS protein (acyl- and aryl-CoA synthetase) (Chang et al. 1997, Gulick 2009). ACS metabolite intermediates are involved in beta-oxidation and phospholipid biosynthesis. ACS proteins have also been implicated in cell signaling (Korchak et al. 1994), protein transportation (Glick and Rothman 1987), protein acylation (Gordon et al. 1991), and enzyme activation (Lai et al. 1993). Importantly, ACSs are involved in pathogenicity (Banchio and Gramajo 2002, Barber et al. 1997, Soto et al. 2002). We plan to characterize the role of the PD1311 protein in order to understand how the deletion strain is avirulent and functions as a biological control. Additionally we plan to explore the general mechanism by which the deletion strain suppresses wild-type *Xf*-induced PD. Basic understanding of its function will facilitate development and acceptance as a viable biological control.

Objective 2a. Elucidate the role of PD1311 protein.

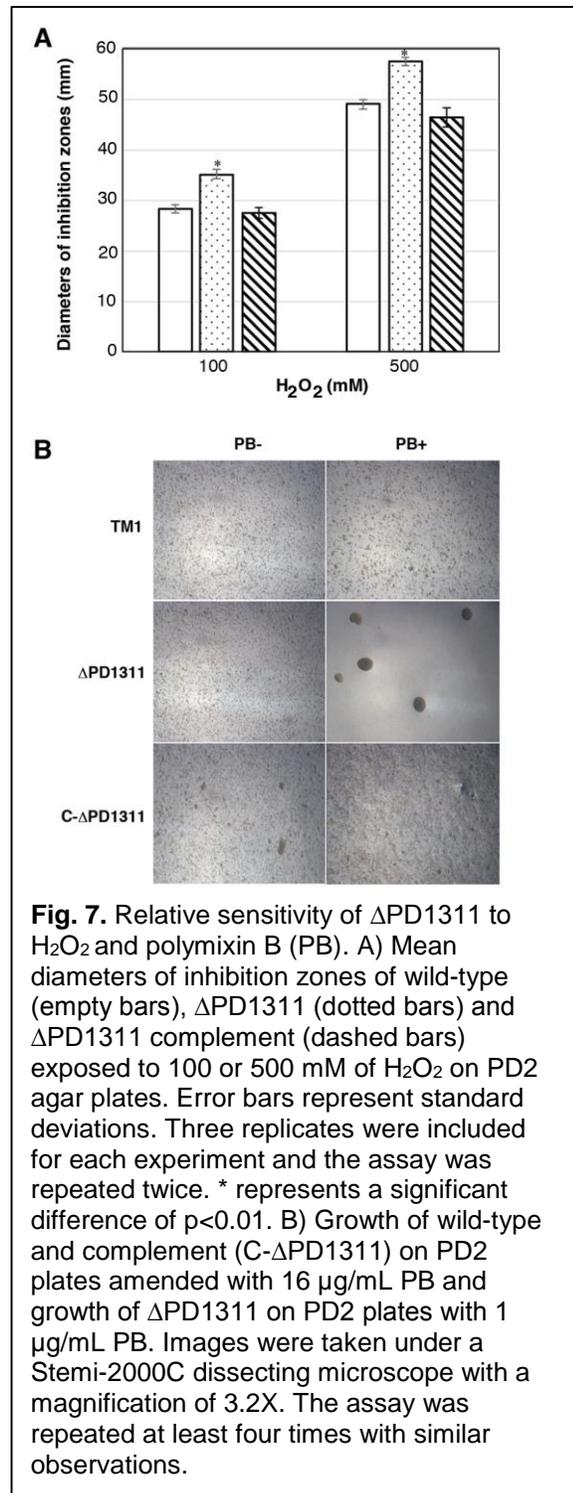
ACS proteins metabolize fatty acids through a two-step process to form a fatty acyl-CoA precursor utilized in any downstream metabolic pathways (Roche et al. 2013, Watkins 1997, Weimar et al. 2002). To confirm enzymatic activity, we expressed and purified a PD1311-His tag protein, and we tested it for ligase activity using acetate as the substrate. Acetate is the simplest substrate for fatty acid synthetase reaction, as a two-carbon (C2) chain length molecule. We used a standard colorimetric assay that measures acyl-CoA production (Kuang et al. 2007). The PD1311 protein exhibited a functional ATP/AMP binding domain that performed the following reaction: ATP + acetate + CoA is converted to AMP + pyrophosphate + acetyl-CoA. Therefore we confirmed that the protein is functional.

The deletion of the PD1311 gene is non-lethal, suggesting that it has a role in non-essential fatty acid metabolism. One possibility is that PD1311 plays a role in DSF production, however, our preliminary results do not support that role (data not shown). An alternative potential role for the PD1311 protein is in precursor production of lipopolysaccharide (LPS). LPS is found on the outer membrane of gram-negative bacteria and is composed of a lipid A innermost component, a core saccharide, and an outer most O-antigen. Upstream of PD1311, are three genes annotated as LPS-associated enzymes: lipid A biosynthesis N-terminal domain protein (PD1312), dolichol-phosphate mannosyltransferase (Dpm1) (PD1313), and WbnF nucleotide sugar epimerase (PD1314) (Simpson et al. 2000). Dolichol-phosphate mannosyltransferase proteins are involved in N-linked oligosaccharides in the LPS core (Kapitonov and Yu 1999), while nucleotide sugar epimerases are involved in O-antigen synthesis (Lam et al. 2011). LPS is a known major virulence factor of *Xf*, and changes in LPS integrity renders bacteria more susceptible to environmental stress and defective in virulence (Clifford et al. 2013).

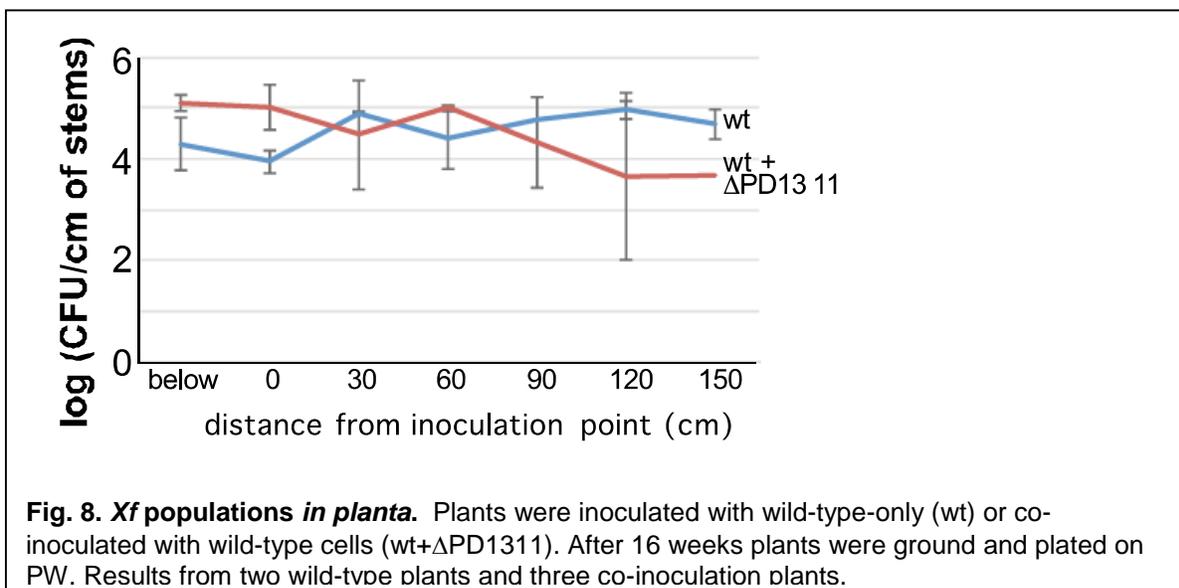
Considering the avirulent phenotype of Δ PD1311 on grapevines, PD1311 may be involved in lipid A biosynthesis or membrane production. Therefore, the Δ PD1311 cells may be more sensitive to environmental stresses such as oxidative stress and cationic antimicrobial peptide polymyxin B (PB). When wild-type and Δ PD1311 cells were exposed to hydrogen peroxide on agar plates in a Kirby-Bauer type assay, the zone of inhibition was greater for the mutant strain than wild-type cells (**Fig. 7A**). In addition, Δ PD1311 cells were more sensitive to PB than wild-type or Δ PD1311 complement cells. While both wild-type and Δ PD1311 complement cells grew on plates supplemented with 16 μ g/mL PB, almost all Δ PD1311 cells were killed when plated on PW agar supplemented with 1 μ g/mL PB (**Fig. 7B**). We are exploring how this sensitivity may be associated with the possible modification of the outer cell envelope of Δ PD1311 to gain better understanding of its avirulence on grapevines.

Objective 2b. Examine impact of the Δ PD1311 strain on wild-type Xf in vitro and in planta.

To have better grounding on why Δ PD1311 acts as a biological control, we need to explore the mechanism by which the mutant strain impacts wild-type cells. We have preliminary results showing that the wild-type induced disease can be limited only when Δ PD1311 was inoculated two weeks before the pathogen (**Fig. 5**). Therefore we would like to know how the two strains spread through the plant when both are inoculated. In multiple trials, we found that six weeks post inoculation, Δ PD1311 was not detected in the plants either by direct plating with ground plant shoot pieces or by ELISA test with petioles sampled at multiple locations on grapevines, including the inoculation point (data not shown). It appears that Δ PD1311 cells are either dead in the vines or decline to a level below detection. Therefore, bacterial populations determined from dilution plating with tissues from the co-inoculated vines only represent those of the wild-type *Xf*. Δ PD1311 does not secrete a toxin that affects wild-type populations (**Fig. 8**); we grew



wild-type cells in supernatant from Δ PD1311 cells and found no growth changes (data not shown). Understanding how the mutant cells impact wild-type *Xf* is important for understanding not only how the biological control is achieved but also how the treatment would be most effectively applied in the field.



VIII. SUMMARY OF ACCOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE.

Concerning objective 1, we confirmed that Δ PD1311 is avirulent, and we found that it can significantly reduce PD development by wild-type *Xf*. Preliminary data suggests that Δ PD1311 attaches to insect hindwings equal to wild-type cells and therefore could possibly be distributed by the vector. For objective 2, our preliminary results show that the mutant has greater sensitivity to chemical environments (hydrogen peroxide, antimicrobial peptides), which may contribute to its avirulent phenotype and help explain the role of the protein in the bacterium. Overall, this work will help further our 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).

- Johnson KL, Cursino L, Athinuwat D, Burr TJ, Mowery P. 2015. Potential complications when developing gene deletion clones in *Xylella fastidiosa*. *BMC Res. Notes*. 8: 155.
- Cursino L, Athinuwat D, Patel K, Galvani CD, Zaini PA, Li Y, De La Fuente L, Hoch HC, Burr TJ, Mowery P. 2015. Characterization of the *Xylella fastidiosa* PD1671 gene encoding degenerate c-di-GMP GGDEF/EAL domains, and its role in the development of Pierce's disease. *Plos One*. 10: e0121851.
- Burr, T.J., Mowery, P., Cursino, L., and K. Johnson. Development of a biological control for Pierce's disease. Proceedings of the Pierce's Disease Research Symposium 2015, pp. 9-17. Proceedings.
- Burr, T.J., Mowery, P., Cursino, L., and K. Johnson. Development of a biological control for

Pierce's disease. Proceedings of the Pierce's Disease Research Symposium 2014, pp. 42-49. Proceedings.

Burr, T.J., Mowery, P., Cursino, L., and Hao, L. 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 2014, pp. 42-49. 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., Athiawat, 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., Athiawat, 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., Athiawat, 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.

Hao L, Johnson K, Cursino L, Mowry P, Burr TJ. 2016. Characterization of the *Xylella fastidiosa* PD1311 gene mutant and its suppression of Pierce's disease on grapevines. Submitted.

Zaini PA, Hao L, Hoch HC, Burr TJ, Mowery P. 2016. Grape cultivar and sap culture conditions affect the development of *Xylella fastidiosa* phenotypes associated with Pierce's disease development. Submitted.

Presentations and Posters.

Mowery P. *Xylella fastidiosa* chemosensory-like involvement in Pierce's disease." American Society of Phytopathology, Pasadena, CA, 2015. Presentation.

Hao L, Johnson K, Cursino L, Burr TJ, Mowery P. *Xylella fastidiosa* PD1311 deleted strain as promising Pierce's disease biological control. American Society of Phytopathology, Pasadena, CA, 2015. Poster.

Burr TJ. PD1311, a virulence factor required for Pierce's disease and its utility in development of a biological control. Pierce's Disease Research Symposium, Sacramento, CA, 2014. Presentation.

Burr TJ. How *Xylella fastidiosa* is able to move in plants. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Presentation.

Johnson K, Mowery P, Burr TJ. Impact of aggregation on development of *Xylella fastidiosa* mutant clones. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Poster.

Mowery P, Johnson KL, Cursino L, Burr TJ. 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 KL, Cursino L, Burr TJ. *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 mutant strain is avirulent. Based on sequence analysis, PD1311 is predicted to encode an acyl-CoA synthetase, which is a class of enzymes involved in many different processes including secondary metabolite production. We have characterized Δ PD1311 and found phenotypes consistent with reduced virulence. In addition to Δ PD1311 being avirulent it also reduces the virulence of wild-type *X. fastidiosa*. Therefore, we propose that the Δ PD1311 has potential as a biological control for PD.

XI. LAY SUMMARY OF PROJECT ACCOMPLISHMENTS.

We discovered that deleting the *X. fastidiosa* Temecula 1 gene, PD1311, results in a strain that does not induce Pierce's Disease. We are conducting research to determine how PD1311 plays such a central role in symptom development. Given the agricultural importance of Pierce's Disease, it is critical to understand how PD1311 exerts its effects. Additionally, we have evidence that the PD1311 mutant has potential as a biological control. When grape plants were inoculated with the mutant prior to wild-type *X. fastidiosa*, disease development becomes significantly reduced. Options for managing Pierce's Disease are limited, which makes development of new control strategies critically important. Together the results from these aims will expand our understanding of Pierce's Disease and provide information in relation to controlling the disease.

XII. STATUS OF FUNDS.

\$107,711.05 of the funds are left.

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

No intellectual property has resulted from research done under this grant. However further development of the PD1311 mutant could result in a commercially viable control for PD.

XIV. LITERATURE CITED.

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