I. FINAL REPORT FOR UC PD RESEARCH GRANT NUMBER #2010-34442-21101.

II. PROJECT TITLE.

Exploiting a Chemosensory Signal Transduction System that Controls Twitching Motility and Virulence in *Xylella fastidiosa*

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

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V. INTRODUCTION.

Regulatory pathways in Xylella fastidiosa. Xylella fastidiosa (*Xf)* is a non-flagellated, xylem-restricted Gram-negative bacterium that moves within grapevines via type IV-mediated twitching motility (Meng et al. 2005). Several genes associated with pili biogenesis in *Xf* have been identified as required for twitching motility (Meng et al. 2005; Li et al. 2007). In addition, genes associated with multiple regulatory networks involved in twitching motility, biofilm, and virulence have been identified. One involves the *pilR*/*pilS* gene pair that belongs to the family of two-component regulatory systems that has been described in many bacterial species (Winther-Larsen and Koomey 2002). PilS is a predicted sensory protein that when stimulated by appropriate environmental signals, activates PilR through kinase activity. PilR then regulates transcription of *pilA,* which encodes the major pilin protein of the type IV pilus. We previously reported that an *Xf pilR* mutant lacks type IV pili and does not exhibit twitching-mediated motility (Li et al. 2007). Another regulatory network involving cell-cell signaling is associated with production of a diffusible signal factor (DSF) that regulates biofilm formation, disease development, and the ability of *Xf* to be transmitted from its insect vector (Newman et al. 2004). Mutants of *Xf* that do not produce DSF produce no biofilm layers inside the vector and are hypervirulent *in planta.* The hypervirulence results from the inverse relationship between DSF and motility by unknown mechanisms (Chatterjee et al. 2008b).

The canonical Escherichia coli chemotaxis system. Bacteria sense and respond to changes in their environment, integrating the signals to produce a directed response. *E. coli* is the chemotaxis model system for Gram-negative bacteria (Hazelbauer et al. 2008). *E. coli* chemotaxis is regulated by a ternary signaling complex composed of chemoreceptors (methyl-accepting chemotaxis proteins or MCPs), a histidine kinase (CheA), and a coupling protein (CheW). MCPs are homodimeric, transmembrane

proteins that are defined by a highly conserved α-helical signaling domain. MCPs bind ligand in the periplasmic domain and interact with the CheA kinase through CheW in the cytoplasmic signaling domain. When MCP ligand is absent, the CheA kinase autophosphorylates and transfers the phosphate group to the response regulator protein, CheY, which in turn interacts with the flagella switch proteins.

Until recently only a handful of bacterial chemotaxis systems were known. Advances in genomic sequencing have revealed that the chemotaxis two-component signaling system is evolutionary conserved (Alexander and Zhulin 2007). To date, genetic evidence suggests that there are chemotaxis systems in over 150 bacterial species, including *Xf*. While the numbers are increasing, few bacterial chemotaxis systems have been extensively explored *in vivo*.

Xf chemosensory system and Pierce's disease (PD). We identified a chemosensory regulatory pathway in *Xf* (Fig. 1). Chemotaxis-like genes do not necessarily correlate with a chemotactic phenotype, as they have evolved to regulate other processes such as pili formation, gene transcription, and exopolysaccharide production (Kirby 2009). Genome comparisons indicate that the chemotaxis genes share a high degree of similarity to *Pseudomonas aeruginosa*. However, while chemotaxis proteins appear to be critical for extension of the *P. aeruginosa* type IV pilus (DeLange et al. 2007), in *Xf* they regulate twitching without altering the pili (Cursino et al. 2011) and therefore movement in response to cues from environmental signals.

Genetic examination reveals that most bacteria with chemotaxis-like genes have multiple putative chemoreceptors (Alexander and Zhulin 2007). For instance, *E. coli* has five chemoreceptors and *P. aeruginosa* has 26. *Xf* strains are members of a subset of bacteria with only one chemoreceptor gene (an exception may be strain Ann-1 which may have additional truncated chemoreceptor). *Xf* may only require one chemoreceptor (PilJ) due to its highly specific environmental niche within plant xylem vessels where it is likely to encounter relatively few chemical and environmental signals as compared to free-living bacteria. Furthermore, chemoreceptors can respond to multiple stimuli (Falke and Hazelbauer 2001; Hazelbauer et al. 2008), and therefore *Xf* may respond to a range of ligands. Sequences of MCP ligand binding domains are not conserved and thus direct binding assays are required to identify the *Xf* ligand(s). Interestingly, the receiver domains of PilJ orthologs found in the different sequenced *Xf* strains are nearly identical and therefore common molecule(s) may drive motility within all susceptible plant hosts. Confirming that PilJ is a functional chemoreceptor and identifying its ligand(s) will provide a foothold into developing methods to prevent or limit *Xf* pathogenesis in grapevines and other susceptible plant hosts.

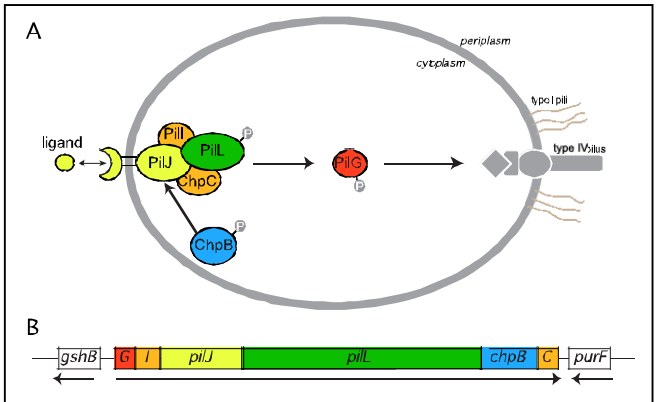

Fig. 1. **The *X. fastidosa* Pil-Chp operon.** A) Model of operon protein products regulating *X. fastidiosa* twitching motility. The chemoreceptor PilJ senses environmental signal(s). ChpC/PilI coupling proteins link PilL to PilJ. PilL phosphorylates PilG, which modifies the type IV pili motor proteins. ChpB may demethylate PilJ. B) The Pil-Chp operon genes. Arrows indicate direction of transcription.

We showed that the *Xf* Temecula chemosensory system is involved in Pierce's disease development (Cursino et al. 2011).

The genes are in an operon, named Pil-Chp, as is common for many chemotaxis systems (Lovdok et al. 2009). A transposon mutation in *pilL* (CheA kinase ortholog) was twitching minus and formed reduced biofilm, suggesting a direct connection between motility and biofilm formation. Unlike chemotaxis mutants in *P. aeruginosa* (DeLange et al. 2007), the *Xf* Pil-Chp mutant was not altered in type IV pili production (Cursino et al. 2011). Most significantly, inhibition of the chemosensory system is capable of preventing disease symptoms. Understanding how the *Xf* chemosensing regulatory network functions and interacts is central to determining how to counter plant colonization and PD expression.

VI. LIST OF OBJECTIVES.

Objective 1. Complete the characterization of the single chemosensory regulatory system of *X. fastidiosa* and its function in Pierce's disease. Toward this end we will:
   a. Characterize the genes of the *X. fastidiosa* chemosensory operon.
   b. Determine the cellular localization of the chemosensing receptor, PilJ.
   c. Examine the effect of host environment on regulation of the chemosensory system.

Objective 2. Identify environmental signals that bind the receptor, PilJ, to activate the chemosensory response. Toward this end we will:
   a. Identify candidate signals that induce a *X. fastidiosa* twitching response on defined media and/or an *E. coli* swimming response supported by a chimeric form of PilJ in a strain lacking chemosensing receptor genes.
   b. Verify signals that bind to PilJ and induce *E. coli* motility for their effect on wild-type *X. fastidiosa* twitching motility and biofilm formation.

VII. ACTIVITIES CONDUCTED TO ACCOMPLISH EACH OBJECTIVE.

Objective 1a). Characterize the genes of the X. fastidiosa chemosensory operon.

Building from our initial chemosensory-like operon findings (Cursino et al. 2011), we explored the role of the individual genes in the Pil-Chp operon (Anthinuwat et al. drafted). We performed non-polar deletions of each operon gene according to Shi et al. 2009 with slight modifications. Using a plate assay (a visible fringe around a colony correlates with twitching motility) (Meng et al. 2005), microfluidic chambers (De La Fuente et al. 2007a), and movement *in planta* (Cursino et al. 2011), we found that the first four Pil-Chp operon genes (*pilI*, *pilG*, *pilJ*, and *pilL*) control motility while the last two (*chpB* and *chpC*) do not (Fig. 2A). Mutants show a complex relationship to aggregation (Fig. 2B), all mutants play a role in biofilm formation (Fig. 2C), and most importantly, are involved in virulence (Fig. 2D). For technical reasons this research has been delayed for publication, however those issues have been resolved and we are repeating the *in planta* this summer to confirm our results. We anticipate submitting the manuscript fall 2013.

Our studies show the surprising finding that the *Xf* Pil-Chp operon regulates biofilm formation, which is critical to virulence and relies on type I pili and non-fimbrial adhesins (Guilhabert and Kirkpatrick 2005; Li et al. 2007; Fiel et al. 2007). To explore the role of the Pil-Chp genes on type I and type IV pili, we examined the pili of Pil-Chp mutants by transmission electron microscopy (TEM). We noted three important observations (data not shown). First, we discovered that the non-motile mutants (*pilG*, *pilI*, *pilJ,* and *pilL)* were either hyperpiliated or lacked type IV pili. Motile mutants (*chpB* and *chpC*) appeared to have the wild-type pili phenotype. Therefore the non-motile phenotype may

result from type IV pili being unable to extend and/or retract. Second, we observed that the non-motile mutants lack type I pili which may explain, in part, their aggregation and biofilm formation phenotypes. Third, since *chpB* and *chpC* appeared to have wild-type levels of pili but produced altered levels of aggregation and biofilms, the Pil-Chp operon may also regulate non-fimbrial adhesins. To further explore these findings, examined if the Pil-Chp operon genes regulate gene expression. Our mRNA studies suggest the Pil-Chp operon does regulate pili and adhesins (data not shown), although further studies need to be completed to understand this apparently complex relationship. Understanding the role of the Pil-Chp operon in aggregation and biofilm formation may provide new targets for blocking PD progression.

Objective 1b). Determine the cellular localization of the chemosensing receptor, PilJ.

Localization of chemoreceptors is thought to be integral to the signaling process (Shaprio et al. 2009), and therefore important to regulation of the chemotactic response. The cellular locations of chemotaxis receptors have been studied in only a few organisms (Maddock and Shapiro 1993; Harrison et al. 1999; Bardy and Maddock 2005). In *E. coli,* chemoreceptors cluster at one pole of the bacterium and are physically at a distance from the flagella, which cover the cell body (Maddock and Shaprio 1993). In *P. aeruginosa*, the similarly named chemoreceptor to the *Xf* receptor, PilJ, is found at both poles (DeLange et al. 2007). We have shown that the *Xf* chemoreceptor, PilJ, localize at one pole (Fig. 3)

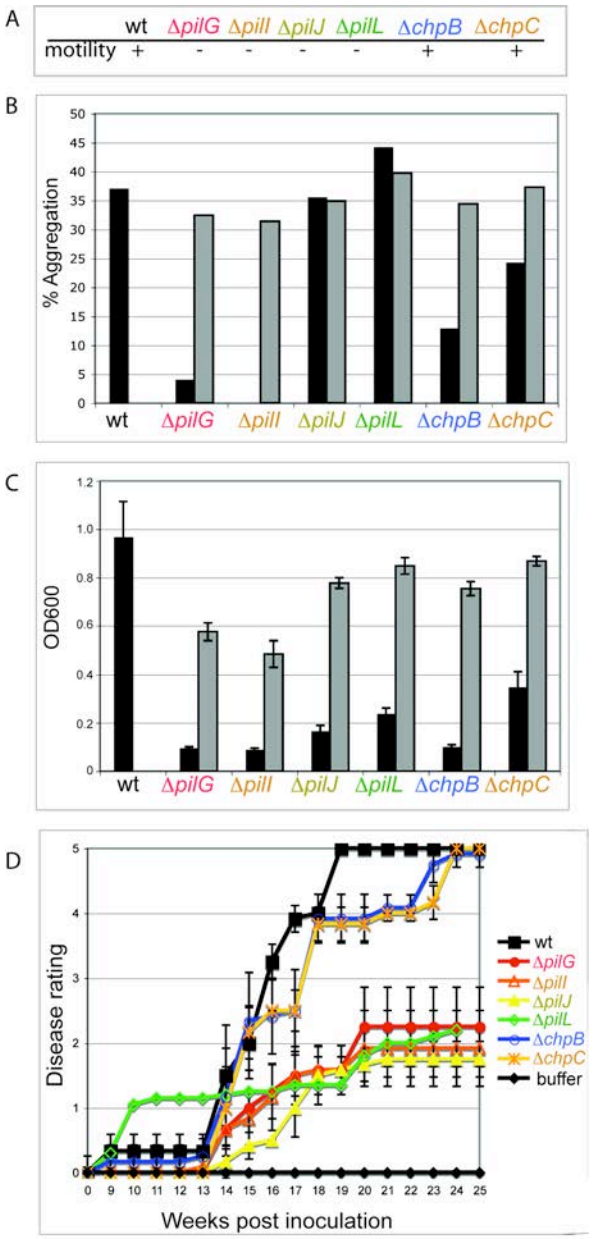

Fig. 2. Phenotypes of Pil-Chp operon gene non-polar mutants. A) Motile ("+") and non-motile ("-") *Xf*, as observed by colony fringe (Meng et al. 2005), microfluidic chambers (De La Fuente et al. 2007a), and *in planta* (Cursino et al. 2011). B) Aggregation by wild-type, mutants (black bars), and complemented mutants (gray bars) per established procedures (Burdman et al. 2000). C) Biofilm formation by wild-type, mutants (black bars), and complemented mutants (gray bars) per established procedures (Zaini et al. 2009). D) Disease assessment of *Vitis vinifera* L. cv. Cabernet Sauvignon grapevines inoculated with wild-type, mutants, or buffer. Disease scale: 0 = fully healthy; 5 = dead (Guilhabert and Kirkpatrick 2005). For A-D, Pil-Chp genes listed in the order found in the operon and the same color as found in Fig. 1.

(Anthinuwat et al. drafted). This result suggests that PilJ organizes and functions like other transmembrane receptors. Understanding the localization of PilJ is another key component of the chemosensory system and may offer insights as to how regulation of this important process can be modified for mitigating infection by *Xf.*

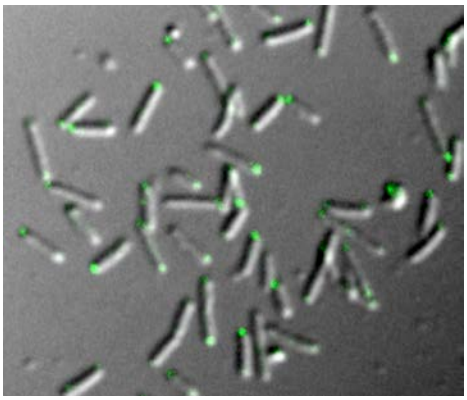

Fig. 3. PilJ is polar-localized. Microscope images of wild-type *Xf* cells with anti-PilJ antibody.

Objective 1c). Examine the effect of host environment on regulation of the chemosensory system.

Xf lives both in an insect vector and in plants, and in planktonic and biofilm states (Chatterjee et al. 2008a). Presumably *Xf* only needs to express the chemotaxis proteins when it migrates within the plant host. Recently, the gene expression profile of *Xf* upon exposure to plant polysaccharides pectin and glucan was assessed (Killiny and Almeida, 2009). The Pil-Chp operon expression levels were not altered significantly under these conditions. Components in grapevine sap and signaling molecules produced by *Xf* were not examined.

We examined the expression of key genes associated with type IV pili in sap from PD-susceptible and -resistant grapevines. We discovered that type IV pili genes, including the Pil-Chp operon, are not expressed in PD-resistant sap (data not shown), suggesting that motility is regulated by a chemical component in PD-susceptible sap.

Two additional projects that have come from examining motility and virulence factors: regulation by the *chpY* gene and the potential biocontrol by the PD1311 deleted strain. The *chpY* gene putative protein has GGDEF and EAL homologous regions, which are domains involved in regulating cyclic di-GMP (Ryan et al. 2006). However the putative ChpY GGDEF and EAL regions appear to be non-functional, as they lack the expected enzymatic amino acid residues (Cursino et al. drafted). Mutation of *chpY* results in reduced cellular motility *in vitro* but not *in vivo* and increased biofilm formation (data not shown). Grapevines inoculated with the *chpY* mutant had increased PD progression compared to a wild-type *Xf* infection (Fig. 4). To test whether increased biofilm formation was related to increased expression of biofilm forming *gum* genes (Roper et al. 2007), we performed RT-PCR and found that *gumD and gumJ* had increased expression in the *chpY* mutant as compared to wild-type cells (data not shown). Similarly, extracellular polymeric substance (EPS) production was higher in the

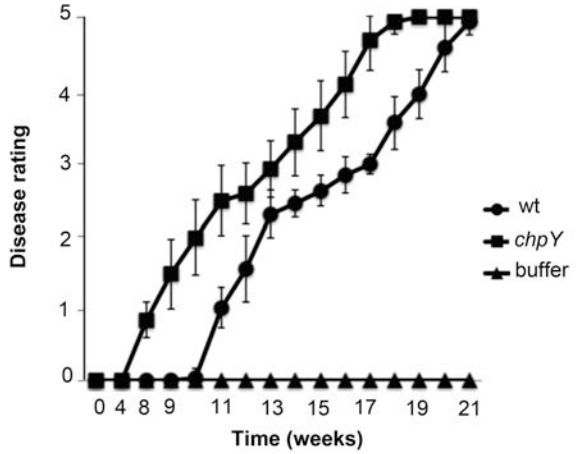

Fig. 4. Severity of Pierce's disease with *chpY* deleted strain. Average of 10 vines inoculated with Temecula 1 (circle), *chpY* mutant (square), and citrate-succinate negative control buffer (triangle). Experiment was repeated four times. Error bars represent standard deviation of the means of a representative experiment.

chpY mutant strain as compared to wild-type cells (data not shown). This work has been drafted and we are in the process of re-submitting it.

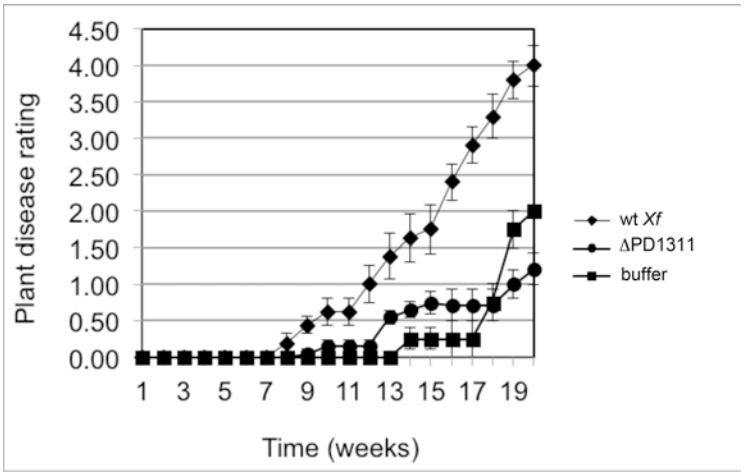

Fig. 5. PD1311 deleted strain is virulent. Grapevines were inoculated with wild-type *Xf* (diamond), ΔPD1311 strain (circle), and buffer as a negative control (square). Symptoms were monitored on 10 plants for each treatment over a period of 20 weeks and rated on a scale of 0-5 (Guilhabert & Kirkpatrick 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.

We explored a new gene potentially involved in PD, PD1311, that was a putative acyl-CoA synthetase (ACS). The most studied bacterial ACS is the *E. coli* FadD*,* which catalyzes exogenous long-chain fatty acyl-CoA from fatty acid, coenzyme A, and ATP (Black 1992). ACS metabolite intermediates are involved in a number of functions including virulence factors (Banchio & Gramajo 2002; Soto et al. 2002; Barber et al. 1997). We discovered that deleting PD1311 results in loss of virulence (Fig. 5), indicating that PD1311 is fundamental for PD development. In addition, when the PD1311 deleted and wild-type strains are grown together, the wild-type strain has reduced biofilm production (data not shown). Given our findings with the PD1311 mutant, we are testing if this strain has potential as a biocontrol for PD.

Objective 2a). Identify candidate signals that induce a X. fastidiosa twitching response on defined media and/or an E. coli swimming response supported by a chimeric form of PilJ in a strain lacking chemosensing receptor genes.

We have shown that *Xf* type IV pilus twitching motility is controlled by a chemosensory-like system (Cursino et al. 2011). Therefore it is highly likely that a molecule exists that binds PilJ to induce the intracellular signaling cascade leading to the twitching response. Chemoreceptors can respond to multiple stimuli (Falke and Hazelbauer 2001; Hazelbauer et al. 2008), and therefore PilJ in *Xf* may bind a range of stimulators and/or inhibitors.

After many attempts, we believe we have finally developed an assay that will allow us to identify the ligand. To screen for a PilJ chemoreceptor ligand, we identified a medium that allows for normal growth without supporting motility (PW minus BSA and lacking soytone). We are testing this medium spotted with xylem fluid to recover motility and confirm the assay. We hope to screen candidate components from the predominant amino acids and sugars reported in grapevine sap (Andersen and Brodbeck 1991; Andersen et al. 2007). Once identified, we will determine concentrations of stimuli required to induce motility. This information should open direct methods to block motility and significantly limit disease.

Objective 2b). Verify signals that bind to PilJ and induce E. coli motility for their effect on wildtype X. fastidiosa twitching motility and biofilm formation.

Xf type IV pilus twitching motility has been most clearly studied in microfluidic

chambers fabricated to mimic xylem vessels (De La Fuente et al. 2007b). Once we identify a candidate compound, we will use a two-channel microfluidic chamber design so we can simultaneously compare motility between the 'tester' and 'control' situation. If the candidate compound stimulates movement, twitching will increase in the 'tester' lane. Additionally, we will alter the amount of compound to identify the optimal ligand concentration. Researchers have used a similar approach studying chemotaxis-influenced swimming behavior in *E. coli* and *P. aeruginosa* (Jeong et al. 2010; Wu et al. 2006).

VIII. SUMMARY OF ACCOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE.

Concerning aim 1a, we have analyzed the Pil-Chp chemosensory genes and found a complex relationship between the genes and motility and biofilm formation. For aim 1b, we have identified the localization of the chemosensory receptor, PilJ, and shown that it acts like most chemoreceptors, indicating that it is a viable target. We have also discovered that PilJ may play a novel role in virulence, which may provide new avenues for limiting disease. Turning to aim 1c, we have shown the chemotaxis-like genes are expressed in sap from PD-susceptible plants but not PD-resistant plants. In addition we have identified and characterized a gene, *chpY*, which regulates multiple responses, including motility. Furthermore, we have identified a strain deleted for PD1311 is avirulent, reduces biofilm formation by wild-type cells, and will evaluate as a PD biocontrol. For aim 2, we believe we have developed a high-throughput medium-based assay for identifying the chemotaxis ligand. Such knowledge can be directly translated into methods to prevent motility and therefore limit PD symptoms.

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

Publications.

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.

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. The influence of *Xylella fastidiosa chpY* on pathogenicity and Pierce's disease. Drafting for resumbission in June 2013.

Athinuwat, D., Johnson, K., Galvani, C.D., Cursino, L., Hoch, H.C., Burr, T.J., and P. Mowery. Analysis of the Pil-Chp Operon that Controls Twitching Motility and Virulence in *Xylella fastidiosa*. Drafted for submission in Fall 2013.

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.

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.

Presentations and Posters.

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

Mowery, P. The Motility of *Xylella fastidiosa*. Plant Pathology and Plant-Microbe Biology Seminar Series. Cornell University. Ithaca, NY, 2011. Presentation.

Mowery, P. The *Xylella fastidiosa* chemoreceptor PilJ and its functions. Receptor Fest Meeting, Salt Lake City, UT, August 2011. Presentation.

Athinuwat, D., Galvani, C., Mowery, P., Parthuangwong, S., Hoch, H.C., and Burr, T.J. Chemosensory regulation of Type IV pili of *Xylella fastidiosa* is essential for twitching motility and pathogenesis. III International Symposium on Tropical Wines, Chaing Mai, Thailand, 2011. Poster.

Athinuwat, D., Mowery, P., Galvani, C., Cursino, L., Hoch, H.C., and Burr, T.J. Characterization of a single chemosensory gene cluster in *Xylella fastidiosa* Pierce’s disease pathogen of grape. American Phytopathological Society-International Plant Protection Congress Joint Meeting, Honolulu, HI, 2011. Poster.

Athinuwat, D., Galvani, C., Cursino, L., Schenk, A., Hoch, H.C., Burr, T.J. and Mowery, P., Analysis of the Pil-Chp operon genes regulating virulence in *Xylella fastidiosa*. Pierce’s Disease Research Symposium, December 2011. Poster.

Shi, X., Cooksey, D.A., Mowery, P., Burr, T.J., and Hoch, H.C. Continued assessment of *Xylella fastidiosa* fimbrial adheins as important virulence factors in Pierce’s disease: influence of xylem sap. Pierce’s Disease Research Symposium, Sacramento, CA, 2011. Poster.

X. RESEARCH RELEVANCE STATEMENT.

This project is based, in part, on results from our previous studies in which we demonstrated that type IV pili are involved in biofilm formation and in long distance migration of *Xylella fastidiosa* (*Xf*) within xylem vessels through twitching motility, and from our recent discovery that a chemosensory signal transduction system controls twitching motility. From this grant we have i) demonstrated that *Xf* has a chemosensory operon, ii) observed that disruption of the operon blocks twitching motility, iii) determined that *Xf* chemosensory-like operon genes regulate symptoms *in planta*, and therefore the chemotaxis-like system is important for PD development, iv) determined the polar localization of the *Xf* chemosensory receptor, PilJ, vi) discovered novel function for the PilJ chemoreceptor, and v) developed a screen for identity of the motility stimuli. In addition we have identified i) a gene regulating multiple pathogenic responses (*chpY*), including motility, and ii) a new gene required for virulence and mutant that may act as a PD biocontrol (PD1311). Together, this research will facilitate discovery of strategies to block PD. Therefore the results of this investigation will enhance our understanding of host colonization and movement of *Xf* in xylem vessels, with the ultimate objective of disease control.

XI. LAY SUMMARY OF PROJECT ACCOMPLISHMENTS.

It has been established by our laboratory, and others, that motility by *Xylella fastidiosa* in grapevines is correlated with disease severity. This project involves studying the chemical sensing pathway by which *X. fastidiosa* is able to control its movement and virulence within the plant environment. We examined 1) a gene cluster essential for cell movement and virulence, 2) the initial protein regulating the signaling response in terms of its cellular location and novel function, and 3) the chemical stimuli

that is critical important for developing disease symptoms. In addition we examined a putative motility regulating protein. Also significantly, we identified a *X. fastidiosa* mutant strain that does not induce disease and represses a disease step in virulent strains. We are testing this new strain for its ability to be a Pierce's disease biocontrol. These results give insight into targets for preventing Pierce's disease.

XII. STATUS OF FUNDS.

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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