

**I. Project title:** EXPLOITING A CHEMOSENSORY SIGNAL TRANSDUCTION SYSTEM THAT CONTROLS TWITCHING MOTILITY AND VIRULENCE IN *XYLELLA FASTIDIOSA*

## **II. Principal investigators and cooperators**

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## **III. List of objectives and description of activities conducted to accomplish each objective**

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:

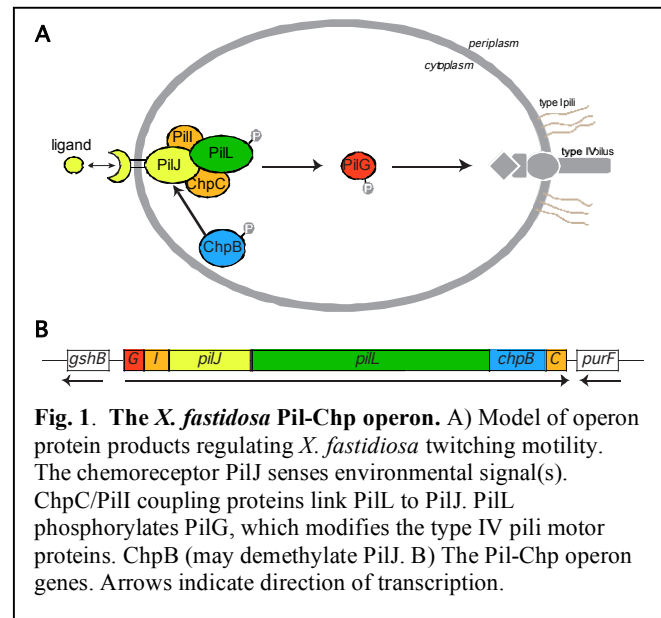
- a. Identify candidate signals that induce a *X. fastidiosa* twitching response on defined media and/or an *E. coli* swimming response supported by a chimeric form of PilJ in a strain lacking chemosensing receptor genes.
- b. Verify signals that bind to PilJ and induce *E. coli* motility for their effect on wildtype *X. fastidiosa* twitching motility and biofilm formation.

## METHODOLOGY TO ACCOMPLISH OBJECTIVES:

### Objective 1. Complete the characterization of the single chemosensory regulatory system of *X. fastidiosa* and its function in Pierce's disease.

#### a. Characterize the genes of the *X. fastidiosa* che operon.

Motility is critical to the pathogenicity of *X. fastidiosa* (*Xf*) (Chatterjee et al. 2008; Cursino et al. 2011). Motile bacteria can perform chemotaxis in which stimuli bind a chemoreceptor and direct movement (Hazelbauer et al., 2008). We discovered that *Xf* has a chemotaxis-like system critical for its movement and PD symptoms (PD) *in planta* (Fig. 1) (Cursino et al., 2011). Unlike the majority of bacterial chemotaxis motility systems (Alexander and Zhulin, 2007) *Xf* Temecula has only one chemotaxis operon (Pil-Chp), one receptor, and exists in a limited environment. These traits provide a unique opportunity to develop strategies for interfering with motility and methods to prevent PD.



To further explore the role of the Pil-Chp operon, we performed non-polar deletions of each operon gene (drafting for publication). 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., 2007), and by measuring progression of movement from the inoculation point *in planta* (Cursino et al., 2011), we found that the first four genes (*pilI*, *pilG*, *pilJ*, and *pilL*) control motility while the last two (*chpB* and *chpC*) do not (Fig. 2a). None of the phenotypes were the result of growth impairment, as all grew like wild-type *Xf* (data not shown). In addition we discovered that a subset of the Pil-Chp operon plays a role in aggregation (*pilG*, *pilI*, *chpB*, and *chpC*), the entire Pil-Chp operon is involved in biofilm formation, and most importantly, the entire Pil-Chp operon is involved in virulence (Fig. 2b-d).

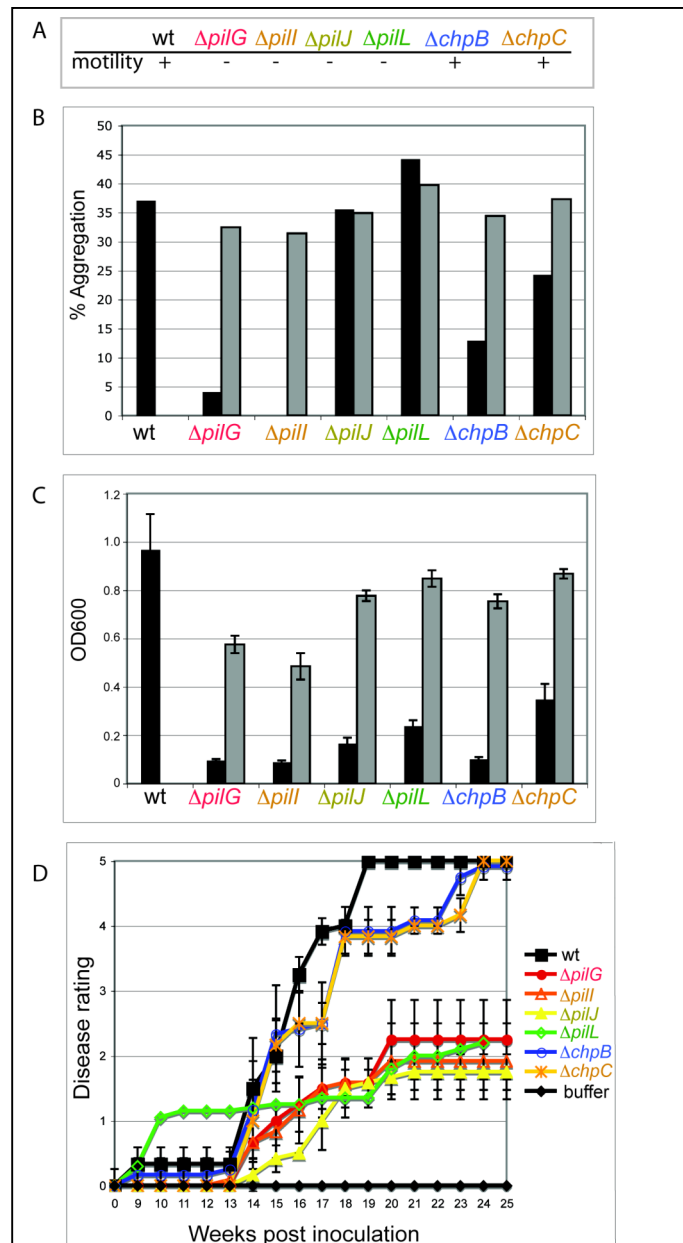
Our studies indicate the surprising finding that the *Xf* Pil-Chp operon regulates aggregation and biofilm formation. These two processes are critical to virulence and rely on type I pili and non-fimbrial adhesins (Guilhabert and Kirkpatrick, 2005; Li et al., 2007; Fiel et al., 2007). To explore the 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. First, we discovered that the non-motile mutants (*pilG*, *pilI*, *pilJ*, and *pilL*) were either hyperpiliated or lacked type IV pili (data not shown). Motile mutants (*chpB* and *chpC*) appeared to have wild-type pili. 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

three findings, we are currently exploring whether the Pil-Chp operon genes regulate gene expression, protein production, or adhesin assembly using a combination of mRNA studies and Western blot analysis. Our pilot 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.

#### b. Determine the cellular localization of the chemosensing receptor, PilJ.

Localization of chemoreceptors is thought to be integral to the signaling process (Shapiro 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). We have shown that the *Xf* chemoreceptor, PilJ, localizes at one pole (drafting for publication). This result suggests that PilJ organizes and functions like other transmembrane receptors, and therefore is an important target for blocking PD progression.

While localized like other chemoreceptors, we have preliminary results suggesting that the PilJ chemoreceptor may have a novel function. Traditionally, chemoreceptors are methylated in order to support ligand adaptation (Hazelbauer et al., 2008). Methyl groups are added by methyltransferases and removed by methylesterases. The putative methylesterase ChpB does not play a role in motility, however it is important for aggregation, biofilm formation, and



**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., 2007), 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 (Guilhbert 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.

virulence. Therefore, PilJ methylation may play a role in one or more of these non-motility responses.

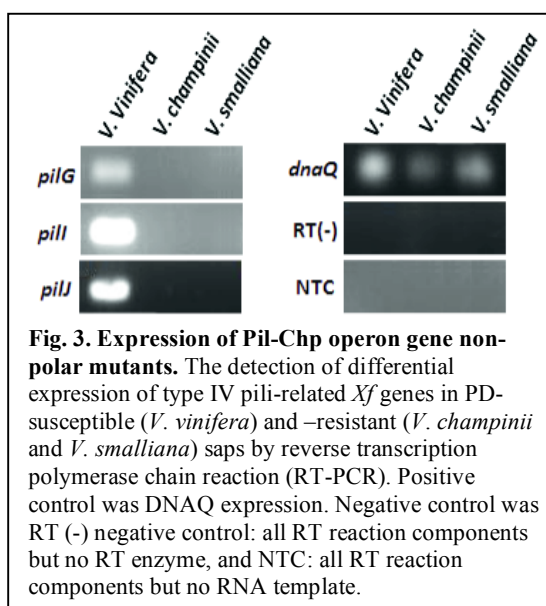
Our preliminary results on methylation were assessed using SDS-PAGE gel shifts; chemoreceptors are known to migrate faster on gels when methylated (Boyd and Simon, 1980; Engstrom and Hazelbauer, 1980; Chelsky and Dahlquist, 1981). We analyzed cell lysates from wild-type and *ΔchpB* strains by Western blot analysis with an anti-PilJ antibody (data not shown). The study revealed a shift, suggesting that ChpB is a functional methylesterase. We are now working to determine if this response is critical for non-motility steps in virulence. From these studies we hope to uncover new steps in the virulence response that will lead to novel approaches to disease control.

### c. Examine the effect of host environment on regulation of the chemosensory system.

An organism's life state can alter the expression of chemosensing genes (Yost et al., 2004; Nielsen et al., 2006; Beyhan et al., 2006). *Xf* lives both in an insect vector and in plants, and in planktonic and biofilm states (Chatterjee et al., 2008). Presumably *Xf* needs to express the chemotaxis proteins only 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 peron 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 resistant sap (Fig. 3), suggesting that motility is regulated by a chemical component in PD-susceptible sap (drafting for publication).

Two potential signaling molecules that may affect Pil-Chp operon expression are diffusible signal factor (DSF) and cyclic-di-GMP. Concerning DSF, our results (Burr et al., 2008) along with results from others (Chatterjee et al., 2008) indicate that the quorum signaling molecule, DSF, blocks twitching motility. However the mechanism has not been elucidated. The *chpY* gene lies downstream of the Pil-Chp operon and has homology to the *Xf pilG* gene that codes for a phospho-shuttle protein. It also has GGDEF and EAL regions that are known to regulate 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. Deletion of *chpY* results in reduced cellular motility, unaltered pili biogenesis, and increased biofilm formation (Burr et al 2008). To determine if there was a regulatory relationship between the Pil-Chp operon and *chpY*, we performed RT-PCR on the Pil-Chp *pilL* gene in the *chpY* null strain. When *chpY* is deleted, *Xf* shows no changes in *pilL* expression (data not shown). However, *chpY* does have an effect on PD. Grapevines inoculated with the *chpY* mutant had increased PD progression compared to a wt *Xf* infection (data not shown), which may stem from the *chpY*-induced



**Fig. 3. Expression of Pil-Chp operon gene non-polar mutants.** The detection of differential expression of type IV pili-related *Xf* genes in PD-susceptible (*V. vinifera*) and -resistant (*V. champinii* and *V. smalliana*) saps by reverse transcription polymerase chain reaction (RT-PCR). Positive control was *dnaQ* expression. Negative control was RT (-) negative control: all RT reaction components but no RT enzyme, and NTC: all RT reaction components but no RNA template.

increased biofilm formation or reduced twitching motility. 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 a three-fold increased expression in the *chpY* mutant as compared to wild-type cells. Similarly, extracellular polymeric substance (EPS) production was three-fold higher in the *chpY* mutant strain as compared to wild-type cells. We are continuing our studies to elucidate how *chpY* regulates these biofilm formation and reduced motility.

**Objective 2. Identify environmental signals that bind the receptor, PilJ, to activate the chemosensory response.**

**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.**

We have shown that *Xf* type IV pilus twitching motility is controlled by a chemosensory system (Hoch et al., 2008). 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.

Our original chimeric approach had technical issues, however, we developed a different assay that will allow us to identify the ligand. Wild-type cells create a fringe on PW medium lacking BSA (Li et al., 2007). A fringe growing from the colony is consistent with twitching motility (Meng et al., 2005). To screen for a PilJ chemoreceptor ligand, we identified a medium that allows for normal growth without supporting motility. *Xf* grown on PW minus BSA and lacking soytone meets our desired conditions. Our preliminary studies show that *Xf* colonies grown on this medium and spotted with drops of aqueous soytone develop a soytone-dependent fringe (data not shown). We will next test the response by spotting the medium with xylem fluid. We will then 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.

**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.**

*Xf* type IV pilus twitching motility has been most clearly studied in microfluidic chambers fabricated to mimic xylem vessels (De La Fuente et al., 2007). 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).

It is possible that more than one signal will be discovered that serves as a PilJ binding ligand to induce motility. Once signals are identified, chemically related compounds will be tested for their ability to interfere with natural ligand binding. Initially this will be done using the methods

described above. As previously indicated, the overall goal is to develop a strategy for interfering with signal-PilJ binding that can be implemented as a means to mitigate damage caused by PD.

#### **IV. Summary of major research 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, aggregation, and biofilm formation. We are now exploring the role of the genes on fimbrial and non-fimbrial adhesins to uncover how the operon regulates aggregation and biofilm formation, which are critical steps in the virulence process. 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 initiated studies on how environmental conditions alter gene expression of the operon; it is expressed in sap from PD-susceptible plants but not PD-resistant plants. Further analysis is underway. For Aim 2, 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.

#### **V. Publications or reports resulting from the project**

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.

Athinuwat, D., Galvani, C.D., Cursino, L., Losito, E.M., Hoch, H.C., Burr, T.J., and P. Mowery. Analysis of the Pil-Chp Operon that Controls Twitching Motility and Virulence in *Xylella fastidiosa*. Drafting for submission.

Shi, X.Y., Mowery, P., Burr, T.J., and H.C. Hoch. Behavior of *Xylella fastidiosa* in Xylem Sap from Pierce's Disease Resistant and Susceptible Grapevines. Drafting for submission.

#### **VI. Presentations on research**

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, Sacramento, CA, December 2011. Poster.

Mowery, P., T.J., Burr, Hoch, H.C., Cursino, L., Athiawat, D., and Galvai, 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.

## **VII. 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 *X. 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 operon genes induce reduced symptoms *in planta*, and therefore the chemosensory system is important for PD development, iv) uncovered that the operon regulates not only type IV pili but also type I pili and non-fimbrial adhesins, v) determined the polar localization of the *Xf* chemosensory receptor, PilJ, vi) discovered novel function for the PilJ chemoreceptor, and vii) developed a screen for identification of the motility stimuli. Together, this research will facilitate discovery of strategies to block chemosensing as a means of disease suppression. 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.

## **VIII. Lay summary of current year's results**

It has been established by our laboratory and others that motility of *X. 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 (twitching motility) 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 critically important for developing disease symptoms. These results give insight into targets for preventing Pierce's disease.

## **IX. Status of funds**

Only a portion of the first year funds have been spent given that they were only recently released. So far, \$23,099 of the \$158,574 has been spent. An additional \$54,366 is for a new postdoctoral associate (Dr. Kameka Johnson), \$45,333 for a research assistant (Cheryl Galvani), and \$10,000 for two summer students through August 2013. This will leave \$25,776 for salaries of other personnel and for purchasing of supplies and expenses.

## **X. Summary and status of intellectual property produced during this research project**

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

## **LITERATURE CITED:**

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