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

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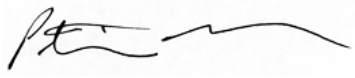

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III. List of objectives and description of activities conducted to accomplish each objective

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.

Unlike the majority of bacterial chemotaxis (*che*) motility systems,[1] *X. fastidiosa* (*Xf*) has only one chemotaxis receptor (with the exception of Ann1 which may have a truncated ortholog). This provides a unique opportunity to develop strategies for interfering with the regulation of the system (inhibit motility) and developing methods to prevent Pierce's disease (PD). We previously determined that the *Xf che* cluster is an operon,[2-4] a common trait found among chemosensing systems.[5] To understand the role of the chemosensing system, we deleted the putative kinase, *pilL* (Fig. 1) by gene replacement.[6] We confirmed the deletions by PCR and DNA sequencing. The mutant gave four important and related phenotypes. First, it resulted in loss of type IV pilus controlled twitching motility (Fig. 2). Second, unlike the similarly named gene cluster in *P. aeruginosa*,[7] the *pilL* mutant did not show apparent physical changes in type IV pili

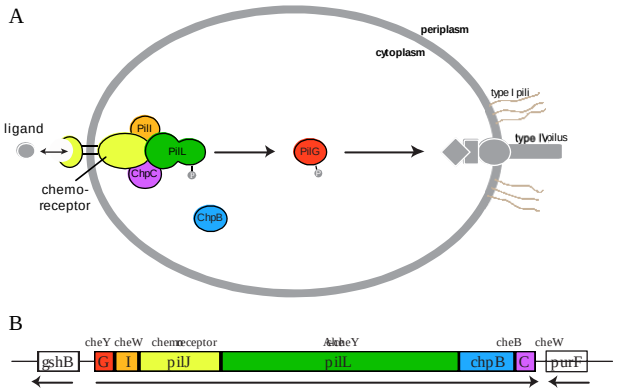

Fig. 1. The *Xf* chemotaxis system. A) Model for chemosensory regulation of twitching motility in *Xf*. The chemoreceptor PilJ senses environmental signal(s). ChpC/PilI couples PilL to PilJ. PilL phosphorylates its hybrid CheY-like receiver domain and PilG. ChpB is homologous to ligand adaptation proteins. B) The Pil-Chp chemosensory operon genes with *E. coli* homologous genes shown above and direction of transcription shown below.

Table 1. Chemosensory system in *Xf*.

***che* Gene**[a]	**Synonym**[b]	**Predicted function**	**Phenotype**
pilG	CheY	Response regulator	• No twitching motility • Reduced attachment • Reduced biofilm formation
pilI	CheW	Coupling protein	• No twitching motility • Reduced attachment • Reduced biofilm formation
pilJ	MCP	Chemoreceptor	• No twitching motility • Mildly reduced biofilm formation
pilL	CheA	Histidine kinase	• No twitching motility • Reduced biofilm formation
chpB	CheB	Methylesterase	• Reduced attachment • Reduced biofilm formation
chpC	CheW	Coupling protein	• Mildly reduced biofilm formation

[a] Listed in gene order found in the chemosensory operon.
[b] Most similar protein or domain found in the chemosensory system of *E. coli*.

(data not shown), indicating that the *che* operon regulates chemosensing and not type IV pili formation. Third, biofilm formation was greatly reduced in the *pilL* mutant (data not shown). Fourth, and most importantly, disruption of the chemotaxis sensory system resulted in significantly reduced PD symptoms *in planta* (Fig. 3).

Given the importance of the *che* operon to PD development, blocking *Xf* chemotaxis is a promising target for preventing disease progression. Therefore we wish to develop a more complete understanding of the system by finishing the characterization of the *che* genes (Fig. 1, Table 1). To that end we performed non-polar deletions of each gene and rescued phenotypes with complementation. Given that these genes are part of an operon, we might expect all of them to be critical for chemotaxis. However, we found that mutating genes *pilI*, *pilG*, and *pilJ* eliminated motility while deleting *chpB* and *chpC* did not. Growth curves showed that the phenotypes did not result from inhibition of growth as all grew like wild-type *Xf*. We also examined aggregation and biofilm formation (Table 1). Mutants of *pilI*, *pilJ*, and *chpC* aggregated similar to wild-type cells, however to our surprise *pilG*, *pilI*, and *chpB* remained in a planktonic state. All chemotaxis mutants were found to have reduced biofilm formation.

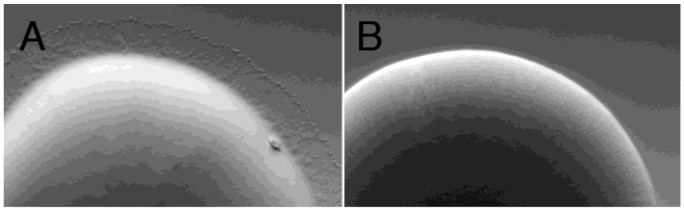

Fig. 2. **The *Xf* chemotaxis system regulates motility.** Colony fringe phenotype of the wild-type *Xf* (A) is indicative of twitching whereas the *pilL* mutant (B) has no fringe.

Based on our results, the *Xf* Pil-Chp operon appears to have at least three functions. First, it regulates motility using the first four genes in the operon: *pilG*, *pilI*, *pilJ*, and *pilL*. We predict that PilJ binds to an unknown ligand leading to changes in the phosphorylation state of PilL, which couples to PilJ via PilI (Fig. 1). PilL phosphorylates PilG, which in turn phosphorylates the type IV pili motor proteins. Second, the Pil-Chp operon plays a role in aggregation using the *pilG*, *pilI*, and *chpB* protein products. Third, the Pil-Chp operon is involved in biofilm formation for which it uses *pilG*, *pilI*, and *chpB*, and to a lesser extent *pilJ* and *chpC*.

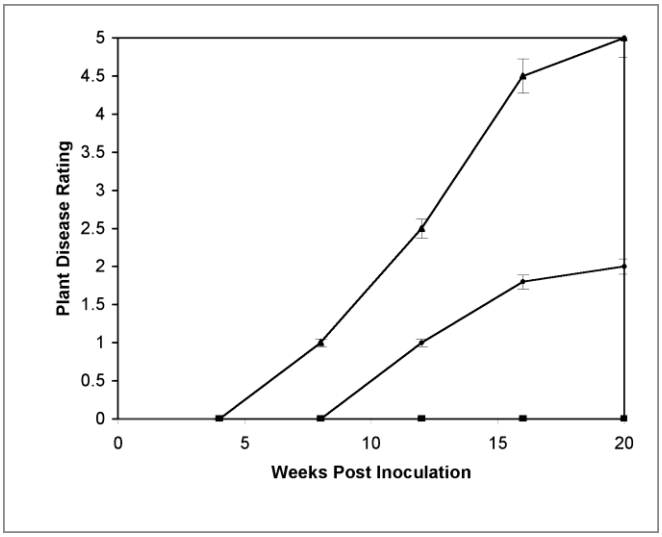

Fig. 3. *Xf* motility is important for PD development. PD assessment of *Vitis vinifera* L. cv. Cabernet Sauvignon vines inoculated with wild-type (triangle), *pilL* mutant (circle), or buffer control (square) and assessed over 20 weeks. 0=no disease, 5= defoliation

<u>b. Determine the cellular localization of the chemosensing receptor, PilJ.</u>

Localization of the chemoreceptors is thought to be integral to the signaling process,[8] and therefore an important component regulating the chemotactic response. The cellular locations of

chemotaxis receptors have been studied in only a handful of organisms.[9-11] 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.[9] In *P. aeruginosa*, the similarly named chemoreceptor to the *Xf* receptor, PilJ, is found at both poles.[7] In *Xf*, both type I and type IV pili uniquely reside at one (and the same) pole.[12] We are determining if the *Xf* chemotaxis receptor PilJ co-localizes with the pili by two methods. First, we expressed *Xf* PilJ protein for antibody production to label the chemotaxis receptor, as previously reported.[9-11,13] Immunocytological localization showed localization to a single cell pole (Fig. 4). We are currently testing for co-localization with the pili. Second, localization has been examined in *E. coli* using chemoreceptors fused to fluorescent tags.[7,14-15] Similarly, we are transforming *Xf* deleted for *pilJ* with a *pilJ-gfp* (green fluorescent protein) gene for visualization. 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*.

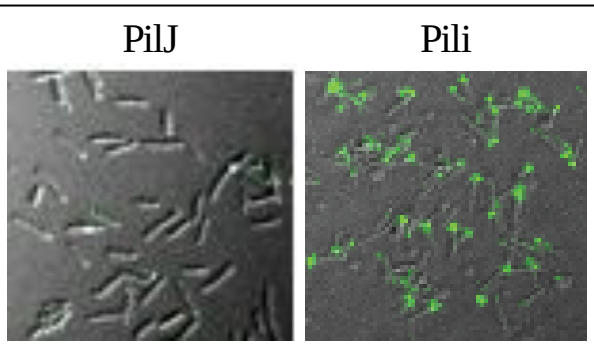

Fig. 4. PilJ is polar-localized. Microscope images of wt *Xf* cells with anti-PilJ antibody (left) or anti- *Xf* antibody (right) that highlights the pili.

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.[16-18] *Xf* lives both in an insect vector and in plants, and in planktonic and biofilm states.[19] 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 accessed.[20] The *che* operon expression levels were not altered significantly under these conditions. Components in grapevine sap and signaling molecules produced by *Xf* were not examined.

We recently examined the expression of key genes associated with type IV pili in sap from PD-resistant and -susceptible grapevines. We discovered that type IV pili genes, including the *che* operon, are not expressed in resistant sap (data not shown), suggesting that motility is regulated by a chemical component in PD-susceptible sap. This result is being further examined in objective 2a.

Two potential signaling molecules that may affect *che* operon expression are DSF and cyclic-di-GMP. Concerning DSF, our results[21] along with results from others[22] indicate that the quorum signaling molecule, DSF, blocks twitching motility. However the mechanism has not been elucidated. One potential mediator is ChpY, which has a GGDEF and EAL domain that has been associated in other bacteria with motility, attachment, and biofilm production.[23-24] Transposon insertion in the *X. fastidiosa chpY* impairs twitching motility and biofilm formation (data not shown). DSF also appears to up regulate biofilm formation and cyclic-di-GMP production.[22] Interestingly, it has been shown that increased cyclic-di-GMP levels in *Vibrio cholerae* induces decreased expression of its chemotaxis genes.[18] Therefore, we will examine the effect of DSF and cyclic-di-GMP on *che* operon expression. We will perform these studies by examining a)

protein levels by immunoblotting with the anti-PilJ antibodies mentioned in objective 1b, and b) mRNA levels using RT-PCR.

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.[2] 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,[25-26] and therefore *Xf* may bind a range of attractants and/or repellents.

The initial candidate PilJ ligand compounds to be screened will be the predominant amino acids and sugars reported in grapevine sap from PD-susceptible vines. The chemistry of grapevine sap has been reported for a few cultivars.[27-28] Products that are produced by *Xf* when grown in grapevine sap will also be tested. Examples are quorum sensing products DSF and cyclic-di-GMP discussed in objective 1c.

We will screen for the PilJ ligand by growing *Xf* cells in defined medium that does not support motility, selectively adding candidate components, and measuring a motility response (the *E. coli* screen will not be employed due to technical difficulties). A number of defined media have been developed for growing *Xf* such as XDM_2, CHARD2, and XfD2.[29-31] A vastly improved 'semi-defined' medium for growing *Xf* was recently reported.[20] Should *Xf* not be motile on one of these media, as assessed by the development of a colony fringe,[12,32] it will be used to screen for PilJ ligands. If *Xf* exhibits twitching motility on all of the media we will attempt to use a buffer only medium in which *Xf* may function (exhibit twitching motility in microfluidic chambers using time lapse microscopy over a period of 15-60 min) long enough to screen for stimuli and test motility in resistant sap with PD-susceptible components added.

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.

Chemical signals that restore movement will be used to confirm effects on the wild-type *Xf*. If a *Xf* motility minus medium is confirmed it will be utilized in the assays. In the case where we find differential motility on specific media (Objective 2a), we will screen for incremental increases twitching movement. In all experiments we will compare the wild-type strain with the chemoreceptor (*pilJ*) mutant generated. We will use two different approaches:

i) Microfluidic chamber assays. *Xf* type IV pilus twitching motility has been most clearly studied in microfluidic chambers fabricated to mimic xylem vessels by our group since 2003.[12,33] We will use a microfluidic chamber design that permits visualization of two channels simultaneously,[34] providing the ability to compare motility and biofilm formation between a 'tester' and a control situation. If the selected chemical signal activates movement, an increase in twitching movement should be observed in the channel with higher concentrations of the chemical. A similar approach has been used by other researchers studying chemotaxis-influenced swimming behavior of *E. coli*.[35]

ii) Solid medium assays. We will prepare Petri dishes with medium that supports *Xf* growth with the least amount of fringe. Fringe around the bacterial colony is directly correlated with type IV pilus twitching motility.[12,32] Wells will be made in the solid medium and filled with the chemical signals that diffuse into the medium. If the chemical signal is affecting motility, we expect to observe the development of a fringe (or a wider fringe) toward the well containing the recognized signal.

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

At this point we have nearly completed our analysis of the Pil-Chp chemosensory genes (objective 1a) and found a complex relationship between the genes and motility, aggregation, and biofilm formation. Additionally, we have identified the localization of the chemosensory receptor, PilJ, and are currently determining if it co-localizes with the pili (aim 1b). Concerning 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. Experiments to address the goals of aim 2 are planned for the near future.

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 *pilL* as a Competent of a Chemosensory Operon that Controls Twitching Motility and Virulence in *Xylella fastidiosa*. Accepted with revisions at *Molecular Plant-Microbe Interactions*.

VI. Presentations on research

Mowery, P., Shi, X., Atinuwat, D., Cursino, L., Galvani, C., Hoch, H.C., and Burr, T.J. Examining the chemosensory system that controls twitching motility and virulence in *Xylella fastidiosa*. Presentation. Pierce's Disease Research Symposium, December 2010.

Athinuwat, D., Mowery, P., Cursino, L., Galvani, C., Hoch, H.C., and Burr, T.J. Chemosensory gene mutations in Xylella fastidiosa confers affected twitching motility, biofilm formation, and aggregation. Poster. Pierce's Disease Research Symposium, December 2010.

Hoch, H.C., Burr, T.J., Mowery, P., Cursino, L., Athiuwat, D., Galvai, C., Losito, E., and Patel, K. Exploiting a chemosensory signal transduction system that controls twitching motility and virulence in *Xylella fastidiosa*. Proceedings of the Pierce's Disease Research Symposium 2010, p. 92-96.

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 *Xf* within xylem vessels through twitching motility, and from our recent discovery that a chemosensory signal transduction system controls twitching motility and not pili biogenesis. From this grant i) we demonstrated that *Xf* has a chemosensory operon, ii) we observed that disruption of the operon blocks twitching motility, iii) we determined that *Xf* with a mutation in the chemosensory operon

induces reduced symptoms *in planta*, and therefore the chemosensory system is important for PD development, and iv) we determined the localization of the *Xf* chemosensory receptor, PilJ. 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 lab and others that motility of *Xf* 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 within the plant environment. We examined a gene cluster essential for cell movement (twitching motility), we identified where the initial protein regulating the signaling response is located in the cell, and we determined that chemical sensing is 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, $24,084 of the $119,420 has been spent. An additional $37,740 has been committed for a new postdoctoral associate (Dr. Dongping Wang), $7800 for a part-time employee (Barbara DeHaven) who will be taking care of all greenhouse plant experiments and doing routine laboratory tasks, and $5,000 for a summer student. This will leave $64,624 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.

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