EXPLOITING A CHEMOSENSORY SIGNAL TRANSDUCTION SYSTEM THAT CONTROLS TWITCHING MOTILITY AND VIRULENCE IN *XYLELLA FASTIDIOSA*

Principal Investigator:
Patricia Mowery
Department of Biology
Hobart & Wm. Smith Colleges
Geneva, NY 14456
mowery@hws.edu

Principal Investigator:
Thomas J. Burr
Department of Plant Pathology
Cornell University, NYSAES
Geneva, NY 14456
tjb1@cornell.edu

Principal Investigator:
Harvey C. Hoch
Department of Plant Pathology
Cornell University, NYSAES
Geneva, New York 14456
hch1@cornell.edu

Researcher:
Luciana Cursino
Department of Biology
Hobart & Wm. Smith Colleges
Geneva, New York 14456
cursino@hws.edu

Researcher:
Dusit Athinuwat
athinvoa6@hotmail.com

Researcher:
Cheryl Galvani
galvani.cheryl@yahoo.com

Reporting Period: The results reported here are from work conducted August 2010 to August 2011.

ABSTRACT
Previously we demonstrated that twitching motility in *Xylella fastidiosa* is dependent on an operon, named Pil-Chp, encoding signal transduction pathway proteins (PilG, PilI, PilJ, PilL, ChpB and ChpC), which is related to the system that controls flagella movement in *Escherichia coli*. We report three advances in examining this chemotaxis system. First, we have examined the operon genes more closely. We have discovered that the operon is essential for the twitching phenotype, biofilm formation, and Pierce's disease (PD) development. Examining each Pil-Chp gene individually we learned that the first four genes are critical for twitching and that all genes play a role in biofilim formation and PD symptoms. Second, we have tentatively found a twitching minus medium to which we can add known sap components, examine for twitching recovery, and determine the component driving motility. Third, we have continued our examination of *chpY*, a gene similar to *pilG*, which plays a role in PD development.

LAYPERSON SUMMARY
This project involves studying the chemical sensing pathway by which the plant pathogen *Xylella 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 are determining what chemical signals are important for developing disease symptoms. These results give insight into targets for preventing Pierce's disease.

INTRODUCTION
Bacteria sense and respond to changes in their environment, integrating the signals to produce a directed response. *Xylella fastidiosa* (*Xf*) is a non-flagellated, xylem-restricted Gram-negative bacterium that moves within grapevines via twitching motility that employs type I and type IV pili (Meng et al. 2005). Movement appears to be controlled by a system with similarities to that first reported in *E. coli*, in which a group of *che* genes regulates the rotational movement of flagella. Transmembrane chemoreceptors bind chemical stimuli in the periplasmic domain and activate a signaling cascade in their cytoplasmic portion to ultimately control the direction of flagella rotation (see review Hazelbauer et al. 2008). We previously found that the homologous gene cluster is an operon (named Pil-Chp) that regulates type IV pili, and that disruption of the operon leads to a decrease in Pierce's disease (PD) symptoms (**Figure 1**). Herein, we further characterize the genes in the Pil-Chp operon and describe our advances in understanding the role of *pilJ* signaling in *Xf*.

OBJECTIVES
1. Complete characterization of the single chemosensory regulatory system of *Xf* and its function in PD. In particular, we will focus on its role in mediating bacterial movement and biofilm formation. Toward this end we will:
   a. Obtain *Xf* mutants in the *pilJ* gene that encodes the single methyl-accepting chemotaxis protein in *Xf*.
   b. Assess virulence and motility of *pilJ* mutants in grapevines, as well as previously created mutants deficient in related chemosensory genes, *pilL* and *chpY*.
2. Identify environmental signals that bind PilJ and activate chemosensory regulation. Toward this end we will:
   a. Express PilJ or a chimeric form of PilJ in a strain of *E. coli* previously deleted of all methyl-accepting chemotaxis protein genes.
   b. Subsequently, candidate signals will be screened using the above *E. coli* system for activation of motility.

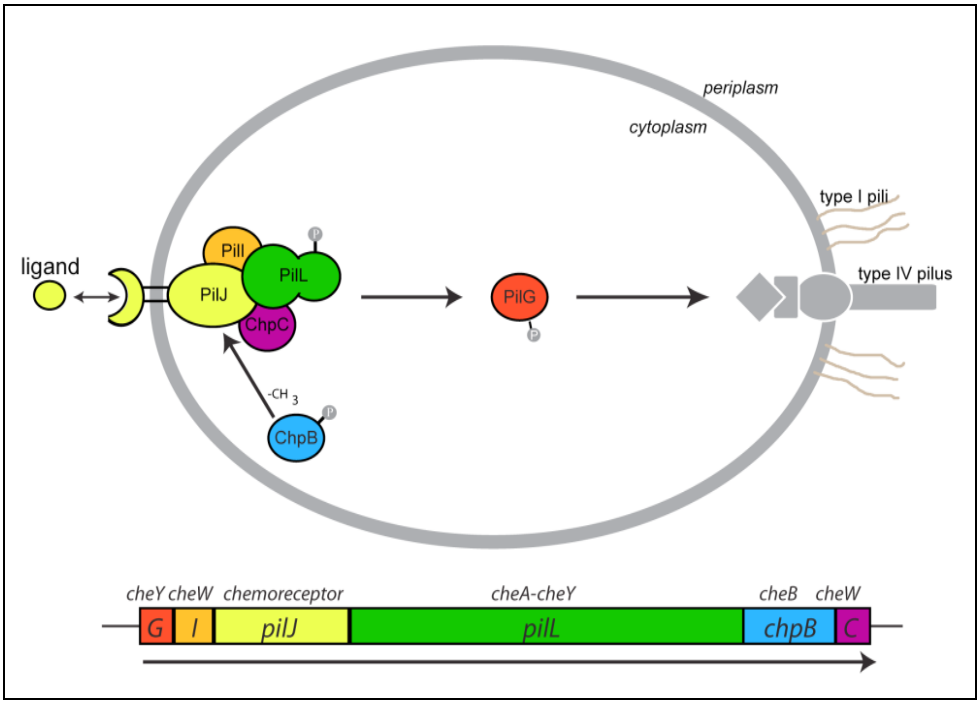

Figure 1. **The *Xf* Pil-Chp operon.** A) Model for operon protein products regulating twitching motility in *Xf*. The chemoreceptor PilJ senses environmental signal(s). ChpC/PilI couples PilL to PilJ. PilL phosphorylates its hybrid CheY-like receiver domain and PilG. ChpB is homologous to ligand adaptation proteins and may act as a phospho-transfer protein or ligand adaptor modulator. B) The Pil-Chp operon genes with *E. coli* homologous genes shown above and direction of transcription shown below.

RESULTS AND DISCUSSION

Construction of the *Xf* Pil-Chp operon null mutant strains. The Pil-Chp operon was disrupted previously with a polar transposon mutation in the *pilL* gene (Cursino et al. 2011). We subsequently constructed non-polar, allelic exchange mutants of all Pil-Chp operon genes, including *pilL*, according to Chatterjee *et al*. 2008 with slight modifications. The disruption of each gene in marker-exchange mutants was confirmed by PCR (not shown).

Construction of plasmids to complement the *Xf* Pil-Chp operon null mutant strains. To complement the non-polar gene disruptions, we constructed *Xf*-compatible plasmids containing the chemotaxis operon promoter region (Hoch et al. 2010). We then cloned the various Pil-Chp genes into these constructs and transformed the constructs into the null mutants. Successful transformation was confirmed by PCR (data not shown).

Twitching motility of the *Xf* Pil-Chp operon null mutant strains. We observed that the Pil-Chp transposon polar mutant was twitching minus on both PW agar surfaces and in microfluidic chambers (Cursino et al. 2011). PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of type IV pili twitching motility function (Meng et al. 2005). This mutant retained type IV pili (Cursino et al. 2011), indicating that the Pil-Chp operon regulates twitching and not pili formation. Results were confirmed in microfluidic chambers (Cursino et al. 2011). Examination of the non-polar Pil-Chp operon gene mutants on PW agar and in chambers revealed that *pilG*, *pilI*, *pilJ*, *pilL* mutants are twitching minus (data not shown). Complementation of these genes recovered twitching (data not shown). Interestingly, the *chpB* and *chpC* mutants performed twitching motility like wild-type cells (data not shown).

Biofilm formation by the *Xf* Pil-Chp operon null mutant strains. Using a crystal violet assay (Zaini et al. 2009), we found that the Pil-Chp transposon polar mutant produced less biofilm compared to wild-type *Xf* cells (Cursino et al. 2011). Additionally, all of the non-polar Pil-Chp operon gene mutants produced less biofilm, even twitching plus mutants *chpB* and *chpC* (**Figure 2**).

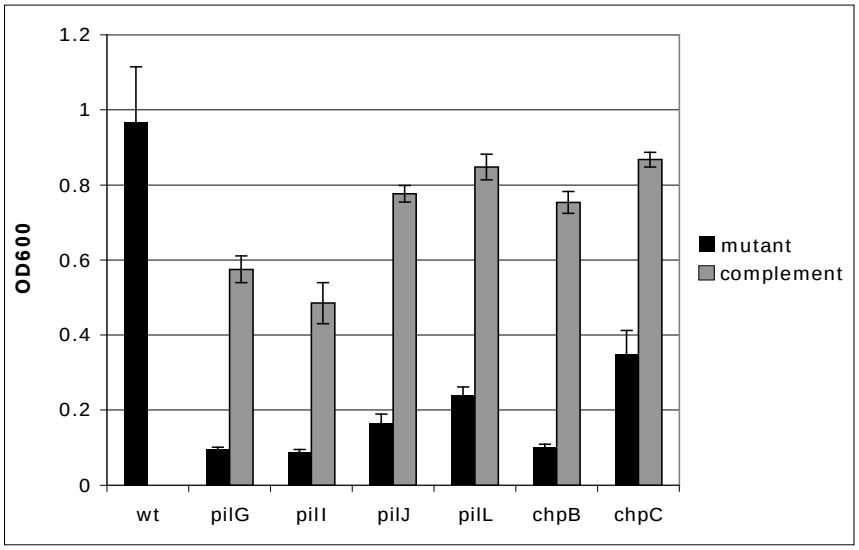

Figure 2. Biofilm formation with Pil-Chp operon mutants. Biofilm formation by wild-type, Pil-Chp non-polar gene mutants, and complemented mutants in *Xf* cells grown in culture flasks following 10 days of growth with agitation. Quantitation of biofilm formation was performed by the crystal violet assay (Zaini et al. 2009).

***In planta* results with the *Xf* Pil-Chp operon null mutant strains.** We discovered that the Pil-Chp transposon polar mutant cells induced less PD symptoms *in planta* compared to inoculation with wild-type cells (Cursino et al. 2011). Twitching minus non-polar Pil-Chp operon mutants *pilG*, *pilI*, and *pilJ* induced less PD disease *in planta* and symptoms plateaued after 20 weeks (**Figure 3**). Conversely, the Pil-Chp operon twitching plus *chpB* and *chpC* mutants induced full PD symptoms, however the disease was delayed compared to wild-type cells. We are currently testing the non-polar *pilL* mutant *in planta*.

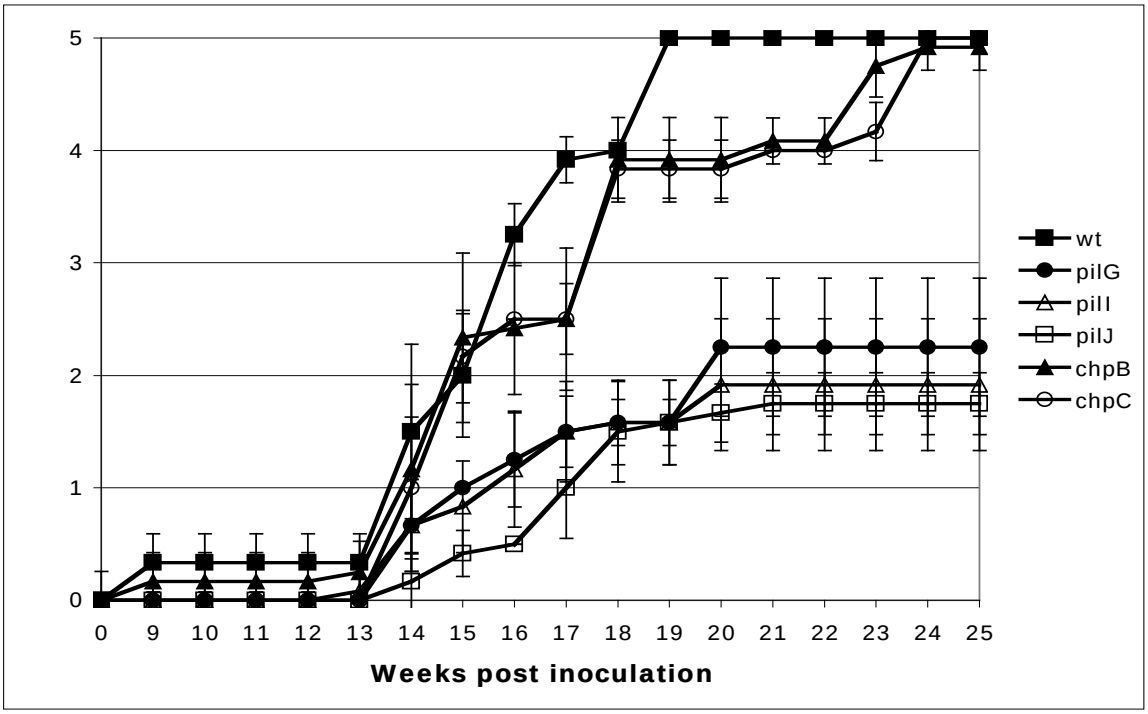

Figure 3. PD development in Pil-Chp operon gene mutants. *Vitis vinifera* L. cv. Cabernet Sauvignon vines were inoculated with wild-type or Pil-Chp non-polar gene mutants and PD disease was assessed over 25 weeks. Plants inoculated with the *pilL* mutant are currently being analyzed. PD rating on a scale of 0-5 (Guilhabert and Kirkpatrick 2005).

Identifying the chemosensory attractant. To examine what component(s) in sap drives the motility response, numerous assays were attempted (Hoch et al. 2009, Hoch et al. 2010). We desired a medium that supports *Xf* growth at wild-type levels but on which colonies do not develop fringe. We could identify the motility stimuli by adding sap components to the medium and testing for fringe recovery. We have found that *Xf* cells grow at wild-type levels in PW medium without soytone but fail to produce a fringe on this medium (data not shown). We are currently developing this method and will begin testing sap components.

Pil-Chp operon and *chpY* gene. 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. In the similarly organized P*seudomonas aeruginosa* Pil-Chp operon, downstream genes produce proteins that are proposed to associate with the Pil-Chp operon protein products (Whitchurch et al. 2004). In addition to the *pilG*-like domain, *chpY* has GGDEF and EAL regions that is 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 (data not shown). 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 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 (**Table 1**). The *gumD and gumJ* were found to have 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 also examined the production of extracellular enzymes in the *chpY* null strain, as these enzymes are known pathogenic factors (Thowthampitak et al. 2008). For the enzymes tested (carboxymethylcellulose, polygaracturonase, protease, and pectin methylesterase) no differences were found between the wild-type and the *chpY* mutant strains (data not shown).

Table 1. Effect of *chpY* on the expression of gum genes. The levels of gene expression in *Vitis vinifera* sap were tested by RT-PCR and normalized to *dnaQ* expression. The experiments were performed three times, with three replicates each. The standard deviations of the normalized means are shown.

Strains	**Genes**		
	chpY	*gumJ*	*gumD*
Wild-type	1.09±0.5	1.36±0.1	1.18±0.2
chpY mutant	ND	2.61±0.4	2.83±0.3
Complemented *chpY*	2.17±0.3	1.25±0.2	1.02±0.1

CONCLUSIONS

Our results with the Pil-Chp mutants show that the operon is required for twitching motility in *Xf*. Interestingly, some of the genes in the operon may not play a role in twitching motility but all play a role in biofilm formation and PD development. We appear to have found an assay to determine the chemical stimuli in grape sap driving motility. Additionally, we report that although *chpY* gene protein product does not regulate the Pil-Chp operon, it does contribute to PD development by upregulating the *gum* genes, which leads to an increased biofilm phenotype.

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FUNDING AGENCIES

Funding for this project was provided by the USDA-funded University of California Pierce's Disease Research Grants Program.