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

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ABSTRACT

Previously we demonstrated that twitching motility (TM) in *Xylella fastidiosa (Xf)* is dependent on an operon 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 four advances in examining this system. First, the gene *pilJ*, putatively coding for a methyl-accepting chemotaxis protein (MCP), is shown to be essential for TM as an insertional mutation in this gene resulted in a twitching-minus phenotype on agar. Further characterization of such mutant is underway. Second, we have results on Pierce's disease (PD) reduction with previous mutants in the operon gene *pilL*. Third, we have made advances on identifying the *Xf* chemotaxis attractant using a chimera protein fusing the *Xf* MCP, *pilJ*, to an *E. coli* MCP. Fourth, we have begun exploring the localization of PilJ in relation to the *Xf* pili.

LAYPERSON SUMMARY

This project involves studies targeting the regulation of chemical sensing pathways by which *Xylella fastidiosa* is able to control its movement within the plant environment. Several genes and gene products of this chemical sensing pathway are being examined. We found that one gene in particular is essential for cell movement (twitching motility), and we found differences in grapevine saps that may influence the sensing pathway.

INTRODUCTION

Bacteria sense and respond to changes in their environment, integrating the signals to produce a balanced response. *Xylella fastidiosa* (*Xf)* is a non-flagellated xylem-restricted Gram-negative bacterium that moves within grapevines via TM that employs type I and type IV pili (Meng et al. 2005). Movement appears to be controlled by a chemosensory system similar to that first reported in *E. coli* where a group of *che* genes regulated the rotation movement of flagella. These proteins work by means of a phosphorylation cascade to ultimately control the direction of flagella rotation (Hazelbauer, 2008). We previously described the new operon involved in TM and likely to be responsible for the chemosensory regulation of type IV pili in *Xf* (**Figure 1**). Herein, we further characterize this operon creating a mutation in *pilJ* and analyzing the effect of a *pilL* mutant *in planta* and describing our advances in understanding the role of *pilJ* signaling in *Xf*.

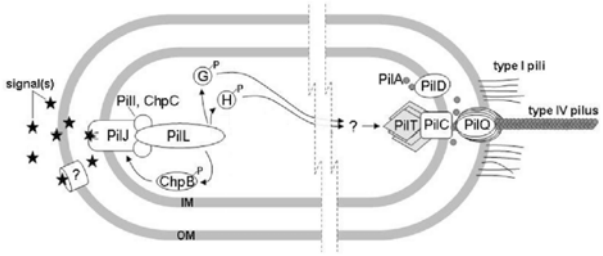

Figure 1. Model for chemosensory regulation of twitching motility in *Xf*. PilJ, the single polar methyl-accepting chemotaxis protein senses environmental signal(s). PilL phosphorylates , PilG, PilH and ChpB. ChpC and PilI couples PilL to PilJ. ChpB might mediate adaptation to a constant chemical concentration by adjusting the methylation level of the receptor. Some aspects still unknown are, for example, the nature of the signal(s) and whether they diffuse or are actively transported across the outer membrane. For schematic purposes not all pili components are shown. (from Burr *et al*. 2007)

OBJECTIVES

1. Complete characterization of the single chemosensory regulatory system of *Xf* and its function in PD and, in particular, focus on its role in mediating bacterial movement and biofilm formation. Toward this 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*.
2. Identify environmental signals that bind PilJ and activate chemosensory regulation. Toward this 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.

RESULTS

Construction of null mutants strains of *Xf* for the chemosensory operon. The construction of an allelic exchange mutant for *pilJ* gene in *Xf* was performed according to Chatterjee *et al*. 2008 with slight modifications. The disruption of the *pilJ* locus in marker-exchange mutants was confirmed by PCR (not shown).

Twitching motility. Examination of *pilJ* on PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of type IV pili twitching motility function (**Figure 2**)

Virulence and movement on grapevines. The *pilL* mutants were inoculated into Cabernet sauvigon grapevines. Symptom expression was assessed 12 weeks following inoculation (**Figure 3**). *pilJ* mutant will be assayed in the Spring of 2010.

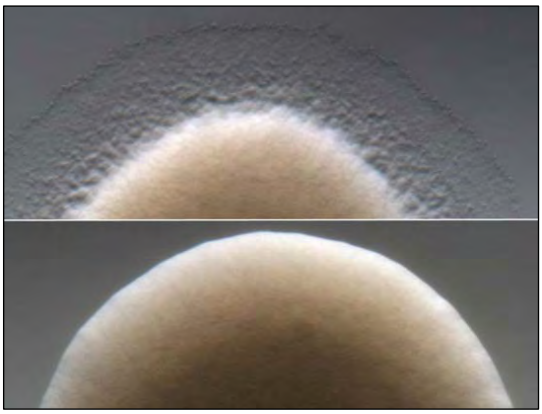

Figure 2. Example of colony morphologies of *pilJ* mutant (lower) and Wild-type Temecula isolate (upper) grown on PW agar for 5 days.

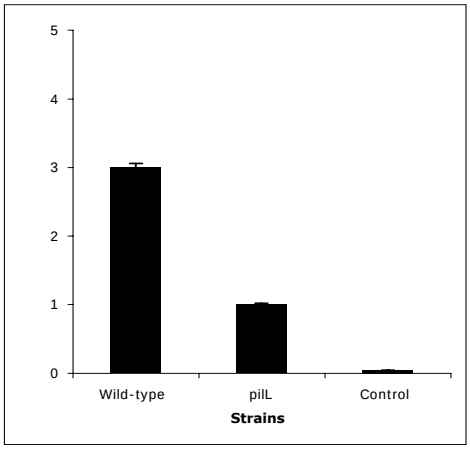

Figure 3. PD expression in greenhouse grown Cabernet sauvignon vines 12 weeks following inoculation.

Complementation of *Xf* chemoreceptor in *E. coli*. Chemoreceptors are transmembrane proteins that bind ligand in the periplasmic domain and associate with a kinase, CheA, in their cytoplasmic portion. Changes in ligand binding result in a phosphorylation cascade: CheA autophorphorylation, CheA phosphorylates a shuttle protein (CheY), CheY modifies flagella proteins, and the flagella alter their rotation and the bacteria moves from random tumbling ("tumble") to smooth swimming ("run") (Hazelbauer, 2008). The putative *Xf* chemoreceptor, *pilJ*, was cloned and expressed in an *E. coli* strain lacking chemoreceptors. To determine if PilJ complemented the *E. coli* system and activated the *E. coli* CheA, we used the ligand-independent pseudotaxis assay (Wolfe, 1989; Ames, 1996). In this assay, cells lacking both chemoreceptors and the normal ligand adaptation system are locked in a smooth swimming response (Bibikov, 2004). In agar at levels to support swimming (0.25%), these cells become trapped in the agar pores, unable to tumble and leave agar dead ends. When *E. coli* chemoreceptors are expressed in these cells, the receptors interact with the CheA kinase and allow the cells to alternate between smooth swimming and tumbling. As a result, the cells can maneuver through the agar alleyways and form a swarm on the plate. PilJ failed to form a swarm, indicating that PilJ does not activate the CheA and complement the *E. coli* chemotaxis system (**Figure 4**).

Construction of a chimeric chemoreceptor. To facilitate PilJ functioning in an *E. coli* system, we constructed chimeric chemoreceptors that contain the periplasmic ligand binding domain of the *Xf* PilJ fused to the cytoplasmic signaling domain of the *E. coli* serine chemoreceptor, Tsr (**Figure 5**). The chimeras should only detect the PilJ ligand as we swapped the periplasmic serine binding site of Tsr with the proposed PilJ ligand binding site. The chimera also should successfully activate the *E. coli* CheA kinase as the CheA binding site is maintained in the Tsr cytoplasmic portion. We constructed two chimeras; one chimera linked PilJ and Tsr in the transmembrane domain and the other, based on published MCP chimeras (Kristich, 2004), linked in the HAMP domain (Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases). The two fusion proteins activate the *E. coli* signaling pathway as measured in the pseudotaxis assay (Wolfe, 1989; Ames, 1996) (**Figure 5**). Western blot analysis, using antibodies to the *E. coli* chemoreceptor portion (Ames, 1994), suggest that the chimeric chemoreceptors are produced at 10-15 times lower levels than wild-type *E. coli* Tsr (data not shown). PilJ contains codons rare to *E. coli*, which presumably affects protein production. We are currently modifying the promoter region to enhance protein production.

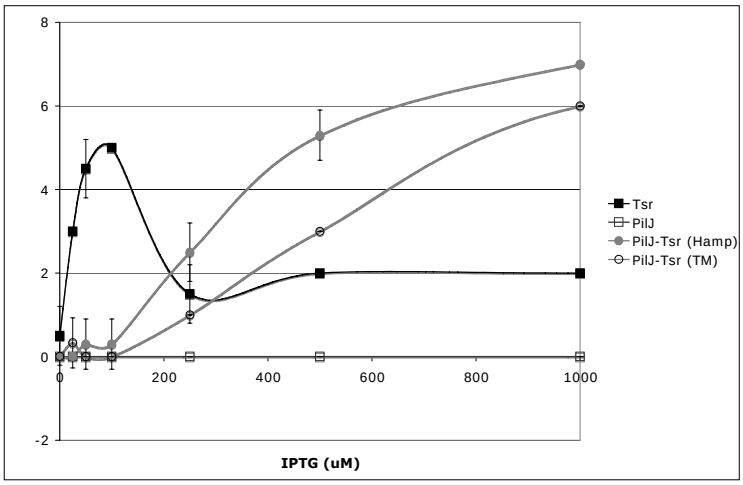

Figure 4. Pseudotaxis of cells expressing PilJ constructs. Constructs were expressed in *E. coli* strain UU1535 (*Δmcp, Δaer, ΔcheR/B*) and patched onto 0.25% tryptone agar plates with 50ug/mL ampicillin and varying concentrations of IPTG inducer. Cells grew for 20 hours at 30°C.

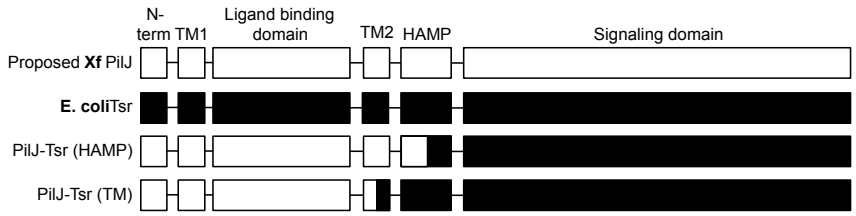

Figure 5. *E. coli* chemoreceptors (including serine receptor, Tsr) contain a periplasmic ligand binding domain and a cytoplasmic HAMP (Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases) domain and signaling domain. *Xf* PilJ is proposed to have similar domains. We constructed chimeras that connect the PilJ to Tsr through the proposed HAMP domain or transmembrane (TM) domain. These chimeras should bind *Xf* PilJ ligand but successfully interact with *E. coli* cytoplasmic proteins involved in chemoreceptor signaling.

Identifying the attractant. In order to confirm that PilJ binds to a molecule in grape sap, we tested the chimeras using a standard swarm plate assay (Adler, 1966). In this qualitative assay, cells are patched onto soft agar plates with a uniform amount of potential attractant. Cells expressing the ligand adaptation system (as oppose to the pseudotaxis assay) alternate between running and tumbling (Eisenbach, 2007). When chemoreceptors bind attractant the cells run smoothly until Brownian motions force them to tumble and reorient. As the cells "sense" ligand via the chemoreceptors, consume the attractant, and migrate through the plate they create a visible smooth ring at the interface between metabolized ligand and available attractant. Behind the ring, the attractant is depleted because the cells have consumed it; in front of the ring the attractant is abundant. Cells lacking chemoreceptors or cells patched onto plates without a chemoreceptor ligand are "blind"; do not experience extended runs, do not progress as far from the original patch, and fail to create a smooth ring even though the media may contain desired nutrients.

When we tested the PilJ-Tsr chimeras in the swarm plate assay, they produced a noticeable ring on soft agar plates containing *Vitis riparia* sap harvested in New York (**Figure 6**). The *E. coli* serine chemoreceptor, Tsr, also formed a ring indicating that *V. riparia* contains detectable levels of serine. Tsr supported a larger swarm ring than the chimeras, which may reflect the different levels of a) PilJ-Tsr chimera attractant compared to serine and/or b) PilJ-Tsr chimera and Tsr proteins as discussed above. As expected, PilJ did not support a swarm as the earlier tests showed that it failed to interact with the *E. coli* kinase CheA. Based on these results, we are currently testing various known components of sap (Anderson, 2007) to identify the PilJ ligand/s. As chemoreceptors can have multiple ligands (Kondoh, 1979) and attractants may not be metabolized (Topp, 2007), we are also testing the PilJ chimeras in established chemotaxis assays that do not require consumption of the ligand (Adler, 1973; Grimm, 1997; Yu, 1997).

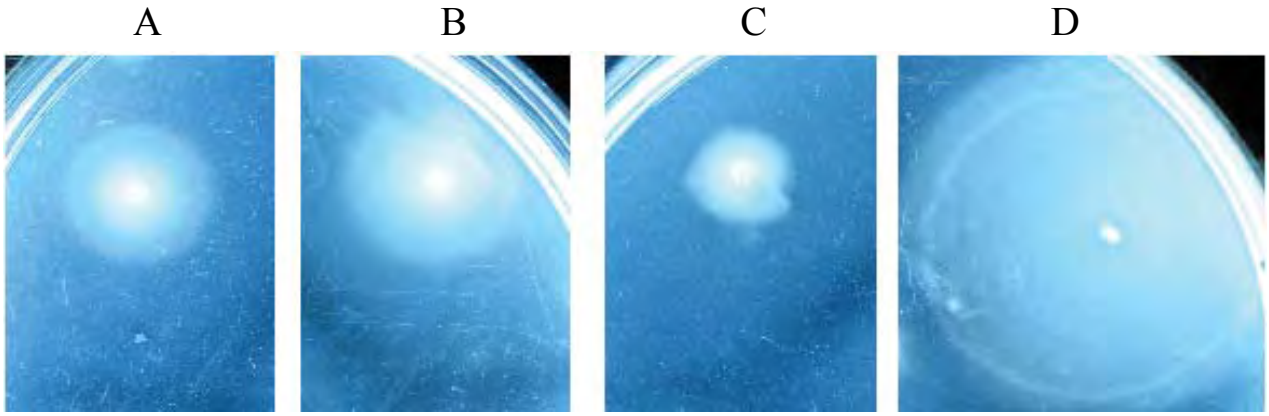

Figure 6. PilJ induces chemotaxis response to *V. riparia* sap. Constructs were expressed in *E. coli* strain, UU1250 (*Δmcp, Δaer*), patched onto swarm plates containing 80% sap, and grown for 18 hours at 30°C. Constructs were A) chimera PilJ-Tsr (TM), B) chimera PilJ-Tsr (HAMP), C) *Xf* PilJ, and D) *E. coli* serine chemoreceptor Tsr.

Localization of the chemoreceptor. Chemoreceptor localization has been studied in only a handful of organisms and found to be polar or cytoplasmic (Maddock, 1993; Harrison, 1999; Bardy, 2005; DeLange, 2007). In *E. coli,* chemoreceptors cluster into a polar lattice that presumably allows the receptors to work in concert and amplify the signal (Parkinson, 2005). As a result, the chemoreceptors are physically at a distance from the flagella. Likewise in *Pseudomonas aeruginosa* the chemoreceptor PilJ is polar localized (DeLange, 2007). We wish to learn if the *Xf* PilJ is a) polar localized, b) at one or both poles, and c) if it co-localizes with the pili. To answer these questions, we are taking two approaches. First, we are expressing PilJ protein for antibody production, which can then be used to label the chemoreceptor as previously reported (Maddock, 1993; Harrison, 1999; Bardy, 2005; Lamanna, 2005). Additionally, similar to earlier chemotaxis studies (Wadhams, 2002; Homma, 2004; DeLange, 2007) we will visualize the physical location of PilJ using a GFP-tagged PilJ protein. From these studies, we hope that determining the physical organization of the chemoreceptor system will help elucidate the nature of the *Xf* chemosensory signaling system.

CONCLUSIONS

Our results with a construction of a mutation in *pilJ*, and the previously reported *pilL*, show that these genes are required for twitching motility in *Xf.* They also play a role in virulence as vines inoculated with *pilL* showed less disease. Currently we are studying other phenotypes of the *pilJ* mutant including growth and biofilm formation. We are attempting to find the chemoattractant in grape sap and the localization of PilJ in *Xf.* We are also creating three new mutants *(pilG, pilI* and *chpC*) to further characterize the chemosensory operon.

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