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

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ABSTRACT

Here we provide evidence that twitching motility (TM) in *Xylella fastidiosa* (*Xf*) is dependent on a cluster of signal transduction pathway proteins (*tonB, pilG, pilI, pilJ, pilL, chpB* and *chpC*), which is related to the system that controls flagella movement in *Escherichia coli*, and highly similar to chemosensory system controlling TM in *Pseudomonas aeruginosa*. The gene *pilL*, coding for a putative kinase, is shown to be essential for TM as an insertional mutation in this gene resulted in a twitching –minus phenotype in agar and inside microfluid chambers, and reduced biofilm formation. We constructed a new site-directed mutant on *pilL* (called $pilL_2$), which confirms the minus phenotype observed for this gene. This second mutation on *pilL* affects biofilm formation as well. We demonstrate that *pilG-chpC* region is organized as an operon. In addition, we showed that *tonB* gene is also required for TM and complementation experiments restore the TM phenotype of *tonB* mutant. TEM revealed that type IV (and type I) pili are present on all mutants in the cluster, indicating that none of the chemosensory-related genes affects the pili production and instead are likely to be involved in the sensory regulation of TM. We also report our advances on the heterologous complementation of swarming motility phenotype in *E. coli* methyl-accepting chemotaxis protein (MCP) mutant using the *Xf* chemoreceptor, *pilJ*.

INTRODUCTION

Bacteria sense and respond to changes in their environment, integrating the signals to produce a balanced response. *Xf* is non-flagellated xylem-restricted gram-negative bacterium that moves inside of grapevines via TM that employs type I and type IV pili (Meng et al. 2005). Movement controlled by a chemosensory system was first reported in *E. coli* where a group of *che* genes regulated the rotation movement of its flagella. These proteins work by means of a phosphorylation cascade to ultimately control the direction of flagella rotation (Blair,1995). In *P. aeruginosa* the chemosensory regulation of type IV pili is controlled by genes in the clusters *pilGHIJK* and *chpABCDE* (Whitchurch, 2006). We previously described the new cluster of genes involved in TM likely to be responsible for the chemosensory regulation of type IV pili in *Xf*. (**Figure 1**). Herein, we further characterize this cluster reproducing mutations in *pilL* and describing a new gene *tonB* as part of this cluster.

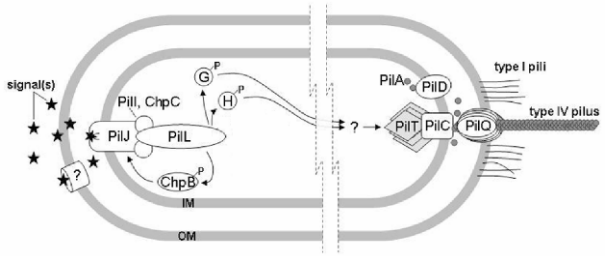

Figure 1. Model for chemosensory regulation of twitching motility in *X. fastidiosa*. PilJ, the single polar methyl-accepting chemotaxis protein senses environmental signal(s). PilL phosphorylates , PilG, PilH and ChpB. ChpC and PilI couples PilL to PilJ. ChpB mediates adaptation to a constant chemical 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 Pierce's disease and, in particular, we will 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 cluster. The construction of an allelic exchange mutant for *pilL* gene in *Xf* was performed according to Chatterjee et al. 2008 with slight modifications. The disruption of the *pilL* locus in marker-exchange mutants was confirmed by sequencing and PCR. The mutation occurred in the codon 968. The *pilL* mutant was designated as $pilL_2$. The construction of a null mutant for the *pilJ* gene is underway.

Growth, biofilm, and pilus formation. We previously described a *tonB* mutant (Burr et al. 2007) and here we show that complementation analysis of the *tonB* mutant was accomplished by cloning the gene in pBBR1MCS-5 followed by transformation. No significant differences in growth rates between the mutant and complemented mutant were observed when compared to wild-type (**Figure 2**). Therefore, the lack of twitching observed in mutants was not correlated with growth. The development of biofilms by wild-type, *pilL*, $pilL_2$, *tonB* mutant, and complemented *tonB* are shown in **Figure 3**. The *tonB* mutant formed significantly less biofilm than the wild-type strain, and biofilm formed by complemented *tonB* was similar to the wild-type (not shown). Similarly, the *pilL* and $pilL_2$, mutants formed less biofilm than the wild-type. Electron microscopy revealed that *pilL,* $pilL_2$, and *tonB* mutants as well as the complemented *tonB* possess type I and type IV pili confirming that these genes are not involved in pili biogenesis. The twitching phenotype by *tonB* mutant therefore is due to the absence of a functional TonB protein, which is predicted to be accessory to the type IV pilus machinery contributing to the release of pili subunits. Similarly, we predict the abolishment of twitching in the *pilL* mutant is due to lack of histidine kinase binding to the chemoreceptor.

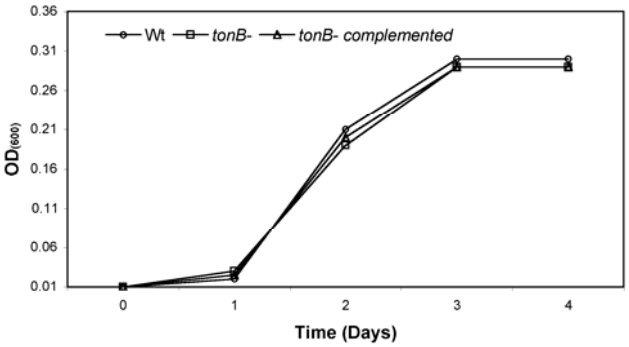

Figure 2. Growth curves of *Xf* wild-type, *tonB* mutant and complemented mutant from a 10-day period (4-day data shown). Experiments were repeated at least three times using five replicates each.

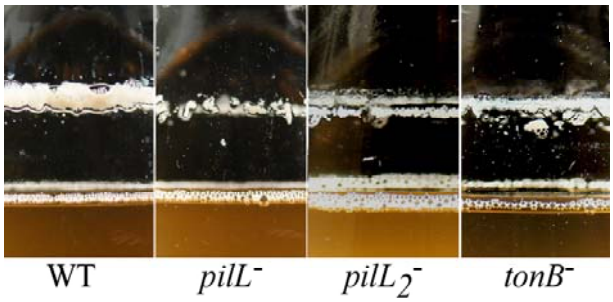

Figure 3. Biofilm formation by *Xf* grown for 10 days in culture flasks. The *tonB*, *pilL* and $pilL_2$ mutant biofilm layers were significantly smaller than *Xf* wild-type.

Twitching motility. Examination of *pilL,* $pilL_2$, and *tonB* mutants on PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of twitching function (**Figures 4a and 4c**). Complementation *tonB* (C) showed the restored fringe phenotype similarly observed in the wild-type (**Figures 4b and 4d**)

We measured the speed of movement of *Xf* wild-type, *pilL* and *tonB* mutant and *tonB*-complemented on PW agar surface. The complemented mutant shows a slight reduction in the speed of movement when compared to the wild-type, but TM phenotype was restored (**Figure 5**).

Transcriptional analysis of the chemosensory cluster. We also investigated the effect of the transposon insertion in *pilL* on the transcription of neighboring coding sequences by semi-quantitative RT-PCR. This analysis showed not only that wild-type expression levels are retained in the mutant (in this case *pilL)* but also that *pilG -chpC* comprise an operon (**Figure 6**). Transcriptional analysis of *tonB* and *tonB*-complemented are underway.

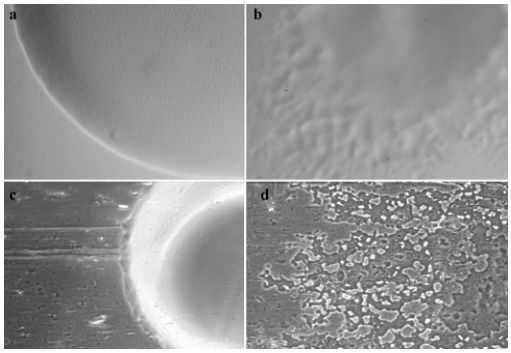

Figure 4. Colony morphology of *tonB* mutant (a, c) and *tonB*-complemented (b, d) grow on PW agar surface (a, b) and on cellophane overlaid on agar (c, d) for 4 days. TM fringe is pronounced in b and d.

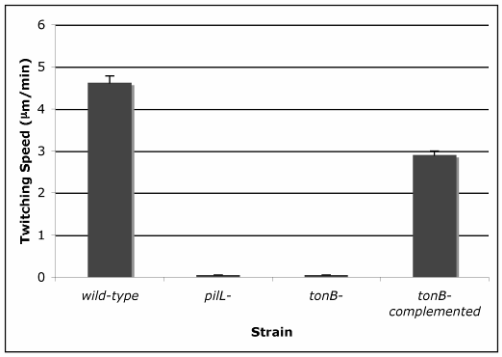

Figure 5. Speed of twitching movement of *X. fastidiosa* wild-type, *tonB* mutant, and complemented *tonB* mutant cells on PW agar. Values shown are means of at least 15 cells.

Virulence and movement on grapevines. The *pilL*, *pilL*$_2$, and *tonB*, mutants were inoculated into grapevines in September 2008. The virulence and movement will be assessed in about 12 weeks. *pilJ* and *tonB*-complemented will be assayed in the Spring of 2009.

Complementation of *Xf* chemoreceptor in *E. coli*. The putative *Xf* chemoreceptor, *pilJ*, was cloned and expressed in an *E. coli* strain lacking all chemoreceptors and methylating proteins, UU1535 (Bibikov, et al. 2004). SDS-PAGE analysis of whole cell lysates suggested that limited PilJ protein was produced, presumably due to differences in codon usage between the two organisms (not shown). We are currently working towards eliminating this problem.

Construction of a chimeric chemoreceptor. A chimeric chemoreceptor was constructed that contains the periplasmic ligand binding domain of the *Xf* putative chemoreceptor PilJ fused to the cytoplasmic signaling domain of *E. coli* chemoreceptor Tsr. The construct was expressed in an *E. coli* strain UU1535 (Bibikov et al. 2004), and Western blot analysis, using antibodies to the *E. coli* chemoreceptor portion (Ames and Parkinson. 1994), suggested that the chimeric chemoreceptor was produced, although at lower levels than wild-type *E. coli* chemoreceptor. Again differences in codon usage were suspected to be affecting protein production. Over-expression of the chimera protein revealed its ability to activate the chemotaxis kinase as measured by pseudotaxis (Wolfe and Berg, 1989; Ames and Parkinson, 1996), an assay measuring expansion of the colony on a soft agar plate (**Figure 7**). We are currently examining if the chimera supports chemotaxis.

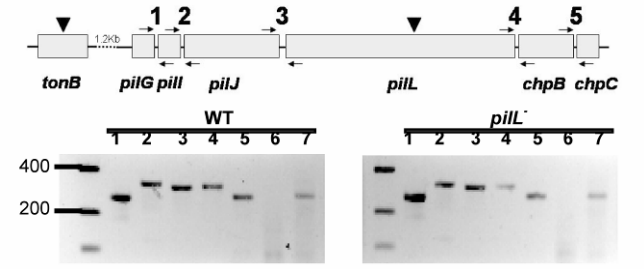

Figure 6. RT-PCR showing operon structure of *pil-chp* cluster. Total RNA treated with DNAse was used to amplify fragments indicated by black arrows in the top diagram. (1) *pilG-pilI*; (2) *pilI-pilJ*; (3) *pilJ-pilL*; (4) *pilL-chpB*; (5) *chpB-chpC*; (6) *pilG-pilI* with no reverse transcriptase but with DNA polymerase; (7*) pilG-pilI* fragment amplified from genomic DNA.

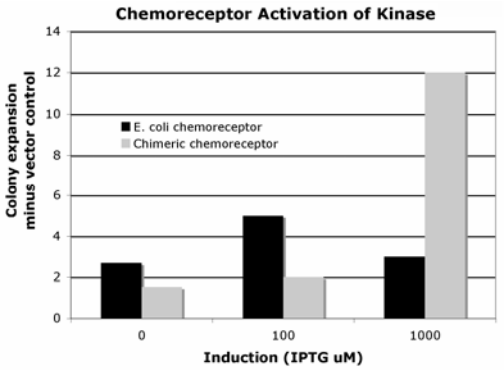

Figure 7. Expansion of colonies (pseudotaxis) in mm on soft agar plates indicates activation of the chemotaxis kinase. *E. coli* chemoreceptor optimal induction at 100μM IPTG while chimeric protein required ten-fold more induction. Chemoreceptors were expressed in an *E. coli* strain lacking chemoreceptors and methylating proteins, and grown on tryptone soft agar plates incubated for 22.5 hours at 30 C.

CONCLUSIONS

Our results with the complementation of *tonB* and a construction of a second mutation in *pilL*, *pilL*$_2$, show these genes are required for twitching motility in *Xf*. They also play a role in biofim formation i.e. the mutation reduces the amount of biofilm and may play a role in virulence. Initial studies with the chemoreceptor suggest that both the *Xf* chemoreceptor, *pilJ*, and chimeric protein express in *E. coli* and that the chimeric protein successfully interacts with the chemotaxis kinase. This project is in initial stages and over the next nine months we will finish investigating *pilL* and the roles of *pilJ*. We will also be able to begin exploring the signals that trigger the chemosensory regulation in *Xf*.

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