THE ROLES THAT DIFFERENT PILI CLASSES IN *XYLELLA FASTIDIOSA* PLAY IN COLONIZATION OF GRAPEVINES AND PIERCE'S DISEASE PATHOGENESIS: CHEMOSENSORY CLUSTER CONTROLLING TWITCHING MOTILITY

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ABSTRACT

We provide evidence that twitching motility in *Xylella fastidiosa* (*Xf*) is controlled by a signal transduction pathway (pilG-chpC cluster), which is highly similar to chemosensory systems controlling flagella rotation in several bacteria including *Pseudomonas aeruginosa*. The gene pilL is shown to be essential for twitching motility as three different insertional mutations in this gene resulted in a twitching defective phenotype. We have also identified a cheY homolog, cheY2, in this pathway and show it is required for wildtype twitching motility. The cheY2 mutant was complemented by expression of the wildtype gene cloned in pBBRMC5. It was demonstrated that cheY2 is also involved in biofilm formation as the mutant produced a reduced biofilm. Transmission electron microscopy revealed type IV (and type I) pili are present in the cheY2 mutant and its complement. In silico analysis of CheY2 predicts its role in a chemosensory transduction signal cascade. In addition we identified a gene near the pilG-chpC cluster, tonB3, that is also related to twitching motility. We also report the recent advances in the production of monoclonal antibodies against *Xf* pili.

INTRODUCTION

Twitching motility (TM) functions in host colonization of many gram-negative bacteria. *Xylella fastidiosa* (*Xf*) has both type I pili and type IV pili, and exhibits twitching motility and biofilm formation (Meng et al, 2005). We have identified several genes in *Xf* associated with pili development and their associated phenotypes (ie. fimT, pilB, pilQ, pilR, pilX and pilY1) (Li et al, 2007). In P. aeruginosa (Pa) TM is regulated by a chemosensory system that involves two gene clusters pilG-K and chpA-E, that are analogous to bacterial chemotaxis systems that control swimming motility in response to environmental stimuli (Whitchuch et al, 2004). Chemosensory systems are composed of sensory receptors networking with components of cytoplasmic phosphorylation and dephosphorilation cascades (Mariconda et al, 2006). Here we describe three new genes in *Xf* that are associated with expression of TM. Two of them, cheY2 and pilL, are predicted to be part of a putative chemosensory system that controls TM in *Xf*; the third is tonB3 that may be responsible for the transport of pili subunits outside of the cell. CheY2 (ORF XP1757) was previously annotated as unnamed and part of a two-component regulatory system. In this report we predict by deduced protein structure that CheY2 most likely functions as a phosphoacceptor.

We also report our progress on the production of monoclonal antibodies against *Xf* pili that we propose to use for development of diagnostic tests and eventually in the development of novel controls for Pierce's Disease.

OBJECTIVES

1. Identify the putative TM chemosensory cluster and phenotype-associated genes.
2. Characterize three additional genes that are likely to be involved in the chemosensory control of TM and related functions.
3. Develop monoclonal antibodies against *Xf* pili.

RESULTS

Screening and sequence analysis of twitching mutants. We previously generated a library of TM mutants through transposon (EZ::TN Transposome system) mutagenesis of the *Xf* Temecula genome (Li et al., 2007). Sequence analysis identified three mutants (2A5, 17A8 and TM25), that contain transposon insertions in the ORF XP0874 (gene pilL) (Figure 2A). The mutants showed a smooth colony margin i.e. a complete lack of peripheral fringe in PW medium (Figure 1). We also identified another mutant (TM26) which has transposon insertion in the region immediately downstream of pilL, ORF XP0869 (predicted gene tonB) (Figure 1B; Figure 2A). A third mutant (3E10) resulted from a transposon insertion in the ORF XP1757 was annotated as part of a two-component regulatory system we designate as cheY2 (Figure 1; Figure 2B).

Sequence analysis of the *Xf* putative chemosensory cluster and related genes. The pilL mutant resulted from insertions in different regions of the ORF XP0874. This 5178 bp ORF encodes a 1726 aa multidomain protein that belongs to the family of CheA-like histidine kinases. The predicted protein is believed to result from a fusion of PilL (the Tfp sensor histidine kinase response regulator) and ChpA (Tfp chemotaxis-related protein kinase). CheA-like proteins contain a CheY docking domain, an autocatalytic histidine kinase domain that is required for ATP binding (HATPase) and a CheW-like domain that

is involved in mediating CheA interactions with CheW (ChpC in *Xf*). *Xf* PilL/ChpA is a predicted fusion protein that possesses four histidine-containing phosphotransfer domains in its N-terminal - HPt (Figure 2B). Upstream of pilL resides a tonB homolog (XP0869); a 900 bp ORF divergently transcribed, encoding a predicted 300 aa protein annotated as a TonB- dependent receptor. TonB interacts with outer membrane receptor proteins in the process to transduce cytoplasmic membrane energy. The TonB system is responsible for energizing transport events at the outer membrane using the proton-motive force of the cytoplasmic membrane (Postle, 2007). *Xf* contains thirteen TonB-dependent receptor copies in its genome; the first is ORF XP0008 and the last is the ORFXP2172. We named the ORF XP0869 tonB3, as it is the third tonB gene from the beginning of genome. Upstream of pilL resides pilG (XP0871), a 417 bp ORF that encodes a predicted 139 aa CheY-like protein. Downstream is gene is pilI (XP0872) a 531 bp ORF that is predicted to encode a 177 aa CheW-like protein that forms a complex with CheA (PilL/ChpA in *Xf*) that together functions as an allosteric enzyme (Fan, 2006). In P.a, the gene downstream of pilG is pilH (Figure 2. A). In *Xf* however, pilH (XP1715) comprises a 387pb ORF encoding a 129 aa protein located 8.4Mb downstream from the main putative chemosensory cluster.

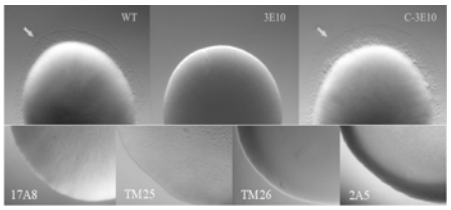


Figure 1. Colony morphologies of Wild-type *Xf* and the mutants grown on modified PW agar for 4 days.

The last gene upstream of pilL is pilJ (XP0873) an ORF comprised of 2043 bp that encodes a putative 681 aa protein predicted to be a methyl accepting chemotaxis protein (MCPs) that is known to be coupled via the adaptor protein CheW (ChpC in *Xf*) to the multidomain histidine protein kinase CheA. Downtrean of pilL are the genes chpB (XP0875 – 1170 bp) and chpC (XP0876 - 471bp) (Figure 2). The protein ChpB (390 aa,) is homologous to the CheB methylesterases, that in chemotaxis systems function to demethylate the sensory MCPs and ChpC, a CheW homolog that is believe to couple other MCPs with ChpA in P.a. *Xf* does not have chpD or chpE or their homologs.

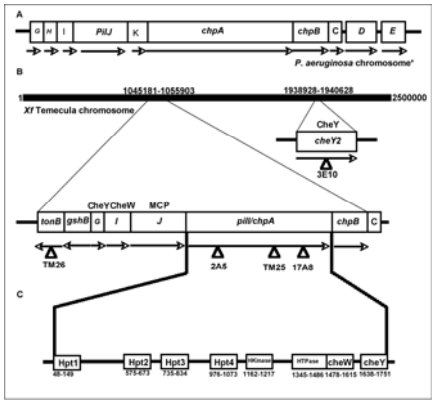
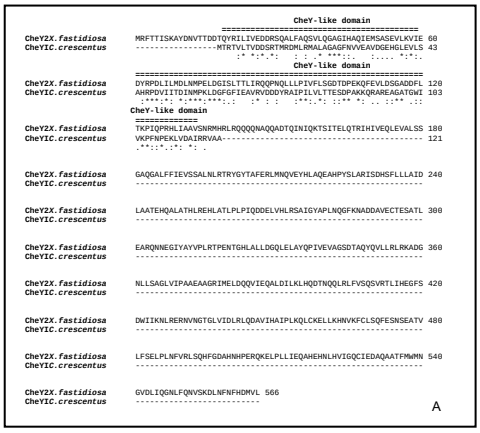


Figure 2. Organization of *pilG-chpC* cluster in *Xf* and *Pa.* (a) *Pa.* PilG-K and ChpA-E clusters (b) *Xf* chromosome and the organization of the chemosensory cluster and its domains. Open arrows denote genes disrupted in this study.

Sequence analysis of CheY2. Mutant 3E10 carries a transposon insertion in ORF XP1757. This 1701 bp ORF is predicted to encode a cytoplasmatic 567 aa protein, that also belongs to the CheY family. It has a CheY-like receiver domain and an N-terminus comprised of amino acids 20 to 133 (Figure 3). We named this protein CheY2, with PiLG being the first CheY-like protein in this chemosensory system. CheY2 showed high identity with homologs in strains of *Xf*; 98% identity with *Xf* Ann-1 (EAO33015), 97% identity with *Xf* Dixon (ZP_00650939) and 96% identity with *Xf* 9a5c (NP_297691). In all strains the gene was annotated as part of a two component response regulator. CheY2, CheY and CheY-like proteins exhibited low identities with CheY and CheY-like proteins in E. coli (20%), P.a (21%) and 31% identity to CheYI of Caulobacter crescentus. The alignment of *Xf* CheY2 and C. crescentus CheYI is shown in the Figure 3A. We used 3D modeling to examine the putative structure of *Xf* CheY2. *E. coli* CheY is a 129-residue protein, arranged in an alpha/beta parallel motif with five-stranded parallel β-sheets surrounded by five α-helices. The β-strands and α-helices alternate along the sequence and are connected by loops (Sola et al, 2000) where the central β-sheet plays the major role. Based on sequence similarity with other response regulators and CheY1 from C. crescentus, the conserved backbone β-α-loop was predicted (β2-α1-loop) for CheY2 (Figure 3B). This putative conformational structure of CheY2 is predicted to function as the part the twitching chemosensory system of *Xf*.



Agregation, growth and biofilm formation. Complementation analysis of the CheY2 mutant, 3E10, was performed by cloning the gene into plasmid pBBRMC5 and then electroporating it into competent cells of the mutant. No significant differences in growth rates between the mutant and its complement were found when compared to *Xf* Temecula wild-type (not shown).

Figure 3. Similary and modeling of CheY2. (a). sequence aligments of CheY2 (*Xf*) and CheY1 (*C. crescentus*) used for structural modeling. (b). Ribbon structures of the two β-sheets and the α-helix predicted for CheY2.

Differences in the dynamic formation and dispersal of bacterial aggregates and cell movement were monitored by light microscopy and in microfluid chambers. The CheY2 mutant showed aggregation patterns different from wildtype, which shows mostly small transient aggregates. The CheY2 mutant forms large clumps of transient aggregates that move slower than wildtype aggregates. The development of biofilms

by mutant, complement and wildtype in PD2 cultured in glass flasks with continuous agitation are shown in the Figure 4. The CheY2 mutant forms less biofilm than wildtype. The biofilm formed by the complemented mutant was visibly more pronounced as compared to the wildtype or mutant (Figure 4). Furthermore, it was noted that the biofilms formed by both 3E10 and C-3E10 were more easily removed from the glass surfaces by swirling in distilled water than the wild-type (data not shown).

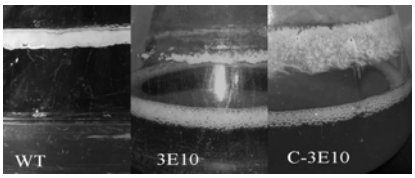


Figure 4. Biofilm formation of *Xf*. Wild-type, 3E10 and C-3E10 cells. Following 10 days of growth in culture with agitation.

Type I and IV Pili were observed on the CheY2 mutant and complement (Figure 5). We predict the lack of twitching phenotype in the mutant is due to absence of a functional CheY2 protein, responsible for initiating the signal transduction cascade associated with pili retraction.

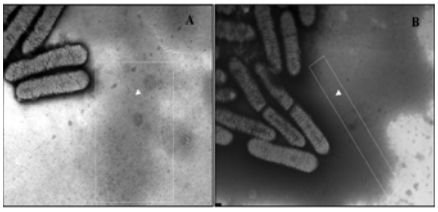


Figure 5. Electron micrographs of *Xf* cells. Transmission electron microscopy micrographs of 3E10 (a) and C-3E10 (b) cells negatively stained. White arrows inside box type IV pili.

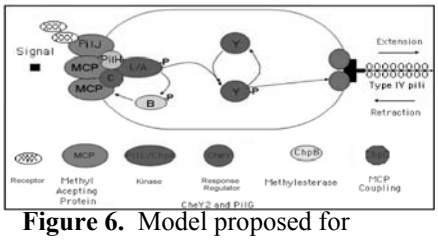


Figure 6. Model proposed for signal transduction pathway of *Xf* chemosensory system for twitching motility.

Twitching motility chemosensory model. We propose a model for TM chemosensory in *Xf* (Figure 6); receptors at the cell surface detect changes in the concentrations of attractants and generate shifts in the level of phosphorylation of diffusible signaling proteins CheY2 and PilG. Phosphorylated CheY2 and PilG modulate the extension and retraction of Tfp, thus affecting twitching behavior of the cell. The entire signal transduction pathway includes chemotaxis proteins: PilL/ChpA is a kinase that phosphorylates the response regulators CheY2 and PilG and also the methylesterase ChpB; ChpC is an adaptor protein coupling PilL/ChpA to MCPs (PilJ); ChpB, a methylesterase, mediates adaptation to a constant attractant concentration by adjusting the methylation level of receptors.

Production of monoclonal antibodies against *Xf*. Monoclonal antibodies (MAb's) will be used for further characterization of *Xf* pili. In addition, they may be useful in inhibition of migration and colonization of *Xf* in vitro and in planta, and in development of a field-applicable diagnostic biodetection sensor with enhanced sensitivity and speed of detection. We have thus proceeded to produce MAb's toward various surface proteins of *Xf*. Thus far, mice have been immunized with whole live cells of *Xf*, and they demonstrate excellent immunogenic reactions with the blood serum (both by ELISA and by immunocytochemistry—see Figure 7). We are scheduled to screen for MAb producing cell lines during October-November 2007.

CONCLUSIONS

We provide evidence that the *Xf* proteins, PilL/ChpA and CheY2 are part of a complex chemosensory system that controls type IV pilus-mediated motility in *Xf*. This predicted chemosensory system contains many modules that are similar to the chp system of P. aeurginosa (PilG-ChpC). The CheY mutant possesses Tfp but does not twitch again suggesting its role in the signal transduction pathways that control TM. Indeed, time-lapse video microscopy suggests that the CheY2 has lower rate of cellular movements and abnormal frequency of cellular reversals during twitching motility. The in silico study of CheY2 predict the conformational structure would confer the capacity to function as a phosphoacceptor in the cascade of signal trasnduction in TM leading ultimately to the movement of extension and retraction of the Tfp. We also have observed reduced biofilm formation by the mutant and an increase in the biofilm formation following complementation. This outcome may result from an overexpression of CheY2 possibly interfering not only with cell movement and direction but also cellular attachment. Within the vicinity of pilG-chpC cluster we have identified another gene tonB3, that also affects the TM in *Xf* (Figure 2B); it may be involved in some aspect of the transport and secretion of pili subunits or of a component required for their formation. TonB is known to provide energy to efflux systems for export molecules out of the cell (14). Further research is required to understand the role of *Xf* tonB3 in TM. As our knowledge of *Xf* Tfp production, regulation and functioning advances, the coordinate regulation of Tfp as a virulence factor is being elucidated. An understanding of how this regulation occurs is central to determining how to counter movement and plant colonization. The targeting of genes

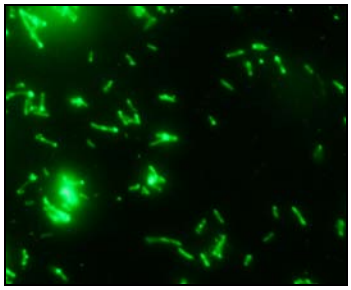


Figure 7. Immunocytochemical detection of *Xf* surface antigens with mice blood serum. Cell surfaces and, in many instances, a 'bright' polar spot (= type I pilus domain) are detected.

required for pathogenicity including colonization may be an effective strategy. The examination of the role of other genes from pilG-chpC cluster and their relation to TM is a crucial step in this process. The production of monoclonal antibodies against *Xf* pili will certainly help contribute to this step as well as to the development of diagnostic tools and eventually for the development of novel controls for Pierce's Disease.

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