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Xylella fastidiosa pil-chp operon is involved in regulating key structural genes of both type I and IV pili

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Summary

***Xylella fastidiosa* is the causal agent of Pierce's disease (PD) in grapevines. It has type I and type IV pili, which are both virulence factors involved in the PD-associated processes of motility, aggregation, and biofilm formation. Many questions remain as to how the two pili are regulated. We previously identified a *X. fastidiosa pil-chp* chemosensory-like cluster as an operon composed of genes *pilG-I-J-L-chpB-C*. In this study, we deleted *pilG* (resulting in a Δ*pilG-I* strain) and *pilJ* and discovered that both mutants (Δ*pilG-I* and Δ*pilJ*) had reduced virulence after 24 weeks post-inoculation, whereas Δ*chpB* and Δ*chpC* did not. Both Δ*pilG-I* and Δ*pilJ* lost motility and were impaired in biofilm formation in rich artificial media and xylem sap. Gene expression was significantly downregulated for representative fimbrial adhesin and motility genes in Δ*pilG-I*, and to a lesser extent in Δ*pilJ*. Our data suggest that Pil, but not Chp, proteins are virulence factors, and *pilG-I-J* are involved in transcriptional regulation of type I and IV pili virulence genes and therefore motility and biofilm formation. To our knowledge, this is the first report of a chemotaxis-like operon involved in the regulation of key structural genes of both type I and type IV pili.**

Key words: *Xylella fastidiosa*, *pil-chp*, Pierce's disease, PilG, PilJ, type I pili, type IV pili.

Introduction

Xylella fastidiosa is a Gram-negative, non-flagellated bacterium that causes economically devastating diseases in plants such as citrus, almond, and coffee, and causes Pierce's disease (PD) in grapevines (Hopkins and Purcell 2002). Sharpshooter vectors inject *X. fastidiosa* into host plants' xylem where the bacteria are proposed to migrate, aggregate, and form biofilms, that prevent movement of water and nutrients and induce disease (Chatterjee *et al.* 2008a). While disease is a complex process that may also involve bacterial secreted effector proteins (Lee *et al.* 2014, Zhang *et al.* 2015, Nascimento *et al.* 2016) and parameters impacting host susceptibility (Newman *et al.* 2003, Leite *et al.* 2004, Krivanek *et al.* 2006, Andersen *et al.* 2007, Riaz *et al.* 2008, Cheng *et al.* 2009, Basha *et al.* 2010, Sun *et al.* 2011, Yang *et al.* 2011, Shi *et al.* 2013, Sun *et al.* 2013), inhibiting migration and biofilm formation alters the progression of PD (Cursino *et al.* 2009, 2011, 2015, Shi *et al.* 2009, Matsumoto *et al.* 2012, Killiny *et al.* 2013). Two key players in motility and biofilm development are the *X. fastidiosa* pili. The bacteria express both type I and type IV pili (Meng *et al.* 2005, de la Fuente *et al.* 2007); type I pili are short filamentous proteins protruding from the cell surface that are involved in cell-to-cell aggregation and biofilm formation (Feil *et al.* 2007, Meng *et al.* 2005), while type IV pili are long filaments that extend and retract from one cell pole to facilitate twitching motility (Meng *et al.* 2005, Mattick 2002). We have previously reported that *X. fastidiosa* motility is regulated by a *pil-chp* chemotaxis-like cluster that is an operon (Cursino *et al.* 2011).

Chemotaxis is a method by which motile organisms sense stimuli and move towards attractants or away from repellents (Parkinson *et al.* 2015). In the Gram-negative chemotaxis model system of *Escherichia coli*, transmembrane chemoreceptors bind chemical stimuli in the periplasmic domain and undergo conformational changes in the cytoplasmic portion resulting in phosphorylation changes of the kinase, CheA (Parkinson *et al.* 2015). The CheA kinase associates with the chemoreceptors through a coupling protein, CheW. The shuttle protein CheY transfers the CheA phosphate to the flagella motor to change its rotation and thus the motion of the bacterium. The system adapts to changing ligand concentrations by undergoing methylation modifications at specific sites on the chemoreceptor cytoplasmic domain. CheR is the methyltransferase, and CheB, which is phosphorylated by CheA, acts as the methylesterase.

The *X. fastidiosa pil-chp* operon contains six genes in the following 5' to 3' order (with the homologous protein names in *E. coli* in parenthesis): *pilG* (CheY), *pilI* (CheW),

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pilJ (chemoreceptor), *pilL* (CheA-CheY fusion protein), *chpB* (CheB), and *chpC* (CheW). We previously created a *X. fastidiosa pil-chp* operon polar mutant disrupted in the last three genes (*pilL-chpB-chpC*) (CURSINO *et al.* 2011). The deletion resulted in loss of twitching motility, reduced biofilm formation, and less virulence in grapevines. Given the importance of the *pil-chp* operon to Pierce's disease, we examined additional genes in the operon, and now report that Pil, but not Chp, proteins are virulence factors, and *pilG-I-J* are involved in regulating both type I and type IV pili key structural gene expression at the transcriptional level, thereby regulating motility and biofilm formation.

Material and Methods

Sequences: Sequence comparisons were made *via* BLAST (ALTSCHUL *et al.* 1990). Sequence accession numbers were as follows: *Xylella fastidiosa* Temecula 1 (TM1) PilG (AAO02713), *X. fastidiosa* Temecula 1 PilI (AAO02714), *X. fastidiosa* Temecula 1 PilJ (AAO02715), *X. fastidiosa* Temecula 1 CheW (AAO28718), and *Escherichia coli* K-12 CheY (WP_061354929).

Strains, collection and storage of xylem sap and culture conditions: TM1 cells were maintained at 28 °C on PWG plates (Periwinkle Wilt plates with 8 $g \cdot L^{-1}$ gelrite) (DAVIS *et al.* 1981) modified by omitting phenol red and adding 25 $mL \cdot L^{-1}$ bovine serum albumin (BSA) fraction V solution (Life Technologies, Grand Island, NY, USA). All mutant strains were cultured similarly but with addition of 30 $\mu g \cdot L^{-1}$ of kanamycin respectively. *Vitis vinifera* sap (80 %) water agar plates were made by mixing 1 mL of autoclaved water agar (0.6 g agar in 10 mL of distilled water) with 4 mL of filter sterilized 100 % *V. vinifera* sap. All strains were stored at -80 °C in modified PD2 broth (DAVIS *et al.* 1981) containing 20 % glycerol. Grapevine xylem fluid was collected in early spring from bleeding *V. vinifera* vines grown in a research vineyard at New York State Agricultural Experiment Station in Geneva, NY. Sap collection and storage procedures were as previously reported (ZAINI *et al.* 2009). Sap was aliquoted and stored at -80 °C upon collection and was filter-sterilized prior to use with a 0.2 µm pore-membrane (Corning, NY).

Construction of the mutant strains: Δ*pilG* (PD0845), Δ*pilJ* (PD0847), Δ*chpB* (PD0849), and Δ*chpC* (PD0850) strains were generated with a double cross-over homologous recombination method as previously described (SHI *et al.* 2009, JOHNSON *et al.* 2015). Two different PCRs (polymerase chain reactions) amplified the 500 bp upstream and downstream flanking the sequence of each target gene with primers pilXA/chpXA and pilXB/chpXB (upstream) and pilXC/chpXC and pilXD/chpXD (downstream), including an *Asc*I recognition site in primers pilXB/chpXB and pilXC/chpXC (Table S1). The upstream and downstream fragments were mixed and further amplified by PCR with primers pilXA/chpXA and pilXD/chpXD to generate the final, mutagenized 1.0-kb fragment, which was cloned into pUC19 to make pUC19-*pilG*, pUC19-*pilJ*, pUC19-*chpB*, and pUC19-*chpC*. The resulting products were verified by PCR and DNA sequence analysis of the upstream and downstream regions (Cornell Core Sequencing Facility, Ithaca, NY, USA) and compared with the genomic sequences of TM1. A kanamycin cassette from pGEM-T-KM (Promega, Madison, WI, USA) was excised and cloned into the *Asc*I site of the 1.0-kb PCR fragment in pUC19-*pilG*, pUC19-*pilJ*, pUC19-*chpB*, and pUC19-*chpC*, and resulting strains were verified by PCR and sequencing. Transformation into *X. fastidiosa* was performed by electroporation as previously described (CURSINO *et al.* 2011) with modifications. Briefly, 2 µL of each construct and 0.5 µL of TypeOne restriction inhibitor (Epicentre, Madison, WI, USA) were added to 50 µL of *X. fastidiosa* electrocompetent cells and subjected to electroporation at 2.5 kV, 25 µF, and 200 Ω. The electrocompetent cells alone and PD2 broth with no bacterial cells served as negative controls. Electroporated cells were added to 1 mL fresh PD2 broth and incubated at 28 °C for 24 h. Transformants were selected on PW agar containing 10 $\mu g \cdot L^{-1}$ kanamycin for 7-10 d. The insertions of kanamycin in each target gene were confirmed and the DNA was sequenced at the Cornell Core Sequencing Facility.

Pathogenicity assay and *in planta* bacterial detection: Pathogenicity assays were performed using the PD-susceptible grape species, *V. vinifera* as described previously (MENG *et al.* 2005). Briefly, strains were grown on PW or PWG plates (with kanamycin for mutant strains) for approximately seven days, harvested separately, and five microliters of bacterial suspension in phosphate buffered saline solution (PBS) of approximately OD_{600} = 2.0 was pin-prick inoculated into the base of the shoot. PD development was recorded based on a 0-5 disease rating system as previously described (GUILHABERT and KIRKPATRICK 2005). Plants inoculated with buffer-only were included as the negative control. Six plants were included for each treatment in trial 1 and ten plants were included for each treatment for both trial 2 and 3 respectively.

The presence of *X. fastidiosa* was determined by a commercially available ELISA (enzyme linked immunosorbent assay) test (Agdia, Elkhart, IN, USA). Briefly, petioles at 0, 30, and 150 cm above the inoculation point from inoculated vines were sampled and processed following the manufacture's instruction. *X. fastidiosa* TM1 cells collected from PWG plates were used as positive controls and petioles from un-inoculated vines were negative controls.

Motility and biofilm assays: The motility assay on rich media plate was performed as previously described (MENG *et al.* 2005). Briefly, five-day-old TM1, Δ*pilG-I* or Δ*pilJ* grown on PWG, or PWG plates with kanamycin were collected separately with a 1 µL sterile loop (Fisher Scientific, Waltham, MA, USA), carefully spotted onto PWG minus BSA plates without dragging the loop, and incubated at 28 ºC. At day one and five post-inoculation (p.i.), colonies were observed under an Olympus SZX12 dissecting microscope (Tokyo, Japan) for the presence of a peripheral fringe as previously described (MENG *et al.* 2005). Pictures were taken with a Nikon camera using software NIS-elements F 4.00.00 with 90X magnification (Tokyo, Japan). The motility assay on sap agar plate was done similarly as described above, except cells were

spotted onto 80 % *V. vinifera* sap water agar plates and observed with a Stemi 200-C dissection microscope with 4.5 X magnification (Zeiss, Oberkochen, Germany), and pictures were taken with an Infinity 2 Lumenera camera using software from the same company (Lumenera, Ottawa, ON, Canada). At least three replicates from each strain were included on each plate, and three plates were included for each assay. The experiment was repeated three times.

Biofilm quantification in PD2 broth was done as previously described in 96-well plates (Zaini *et al.* 2009). Twenty-four replicates were included for each treatment, and the assay was repeated three times. Biofilm quantification in 100 % sap was done in a similar way, except cells from each strain were acclimated to 100 % *V. vinifera* sap, as described previously (Zaini *et al.* 2009), before being inoculated into glass tubes (same inoculum as the assay with PD2 broth, except in a total volume of 2 mL of sap per tube). At least five replicates were included for each treatment, and the assay was repeated twice.

Gene expression analysis: For gene expression analysis in the Table, bacteria were inoculated into 30 mL of PD2 broth to an OD_{600} of ~0.013 and incubated at 28 °C, 185 rpm. After 3 d of inoculation, cells were collected for RNA extraction as previously described (Hao *et al.* 2012). Total RNA was evaluated using the Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) to ensure good quality. Contaminating genomic DNA was removed with Turbo DNA-free with the stringent protocol as instructed (Fisher Scientific) and confirmed by performing real-time RT PCR (qPCR) using one-step universal supermix (Bio-Rad Laboratories) without adding reverse transcriptase. RNA samples with yield quantification cycle (Cq) values larger than 32 were judged to be sufficiently free of contaminating DNA for further analysis. Primer amplification efficiencies were determined using a serial dilution of *X. fastidiosa* genomic DNA.

Suitable internal reference gene primers with efficiencies close to 100% and stable expression within TM1 and mutant samples were chosen (Table S1). The stability of reference transcripts was validated using the BestKeeper program (Pfaffl *et al.* 2004). We evaluated five *X. fastidiosa* genes [*dnaQ* (PD1217), *nuoA* (PD0248), *petC* (PD1775), *dnaE* (PD0165), *mreD* (PD0559)] and 2 of them, *nuoA* and *petC* were used as reference genes to normalize target gene expression.

qRT-PCR (quantitative RT-PCR) was performed with 150 ng RNA per reaction and one-step universal supermix (Bio-Rad Laboratories) using the Bio-Rad-CFX PCR detection System, and the results were analyzed using the Bio-Rad-CFX manager (Bio-Rad Laboratories). The mean Cq of each target transcript was normalized to the Cq of the reference transcripts using the following formula: $2^{q\text{ internal standard} - Cq\text{ target}}$. Two biological replicates were included for each experiment and each experiment was repeated at least two times.

For RT-PCR assay in Fig. S2, TM1, Δ*pilG*, or Δ*pilJ* cells grown for ~ 7 d on PWG plates were collected for RNA extraction and contaminating DNA was removed as described above. First strand of cDNA was synthesized with the SuperScript III first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and subsequent PCR was performed with Go Taq Flexi PCR kit (New England Biolabs, Ipswich, MA) as instructed using each cDNA template combined with the following internal primers for detection of *pilG*, *pilI*, *pilJ* and *pilL* expression respectively (Table S1). Seven microliters of the PCR products were analyzed with gel (1 %) electrophoresis and visualized with Gel Doc™ XR (Bio-Rad Laboratories) with Image Lab™ software. At least two RNA samples per mutant strain independently extracted from different batches of cells were analyzed.

Statistical analysis: All statistical analyses were performed using the R program. Student's t-test was performed for pathogenicity assays, while post hoc comparisons between the treatments were performed for other assays using Tukey's HSD (honest significance difference) to correct for multiple comparisons. A residual analysis was performed to assess the validity of the assumptions of normality and homogeneous variances. Gene expression data were log transformed to be qualified for the assumptions of normality and homogeneous variances. A *p*-value less than 0.05 was considered to be significant.

Table

Fold of gene expression changes in Δ*pilG-I* and Δ*pilJ* relative to TM1

Strains [a]	Genes[b]			
	pilA	*fimA*	*hxfB*	*xadA*
Δ*pilG-I*	0.01 ± 0.00[c]	0.09 ± 0.05[c]	0.29 ± 0.42[c]	51.94 ± 28.22[c]
Δ*pilJ*	0.33 ± 0.39[c]	0.46 ± 0.24[c]	0.59 ± 0.64	1.13 ± 0.58

[a] Shown were fold of expression changes relative to TM1 by real-time RT-PCR. TM1= *X. fastidiosa* wild-type strain Temecula 1; Δ*pilG-I* = TM1 deleted of *pilG* and also lacking expression of *pilI*; Δ*pilJ* = TM1 deleted of *pilJ*.

[b] To normalize target gene expression, reference genes were *nuoA* (encodes NADH dehydrogenase subunit A) and *petC* (encodes ubiquinol cytochrome C oxidoreductase cytochrome C1 subunit). Two biological replicates were included for each treatment and the experiment was repeated at least twice.

[c] Represents a significance change in expression with $p < 0.05$ compared to that of TM1 by Tukey's HSD test.

Results and Discussion

We analyzed the annotated proteins sequences of PilG, PilI, and PilJ in order to make predictions concerning their structure and/or function; PilL, ChpB, and ChpC were detailed previously (CURSINO *et al.* 2009). When PilG was aligned with *E. coli* CheY, the PilG Asp64 residue corresponded to the highly conserved CheY Asp57, which is a phospho-accepting residue that is necessary for signal transduction (Fig. S1) (SANDERS *et al.* 1989), suggesting that PilG may be a functional protein. The PilI protein is predicted to be a 176 amino acid CheW homologous protein (VAN SLUYS *et al.* 2003). While the *X. fastidiosa pil-chp* operon is also predicted to have a second C-terminal *cheW* gene, *chpC* (CURSINO *et al.* 2011), multiple CheW proteins in a chemotaxis system are not unusual (WUICHET and ZHULIN 2010).

The PilJ protein is predicted be a putative transmembrane chemoreceptor with a large 269 amino acid periplasmic domain (VAN SLUYS *et al.* 2003) that would be sufficient for ligand binding. The *X. fastidiosa* PilJ was previously classified as belonging to the 40H chemoreceptor class, which have a cytoplasmic alpha-helical structure with 40 amino acid heptad repeats and lack methylation sites for CheB/CheR modification (ALEXANDER and ZHULIN 2007). This classification parallels our findings in that the PilJ protein lacks a C-terminal CheB/CheR docking site (WU *et al.* 1996, BARNAKOVA *et al.* 1999), the *pil-chp* operon does not contain a *cheR* gene, and the *chpB* is predicted to encode a non-functional protein (CURSINO *et al.* 2011).

To examine the role of additional *pil-chp* operon genes in *X. fastidiosa* virulence, we constructed gene deletion mutants of *pilG* and *pilJ*, as within chemotaxis proteins, chemoreceptors (PilJ) function as the initial signal input protein and CheY proteins (PilG) function as the final output protein that phosphorylates its target proteins (PARKINSON *et al.* 2015). We also made single gene deletion mutants of *chpB* and *chpC* to learn if the two *chp* genes contributed to the disease phenotype from the previous *pilL-chpB-chpC* polar mutant (CURSINO *et al.* 2011).

We examined the ability of these mutants to cause PD on grapevines. In trial 1 in year 1, at 25 weeks post inoculation (w.p.i.), 100 % of Δ*chpB* and Δ*chpC* inoculated plants developed severe to full PD symptoms (GUILHABERT and KIRKPATRICK 2005), similar to those of the wild-type *X. fastidiosa* Temecula 1 (TM1) treatment, in which 100 % of plants developed full PD symptoms (Fig. 1). In contrast, all Δ*pilG* and Δ*pilJ* inoculated plants developed some levels of PD; however, the disease severity was much lower at week 25 than that of the TM1 treatment, with an average disease rating being 2.3 ± 0.6, and 1.8 ± 0.3, respectively ($p < 0.01$).

Because the *pilG* and *pilJ* genes are in an operon, and therefore mutant constructs could have a polar effect, we examined the expression of the *pilG*, *pilI*, *pilJ,* and *pilL* genes in the Δ*pilG* and Δ*pilJ* mutants by reverse transcriptase polymerase chain reaction (RT-PCR). As shown in Fig. S2, *pilG* was not expressed in Δ*pilG* as expected; however, the *pilI* gene was also not expressed. Meanwhile, *pilJ* and *pilL* were expressed in Δ*pilG*. These findings suggest that deletion of *pilG* caused a polar effect affecting downstream *pilI* expression. Therefore, we will subsequently refer to Δ*pilG* as Δ*pilG-I*. On the other hand, the *pilJ* deletion did not result in a polar mutation as the adjacent *pilI* and *pilL* genes were both expressed in Δ*pilJ*.

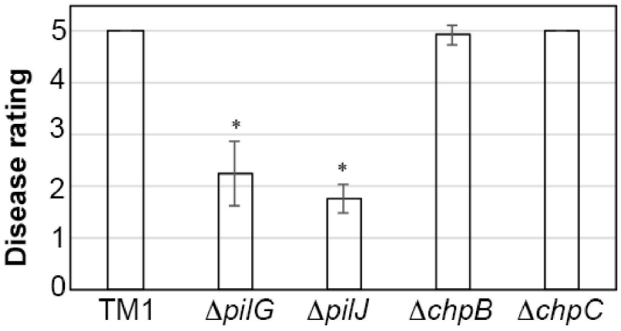

Fig. 1: Virulence of TM1, Δ*pilG*, Δ*pilJ*, Δ*chpB* and Δ*chpC* in grapevines in trial 1 at 25-weeks post-inoculation. Shown are mean disease ratings of vines inoculated with TM1 (wild-type), Δ*pilG*, Δ*pilJ*, Δ*chpB,* and Δ*chpC* respectively. A 0-5 scale disease rating system was used as described previously (GUILHABERT and KIRKATRICK 2005). A total of 6 plants were included in each treatment. Error bars were standard deviations. Plants in the negative control group (buffer-inoculated) remained healthy throughout the experiment. TM1= *X. fastidiosa* wild-type strain Temecula 1; Δ*pilG* = TM1 deleted of *pilG*; Δ*pilJ* = TM1 deleted of *pilJ*; Δ*chpB* = TM1 deleted of *chpB*; Δ*chpC* = TM1 deleted of Δ*chpC*. * represents a $p < 0.01$ compared to TM1 by student's t-test.

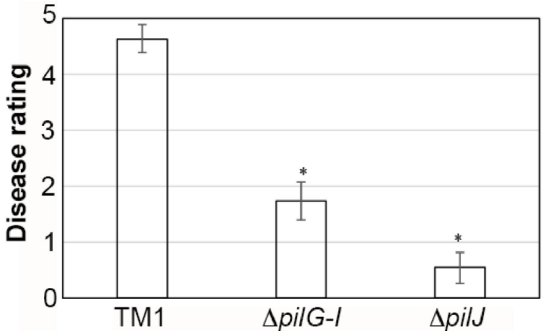

Fig. 2: Virulence of TM1, Δ*pilG-I*, and Δ*pilJ* in grapevines in trial 2 and 3 at 24-weeks post-inoculation. Shown are mean disease ratings of vines inoculated with TM1, Δ*pilG-I*, and Δ*pilJ* respectively. A 0-5 scale disease rating system was used as described previously (GUILHABERT and KIRKATRICK 2005). A total of 20 plants were included in each treatment (10 per trial). Error bars were standard errors. Plants in the negative control group (buffer-inoculated) remained healthy throughout the experiment. TM1= *X. fastidiosa* wild-type strain Temecula 1; Δ*pilG-I* = TM1 deleted of *pilG* and also lacking expression of *pilI*; Δ*pilJ* = TM1 deleted of *pilJ*. * represents a $p < 0.01$ compared to TM1 by student's t-test.

For trials 2 and 3 in year 2 we focused on Δ*pilG-I* and Δ*pilJ*, as Δ*chpB* and Δ*chpC* had shown the same disease outcome as TM1-inoculated vines. Two additional trials of pathogenicity assays were performed in the second year with Δ*pilG-I* and Δ*pilJ*, and the results were similar to that of trial 1 in that both mutants were significantly defective in virulence compared to the TM1 strain at 24 w.p.i. ($p < 0.01$) (Fig. 2). Petioles close to the symptomatic leaves from each treatment were collected at the end of the experiments and

the presence of *X. fastidiosa* was confirmed by ELISA (data not shown) (Hao *et al.* 2016). Therefore, three independent pathogenicity assays performed in two different years demonstrated that PilG-I and PilJ are virulence factors of *X. fastidiosa* required for full PD development.

Next we examined PD-associated twitching motility and biofilm formation. Twitching motility was assessed by the development of a colony fringe on agar plates (Meng *et al.* 2005); colonies from both mutants lacked a fringe on PW without BSA (Periwinkle Wilt without Bovine Serum Albumin) (Davis *et al.* 1981) plates (Fig. 3A). These results indicate the loss of motility, as observed in previous reports (Shi and Lin 2016). In PD2 broth (Pierce's disease 2) (Davis *et al.* 1981), Δ*pilG-I* and Δ*pilJ* produced ~8.7 and ~4.7 fold less biofilm compared to that of TM1 (Fig. 3B). Together these data indicate that both *pilG-I* and *pilJ* are involved in regulating motility and biofilm formation in a rich media environment. We also examined the above phenotypes in grapevine sap, to more closely mimic the *in planta* biochemical environment. On *Vitis vinifera* sap (80 %)-water agar plates, TM1 colonies formed typical fringes (Fig. 3C), representing outward movement of bacterial cells on the edge of the colonies. In contrast, Δ*pilG-I* and Δ*pilJ* colonies had smooth colony, margins indicating the loss of motility. In addition, after seven days of incubation in 100 % sap medium following acclimation, Δ*pilG-I* and Δ*pilJ* produced ~3.2 and ~1.5 fold less biofilm than that of the TM1 cells (Fig. 3D). The phenotypic consistency of Δ*pilG-I* and Δ*pilJ* between nutrient rich (PD2) and poor (sap) media (Bove and

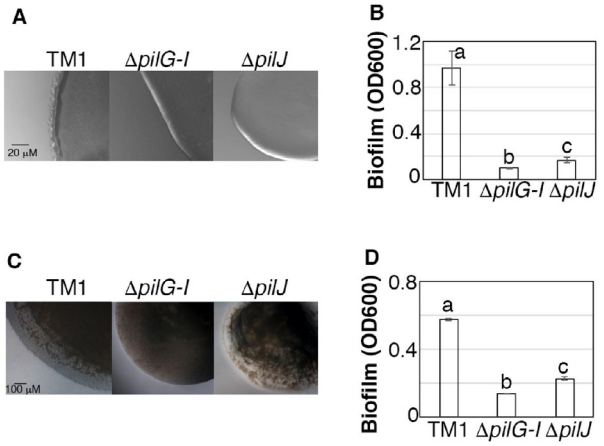

Fig. 3: Motility and biofilm formation of TM1, Δ*pilG-I*, and Δ*pilJ* in different media. **A**) Colony fringes on PW plates (without BSA) 5 days post-inoculation (d.p.i.). **B**) Mean biofilm quantification 5 d.p.i in PD 2 broth in 96-well plates. **C**) Colony fringes on 80 % *V. vinifera* sap agar plates 5 d.p.i. **D**) Mean biofilm formation 7 d.p.i in 100 % *V. vinifera* sap in glass tubes. For A and C, cells from each strain were spot inoculated at least three times on each plate, and three plates were included for each experiment and all experiments were repeated three times. For B and D, all error bars are standard deviations. Twenty-four replicates were included for each strain in B and the experiment was repeated three times. Five replicates were included for each strain in D and the experiment was repeated twice. Different non-capitalized letters represent a significant difference with $p<0.01$ using Tukey's HSD test. TM1= *X. fastidiosa* wild-type strain Temecula 1; Δ*pilG-I* = TM1 deleted of *pilG* and also lacking expression of *pilI*; Δ*pilJ* = TM1 deleted of *pilJ*.

Garnier 2002) implies that the involvement of these genes in regulating motility and biofilm formation in *X. fastidiosa* is likely to be independent of nutrient status of the medium.

We observed that in PD2 broth Δ*pilG-I* exhibited planktonic growth and formed little biofilm (Fig. S3), suggesting an impairment in cell-to-cell aggregation and/or cell-to-surface attachment in Δ*pilG-I*, which could be due to altered expression of the fimbrial type I pili structural gene *fimA* (Meng *et al.* 2005) and/or afimbrial adhesion genes (such as *hxfB*) (Guilhabert and Kirkpatrick 2005, Feil *et al.* 2007). Therefore, we examined the expression of genes known to be associated with aggregation and attachment via real-time RT-PCR. The expression of *fimA* was reduced in both mutants compared to that of TM1 (Table). The afimbrial adhesion gene *hxfB* was downregulated in Δ*pilG-I* and remained unchanged in Δ*pilJ*. We also examined the expression of *pilA* in Δ*pilG-I* and Δ*pilJ*; the *pilA* gene is predicted to encode the major filamentous structural protein of *X. fastidiosa* type IV pili (Da Silva Neto *et al.*, 2008), and deletion of *pilA* leads to a non-motile phenotype *in vitro* (unpublished data). Compared to TM1, *pilA* was downregulated in Δ*pilG-I* and Δ*pilJ*. Together these data indicate that *pilG-I*, and to a lesser extent *pilJ*, are involved in transcriptional regulation of the key structural genes associated with both type I (*fimA*) and type IV (*pilA*) pili biosynthesis and a non-fimbrial adhesion gene (*hxfB*), thereby regulating the virulence responses of twitching motility, aggregation, and biofilm formation.

We also assayed afimbrial adhesion gene *xadA*, which encodes an outer membrane protein secreted as vesicles to coat the xylem vessel surfaces thus to reduce bacterial adherence (Feil *et al.* 2007, Ionescu *et al.* 2014). Interestingly, *xadA* was up-regulated almost 50 fold in Δ*pilG-I* compared to that of TM1; it remained the same in Δ*pilJ* (Table). Thus it is possible that increased production of outer membrane vesicles reduced bacterial attachment and subsequent biofilm formation and therefore lead to less disease. Further studies, such as examination of outer membrane vesicles production in Δ*pilG-I*, may help to explain this phenotype.

In this study, we found that disruption of *pilG-I* or *pilJ* caused significant less virulence in *X. fastidiosa*. The single deletion of the genes downstream of *pilL* (*chpB* and *chpC*), which are the last two genes within the *pil-chp* operon, did not affect *X. fastidiosa* virulence. Whether the deletion of *chpB* caused a polar effect on the expression of *chpC* was not explored. However, the fact that Δ*chpC* displayed wild-type like virulence confirmed that both Chp proteins are not virulence factors in *X. fastidiosa*. Thus the previous finding that the polar Δ*pilL-chpB-chpC* mutant has reduced PD (Cursino *et al.* 2011) most likely relies on the interruption of *pilL* but not *chpB* or *chpC*. Although we did not identify a role of the Chp proteins in grapevines, whether they play a role in transmission by the sharpshooter vector is an area for future study. Thus future examination of other phenotypes, such as attachment or biofilm formation, of these two mutants would provide interesting information.

Chemoreceptor-mediated signaling pathways have been reported to play roles not just in motility, but also in processes such as pathogenicity, biofilm formation, and transcriptional regulation (Kirby 2009, Wadhams and Armitage,

2004, HE and BAUER 2014). To our knowledge, this is the first report of a chemotaxis-like operon being involved in the regulation of key structural genes for both type I and type IV pili. Based on genome sequences *X. fastidiosa* has a single chemoreceptor and chemotaxis-like operon, which may result from an adaptation to a highly specific niche within the vector and plant xylem vessels. Thus the single chemotaxis-like operon may have evolved to directly or indirectly regulate multiple cellular behaviors involved in virulence. Our data from motility and biofilm formation assays support this hypothesis, as both Δ*pilG-I* and Δ*pilJ* are impaired in motility, aggregation, and biofilm formation, which can be explained by downregulation of fimbrial (*pilA* and *fimA*) and *hxfB* afimbrial gene with upregulation of *xadA*. We acknowledge that many other genes in *X. fastidiosa* are also known to play important roles in either motility or biofilm formation such as those also involved in type IV pili biosynthesis, exo- or lipopolysaccharide production (CRUZ and PARKER *et al.* 2014, KILLINY *et al.* 2013, CLIFFORD *et al.* 2013). Future investigation of whether such genes might be regulated by the *pil-chp* operon will help to further understand its genetic function. Although the importance of motility, aggregation, and biofilm formation in *X. fastidiosa* virulence is well recognized, how the type I and type IV pili genes are regulated has remained unknown. *X. fastidiosa* is known to produce quorum sensing molecules, diffusible signaling factors (DSF), to regulate diverse cellular behaviors. Low concentration of DSF favors motility, while high concentrations promote aggregation, biofilm formation, and insect transmission (DE SOUZA *et al.* 2013, CHATTERJEE *et al.* 2008b, NEWMAN *et al.* 2004). Preventing DSF production leads to up-regulation of motility genes and down-regulation of adhesion genes (DE SOUA *et al.* 2013, ALMEIDA *et al.* 2012, WANG *et al.* 2012). Related, in the homologous *P. aeruginosa pil-chp* operon PilJ binds the type IV pilus PilA proteins leading to a phosphorylation cascade that alters motility and induces cAMP-dependent upregulation of virulence factor genes (FULCHER *et al.* 2010, LEIGHTON *et al.* 2015, LUO *et al.* 2015, PERSAT *et al.* 2015, INCLAN *et al.* 2016, JANSARI *et al.* 2016). In other bacteria cAMP is known to regulate type I pili or fimbriae (KALIVODA *et al.* 2008, MÜLLER *et al.* 2009). Whether *X. fastidiosa* type I pili are regulated in a cAMP-dependent manner, cAMP regulation is connected to type IV pili gene expression, or the *pil-chp* operon regulates pili gene expression in a DSF-dependent manner warrants further research.

Conclusion

In summary, we report that the *X. fastidiosa pil-chp* chemotaxis-like operon is involved in regulating key structural genes involved in both type I and type IV pili biosynthesis at the transcriptional level along with other important virulence afimbrial genes. Our data suggest a model in which the *pil* genes positively impact expression of *pilA* (type IV pili), *fimA* (type I pili), and *hxfB* (afimbrial adhesion), and negatively impact expression of *xadA* (outer membrane vesicles); all of these virulence genes play a role in disease development by modulating *X. fastidiosa* motility, aggregation, and biofilm formation. Whether the operon is also involved in regulating the two pili at the translational level needs future experimentation, such as with scanned electron microscopy or Western blot analysis. It has been previously shown that the type I and type IV pili modulate each other; deletion of type I pili increases type IV-driven motility and deletion of type IV pili increases type I-associated biofilm formation (LI *et al.* 2007). Therefore, having the operon regulate both pili may allow for optimal behaviors for survival in plants and/or the vector. Understanding the mechanisms for how the *pil-chp* operon regulates both pili types will help to identify key genes and steps involved in PD and to develop potential targets for disease management.

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