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The chemotaxis regulator *pilG* of *Xylella fastidiosa* is required for virulence in *Vitis vinifera* grapevines

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Abstract Type IV pili of *X. fastidiosa* are regulated by pilG, a response regulator protein putatively involved in chemotaxis-like operon sensing stimuli through signal transduction pathways. To elucidate the roles of *pilG* in pathogenicity of *X. fastidiosa*, the *pilG*-deletion mutant *XfΔpilG* and complemented strain *XfΔpilG*-C were generated. While all strains had similar growth curves in vitro, *XfΔpliG* showed significant reduction in cell-matrix adherence and biofilm production compared with wild-type *X. fastidiosa* and *XfΔpilG*-C. The genes *pilE, pilU, pilT*, and *pilS* were down-regulated in *XfΔpliG* when compared with its complemented strain and wild-type *X. fastidiosa*. Finally, no Pierce's disease symptoms were observed in grapevines inoculated with *XfΔpilG,* whereas grapevines inoculated with the wild-type *X. fastidiosa* and complemented strain of *XfΔpilG*-C developed typical Pierce's Disease (PD) symptoms. The results indicate that *pilG* has a role in *X. fastidiosa* virulence in grapevines.

Keywords *Xylella fastidiosa* · *pilG* · Pathogenicity · Type IV pilus · Twitching motility

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Introduction

Xylella fastidiosa is an important pathogenic bacterium that causes a number of economically important diseases, including citrus variegated chlorosis (CVC) in South America (Chang et al. 1993; Hartung et al. 1994) and Pierce's disease of grapevines (PD) in North America (Purcell and Hopkins 1996; Purcell 1997). *X. fastidiosa* is a Gram-negative non-flagellated bacterium and limited to the water-conducting xylem vessels. PD results in the blockage of xylem vessels, water stress and nutritional deficiencies (Hopkins 1989). The twitching motility of *X. fastidiosa*, a means of flagellum-independent bacterial movement through extension, attachment and retraction of the polar type IV pili (Mattick 2002), has been microscopically characterized in a fabricated microfluidic chambers (Li et al. 2007; Meng et al. 2005). The colonization of xylem vessels is dependent on the ability of *X. fastidiosa* to move within xylem vessels (Meng et al. 2005). The *pilB*, *pilQ*, and *pilR* mutants resulting in the defect of type IV pili and non-twitching phenotypes showed reduced disease symptoms in grapevines (Li et al. 2007; Meng et al. 2005). These suggest that twitching motility provides *X. fastidiosa* not only a means for long-distance intra-plant movement and colonization but also contributes toward virulence.

X. fastidiosa type IV pili possess major structural protein (PilA) and minor proteins involved in formation of the base and/or tip of the pilus (PilE, PilV, and FimT). In addition, proteins, PilB, PilC, PilQ, PilT and PilU are required for pilus assembly and retraction (Mattick 2002;

Simpson et al. 2000). The transcription of *pilA* was regulated by *pilR*, a response regulator in a two-component sensor-regulator pair pilS/pilR system (Winther-Larsen and Koomey 2002). The activity of twitching motility of *X. fastidiosa* was controlled by a chemotaxis-like regulatory system (Cursino et al. 2011), Pil-Chp operon, similar to that in *P. aeruginosa* and *E. coli* (Ferandez et al. 2002; Fulcher et al. 2010; Hazelbauer et al. 2008; Kirby 2009). Like *P. aeruginosa* CheIV (Pil-Chp) cluster (Ferandez et al. 2002; Fulcher et al. 2010), *X. fastidiosa* possesses a single predicated chemosensory system, Pil-Chp operon that regulates the twitching motility of type IV pili (Fulcher et al. 2010; Simpson et al. 2000). Pil-Chp operon of *X. fastidiosa* encodes proteins involved in signal transduction pathways including *pilG, pilI, pilJ, pilL, chpB* and *chpC* as in *P. aeruginosa* and *E. coli* (Cursino et al. 2011; Fulcher et al. 2010; Kirby 2009). Upon binding of the chemical stimuli in the periplasmic domain, the transmembrane chemoreceptors activate a signaling cascade in the cytoplasmic portion and ultimately control bacterial twitching motility (Cursino et al. 2011). A phospho-shuttle protein PilG in Pil-Chp operon of *X. fastidiosa* is homologous to CheY, a response regulator in chemotaxis systems of *E. coli* and *P. aeruginosa*, in which CheY interacts with the flagellar motor proteins (Ferandez et al. 2002; Fulcher et al. 2010). Recent studies indicated that the homologue of chemotaxis regulator, PilG is required for the twitching motility of *X. fastidiosa* since the deleted *pilG X. fastidiosa* strain was deficient in twitching motility (Shi and Lin 2016).

The critical roles of the Pil-Chp operon in the virulence in *X. fastidiosa* were examined recently (Cursino et al. 2011). However, the contributions of *pilG* in Pil-Chp chemotaxis operon toward the pathogenicity of *X. fastidiosa* are not clear. In this study, the functional roles of chemotaxis regulator PilG involving in biofilm, cell adherence and pathogenicity are discussed.

Materials and methods

Bacterial strains

Bacterial strains of *E. coli* and *X. fastidiosa* Temecula (Costa et al. 2004) used in this work are listed in Table 1. *X. fastidiosa* Temecula strain and derivatives were cultured at 28 °C on solid PD2 medium (Davis et al. 1981). When required, PD2 medium was supplemented with 10 μg/ml gentamicin (Gm) and 10 μg/ml chloramphenicol (Cm).

Sequence analysis and domain identifications

Complete genome sequences of *P. aeruginosa* PAO1(AE004091.2), *Xanthomonas citris* (GCA_001498875.1) and *X. fastidiosa* Temecula (NC_004556.1) were obtained from NCBI GenBank. Type IV pilin-related gene homologous analysis was performed with blastn, blastp and tblastn, using *X. fastidiosa* Temecula genome sequence data as a query against the reference genomes. Functional domains were predicted and identified through Conserved Domain Database PROSITE (Sigrist et al. 2005).

Construction of *XfΔpilG* mutant and *XfΔpilG*-C complemented strain

An *XfΔpilG* mutant of *X. fastidiosa* was generated by deleting *pilG* ORF using a double-crossover homologous recombination strategy as described previously (Shi et al. 2007). In brief, two PCRs were performed to generate DNA fragments to the left side (primers pilGA and pilGB) and the right side (primers pliGC and pilGD) of the *pilG* ORF (PD0845) (Table 1). This two PCR strategy ensures that the entire ORF of *pilG* is precisely replaced with Gm cassette without disrupting downstream operon genes. Five μl of the left and the right of amplified DNA fragments were then purified, mixed and denatured at 95 °C for 5 min. Double strands were annealed at overlapping barcode regions in primers pilGB and pilGC (indicated with italics in Table 1) at 25 °C for 10 min. The mixture was then amplified by PCR with a pair of primers pilGA and pilGD to generate a 1.159-kb fragment, which was then cloned into the pGEM-T Easy (Promega, WI) to make pUC0845 (Table 1). A Gm cassette from the pGEM-T-GM (Table 1) was excised and cloned into *AscI* site of a 1.159-kb PCR fragment in pUC0845, resulting in the mutant construct pUC08451 (Table 1). About two micrograms of pUC08451 DNA in a volume of 10 μl was electroporated into electrocompetent cells of *X. fastidiosa* Temecula under the conditions described previously (Shi et al. 2007). Electroporated cells were then incubated in PD2 broth on a shaker at 200 rpm for 24 h and followed by plating cells on PD2 agar medium supplemented with 10 μg/ml Gm. Gm-resistant clones that grew on selective media were identified as potential *XfΔpilG* mutant strains. The

Table 1 Bacterial strains, plasmids, and primers used in this study

Strains	Descriptions	Reference
Escherichia coli DH5	DH1 F$^-$ Φ80*ΔlacZ*ΔM15*Δ*(*lacZYA-argF*)*U169*	
X. fastidiosa(Xf)		
Temecula	*Xf* wild type	
XfΔpilG	Gentamicin (Gm) cassette replacing entire *pilG* ORF (*ΔpilG*::Gm)	This work
XfΔpilG-C	Gm^{r}Cm^r; a fragment including chloramphenicol (Cm) cassette and the *pilG* promoter and ORF of *Xf* insert the chromosome of *XfΔpilG*	This work
Plasmids		
pGEM-T Easy	Ap^r; cloning vector	Promega
pBBR1MCS-5	Gm^r; broad-range plasmid	Kovach et al. 1995
pGEM-T-GM	Ap^r Gm^r; Gm cassette from pBBR1MCS-5 cloned into pGEM-T	This work
pUC0845	Ap^r; mutagenized PCR fragment of the flanking regions of *pilG* ORF of *Xf* cloned into pGEM-T Easy	This work
pUC08451	Ap^r Gm^r; Gm cassette from pGEM-T-GM cloned into *AscI* site of pUC0845	This work
pUC129	Ap^r; cloning vector	New England Biolabs
pUC129PD	Ap^r; the fragment including two pseudogenes PD0702 and PD0703 of *Xf* cloned into pUC129. There are *AscI* and *PacI* site between the fragment of PD0702 and PD0703.	This work
pBBR1MCS	Cm^r; broad-range plasmid	Kovach et al. 1995
pUC129PDCm	Cm^r; Cm cassette from pBBR1MCS-3 cloned into the *PacI* site of pUC129PD	This work
pUC*pilG*$_{Xf\text{-}Exp}$	Ap^r Cm^r; a fragment including the *pilG* promoter and ORF of *Xf* cloned into *AscI* site of pUC129PDCm	This work
Primers		
pilGA	5′-GCGATACTAAGCAACTGTGT-3’	This work
pilGB	5′-*CGGCGCGCCG*GCTCTGAATCTAAATACTGT-3’	This work
pilGC	5′-*CGGCGCGCCG*CCTGACTGTTCATCTGATGC-3’	This work
pilGD	5′-TGCGGACATTCGGGGAGCTA-3’	This work
pilGCh For	5′- TGCTTGCATGCGATGCTAGG −3’	This work
pilGCh Rev	5′- ACCCGGCACTAATGTCACCG −3’	This work
GmF	5′-GAATTGACATAAGCCTGTTC-3’	This work
GmR	5′-CGTTGTGACAATTTACCGAA-3’	This work
pilGXFExpFor	5′- TAAAGGTCAACCTGATTTGA-3’	This work
pilGXFExpRev	5′- CGCATCAGATGAACAGTCAG-3’	This work
CmF	5′-GGATGCATATGATCAGATCTT-3’	This work
CmR	5′-TCACTTATTCAGGCGTAGCAC-3’	This work
PD0702For	5′-CACGCCCGTTATTAATCGAA-3’	This work
PD0703Rev	5′-TAACCTTGTCAGCGTAGATG-3’	This work
Rst31	5′-GCGTTAATTTTCGAAGTGATTCGATTGC-3’	Minsavage et al. 1994
Rst33	5′-CACCATTCGTATCCCGGTG-3’	Minsavage et al. 1994
pUCFor	5′-GTTTTCCCAGTCACGAC-3’	Promega
pUCRev	5′-CAGGAAACAGCTATGAC-3’	Promega
M13For	5′-CGCCAGGGTTTTCCCAGTCACGAC-3’	Promega
M13Rev	5′-TCACACAGGAAACAGCTATGAC-3’	Promega

successful insertion of Gm cassette into *X. fastidiosa* genome was further confirmed by PCR using primers M13For/Rev., pilGChFor/Rev. and CmF/R, respectively (Table 1). The location and sequences of the Gm cassette

in genomic DNA of *X. fastidiosa* was also confirmed through resequencing respective amplicons.

The complemented strain *XfΔpilG*-C was constructed through the chromosome-based genetic complementation strategy as described earlier (Matsumoto et al. 2009). In brief, a 0.693-kp of *fastidiosa* Temecula genomic DNA containing the *pilG* promoter and open reading frame (ORF) was amplified by PCR using the primers pilGXFExpFor and pilGXFExpRev (Table 1). The PCR-amplified fragment was cloned into *Asc*I site of pUC129PDCm to make pUC*pilG*$_{\text{Xf-Exp}}$ (Table 1). The cloned PCR fragment (*pilG* promoter and ORF) in pUC*pilG*$_{\text{Xf-Exp}}$ was confirmed by resequencing amplicons. One microgram of the plasmid pUC*pilG*$_{\text{Xf-Exp}}$ DNA in a volume of 5 μl was then electroporated with 50 μl of *XfΔpilG* electrocompetent cells under the conditions described earlier (Shi et al. 2009). Electroporated *XfΔpilG* cells were plated on PD2 agar medium supplemented with 10 μg/ml Gm and 10 μg/ml Cm. Clones that survived on selected medium were identified as complemented *XfΔpilG* cells. The successful complemented strain *XfΔpilG*-C was confirmed by PCR using primers pUCFor/Rev and CmF/PD0730Rev (Table 1) and validated by resequencing of respective amplicons.

Gene expression assays

Total RNA was extracted from the cells of wild-type, *XfΔpilG,* and *XfΔpilG*-C grown in PD2 liquid media agitated at 200 rpm for 5 days at 28 °C (Chuang et al. 1993; Kustu et al. 1989). The extracted RNA samples were treated with DNA-free DNase (2 U/μl) to remove residual DNA contamination following the manufacturer's instruction (Ambion, TX). RNA samples were then quantified by a spectrophotometer and adjusted to 0.5 μg/μl (Kustu et al. 1989). To confirm that deletion of *pilG* does not cause frameshift mutation on downstream genes (*pilI*, *pilJ*, *pilL, chpB and chpC*), reverse transcription polymerase chain reaction (RT-PCR) was carried out using OneStep RT-PCR (Invitrogen, CA) with corresponding primer pairs (Supplementary Table S1), under the conditions: 45 °C for 60 min and then 95 °C for 5 min, and followed by 30 cycles of 95 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min, and ended at 72 °C for 10 min. PCR products were separated by 1% agarose gel.

To investigate the effect of pilG mutant on genes involved in the type IV twitching motility and virulence, fifteen genes were selected for gene expression assay (Table 2). One μg of total RNA from wild-type, *XfΔpilG,* and *XfΔpilG*-C was synthesized into single strand cDNA via reverse transcription according to manufacturer's protocol (Invitrogen, CA). Quantitative PCR (qPCR) was then carried out using 0.5 μl of cDNA and 2 μl of 5 pmol/μl gene-specific forward and reverse primers (Table 2) in a 20 μl of real-time PCR mixture. PCR was conducted with the following conditions: 50 °C for 2 min, 95 °C for 10 min and followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s and end at 72 °C for 10 min on a IQ5 PCR system using Universal SYBR green super-mix (Bio-Rad, CA). Results were analyzed using $\Delta\Delta C_T$ calculation method (Giampetruzzi et al. 2016; Petriccione et al. 2015) where the $\Delta\Delta C_T$ is the difference in threshold cycle between the target gene and housekeeping gene: $\Delta\Delta C_T = \Delta C_T$ (target gene) - ΔC_T (housekeeping gene). The final result of the calculation is presented as the fold change of target gene expression (mutant or complemented stain) relative to a reference sample (wild-type), normalized to a reference gene (housekeeping gene). The relative gene expression is then set to 1 for reference samples as when $\Delta\Delta C_T$ is equal to 0 (2^0 is equal to 1). There were three biological replicates and each sample was repeatedly measured three times.

Phenotypic analyses

To evaluate the effect of *pilG*-deficiency on cell growth, all *X. fastidiosa* strains were cultured in vitro in PD2 broth at 28 °C from day 3 to day 9. Cell concentration was determined by measuring turbidity at OD_{600nm} (Shi et al. 2009). To analyze the cell attachment, each strain was cultured in a 5 ml Erlenmeyer tube containing 2 ml of PD2 broth. The tubes were maintained in an incubator for 10 days at 28 °C at 120 rpm rotation speed. The cell attachment on the walls of the tube was then assayed using crystal violet dye method (Burdman et al. 2000; Leite et al. 2004). The quantification of biofilm formation was assessed in 96-well microtiter culture plates as described previously (Leite et al. 2004; Shi et al. 2007). All strains were first cultured in PD2 broth at 28 °C without shaking for 4 days. Bacterial cells were then collected and adjusted to an OD_{600nm} of 0.02. About 150 μl from each culture were transferred to a well in 96-well microtiter plates. Bacterium-free PD2 broth was used as negative control. Microtiter plates were incubated at 28 °C without shaking for 12 days. Each treatment

Table 2 Primers used for gene expression analysis

Gene name	Locus [a]	Primers names	Primers 5′ ----------3’	Putative function
csrA	PD0095	PD0095-F PD0095-R	GGAGATTATTAAAATGTTGATC GTTCCAGAAGAACACGCAAG	Virulence gene regulator
pilZ	PD1497	PD1497-F PD1497-R	TTGATGAGTACAACAAGTACGC GTGCGTTGGCTTATCTGAGT	Type IV fimbriae assembly protein
pilE	PD0024	PD0024-F PD0024-R	GATTGAGTTGATGGTTGTGGT CGGATAATTCACCTTGGCTAT	Type IV pilin
pilC	PD1923	PD1923-F PD1923-R	AAGGAGAACATTGAGGCTCTG CTCAGCGACTTTAAACAGCATC	Fimbrial assembly protein
pilB	PD1927	PD1927-F PD1927-R	GCATTGGAGGAAGAGGATAAC CTGTGGAGATATTGCGTGTTT	Pilus biogenesis protein
gacA	PD1984	PD1984-F PD1984-R	ATCATACTCTCGTGCGTGTTG CTCACCCCGTACTGAATAGC	Two-component transcriptional regulator
pilT	PD1147	PD1147-F PD1147-R	GTGATGACATTGGACGAACTC TCTGTGCGACTTTATCCTCAC	Twitching motility protein
pilG	PD0845	PD0845-F PD0845-R	CGCTTGGATGGTTATCAAACTT ACGGATGGCACTTAACAACTC	Pilus protein regulator
pilI	PD0846	PD0846-F PD0846-R	CGGTGTTGGCTATCGTATTGG TACGGTACGCTGTCCTTCC	Pilus biogenesis protein
pilJ	PD0847	PD0847-F PD0847-R	AACAAGAGCGGCGTTATCAAG GCAGTTCATCAACAGCACAGT	Pilus biogenesis protein
chpB	PD0849	PD0849-F PD0849-R	GCTGAATCTGGTCATCGCATT GACTAGCACCACTAAGCAACAG	Chemotaxis response regulator protein
pilU	PD1148	PD1148-F PD1148-R	GAAGGTCCACGAATGCAACT GCCTACGCGTATGTAGTCCT	Twitching mobility protein (Pilus retraction protein)
pilH	PD1632	PD1632-F PD1632-R	CGAGGACTCACCGTCACAAT GCTGAATATTCTTTCCATGA	Type IV pilus response regulator
pilR	PD1928	PD1928-F PD1928-R	CACAAAGCCCCAGCCAACGT GCGTGTGTGCCAAGCGAGCC	Type IV fimbriae expression regulatory protein
pilS	PD1929	PD1929-F PD1929-R	GAATACATCTGGACTGTACTCA ATCCCATTGGTCCGCTGGCA	Sensor protein

[a] Based on SABIA *X. fastidiosa* genome project

had three replications, and experiments were repeated three times.

Pathogenicity assays

The cells of wild-type, *XfΔpilG*, and *XfΔpilG*-C grown on PD2 agar medium for 5 days at 28 °C, were suspended in phosphate buffered saline (PBS) and adjusted to an OD_{600nm} of 0.10 (~ 6.15×10^7 cells/ml). A drop of 20 μl of cell suspension was used to inoculate grapevine, *V. vinifera* L. ‘Chardonnay’. A semi-lignified stem 10 cm above the base was pierced by a needle, allowing the droplet to be taken into the stem vascular system by transpiration flow. Each plant was inoculated twice to ensure successful inoculation and five plants were used for each treatment as previously described (Shi et al. 2009). A phosphate buffered saline (PBS) buffer served as a mock inoculation. Greenhouse temperatures were maintained between 20 °C and 32 °C. An average day/night cycle of 18/6 h was obtained through a combination of ambient and supplemental lighting. Plants were irrigated with 1.9 L/h emitters for 2 min per day (Fritschi et al. 2007). The PD symptoms were rated based on a visual scale from 0 to 5 with zero as healthy and 5 for the most severe as described previously (Guilhabert and Kirkpatrick 2005; Krivanek et al. 2005). The disease index was an average from five replications for each *X. fastidiosa* strain. Experiments were repeated three rounds under the same greenhouse conditions.

Bacterial titer measurement

Ten weeks after inoculation, multiple petiole tissues were harvested above the inoculation point from each vine inoculated with the wild-type, *XfΔpilG*, *XfΔpilG-C* strain, respectively, for DNA extraction. The successful inoculation was confirmed by PCR using *Xf* specific Rst31/33 primers (Minsavage et al. 1994). Bacterial titer measurement was performed at week 20 post inoculation. DNA was extracted again from all experimental plants (Lin et al. 2007). The titers of bacteria in the samples were estimated using qPCR according to the method described earlier (Francis et al. 2006). The means of bacterial titers were obtained from five replicates in each round of experiment.

Statistical analysis

All experiments with various treatments had 3–5 replications and were repeated at least three times. The statistical significance was calculated using ANOVA at 99% ($p < 0.01$) and 95% ($p < 0.05$).

Results

Sequence analysis

Sequence analysis of the Pil-Chp gene cluster revealed homology to the *P. aeruginosa* CheIV chemotaxis operon. Genes in Pil-Chp cluster of *X. fastidiosa* are organized in tandem with *PilG* in farthest upstream followed by *pilI-pilJ-pilL* and *chpB-chpC* parallel to the order of *P. aeruginosa* CheIV operon except for missing *pilH* and *pilK*. The *pilL* is predicted to contain several conserved domains of ChpA proteins. These results agree with the earlier report (Cursino et al. 2011; Wuichet et al. 2007). Conserved domain analysis indicates that *pilG* contains one phosphorylation site, five intermolecular recognition sites and three dimerization interface sites (Fig. 1). *pilG* is predicted to encode a response regulator homologue of CheY protein. This domain receives the signal from the sensor partner in a two-component system with a phosphor-acceptor site that is phosphorylated through histidine kinase homologs, commonly found at N-terminal to a DNA binding effector domain. Sequence analysis also hit OmpR, a DNA-binding response regulator (Fig. 1).

pilG deletion mutant and complemented strain preparation

pilG-knock-out strain *XfΔpilG* was successfully obtained from the colonies grown on agar PD2 medium supplemented with 10 μg/ml Gm antibiotics. The location of Gm cassette was confirmed by resequencing respective locus where the ORF of *pilG* was replaced with Gm cassette. The downstream genes (*pili*, *pilJ*, *pilL chpB and chpC*) in Pil-Chp operon were expressed (Supplementary Fig. S1). The chromosome-based complemented *XfΔpilG* was also obtained from the colonies that survived on the medium supplemented with 10 μg/ml Gm and 10 μg/ml Cm antibiotics. Due to technical limitations, complemented strain *XfΔpilG*

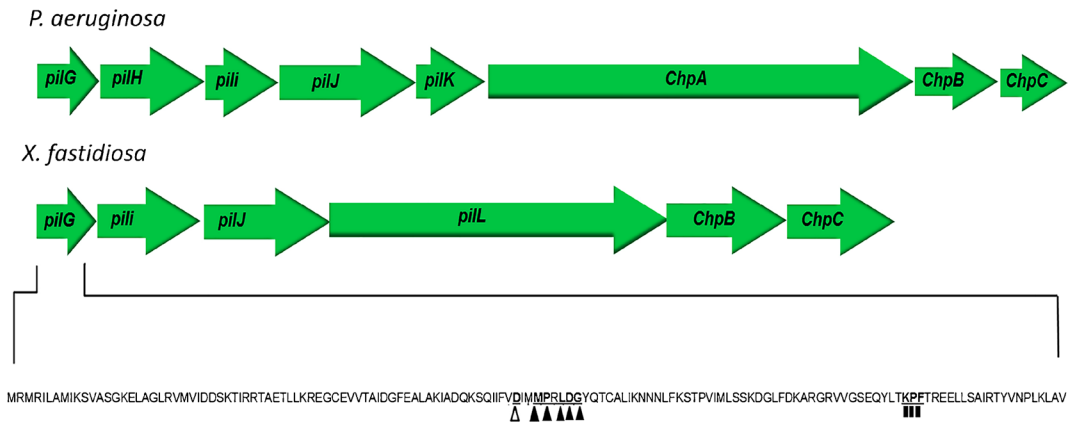

Fig. 1 Organizations of the *pilG-I-J-L* and *chpB-C* gene cluster of *X. fastidiosa* Temecula strain and the *Pseudomonas aeruginosa* CheIV cluster operon. Arrows indicate the direction of transcription of the genes. An open triangle represents phosphorylation site. Solid triangles represent intermolecular recognition sites and solid rectangles represent dimerization interfaces

was generated by inserting the *pilG* gene (promoter and ORF) along with Cm cassette at intergenic loci between two pseudogenes, PD0702 and PD0703. The insertion of *pilG* gene (promoter and ORF) in complemented strain was confirmed by resequencing respective locus. Stable *XfΔpilG* and *XfΔpilG*-C colonies were obtained after five to eight streaks on PD2 agar medium supplemented with said antibiotics. The expression of *pilG* was not detected in *XfΔpilG* but detected in complemented *XfΔpilG*-C (Supplementary Fig. S1).

Cell growth, attachment and biofilm formation

No significant difference in cell growth was observed between wild-type, *XfΔpilG* and *XfΔpilG*-C strains after nine days growth in liquid culture. The growth curves of the *XfΔpilG* mutant and complemented *XfΔpilG*-C strains paralleled wild-type, suggesting that deletion of *pilG* do not affect cell growth. All three strains showed similar growth curves (Fig. 2). *X. fastidiosa* and *XfΔpilG*-C strains attached to the inner surface of walls of the tubes and formed wide rings whereas no cell-attached ring was observed in *XfΔpilG* cells (Fig. 3a). The biofilm formation of *XfΔpilG* was about 5–6 folds less than that of wild-type and *XfΔpilG*-C strain ($P < 0.01$) (Fig. 3b).

Gene expressions associated with Pil-Chp pili system

No significantly differential expression was detected in virulence transcriptional regulator *csrA* and *gcvR,* and two-component system regulator *gacA* in *XfΔpilG* and *XfΔpilG*-C strains compared to wild-type strain (Fig. 4). However, the expression of the Type IV pilin *pilE*, twitching mobility genes *pilU* and *pilT*, and the Type IV fimbriae expression two-component system regulator *pilS* were 2-fold or more down-regulated in strain *XfΔpilG* compared with wild-type and *XfΔpilG*-C strains ($P < 0.05$) (Fig. 4). The housekeeping gene *dnaQ* (DNA polymerase III) was expressed constantly in all strains (data not shown).

Pathogenicity assay

The grapevines inoculated with wild-type *X. fastidiosa* and strain *XfΔpilG*-C developed typical Pierce's disease symptoms while plants inoculated with strain *XfΔpilG* showed no symptoms 12 weeks post inoculation. No PD symptoms were observed in PBS-inoculated control grapevines (Fig. 5). All inoculated grapevines were *X. fastidiosa*-positive and no *X. fastidiosa* was detected in PBS inoculated grapevines.

Bacterial titers

The titers of bacterium inoculated with wild-type, *XfΔpilG* and *XfΔpilG*-C strains were determined using quantitative PCR. The titers of *XfΔpilG* strain were about only 20% of wild-type ($P < 0.001$) while the titers of complemented *XfΔpilG*-C were about 63% of wild-type ($P < 0.05$), significantly higher than mutant strain *XfΔpilG* but lower than that of wild-type (Fig. 6). The data were averaged from five replicates. The experiments were independently repeated three times.

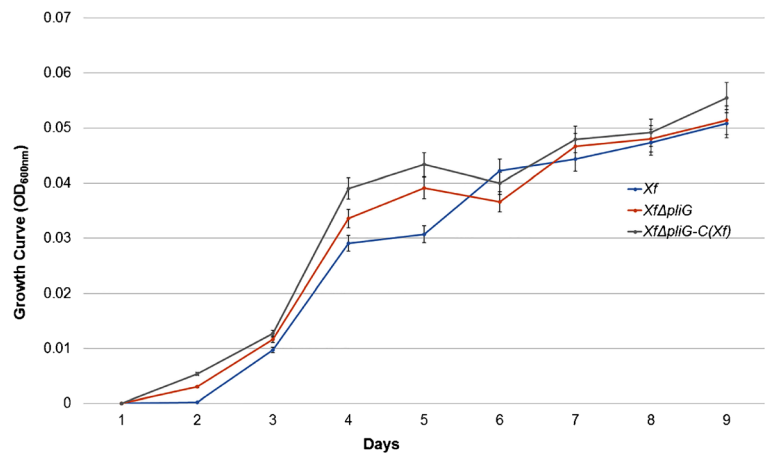

Fig. 2 Growth curves of wild-type *X. fastidiosa*, *XfΔpilG* mutant and *XfΔpilG*-C complemented strains in PD2 broth were measured over 9 days with a spectrophotometer. Data are the average of three replications. The experiments were repeated three times

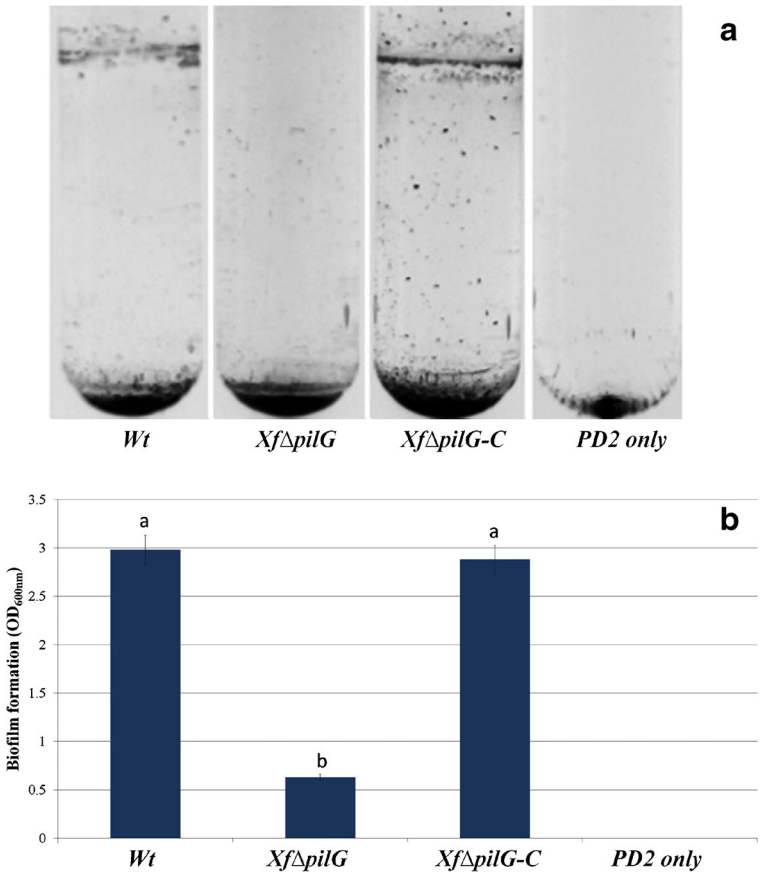

Fig. 3 Cell attachment and biofilm formation analysis of wild-type *X. fastidiosa*, *XfΔpilG* and *XfΔpilG*-C strains in PD2 broth. **a** Cells attached to the inside wall of the glass tubes forming a ring. **b** Quantitative measurement of biofilm formation of wild-type, *XfΔpilG* and *XfΔpilG*-C trains. Data are the average of three replications with error bars indicating standard deviation. Bars with the different lowercase letter are significantly different from each other ($P < 0.01$), no significant difference was indicated as the same letter. The experiments were repeated three times

Discussion

Twitching motility is a flagellum-independent form of bacterial translocation over moist surfaces mediated by type IV pili (Burrows 2012; Mattick 2002). Previously, we have shown that *pilG* plays a key functional role in type IV pilus-dependent twitching motility (Shi and Lin 2016). The results presented in this study show that twitching motility in *X. fastidiosa* is modulated by a two-component regulatory system that possesses a sequence homologous to bacterial chemotaxis system (Bertrand et al. 2010). In contrast to other gram-negative bacteria, such as *P. aeruginosa* and *E. coli,* which contain either multiple chemotaxis operons or multiple chemoreceptors (Ferandez et al. 2002; Fulcher et al. 2010; Hazelbauer et al. 2008; Kirby 2009), only a single chemotaxis operon and chemoreceptor in Pil-Chp operon were identified in *X. fastidiosa* (Cursino et al. 2011; Simpson et al. 2000). Such a unique feature likely reflects its lifestyle that *X. fastidiosa* has specifically adapted to the inside of host xylem vessels where physical and chemical complexities are much less compared to free living bacteria (Cursino et al. 2011). Sequence analysis revealed that the transcriptional orientation of chemotaxis genes is organized into a gene cluster with *pilG* located in the first upstream position (Fig. 1). There are six genes in this operon in an order *pilG*, *pilI*, *pilJ*, *pilL* and *chpB* terminating at *chpC* (Fig. 1). Together with the *pilG-I-J-L* and *ChpB-C*, the Pil-Chp constitutes a signal transduction system analogous to *P. aeruginosa* chemotaxis system that mediates rotation of pili in response to chemical attractants or repellents. In *X. fastidiosa*, *pilG* in Pil-Chp operon is predicted to encode a phospho-shuttle protein (Simpson et al. 2000) which is homologous to a response regulator CheY in *P. aeruginosa* Pil-Chp operon (Ferandez et al. 2002; Fulcher et al. 2010). By using the microfluidic chamber devices, our previous studies provide visual evidence that the *X. fastidiosa pilG* mutant strain is deficient in motility in vitro while the complemented strain fully restored twitching motility indicating that *pilG* is indispensable to Type IV pilus

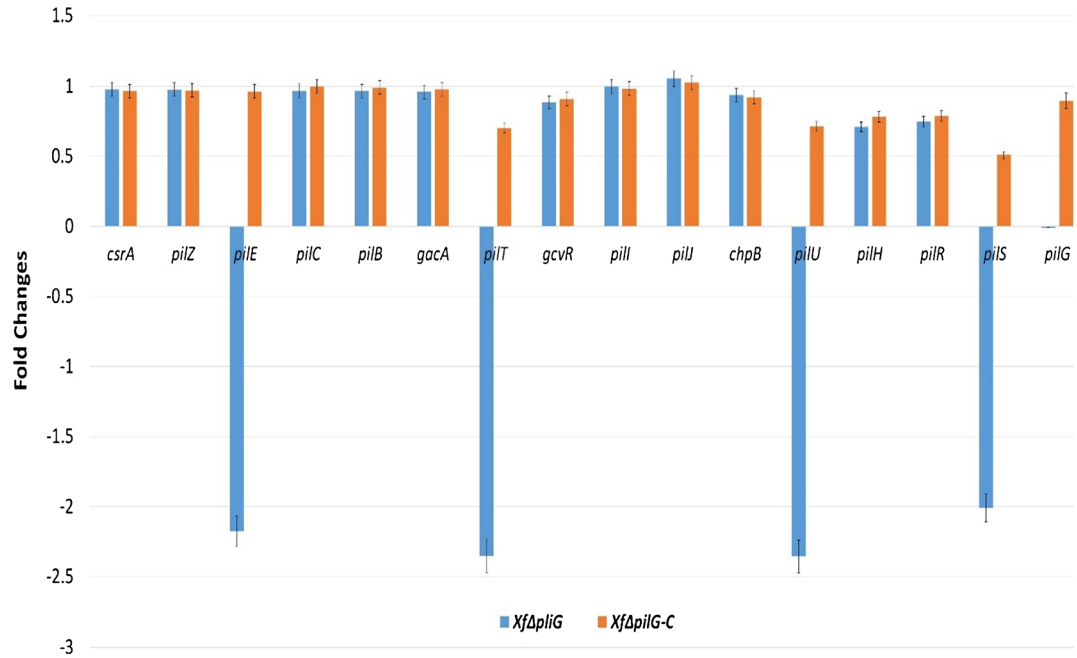

Fig. 4 Differential expressions of several *pil* and regulatory genes involved in the Type IV pili. Expression levels were compared between strain *XfΔpilG* vs wild-type or strain *XfΔpilG*-C vs wild-type. Housekeeping gene *dnaQ* (DNA polymerase III) was used as control. Gene expressions in wild-type were normalized to one. The positive and negative along Y-axis indicate up or down regulation in relation to the wild-type. Data are plotted as fold-change measured by real-time PCR. Each sample was repeated three times

twitching motility in *X. fastidiosa* (Shi and Lin 2016). In host, flagellum-independent *X. fastidiosa* is capable of actively moving against the xylem stream, colonizing grape xylem vessels and subsequently developing PD. Apparently, the twitching motility of the type IV pilus and colonization through biofilm formation and cell-cell aggregation facilitate the intra-plant spread (Meng et al. 2005; Newman et al. 2003; Stevenson et al. 2004). This phenomenon was also observed in other bacterial pathogens, such as *Ralstonia solanacearum* and *P. aeruginosa*

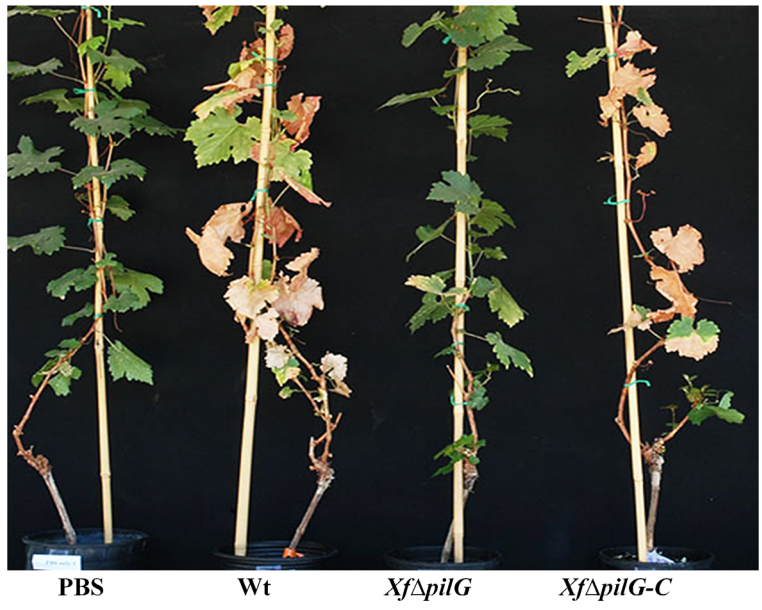

Fig. 5 Pathogenicity assays on Chardonnay grapevines inoculated with PBS (negative control), wild-type *X. fastidiosa*, *XfΔpilG* and *XfΔpilG*-C strains 20 weeks post-inoculation in the greenhouse. Grapevines from each treatment group developed Pierces disease symptoms ranging from healthy to severe. Greenhouse experiments were repeated three times in 2013, 2014 and 2015

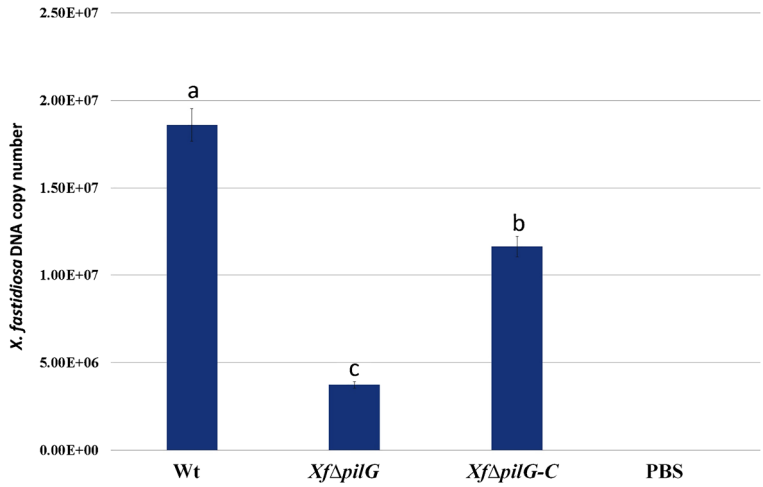

Fig. 6 Populations of wild-type *X. fastidiosa*, *XfΔpilG* and *XfΔpilG*-C strains from Chardonnay grapevine petioles were estimated by q-PCR twenty weeks post-inoculation. Data were means from five replications. Different letters indicate ANOVA-test results of significance ($P < 0.05$). Experiments were repeated three times from 2013 to 2015

which possess a similar twitching-mediated migration mechanism (Darzins 1993, 1994; Liu et al. 2001; Whitchurch et al. 2004). Though the molecular mechanisms of *pilG* regulating the pilus biosynthesis and regulatory response network in *X. fastidiosa* is yet to be revealed, sequence analysis reveals that genes in Pil-Chp operon of *X. fastidiosa* are close in proximity to each other and transcribed with the transcriptional orientation from *pilG* to the transcriptional terminating of *chpC* (Cursino et al. 2011). The biosynthesis, assembly, and function of type IV pili require more than 20 genes (Mattick 2002). Regulation of Type IV pili function involves several signal transduction systems including two-component signaling systems (Bertrand et al. 2010). For example, *pilE* encodes the minor proteins involved in formation of the base and/or tip of the pilus. The expression of *pilE* was down-regulated in *pilG* deleted strain compared to its wild-type and strain *XfΔpilG*-C suggesting that *pilG* has a functional role in regulation of the pilus production. In *X. fastidiosa,* the *pilT* and *pilU* genes are contiguous on the chromosome but reside in a locus separated from other genes involved in pilus biosynthesis and related functions. The gene expressions of *pilU* and *pilT* which encode the proteins required for pilus assembly and retraction (Mattick 2002; Simpson et al. 2000) were also down-regulated in strain *XfΔpilG* while the levels of *pilU* and *pilT* gene expression were restored in the *pilG* complemented strain. Our data further indicated that *pilS,* two component sensor–regulator involved in the regulation of the transcription of *pilA* was also down regulated in *pilG* mutant. The expression of *pilS* was restored close to wild-type levels in complemented strains. As for CheY in *P. aeruginosa* (Darzins 1993, 1994; Whitchurch et al. 2004), *pilG* is a regulator in the chemosensory system in Pil-Chp operon, therefore it seems that *pilG* plays a central role in the coordination of pilus biosynthesis, assembly and retraction in response to the environmental stimuli signals.

In this study, we demonstrate that *pilG* is also associated with several virulence properties including cell attachment, biofilm formation and PD development in grapevines. While virulence genes are critical for determinants of pathogenicity they are not essential for bacterial survival (Rasko and Sperandio 2010). Thus, *pilG* mutant strain showed similar growth curve as wild-type when both grow in rich medium (Fig. 2). Results from in vitro studies indicated that deletion of *pilG* caused significant reduction in cell attachment and biofilm formation whereas the *pilG* complemented strain restored wild-type phenotypes (Fig. 3). *In planta* pathogenicity assessment further confirmed that grapevine inoculated with *pilG* complemented strains developed typical PD symptoms with severity comparable to the wild-type. In contrast, grapevines inoculated with *pilG* mutant strains exhibited no visible symptoms in three independent greenhouse experiments (Fig. 5). The titers of three strains of *X. fastidiosa* well correlated the severity of disease symptoms (Fig. 6). Defect of motility does not necessarily result in loss of virulence. Several *X. fastidiosa* twitching motility-associated mutants have been reported (Li et al. 2007; Meng et al. 2005). Most of these were found in partial reduction in virulence and PD symptoms (Cursino et al. 2009; Meng et al. 2005). More recently, Cursino et al. (2009) reported that *tonB1* mutant showed only 30% of reduction in virulence as compared with its wild-type *X. fastidiosa* although *tonB1* mutant caused motility

deficiency. These results could be largely due to the fact that motility deficiency *X. fastidiosa* could be passively spread through the xylem evapotranspiration stream. In this study, however, we found that the pathogenicity was completely knocked-out in *pilG* mutant. To this regard, combining our in vitro and in vivo data we conclude that *pilG* may have several roles involving multiple regulatory functions and pathogenicity, therefore is a central protein in mediating PD development. In this study, we used a chromosome-based complementation method. Genome-based transformation provides stable and single copy in contrast to the plasmid complementation approach which usually yields high-copy and is unstable. The complemented *pilG* successfully restored wild-type phenotype as confirmed by in vitro and *in planta* experiments. However, it should be noted that gene expression of complemented strain of *pilG* was not quite to the levels of wild-type, presumably due to the fact that complementation was accomplished at the locus rather than native location in *X. fastidiosa* genome. The position effect on gene expression has been reported in *E. coli* (Bryant et al. 2014).

While the exact molecular mechanisms by which PilG regulates the twitching motility and pathogenicity in *X. fastidiosa* require further investigation, results from this study demonstrate that PilG is a critical component of the regulatory hierarchy governing the pathogenicity of *X. fastidiosa* in response to environmental signals. Those results will provide a new target to develop gene-based therapeutics for disrupting the pathogenicity of *X. fastidiosa* to control Pierce's disease.

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Compliance with ethical standards We state there is no potential conflict of interest in regard to this study. This research does not contain any studies with human or animal subjects. The data represented in this article are original and have not been published in the public. Both authors have written, read and consented to the manuscript.

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