Identify key virulence gene as a control target to mitigate Pierce's disease of grape

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ABSTRACT

Xylella fastidiosa (*Xf*) is an important phytopathogen that infects a number of economically important crops including citrus, almonds, coffee and olive. The *Xf* Temecula1 strain causes Pierce's disease of grapevines, a devastating disease to the viticulture industry. We deleted the *pilG* in *Xf* (*XfΔpliG*) and found that the mutant strain was avirulent. Type IV pili of *Xyella fastidiosa* are regulated by *pilG*, a response regulator protein putatively involved in chemotaxis-like operon sensing stimuli through signal transduction pathways. Results demonstrated that *XfΔpliG* showed significant reduction in cell-matrix adherence and biofilm production compared with wild type *X. fastidiosa*. *In planta* experiments showed that no Pierce's disease (PD) symptoms were observed in grapevines inoculated with *XfΔpilG,* whereas grapevines inoculated with the wild type *X. fastidiosa* and complemented strain *XfΔpilG*-C developed typical PD symptoms. These results suggest that *pilG* plays a key role in *X. fastidiosa* virulence. To develop a target-basis of therapeutic control of *X. fastidiosa* virulence, we evaluate the effect of putative anti-virulence molecules on the target gene. Our studies identified several small molecules that exhibit effective suppression on twitching motility and virulence traits under *in vitro* and *in planta* evaluation. This study facilitates the development of a novel target-basis strategy to mitigate the Pierce's disease of grapevines.

Keywords: *Xylella fastidiosa*, Pierce's disease, virulence genes, antivirulence

INTRODUCTION

X. fastidiosa is a Gram-negative non-flagellated bacterium and limited to the water-conducting xylem vessels. Pierce's disease of grapevines (PD) results in the blockage of xylem vessels, water stress and nutritional deficiencies [(5)]. Genome analysis indicates

that *X. fastidiosa* is involved in the mechanisms associated with pathogenicity and virulence such as toxins, antibiotics and ion sequestration systems, as well as bacterium-bacterium and bacterium-host interactions mediated by a range of proteins. Like most phytopathogens, *X. fastidiosa* possesses a wide array of virulence factors to invade their hosts and develop diseases. These virulence factors are precisely regulated to reduce unnecessary expression cost and to maximize fitness. To this regard, the pathogens use environmental cues to regulate their virulence gene expression. Therefore, a dynamic regulation of virulence factor expression is a key strategy utilized by pathogens. Twitching motility is a flagella-independent form of bacterial translocation over moist surfaces. It occurs in a manner of jerky motility. Twitching is mediated by the activity of filament-like structure called type IV pili which extend from the cell's exterior. Active movement mediated by the twitching system has been shown to be an important component of the pathogenic mechanisms.

The twitching motility of *X. fastidiosa* has been microscopically characterized [7, 9]. The colonization of xylem vessels is dependent on the ability of *X. fastidiosa* to migrate within xylem vessels. At least about twenty genes in *X. fastidiosa* genome have been putatively identified to be associated with type IV pili. The *pilB*, *pilQ*, and *pilR* mutants, for example, result in the defect of type IV pili and non-twitching phenotypes that showed reduced disease symptoms in grapevines [7, 9]. 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.

The activity of twitching motility of *X. fastidiosa* was controlled by a chemotaxis-like regulatory system [2,14], Pil-Chp operon, similar to that in *P. aeruginosa* and *E. coli* [3, 4]. Like *P. aeruginosa* CheIV (Pil-Chp) cluster, *X. fastidiosa* possesses a single predicated chemosensory system, Pil-Chp operon that regulates the twitching motility of type IV pili [4, 15]. 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* [2, 4]. Upon binding of the chemical stimuli in the periplasmic domain, the transmembrane chemoreceptors activate a signalling cascade in the cytoplasmic portions and ultimately control bacterial twitching motility [2]. 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 [3, 4]. Recent studies indicated that the

homologue of chemotaxis regulator, PilG is required for the twitching motility of *X. fastidiosa* since the *pilG*-deleted *X. fastidiosa* strain was deficient in twitching motility [14]. The critical roles of the Pil-Chp operon in the virulence in *X. fastidiosa* were examined recently [2, 14].

The mobility mediated by pili genes were reported to play important roles in the pathogenicity of animal and human bacterial pathogens including *Vibrio cholera*, *Neisseria meningitides*, and alkalophic *Bacillus* strains [6, 8, 16]. Recently, small molecule inhibitors targeting to bacterial motility were reported [6, 8, 16]. These molecules specifically bind the domains of the target genes and result in disrupting the function of virulence factors. For example, a small molecule amiloride was found to be able to target the extracellular Na^+-driven flagellar motor resulting in the inhibition of the motility of alkalophic *Bacillus* strains [13, 16]. Rasmussen et al. [13] demonstrated that small molecules Quinazoline and its analogs effectively inhibited the expression of the cholera toxin and the toxin-coregulated pilus responsible for motility but did not affect the cell growth *in vitro*. In *X. fastidiosa,* the twitching motility *mediated by* type IV pili contributes toward virulence via the long-distance intra-plant movement and colonization. Thus, the disruptions of the functions of the type IV pilus genes via small molecule inhibitors that block the twitching motility of *X. fastidiosa* could be a promising strategy to disarm pathogenicity and prevent and/or block diseases development. The goal of this study was to determine the roles of *pilG* in cell growth, attachment, biofilm formation, pathogenicity and to evaluation of the effects of small molecular inhibitors on the twitching motility and the pathogenicity of *X. fastidiosa.* Our results demonstrate that the disrupting the function of the target gene by antivirulence molecules would suppress virulence of *X. fastidiosa*. Therefore, this strategy could provide a novel control method for grape PD management.

RESULTS AND DISCUSSION

The roles of pilG in cell growth, attachment, biofilm formation, and pathogenicity

pilG-knock-out strain *XfΔpilG* and its complemented strain *XfΔpilG*-C were obtained as described previously [14]. The expression of *pilG* was not detected in *XfΔpilG* but was in complemented *XfΔpilG*-C (Data not shown). No significant difference in cell growth was observed between wild-type, *XfΔpilG* and *XfΔpilG*-C strains grown in liquid culture. The growth curves of the *XfΔpilG* mutant and

complemented *XfΔpilG*-C strains paralleled wild-type, all three strains showed similar growth curves suggesting that deletion of *pilG* does not affect cell growth under rich cultural media. However, bacterial populations of mutant *XfΔpilG* strain are significantly lower than that of wild type and complemented strains in infected grapevines, indicating mutation causes the reduction of fitness in host. *In vitro* study showed that *X. fastidiosa* and *XfΔpilG*-C strains attached to the inner surface of walls of the tubes and formed a wide ring whereas no cell-attached ring was observed in *XfΔpilG* cells (Fig. 1A). The biofilm formation of *XfΔpilG* was about 5-6 fold less than that of wild-type and *XfΔpilG*-C strain ($P<0.01$) (Fig. 1B).

In planta pathogenicity assessment further confirmed that grapevines inoculated with *XfΔpilG*-C developed typical PD symptoms with a severity comparable to wild type. In contrast, grapevines inoculated with *XfΔpilG* exhibited no visible symptoms in greenhouse experiments (Fig. 2). The titer of *X. fastidiosa* was well correlated with the severity of disease symptoms. Grapevines inoculated by mutant strain had significantly lower of *Xf* populations than those inoculated by wt while complemented strains showed similar levels to wt (data not shown). Twitching motility is one of the important virulence factors. Several *X. fastidiosa* twitching motility-associated mutants have been reported [7, 9]. Most of these were found only in partial reduction in virulence and PD symptoms [1, 9]. In this study, however, we found that the pathogenicity was completely knocked-out in *XfΔpilG*. To this regard, based on our *in vitro* and *in planta* data we conclude that *pilG* could have critical roles involving multiple regulatory functions and pathogenicity, therefore, it is a central virulence factor in mediating PD development.

Effect of anti-virulence molecules on virulence

Many pathogenic bacteria use a conserved membrane histidine sensor kinase (QseC) to respond to external signals or stimulus in order to promote the expression of virulence factors. Rasko et al [11] identified a small molecule that inhibits the binding of signals to QseC and prevented its autophosphorylation and consequently inhibited QseC-mediated activation of virulence gene expression. This type of small molecule usually is not toxic but specifically disrupt functional domain of the target genes. In addition, such small molecules (< 500 Daltons) are ready to be delivered into the cells. Since molecules only target to functional domains of virulence genes they don't likely impose selection pressure on cell growth. In addition, such molecules usually do not

repress pathogen growth but selectively inhibit the target virulence of pathogens in vitro and in vivo in animal [11]. Similarly, several studies demonstrated that small molecules inhibitors had functional roles in inhibiting the pilus assembly and suppressing bacterial motility [8, 13, 17]. For example, the inhibition of motility with phenamil in *V. cholera* has been shown to have effects on virulence gene expression and mitigation of the disease development [17]. Consequently, these findings suggest that small molecules inhibitors could exert anti-virulence action on virulence traits of pathogens. Results from our study demonstrated that *pilG* mutant exhibited deficiency in twitching motility, reduction in biofilm formation, and virulence [14]. To examine whether antivirulence molecules are capable of disrupting twitching motility and therefore disarm pathogenicity of *X. fastidiosa*, we constructed a custom chemical library (ChemBridge Corp, San Diego, USA) consisting of two thousand putatively small molecule inhibitors. We examined the peripheral fringe of cell morphology, an indication of the capability of type IV pilus-mediated twitching motility [14], and assessed the inhibitory effect of small molecules on the peripheral fringe morphologies of *X. fastidiosa*. From chemical library screening, we have identified several compounds that showed promising inhibitory effects on bacterial twitching motility. For example, one of the compounds, DL-3-Amino butyric acid exerts notable effective inhibition on peripheral fringes at a concentration of as low as 5μM. (Fig. 3). A microfluidic chamber time-lapse recording system further confirmed the suppression of twitching motility treated by selected anti-virulence molecules (data not shown).

To further confirm the effect of small molecular inhibitors on the pathogenicity of *X. fastidiosa,* greenhouse-grown *X. fastidiosa*-infected tobacco plants were used for pathogenicity assay. Plants were mechanically inoculated with *X. fastidiosa.* Two weeks post-inoculation, plants in treatment group were foliar-sprayed with selected inhibitor compounds while plants in control group were sprayed with water. Spraying continued once a week for 4 weeks. Our results indicated that leaves of tobacco plants developed chlorosis and necrosis five weeks post inoculation with *X. fastidiosa* and symptoms continued up to 12 weeks while plants treated with DL-3-Amino butyric acid showed significantly alleviated symptoms (Fig. 4A) indicating treatment of virulence inhibitor mitigate *X. fastidiosa* infection and disease development. Anti-virulence treatment also resulted in lower bacterial titers compared to those of untreated tobacco

plants (Figure. 4B). These results are consistent with the data obtained from *in vitro* evaluations.

CONCLUSIONS

X. fastidiosa possesses a multitude of virulence factors that enable the pathogen to invade to and disseminate of pathogens in host and subsequently cause systemic infections. The virulence factors including cell-to-cell auto-aggregation, biofilm formation, and twitching motility are critical requirements for pathogenicity of *X. fastidiosa* [5, 10, 15] (Purcell and Saunders 1999; Hopkins 1989; Simpson et al. 2000). A conventional strategy to combat bacterium-associated diseases is to kill bacteria by antibiotics or bactericides which directly disrupt essential metabolic pathways such as preventing protein synthesis, cell wall synthesis and DNA replication [12]. While these approaches are usually effective, they impose strong selection pressure on bacteria which facilitates the selection for development of antibiotic resistance populations [12]. Anti-virulence molecular approach specifically targets bacterial virulence genes or factors and selectively disarm of bacterial pathogenicity without affecting bacterial survival. This approach is unlikely to impose strong selection pressure on bacteria for the development of resistance [12]. Taking these results together, the application of virulence-target-based strategy provides a novel means for controlling *X. fastidiosa* or other bacterium-associated economically significant crop diseases.

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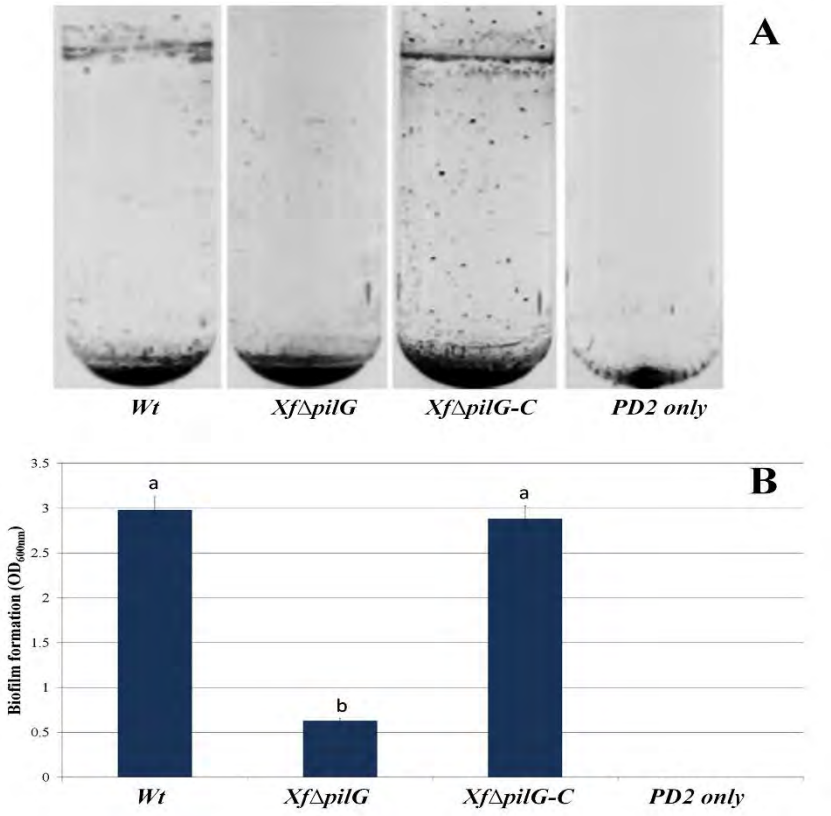

Fig. 1. A. Representative cell attachment of *X. fastidiosa* wild type, *XfΔpilG* and *XfΔpilG*-C in PD2 broth. *X. fastidiosa* wt cells showed an attached ring inside wall of the glass tube, no ring was formed in *XfΔpilG* cells under the same cultural conditions. Complemented strain *XfΔpilG*-C restored ring formation. *Xf*-free PD2 medium was served as a negative control. **B.** Quantitative measurement of biofilm formation of *X. fastidiosa* wild type, *XfΔpilG* and *XfΔpilG*-C trains. Data are the average of three replications with error bars indicating standard deviation. Bars with the same lowercase letter are not significantly different ($P< 0.01$). The experiments were repeated three times.

Fig. 2. Pathogenicity assays on Chardonnay grapevines inoculated with PBS (negative control), *X. fastidiosa* wild type, *XfΔpilG* and *XfΔpilG*-C in the greenhouse, respectively. Twenty weeks post-inoculation grapevines inoculated with wt and *XfΔpilG*-C developed typical PD systems while vines infected with *XfΔpilG* showed very mild or no symptoms. The experiments were repeated three times with at least six plants per treatment group.

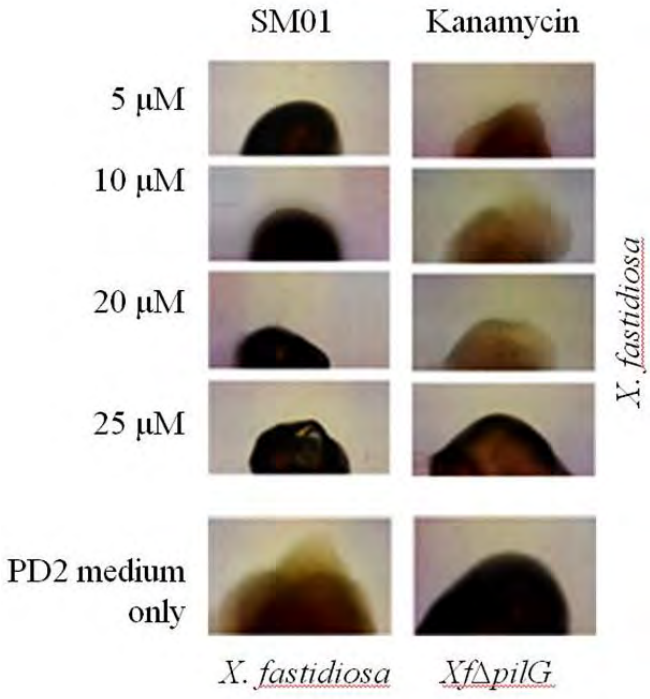

Fig. 3. The peripheral fringes were observed in *X. fastidiosa* colonies grown on PD2 agar medium while *pilG* mutant *Xf*Δ*pilG* showed smooth colony morphologies. When PD2 medium was supplemented with 5μM, 10μM, 20μM and 25 μM of small molecular inhibitor SM01 (DL-3-Amino butyric acid), no peripheral fringes were observed on *X. fastidiosa* colonies. In contrast, the effective concentrations on suppression of peripheral fringe structure were observed on PD medium supplemented with at least 25μM Kanamycin. The experiments were repeated three times.

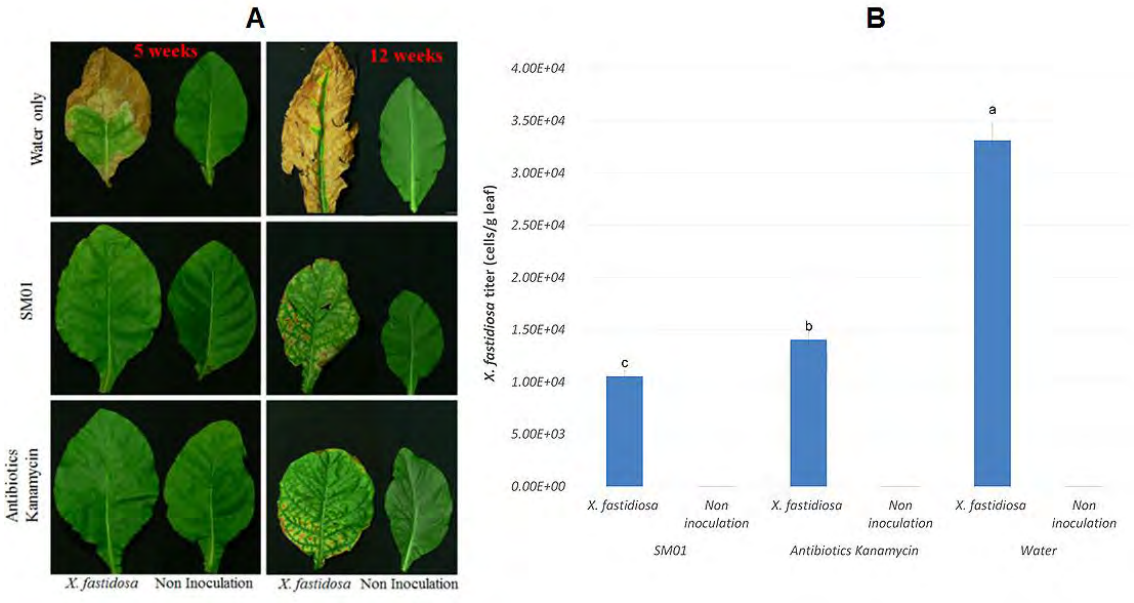

Fig. 4. Pathogenicity evaluation on tobacco plants inoculated with *X. fastidious*. **A.** Progressive development of leaf symptoms on the experimental tobacco plants 5 weeks and 12 weeks after inoculation with *X. fastidiosa* and foliar-sprayed with SM01(DL-3-Amino butyric acid), water or Kanamycin, respectively, Foliar spray was conducted once a week at 50μM for four weeks in the greenhouse. Tobacco plants developed disease symptoms from mild to severe while treatment groups showed alleviated symptoms. Greenhouse experiments were repeated three times. **B)** *X. fastidiosa* titers of tobacco leaves were estimated by ELISA two months post-inoculation. Data were means from five replications. Different letters indicate statistical significance ($P < 0.05$).