Xylella fastidiosa Requires Polygalacturonase for Colonization and Pathogenicity in *Vitis vinifera* Grapevines

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Xylella fastidiosa is the causal agent of Pierce's disease of grape, an economically significant disease for the grape industry. *X. fastidiosa* systemically colonizes the xylem elements of grapevines and is able to breach the pit pore membranes separating xylem vessels by unknown mechanisms. We hypothesized that *X. fastidiosa* utilizes cell wall degrading enzymes to break down pit membranes, based on the presence of genes involved in plant cell wall degradation in the *X. fastidiosa* genome. These genes include several β -1,4 endoglucanases, several xylanases, several xylosidases, and one polygalacturonase (PG). In this study, we demonstrated that the *pglA* gene encodes a functional PG. A mutant in *pglA* lost pathogenicity and was compromised in its ability to systemically colonize *Vitis vinifera* grapevines. The results indicate that PG is required for *X. fastidiosa* to successfully infect grapevines and is a critical virulence factor for *X. fastidiosa* pathogenesis in grapevine.

Additional keyword: pectinase.

Pierce's disease of grapevines (PD) is an economically important disease that affects wine, table, and raisin grape production (*Vitis vinifera*). *Xylella fastidiosa*, the causal agent of PD, is a gram-negative, xylem-limited bacterium that is transmitted by xylem-feeding insect vectors, mainly sharpshooters (family Cicadellidae) (Hopkins 1989). After introduction into the xylem, *X. fastidiosa* can establish either local or systemic infections in plants (Purcell and Saunders 1999). Once *X. fastidiosa* colonizes grapevine xylem tissues, symptoms can be severe, including leaf scorching, desiccated fruit ("raisining" of berries), cordon dieback, and vine death. PD-infected vines have occlusions in the xylem vessels that disrupt water flow throughout the vine (Esau 1948; Fry and Milholland 1990a and b; Goodwin et al. 1988a and b). Besides bacterial cell aggregates and tyloses, the exact origin and composition of the xylem occlusions are not known but likely are composed of host gums, bacterial exopolysaccharide, or degradation products from the host cell wall, singly or in combination.

In systemically infected plant hosts, the exact mechanism by which *X. fastidiosa* breaches the pit membrane is not known. The pit membrane is the primary cell wall that separates vessels or tracheary elements from each other. The plant primary cell wall is composed of pectin, cellulose, hemicellulose, and proteins (Keegstra et al. 1973; Carpita and Gibeau 1993). Pectin polymers regulate the porosity of the cell-wall fabric and, most

likely, pores in the intervessel pit membranes (Baron-Epel et al. 1988; Buchanan et al. 2000; Zwieniecki et al. 2001). Pore sizes have been estimated to be between 5 and 20 nm in several plant species (Choat et al. 2003; Zimmerman et al. 1983). Intact pit membranes in grapevines do not allow passage of particles larger than 5 nm (Labavitch et al. 2004), an exclusion limit that is much too small to allow passage of *X. fastidiosa* cells, whose diameter is 0.3 to 0.5 μ m (Davis et al. 1978). In order for *X. fastidiosa* to systemically colonize the vine, it is probably essential for the pit membrane barrier to be breached, because the average grapevine xylem vessel is only 10 μ m long (Labavitch et al. 2002). The mechanism by which *X. fastidiosa* breaches this barrier is poorly understood. We hypothesize that *X. fastidiosa* utilizes polysaccharide-degrading enzymes to digest cell-wall polymers that make up the xylem pit membranes. Open reading frames (ORF) putatively encoding a polygalacturonase (PG), three endo-1,4 β -glucanases, a cellobiohydrolase, three endo- β -xylanases, and two β -xylosidases were identified in the genome of the PD strain of *X. fastidiosa* (Van Sluys et al. 2003).

PGs are important virulence factors in other plant-pathogenic bacteria, such as *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, and *Erwinia carotovora* (Alghisi and Favron 1995; Collmer and Keen 1986; Dow et al. 1987; Huang and Allen, 2000; Koutajansky 1987; Lei et al. 1985; Prade et al. 1999; Rodriguez-Palenzuela et al. 1991; Schell et al. 1988). Based on the 65% shared amino-acid identity, the *X. fastidiosa* PG is most closely related to the endo-PG of *R. solanacearum*, another xylem-inhabiting bacterium (Salanoubat et al. 2002). In this study, we examined the single copy PG gene (PD1485; Van Sluys et al. 2003) as a potential virulence factor mediating the systemic infection of grapevines by site-directed mutagenesis and evaluated the mutant for alterations in pathogenicity and movement in grapevines.

RESULTS

Construction and confirmation of *pglA* gene replacement by marker exchange mutagenesis.

The wild-type *pglA* gene was disrupted using the Tn903 <kan-2> cassette, and this construct was electroporated into the PD Fetzner strain of *X. fastidiosa*. Kanamycin-resistant colonies were tested for a double crossing over event by polymerase chain reaction (PCR), using the primers pgfwd and pggrev. The resulting PCR product from the *pglA* *X. fastidiosa* mutants was 2,916 bp, representing the wild-type *pglA* (1,695 bp) + Tn903<kan-2> cassette (1,238 bp) (Fig. 1). The insertion site of the gene replacement was identified by PCR and sequence analysis. Primers specific to the DNA flanking the *pglA* gene

were used in combination with the kan-2-fp-1 and kan-2-rp-1 primers. A 1,572-bp PCR fragment was amplified with the pgchkfwd and kan-2-rp-1 primers, and a 1,542-bp fragment was amplified with the pgchkrev and kan-2-fp-1 primers (data not shown). Sequence analysis of the resulting PCR fragments confirmed that marker exchange mutagenesis had occurred in the *pglA* ORF. The wild type and *pglA*⁻ *X. fastidiosa* mutants exhibited similar colony morphology on solid PD3 medium (Davis et al. 1981). Growth curves of the mutant and wild-type cells were similar in PD3 liquid medium (data not shown).

Pathogenicity assay.

Grapevines (10 each) were inoculated with either wild-type *X. fastidiosa*, *pglA*⁻ *X. fastidiosa* mutant, or H₂O. Plants were rated for disease severity weekly, beginning 12 weeks postinoculation. The plants inoculated with the *pglA*⁻ *X. fastidiosa* mutant never developed typical PD symptoms and were indistinguishable from the water-inoculated control vines throughout the course of the experiment (Fig. 2). Wild-type *X. fastidiosa*-inoculated vines developed symptoms beginning 12 weeks postinoculation and exhibited a disease severity progression typical of PD-infected grapevines. Contingency table analysis showed that, beginning at 13 weeks postinoculation, the wild-type disease ratings were statistically different from those of the mutant ($P < 0.01$), with the difference increasing over time (Table 1). In the second replication, using the same number of vines, no PD symptoms developed in the vines

inoculated with the *pglA*⁻ *X. fastidiosa* mutants and disease progression in wild-type *X. fastidiosa*-inoculated vines was the same as in the first experiment.

Movement assay.

X. fastidiosa isolations were performed on petioles sampled from inoculated vines to determine the pathogen's presence or absence and to quantify populations of *X. fastidiosa*. *X. fastidiosa* isolations from the point of inoculation showed that *X. fastidiosa* was present in 80% of the vines inoculated with wild-type *X. fastidiosa* and 50% of the vines inoculated with the *pglA*⁻ mutant, values that were not significantly different ($P > 0.05$) (Table 2). At 14 weeks postinoculation, *X. fastidiosa* was detected at 25 cm above the point of inoculation in 100% of the plants inoculated with wild-type *X. fastidiosa* and in only 30% of the plants inoculated with the *pglA*⁻ *X. fastidiosa* mutant. These results were statistically different ($P < 0.01$), indicating that the mutant had not moved 25 cm upward from the point of inoculation to the extent the wild type had in 14 weeks. At 15 weeks postinoculation, *X. fastidiosa* was recovered 37 cm above the point of inoculation from 100% of the vines inoculated with wild-type *X. fastidiosa* and from only 45% of the vines inoculated with the *X. fastidiosa pglA*⁻ mutant ($P < 0.01$). At 20 weeks postinoculation, a second set of petioles was sampled at 25 cm above the point of inoculation, to determine whether the *pglA*⁻ *X. fastidiosa* population had finally colonized inoculated plants. Further sampling of the wild-type inoculated plants was not possible at this time-

Fig. 1. Confirmation of double exchange integration of the *pglA*::Tn903<kan-2> cassette in the Fetzer *Xylella fastidiosa* chromosome by polymerase chain reaction (PCR). **A**, Diagram of the transcriptional orientation of the *pglA* and flanking genes *metaA* and conserved hypothetical genes. **B**, Lane 1, 1-kb ladder; lanes 2 and 3, PCR products obtained using pgfwd and pgrev primers and DNA from the wild-type Fetzer *X. fastidiosa* strain or the *X. fastidiosa pglA*⁻ mutant, respectively. The PCR products were separated by electrophoresis in a 1.0% agarose gel.

point, due to severe defoliation or vine death. The *pglA*⁻ *X. fastidiosa* mutant could be isolated from plants 20 weeks postinoculation, but the frequency was not statistically different from the isolations performed after 14 weeks postinoculation (Table 2).

In order to assess the genotype of the *pglA*⁻ *X. fastidiosa* cells present in the grapevines inoculated with *pglA*⁻ *X. fastidiosa* mutants, isolations were performed on PD3 medium containing kanamycin. PCR was performed on five individual *pglA*⁻ *X. fastidiosa* colonies, using pgchkrev and kan-2 fp-1 and pgchkfwd and kan-2 rp-1 primers (Fig. 1). The expected 1,572 bp and 1,542 PCR amplicons were obtained, thus confirming the identity of the mutants and that the mutation was stable (data not shown). No PCR fragments indicating the presence of the kanamycin gene were obtained from *X. fastidiosa* colonies isolated from vines inoculated with wild-type *X. fastidiosa*.

Bacterial populations were determined at the point of inoculation, 25, and 37 cm above the point of inoculation at 13, 14, and 15 weeks postinoculation, respectively. Based on a repeated measures analysis of variance (ANOVA) test, the populations of wild-type *X. fastidiosa* were always higher than the *pglA*⁻ *X. fastidiosa* populations at each distance point measured (Table 3).

Cloning and expression of the *pglA* ORF in *Escherichia coli*.

The ORF PD1485 (Van Sluys et al. 2003) was cloned into the expression vector pET-29b (+) to create pMCR5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *E. coli* BL21 (DE3) transformed with pMCR5 confirmed that, following induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), the transformed *E. coli* produced a recombinant protein of approximately 58.2 kDa, the expected size of the *X. fastidiosa* PglA protein. *E. coli* transformed with the empty vector did not accumulate any protein with the predicted size of the recombinant PG (Fig. 3).

Enzymatic activity of the recombinant *X. fastidiosa* PglA.

The crude lysates from *E. coli* transformed with pMCR5 were capable of degrading polygalacturonic acid (PGA), as

indicated by the production of reducing end groups when the lysates were incubated with PGA (Fig. 4). High-performance liquid chromatography (HPLC) analysis showed that this increase in reducing potential was the result of increased concentrations of acidic oligomers, which was confirmed by co-injection of standards (obtained from the Complex Carbohydrate Research Center, Athens, GA, U.S.A) (Spiro et al. 1993) with degrees of polymerization of 10 (elution time = 25.51 min) and 11 (elution time = 26.61 min) (Fig. 5). Additionally,

Table 1. Disease severity of greenhouse-grown grapevines inoculated with wild-type *Xylella fastidiosa* (WT), *pglA*⁻ *X. fastidiosa*, or H₂O (control)^a

Time postinoculation	WT	<i>pglA</i> ⁻	H ₂ O
12 weeks	0.56 ± 0.52	0	0
13 weeks ^b	1.22 ± 0.97	0	0
14 weeks	2.22 ± 1.0	0	0
15 weeks	2.66 ± 1.0	0	0
16 weeks	3.2 ± 0.83	0	0
17 weeks	3.4 ± 0.76	0	0
18 weeks	3.5 ± 0.52	0	0
19 weeks	4.0 ± 0.5	0	0
20 weeks	4.6 ± 0.5	0	0
21 weeks	4.7 ± 0.44	0	0

^a Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). Data represent the mean of the first repetition of the pathogenicity assay. The second repetition obtained similar results.

^b From this timepoint on, significance detected at the 99% confidence level.

Table 2. Percentage of Chardonnay grapevines containing wild-type *Xylella fastidiosa* or *X. fastidiosa pglA*⁻ mutant cells in petiole tissue at various times postinoculation and distances from the point of inoculation (POI)^a

Genotype	POI	Distance above POI			
		25 cm	37 cm	25 cm	25 cm
	13 weeks	14 weeks	15 weeks	20 weeks	
Wild type	80%	100%	100%	ND	
<i>pglA</i> ⁻ :Tn903<kan-2>	50%	30%	45%	50%	

^a Data represent the mean of two experiments inoculating 20 vines with wild-type *X. fastidiosa* or *pglA*⁻ *X. fastidiosa* (80 vines total); ND = not determined because vines were completely defoliated or dead.

Fig. 2. *Vitis vinifera* cv. Chardonnay plants inoculated with wild-type Fetzter *Xylella fastidiosa*, *pglA*⁻ *X. fastidiosa*, or a water control. Plants were inoculated with 40 µl of a 10⁸ CFU/ml bacterial suspension or H₂O. Plants shown are 18 weeks postinoculation.

the peak eluting at 23.20 min tested positive for uronic acid (data not shown).

DISCUSSION

The genome sequence of the Citrus Variegated Chlorosis (CVC) and the PD Temecula strains of *X. fastidiosa* identified

Table 3. *Xylella fastidiosa* populations in leaf petioles collected from Chardonnay grapevines inoculated with wild-type *Xylella fastidiosa* (WT) and *pglA*[−] *X. fastidiosa*

Distance ^a	Weeks ^b	WT (CFU)	<i>pglA</i> [−] (CFU)
POI	13	1.7 (±4.3) × 10 ⁵	1.3 (±2.4) × 10 ⁵
25 cm	14	1.2 (±3.0) × 10 ⁷	2.3 (±4.2) × 10 ⁵
37 cm	15	3.5 (±3.7) × 10 ⁷	1.5 (±4) × 10 ⁶

^a Point of inoculation (POI) or distance above POI in centimeters.
^b Time postinoculation.

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis gel analysis of *Escherichia coli* cell lysates transformed with pMCR5 or the empty pET-29b(+) vector. Lane 1, Molecular-weight ladder; lane 2, *E. coli* transformed with pMCR5 prior to induction with isopropyl β-D-1-thiogalactopyranoside (IPTG); lane 3, *E. coli* transformed with pMCR5 following induction with IPTG at 4°C overnight; lane 4, *E. coli* transformed with pET-29b (+) prior to induction with IPTG; and lane 5, *E. coli* transformed with pET-29b (+) following induction with IPTG at 4°C overnight.

Fig. 4. Degradation of polygalacturonic acid indicated by the production of reducing ends in reaction mixtures of crude *Escherichia coli* lysates containing either the pMCR5 construct (pET29b::PG) or the empty pET29(b)+ vector.

genes that may be involved in pathogenesis, based on their homology and known functions in other plant pathogenic bacteria (Simpson et al. 2000; Vans Sluys et al. 2003). More specifically, the analysis of the *X. fastidiosa* PD Temecula genome revealed ORF encoding putative cell wall degrading enzymes (CWDE), including a PG, three endo-1,4-β-glucanases, a cellobiohydrolase, three endoxylanases, and two β-xylosidases (Van Sluys et al. 2003). We hypothesize that *X. fastidiosa* uses these enzymes to facilitate intervessel migration, because the grapevine pit membrane pore sizes are too small to allow passive movement of the bacteria (Labavitch et al. 2002). The purpose of this study was to determine if the single copy PG gene present in the genome of a PD strain of *X. fastidiosa* encodes a functional PG protein and if this PG is important for movement or mediating pathogenicity in grapevines.

While *X. fastidiosa* contains several genes encoding potential enzymes that could possibly digest the cellulosic and hemicellulosic components of the plant cell wall, it has only one gene encoding a potential pectinase (PD1485). The *X. fastidiosa* PG is theoretically an endo-PG with high homology (65% amino-acid identity) to the endo-PG (PglA) of *R. solanacearum* as well as high homology with the PG orthologs of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, *X. oryzae* pv. *oryzae*, *Agrobacterium vitis*, and *Erwinia carotovora* (Van Sluys et al. 2003). Interestingly, the corresponding PG ortholog found in the *X. fastidiosa* CVC strain contains a frameshift that presumably renders the resulting protein non-functional. Sequencing of the PG gene from 11 strains of *X. fastidiosa* indicated that all the citrus strains examined, as well as the coffee strains that are phylogenetically similar to *X. fastidiosa* CVC, contained the same frameshift in the PG ORF (Van Sluys et al. 2003). All the other *X. fastidiosa* isolates including grape, almond, and mulberry do not contain the

Fig. 5. High-performance liquid chromatograms of *Escherichia coli* lysates incubated with 0.2% polygalacturonic acid and **A**, *E. coli* containing the empty pET29-b (+) vector or **B**, *E. coli* transformed with pMCR5. Arrows indicate oligomers with a degree of polymerization of 5 to 12.

frameshift. The authors proposed that the apparent lack of PG in the *X. fastidiosa* CVC strain might be responsible for the less aggressive nature of the *X. fastidiosa* CVC strain as compared with the PD *X. fastidiosa* strain (Van Sluys et al. 2003).

At present there is only one selectable marker that is expressed in an *X. fastidiosa* PD strain (Guilhabert et al. 2001). This limits current *X. fastidiosa* genetic studies to the use of single-gene as opposed to multiple-gene knockouts in the pathogen. We chose to investigate the role of the single-copy PG because disrupting one of the multiple glucanases or xylanases might not give a measurable effect on *X. fastidiosa* movement or virulence. For example, knocking out only one of the nine pectolytic enzymes of *X. campestris* pv. *campestris* had no effect on virulence, while a mutant deficient in the export of all of the pectolytic enzymes was nonpathogenic (Dow et al. 1987; Dow et al. 1989). In addition, it is known that pectin is a key determinant of pore size in the pit membrane (Baron-Epel et al. 1988; Zwienicki et al. 2001), and this size limitation should restrict *X. fastidiosa* from moving into adjacent vessels (either vertically or horizontally) from the vessel into which it was originally introduced by the insect vector. Therefore, the single-copy PG gene was an obvious candidate to study to better understand the involvement of host wall-degradation processes in *X. fastidiosa* pathogenesis.

The results of the movement assay indicate that the *pglA*⁻ *X. fastidiosa* mutant was impeded in long-distance movement measured at 25 and 37 cm above the point of inoculation, as indicated by lower bacterial titers and lower frequency of *X. fastidiosa* recovery at these points (30 and 45% of the vines inoculated with the *pglA*⁻ *X. fastidiosa* mutant at the 25- and 37-cm points as opposed to 100% of vines inoculated with the wild-type *X. fastidiosa*). We conclude that the difference in movement efficiency is a result of bacteria being introduced into stem xylem pathways that are interrupted by pit membranes that cannot be digested by *pglA*⁻ *X. fastidiosa* due to the absence of PG. These results are similar to those of Huang and Allen (2000), who reported that *R. solanacearum* *pglA*⁻ mutants colonized tomato stems more slowly and at a lower frequency than wild type. We propose that *X. fastidiosa* lacking a functional PG cannot efficiently digest grapevine pit membranes and colonize xylem vessels. The average vessel length in grapevines is only 10 cm, although longer vessels can be found at lower frequencies (Labavitch et al. 2002). Since 10 cm is shorter than the distances assessed in this study, wild-type *X. fastidiosa* that was recovered from points greater than 10 cm most likely would have traversed pit membranes during its vertical movement in the stem. Vessel to vessel pits would need to be degraded during *X. fastidiosa* horizontal movement as well (Zimmerman 1983). Newman and associates (2003) observed *X. fastidiosa* crossing multiple adjacent pit membranes simultaneously and concluded that *X. fastidiosa* must have a means of digesting this barrier rather than relying on naturally occurring tears or perforations in the pits, because it is unlikely that more than one adjacent pit would become naturally compromised. In the few instances in which the *X. fastidiosa* *pglA*⁻ mutant was able to move longer distances, it is probable that the bacteria were introduced into one of the few longer (30 to 50 cm) grapevine xylem vessels and, therefore, would not have needed to cross pit membranes to move from the point of inoculation (Labavitch et al. 2002). Indeed, Thorne and associates (2006) have described passive movement of bacteria and fluorescent beads (1 µm) through open xylem grapevine conduits into leaves and this could contribute to the presence of *pglA*⁻ *X. fastidiosa* in leaves 25 and 37 cm above the point of inoculation. Their study introduced bacteria through the cut ends of grapevine stems, allowing bacteria access to all xylem conduits in the stem, therefore increasing the likelihood that

bacteria would be introduced into one of the few longer vessels. In contrast, in our study, bacteria were introduced locally (pin-prick inoculation), thereby decreasing the chance that bacteria would be introduced into a long vessel. The observation that the *pglA*⁻ *X. fastidiosa* was only recovered from 9 of 20 vines at 37 cm above the point of inoculation (Table 2) suggests that the pin-prick inoculation method does not consistently introduce *X. fastidiosa* cells into grapevine xylem elements that are longer than 10 cm.

Compared with the 100% recovery of wild-type *X. fastidiosa* cells at 25 and 37 cm above the point of inoculation, the *pglA*⁻ *X. fastidiosa* mutant was recovered at a much lower percentage (30 and 45%) (Table 2). When allowed a longer incubation time of 20 weeks postinoculation, the *pglA*⁻ *X. fastidiosa* mutant was recovered at 25 cm above the point of inoculation from four more vines that did not initially yield *X. fastidiosa* after 14 weeks of incubation, suggesting that xylem pit membranes can possibly be degraded by the other *X. fastidiosa* CWDE. However, the number of plants harboring the *pglA*⁻ *X. fastidiosa* at 25 cm after 20 weeks postinoculation was not statistically different from the number of plants harboring the *pglA*⁻ *X. fastidiosa* mutant 14 weeks postinoculation. This observation shows that PG greatly facilitates the movement of *X. fastidiosa* in grapevines but suggests that the other *X. fastidiosa* CWDE may also play a role in systemic colonization. It may be necessary for *X. fastidiosa* to first digest the pectin component of the pit membrane in order to expose the cell-wall polysaccharides that are then targeted by glucanases and xylanases. In support of this hypothesis, in vitro studies of the plant cell-wall breakdown show that the digestion of the hemicellulosic polysaccharides (e.g., glucans) is facilitated by first digesting the pectin portion of the cell wall (Keegstra et al. 1973). A less-likely explanation for this delayed movement might be physical damage to the xylem pit membranes.

Analysis of *X. fastidiosa* populations measured in petioles showed that populations were significantly higher for the wild type than the mutant. Likewise, *R. solanacearum* *pglA*⁻ mutant populations were also found to be lower than wild type in tomato plants (Huang and Allen 2000). *X. fastidiosa* must digest pectin in pit membranes in order to move from vessel to vessel, but this digested pectin is also likely used as a nutrient source that would augment the otherwise dilute nutrient composition of the xylem sap. Because the *X. fastidiosa* *pglA*⁻ mutant cannot break down pectin polymers, it cannot utilize this component of the cell wall as a potential carbon source. This could explain, at least in part, why the *X. fastidiosa* *pglA*⁻ mutant populations were smaller than wild-type populations. No differences were observed between the growth curves of the wild-type *X. fastidiosa* and the *X. fastidiosa* *pglA*⁻ mutant when they were grown in PD3 medium; therefore, this mutant does not appear to be compromised in its ability to metabolize nutrients that are present in its environment. Analysis of xylem sap from grapevines inoculated with wild-type *X. fastidiosa* revealed pectin breakdown products, which indicates that pectin-derived oligosaccharides are available for bacterial energy metabolism (Labavitch et al. 2001). In addition, the *X. fastidiosa* *pglA*⁻ mutant is restricted to the xylem vessels in which it was introduced. Therefore, growth of the population would be limited because the mutant has less access to the xylem sap flow and the nutrients that the sap carries. Additional microscopy studies could provide insights into the colonization and distribution of the *pglA*⁻ mutant versus wild-type *X. fastidiosa* in grapevine xylem elements over time.

Our attempts to detect direct activity of PG in infected grapevine xylem sap and leaf or petiole tissue using radial diffusion assays and spectrophotometric reducing sugar assays

(Gross 1982; Taylor and Secor 1988) were unsuccessful. Levels of PG may be lower at the time of sample collection (pre-symptomatic and postsymptomatic) than our ability to detect activity. We also tried to induce the expression of PG in PD3 media amended with commercially available citrus pectin, grapevine cell walls, or the diffusible signal factor from *X. campestris*; these attempts were unsuccessful as well. PD3 is a nutrient-rich, complex medium, and it is possible that the PG expression is under catabolite repression by the large amounts of glucose found in the starch component of PD3 medium. However, the recombinant *X. fastidiosa* PglA protein, when expressed in *E. coli*, was capable of degrading pectin in vitro, indicating that this gene encodes a biochemically functional PG.

The results of the pathogenicity assay demonstrate that PG is clearly required for the development of PD in grapevines. Grapevines inoculated with the *X. fastidiosa* *pglA*⁻ mutant developed none of the symptoms associated with PD. The movement assay showed that the mutant is not as efficient at systemically colonizing grapevines as wild-type *X. fastidiosa*. Although we did not quantify the specific distribution of the *pglA*⁻ *X. fastidiosa* mutant or wild-type *X. fastidiosa* cells in xylem elements in the inoculated vines, the isolation of lower populations of the mutant compared with wild-type *X. fastidiosa* suggests that the mutant likely colonized fewer xylem elements than the wild type. If fewer elements were colonized, then fewer vessels had the potential to become occluded with bacterial aggregates, which is correlated with the development of PD symptoms (Newman et al. 2003). Other reports indicated that avirulent strains of *X. fastidiosa* were unable to move beyond the point of inoculation in susceptible grapevine cultivars (Hopkins 1985; Fry and Milholland, et al. 1990a.). There is no information to suggest whether these avirulent strains are PG-deficient. The results of this study are consistent with the hypothesis by Hopkins (1989) that *X. fastidiosa* pathogenicity is dependent on systemic movement and successful colonization in the plant.

Several studies have investigated the possible role of various *X. fastidiosa* genes in virulence. Guilhabert and associates (2005) reported that two hemagglutinin-minus mutants were hypervirulent in grapevines, indicating that hemagglutinins are important in attenuating *X. fastidiosa* virulence. Additionally, a mutant deficient in the production of a diffusible signal molecule predicted to be involved in cell-to-cell signaling is hypervirulent in grapevines, thus indicating that *X. fastidiosa* may regulate some of its virulence factors via the *rpf* operon (Newman et al. 2004). *X. fastidiosa* mutants in two of the type IV pili genes are deficient in basipetal movement, but effects on pathogenicity were not reported (Meng et al. 2005). Fimbriae do not appear to be involved in pathogenicity, because mutations in several *X. fastidiosa* fimbriae genes had no effect on pathogenicity (Feil et al. 2003). *X. fastidiosa* possesses two type I secretion systems (Van Sluys et al. 2003). Marker exchange mutagenesis of an *X. fastidiosa* TolC homolog, a structural protein of the type I secretion system, produced mutants that were nonpathogenic in grapevine due to their inability to survive in planta (Reddy and Gabriel, 2005).

We currently do not have the means to complement *X. fastidiosa* in planta to test if the wild-type *pglA* gene can restore virulence and movement to the *pglA*⁻ *X. fastidiosa* mutant. Therefore, complementation tests were not performed. Aguero and associates (2005) transformed *Vitis vinifera* cv. Thompson seedless and cv. Chardonnay with the gene encoding the pear fruit PG-inhibiting protein (PGIP) under the control of the constitutively expressed *Cauliflower mosaic virus* 35S promoter. The PGIP-expressing vines accumulated PGIP in the xylem sap and developed PD symptoms at a slower rate and to a lesser extent than control vines, which provides additional

evidence that PG is an important effector in *X. fastidiosa* virulence. Now that a biochemically active *X. fastidiosa* PG has been expressed in *E. coli*, it should be possible to search for additional plant PGIPs that have higher anti-PG activity than the pear PGIP. If discovered and expressed in transgenic grapevines, the high-activity PGIP may provide even greater attenuation of PD symptoms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and PCR primers.

All bacterial strains, plasmids, and primers used in this study are listed in Table 4.

Media and growth of bacterial strains.

X. fastidiosa was grown at 28°C in PD3 broth and solid medium (Davis et al. 1981). *E. coli* strains were cultured at 37°C in Luria-Bertani medium (LB). When needed, antibiotics were added to media at the following concentrations: kanamycin, 5 µg/ml (selection for insertion into *X. fastidiosa*); kanamycin, 30 µg/ml (selection of transformed *E. coli*); and ampicillin, 100 µg/ml.

DNA manipulations.

Chromosomal DNA of *X. fastidiosa* was isolated using the DNeasy tissue kit as specified by the manufacturer (Qiagen, Chatsworth, CA, U.S.A.). *X. fastidiosa* was transformed by electroporation as previously described (Guilhabert et al. 2001). Isolation of plasmid DNA was performed using the Qiagen miniprep kit. Restriction digests, cloning, subcloning, and transformation of *E. coli* strains were performed by standard procedures (Sambrook and Russell 2001).

PCR amplification, cloning, and disruption of the *pglA* ORF.

PCR was used to amplify the *pglA* (PD1485) ORF from *X. fastidiosa* genomic DNA. For all PCR reactions the following parameters were used: denaturation at 95°C for 1 min (5 min for first cycle), annealing for 1 min at 55°C for primers *pgfwd* and *pgrev* and 60°C for primers *kan-2-fp-1* and *pgchkrev* and *pgchkfwd* and *kan-2-rp-1* (Fig. 1). The extension step was 72°C for 2 min (10-min final cycle). All PCR reactions were carried out for 35 cycles. A 1,695-bp PCR product containing the entire *pglA* ORF was amplified from Fetzter strain *X. fastidiosa* genomic DNA using primers *pgfwd* and *pgrev*. The PCR product was cloned into pCR2.1-TOPO to generate pMCR1 (Invitrogen, Carlsbad, CA, U.S.A.). pMCR1 was linearized utilizing the unique *NheI* restriction site located at position 927 bp of the cloned *pglA* fragment. The annealed *NheI* adaptor was then cloned into pMCR1 to generate pMCR2. pMCR2 was digested with *SacI* and *XbaI* to generate a 1,794 bp fragment that was cloned into pUC18 (Invitrogen), which does not replicate in *X. fastidiosa*, to generate pMCR3. The cloned *X. fastidiosa* *pglA* ORF contains a unique *MfeI* site that generated a cohesive end that is compatible with *EcoRI*. The Tn903<kan-2> cassette and the associated promoter that is expressed in *X. fastidiosa* are contained in a 1,238-bp *EcoRI* fragment (Guilhabert et al. 2001). The *EcoRI* Tn903 cassette was cloned into *MfeI*-digested pMCR3 to create pMCR4. Disruption of the *pglA* ORF was confirmed by sequencing pMCR4.

Electroporation.

Electrocompetent *X. fastidiosa* Fetzter cells were prepared as previously described (Guilhabert et al. 2001). Marker exchange mutagenesis was accomplished by electroporating 2 µg of pMCR4 DNA into electrocompetent *X. fastidiosa* cells as previously described (Guilhabert et al. 2001). Following electro-

poration, transformants were selected on PD3 media containing 5 µg of kanamycin per milliliter.

Confirmation of *pglA* gene replacement.

PCR using the *pgfwd* and *pgrev* primers confirmed that a double crossing-over event had occurred in some of the Kan^r transformants. In order to confirm that the Tn903<kan-2>/*pglA* ORF construct had replaced the wild-type gene, we used PCR primers specific to the DNA flanking the *pglA* gene (*pgchkfwd* and *pgchkrev*) in combination with primers specific to the Tn903<kan-2> cassette (*kan-2-fp-1* and *kan-2-rp-1*). Sequencing of these PCR fragments confirmed that the Tn903<kan-2>/*pglA* ORF integrated and replaced the wild-type *pglA* ORF.

Pathogenicity and movement assays.

In the pathogenicity assay, *Vitis vinifera* cv. Chardonnay grapevines that were grown in the greenhouse for 4 months were pin-prick inoculated using a 20-gauge syringe needle (Hill and Purcell 1995). Each of 10 plants per treatment was inoculated twice on the stem and four internodes up from the base of the plant with 20 µl drops of a 10⁸ CFU/ml solution of wild-type *X. fastidiosa*, *pglA*⁻ *X. fastidiosa* or H₂O. Twelve weeks after inoculation, and weekly thereafter for nine more weeks, plants were rated for PD symptoms on a scale from 0 to 5 (Guilhabert and Kirkpatrick 2005; Hopkins 1985), in which 0 = no PD symptoms, 1 = one or two leaves just beginning to show marginal necrosis, 2 = two to three leaves with significant marginal necrosis, 3 = one half or more of the leaves showing marginal necrosis and a few match sticks (attached petioles whose leaf blade had abscised), 4 = all of the leaves showing heavy scorching and numerous matchsticks, and 5 = a dead vine.

Plants were inoculated in the same manner for the movement assays but using 20 plants per treatment. Populations of *X. fastidiosa* wild type and *pglA*⁻ *X. fastidiosa* mutant were quantified from petioles sampled at the point of inoculation, 25, and 37 cm above the point of inoculation at 13, 14, and 15 weeks postinoculation, respectively. In addition, we isolated the *pglA*⁻ *X. fastidiosa* mutants from petioles 25 cm above the point of inoculation 20 weeks postinoculation. We were unable to harvest petioles from the wild-type inoculated plants at the 20-week timepoint due to severe defoliation and vine death. Petioles were weighed, surface-sterilized with 95% EtOH (30 s) and 1.2% sodium hypochlorite (30 s), and then, rinsed two times in sterile distilled H₂O (30 s) and placed in mesh sample bags (Agdia, Inc., Elkhart, IN, U.S.A.) containing 2 ml of sterile 1× potassium phosphate buffered saline. Samples were macerated with a grinding machine (Bioreba AG, Reinach, Switzerland). Serial dilutions of the macerate were plated on PD3 or PD3 medium containing 5 µg of kanamycin per milliliter, incubated at 28°C, and resulting colonies were counted after 10 days. The pathogenicity and movement assays were replicated twice using two different *pglA*⁻ *X. fastidiosa* clones. The data presented in Table 2 represents the mean values of two separate experiments using two different *pglA*⁻ *X. fastidiosa* clones.

Statistical analysis.

We used a 2 × 2 contingency table analysis at a 99% confidence level to assess if the mutant was hindered in its ability to move in planta as compared with the wild type. Potential differences in the sizes of the bacterial populations of the *X. fastidiosa* mutant and the wild type at different distances from the point of inoculation were determined using a repeated-meas-

Table 4. Plasmids, strains, and primer sequences used in this study

Plasmids, primers, strains	Relevant characteristics or primer sequence ^a	Source
Plasmids		
pUC18	pMB1 derivative, <i>rep</i> (pMB1), <i>bla</i> (Ap ^r), <i>lacZ</i>	Roche
pCR2.1-TOPO vector	pUC18 derivative, kan ^r , <i>bla</i> (Ap ^r), <i>lacZ</i>	Invitrogen
pMCR1	pCR2.1-TOPO vector with 1,695-bp <i>pglA</i> fragment	This study
pMCR2	pMCR1 with <i>NheI</i> adaptor insertion	This study
pMCR3	pUC18 with <i>pglA::NheI</i> adaptor	This study
pMCR4	pMCR3 with <i>pglA::Tn903<kan-2></i>	This study
pET29 b(+)	Km ^r	Novagen
pMCR5	pET-29b(+) with <i>pglA</i> insertion	This study
Primers		
<i>pgfwd</i>	GTGCCATGGCTTCCTTAC ^b	This study
<i>pgrev</i>	TACAGCTTCGAATGGACACA ^c	This study
<i>NheI</i> adaptor	CTAGCCAATTGG ^d	This study
kan-2fp-1	ACCTACAACAAAGCTCTCATCAACC	Invitrogen
<i>pgchkrev</i>	CACAGGATCTGGTCGTGTCT	This study
kan-2-rp-1	GCAATGTAACATCAGAGATTTTGAG	Invitrogen
<i>pgchkfwd</i>	TTCGTGCACACCTTCGACC	This study
<i>pgoxinfwd</i>	GAAATCTGGGGTGACGTTGT	This study
<i>pgoxinrev</i>	CAGACGACGCATGCATATT	This study
<i>pgoxfwd2</i>	TTTCCCATGGACCTTGACCGTTT ^b	This study
<i>pgoxrev5</i>	TTGGACACACGGCCGTTAGATAGGCGAATCA ^c	This study
T7 promoter	TAATACGACTCACTATAGGG	Novagen
T7 terminator	GCTAGTTATTGCTCAGCGG	Novagen
Strains		
<i>Escherichia coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>araleu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (<i>StrR</i>) <i>endA1</i> <i>nupG</i>	Invitrogen
BL21 (DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B</i> <i>m_B</i> ⁻) <i>gal</i> <i>dcm</i> (DE3)	Novagen
<i>Xylella fastidiosa</i>		
Fetzer	...	Napa, CA

^a Primer sequences are presented 5' to 3'; Ap^r and Kan^r indicated ampicillin and kanamycin resistant, respectively.

^b The restriction *NcoI* site is shown in bold.

^c The restriction site *BstBI* is shown in bold.

^d The restriction site, *MfeI*, is shown in bold.

^e The restriction site, *EagI* is shown in bold.

ures ANOVA test. The pathogenicity assay data were analyzed using a 5×2 contingency table analysis at a 99% confidence level.

Cloning the *pglA* ORF into an expression vector.

The *pglA* ORF was PCR-amplified with the Expand high fidelity *Taq* polymerase (Roche Applied Science, Indianapolis, IN, U.S.A.) from genomic *X. fastidiosa* DNA. Primers pgoxwd2 and pgoxrev5 were designed to amplify a product that began at the ATG start codon and ended immediately after the TAA stop codon to prevent the addition of the vector-encoded carboxy terminal 6 histidine tag. The primers also contained the restriction sites *NcoI* and *EagI* (Table 4). The following PCR parameters were used: denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 1 min at 55°C, and extension for 2 min at 72°C. (6-min final cycle). The PCR reaction was carried out for 35 cycles. The *NcoI-EagI* fragment obtained after digestion was then cloned into the pET-29b(+) expression vector to create the plasmid pMCR5. Sequencing with pgoxwd2 and pgrex5 as well as a set of internal primers (pginfwd and pginrev) confirmed that the insertion was cloned in-frame with no introduced mutations.

Expression of a *pglA* fusion protein in *Escherichia coli*.

The pMCR5 construct was transformed into chemically competent *E. coli* Novablue (Novagen, EMD Biosciences, La Jolla, CA, U.S.A.) by heat shock and was plated on LB medium containing 30 µg of kanamycin per milliliter. Plasmids were extracted from transformants and were screened by restriction analysis for the correct insertion of the *pglA* amplicon. A plasmid with the correct size insertion was sequenced using the T7 promoter and T7 terminator and pginchkfwd and pginchkrev primers to confirm that no mutations were introduced during PCR amplification. A plasmid with the correct insertion was used to transform chemically competent *E. coli* BL21(DE3) (Novagen, EMD Biosciences). A fresh transformant was used for protein expression. Single colonies were grown to an optical density at 600 nm (OD_{600}) = 0.5 at 37°C in 2 ml of LB broth containing 30 µg of kanamycin per milliliter. The cells were harvested by centrifugation and were resuspended in 150 ml of LB broth containing kanamycin at 30 µg/ml. The cells were grown at 37°C with vigorous shaking to OD_{600} = 0.5, and then, were induced with IPTG to a final concentration of 0.6 mM. Cultures were then incubated at 4°C overnight with vigorous shaking. Aliquots were removed before and after induction and were analyzed by SDS-PAGE electrophoresis to confirm the presence of the recombinant protein. BL21 (DE3) transformed with pET-29b (+) containing no insert was used as a negative control.

Enzymatic activity

of the recombinant *X. fastidiosa* PglA protein.

PG activity (hydrolysis of PGA) was confirmed by reducing sugar assays using PGA as substrate and examination of digestion products using HPLC. Following expression, *E. coli* cells transformed with either pMCR5 or the empty pET29-b (+) vector were harvested by centrifugation for 15 min at 8,000 rpm. The cell pellet was resuspended in 0.5 M NaCl, 50 mM HEPES, pH 7.5, containing 0.35 mg of lysozyme per milliliter and Complete protease inhibitor cocktail, pH 7.5 (Roche Diagnostics, Mannheim, Germany) and was incubated at room temperature for 30 min. The lysate was sonicated on ice in five, 10-s bursts, followed by gentle stirring at room temperature for 30 min, followed by dialysis at 4°C against two changes of distilled water using 1,000 kDa molecular mass cut-off dialysis tubing. A 1-ml sample of the resulting protein extract from either cells transformed with pMCR5 or the empty pET29-

b(+) vector was incubated with 1 ml of 0.2% PGA (MP Bio-medicals, Solon, OH, U.S.A.) in 0.1 M sodium acetate, pH 5.0. Protein extract incubated without PGA and PGA incubated without protein extract served as enzyme and substrate blanks, respectively. Reactions were performed in screw-cap tubes, and a drop of toluene was added to all the reaction mixtures to prevent microbial growth. Reaction mixtures were incubated at 37°C while shaking at 125 rpm. Aliquots (200 µl) were taken immediately (time 0) and at 24-h intervals during incubation. Each aliquot was assayed for reducing groups using 2-cyanoacetamide, as described by Gross (1982). Assays were performed in triplicate and were repeated three times. HPLC analysis was performed using a Dionex BioLC HPLC module equipped with a pulsed amperometric detector. The acidic oligomers were separated on a Dionex CarboPac PA-1 (4 × 250 mm) anion exchange column using a sodium acetate gradient, as described by Melotto and associates (1994). Additionally, the peak eluting at 23.20 min was collected and assayed for uronic acid content, using the method described by Blumenkrantz and Asboe-Hansen (1973).

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