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PROJECT TITLE: Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery.

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INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-limited bacterial pathogen that is the causal agent of Pierce's Disease (PD) of grapevine (Hopkins and Purcell, 2002, Chatterjee et al., 2008, Purcell and Hopkins, 1996). In order to systemically colonize the xylem, *Xf* must be able to move efficiently from one xylem vessel element to adjacent vessels. These xylem vessels are connected by pit membranes, which are porous primary cell wall interfaces that are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose (Buchanan, 2000, Sun et al., 2011). The pore sizes of these pit membranes range from 5 to 20 nm, and serve to prevent the movement of air embolisms and pathogens within the xylem (Mollenhauer & Hopkins, 1974, Buchanan, 2000). Indeed, these small pore sizes do prevent the passive movement of *Xf* between xylem vessels given that the size of the bacterium is 250-500 x 1,000-4,000 nm (Perez-Donoso et al., 2010, Mollenhauer & Hopkins, 1974). In order to move from one vessel to another, it has been shown through genomic and experimental evidence that *Xf* utilizes Cell Wall-Degrading Enzymes (CWDEs), including a polygalacturonase (PG) and at least one β-1,4 Endoglucanase (EGase), to break down the pit membrane's network (Roper et al., 2007, Perez-Donoso et al., 2010). Furthermore, PG is necessary for pathogenicity in grape and has become a primary target for *Xf* inhibition studies (Roper et al, 2007). However, *Xf* requires both PG and an EGase for pit membrane degradation (Perez-Donoso et al., 2010). Therefore, elucidating the role of EGases in pit membrane degradation is critical for understanding systemic movement within the xylem. The *Xf* genome contains three genes that encode canonical EGases: *egl* (PD2061) *rlpA* (PD1236) and *engXCA2* (PD1851). A fourth annotated EGase, *engXCA1* (PD 1856), putatively encodes a modular hybrid protein that contains both an EGase domain and an expansin domain (Simpson et al., 2000). Expansins are primarily plant proteins that function to non-enzymatically loosen the cell wall during development (e.g., cell elongation, fruit ripening). Recently, expansins have been found in several plant-associated bacteria, most of which have a significant xylem-dwelling phase in their lifestyle (Nikolaidis et al., 2014). It is predicted that these EGases and PG are delivered into the xylem by the Type II Secretion System (T2SS). Preliminary data demonstrate that *X. fastidiosa* with a deficient T2SS display a non-pathogenic phenotype similar to that of the *Xf pglA* mutant that is deficient in production of PG, suggesting that the T2SS is essential for *Xf* pathogenicity. Therefore, our central hypothesis is that *Xf* utilizes other CWDEs and an endoglucanase/expansin hybrid protein in concert with PG to breach the pit membranes and that the majority of these are secreted by the Type II Secretion System. We are determining the role that each of these components plays in pit membrane degradation and systemic movement, and subsequently if they are good candidates for potential inhibition to limit PD development.

OBJECTIVES

1. Characterization of *Xf* host cell wall degrading enzymes and an endoglucanase/expansin protein.
2. Inhibition of *Xf* endoglucanases and the endoglucanase/expansin using endoglucanase-inhibiting proteins.
3. Characterization of the *Xf* Type II secretion system.
4. Inhibition of the *Xf* Type II secretion system.

RESULTS AND DISCUSSION

Objective 1: Characterization of *Xf* host cell wall degrading enzymes and an endoglucanase/expansin.

It was previously determined that *Xf* EngXCA2, which is one of the four putative *X. fastidiosa* EGase-encoding genes is a major contributor to the pit membrane dissolution and the synergistic effects of the PG and the EngXCA2 were sufficient to increase pit membrane pore size (Perez-Donoso et al., 2010). Indeed, recombinant EngXCA2 was capable of digesting carboxymethyl cellulose (CMC) and xyloglucan (XyG) polymers, which both contain β-1,4-linked glucan backbones and are representative of substrates *Xf* would likely encounter in grapevine primary cell walls (Roper, 2006; Perez-Donoso et al., 2010). Given the role EngXCA2 plays in pit membrane degradation, we hypothesize that other predicted EGases produced by *Xf* may impact pit membrane integrity as well. The *egl* gene is predicted to encode a β-1,4 EGase belonging to the glycoside hydrolase family 5 as indicated in the CAZy (Carbohydrate Active Enzyme) database. Glycoside hydrolase family 5 proteins hydrolyze glycosidic bonds between two carbohydrates or a carbohydrate and non-carbohydrate moiety and have activities ranging from EGases to mannanases. *rlpA* putatively encodes a lipoprotein containing a Barwin-related EGase domain belonging to the glycoside hydrolase family 45. This family contains proteins with only EGase activity. The last gene annotated as an EGase is *engXCA1*, which encodes an EGase/expansin hybrid putatively involved in plant cell wall disassembly. This is of particular interest because expansins are primarily found in the plant kingdom and are non-enzymatic proteins that function to loosen the cell wall during plant growth without enzymatic digestion of the wall (Cosgrove, 2000). Expansins facilitate cell wall loosening by binding to their target polysaccharide and disrupting the weak bonds between the cellulosic glucan and the microfibril surface, allowing turgor pressure from within the cell to expand the cell wall (Cosgrove, 2000). Expansin-like proteins with similar structure and function were later found in a few bacterial species that associate with plants likely as a result of cross-kingdom horizontal gene transfer (Nikolaidis et al., 2014). These bacterial expansins are thought to enhance the activity of bacterial CWDEs by loosening the cell wall, thereby promoting wall breakdown, colonization and virulence. Interestingly, orthologs of at least one bacterial expansin (EXLX1) are found in several plant pathogens, including *Xylella, Xanthomonas*, *Ralstonia* and *Erwinia* species (Kerff et al., 2008, Georgelis et al., 2014). While these are phylogenetically diverse bacteria, they all share the commonality that they spend the majority of their lives in the xylem tissue of plants. It is hypothesized that they are involved in host colonization (Kerff et al., 2008). In the *Xf* pathosystem, they could potentially weaken the wall and more readily expose carbohydrate targets for digestion by the suite of other *Xf* CWDEs.

Characterization of the *Xf* EGase/Expansin hybrid protein.

The gene *engXCA1* was cloned from the *X. fastidiosa* Temecula 1 genome into the pET200 Directional TOPO expression vector (Fig. 1A). The plasmid construct (pET200::*engXCA1*) was then transformed into the *E. coli* strain BL21 Star, and recombinant protein expression was induced with 1 mM IPTG for six hours at 37°C. The bacterial cells were lysed using the B-PER lysis reagent containing lysozyme and DNaseI (ThermoFisher) and the lysate was run on an SDS-polyacrylamide gel (Fig. 1B). The lysate was analyzed by Western Blot using a monoclonal α-His-tag primary antibody and a polyclonal alkaline phosphatase (AP) secondary antibody (Fig. 1C). The Western Blot was developed using an AP development kit (Bio Rad), and the protein sequence was confirmed by Mass Spectrometry. Analysis of the soluble and insoluble lysate fractions determined that expression at 37°C did not favor soluble recombinant protein, so conditions were optimized to facilitate the presence of recombinant protein in the soluble fraction. The samples were incubated for four hours at either 25°C or 18°C in the presence of 0.1 mM IPTG, and GelQuant.NET software provided by biochemlabsolutions.com was used to calculate the band intensity for the soluble fraction relative to the insoluble fraction (Fig. 1D). After

incubation at 25°C, the soluble fraction contained 7.3% of the total recombinant protein, while incubation at 18°C yielded 22.7% of the total recombinant protein in the soluble fraction.

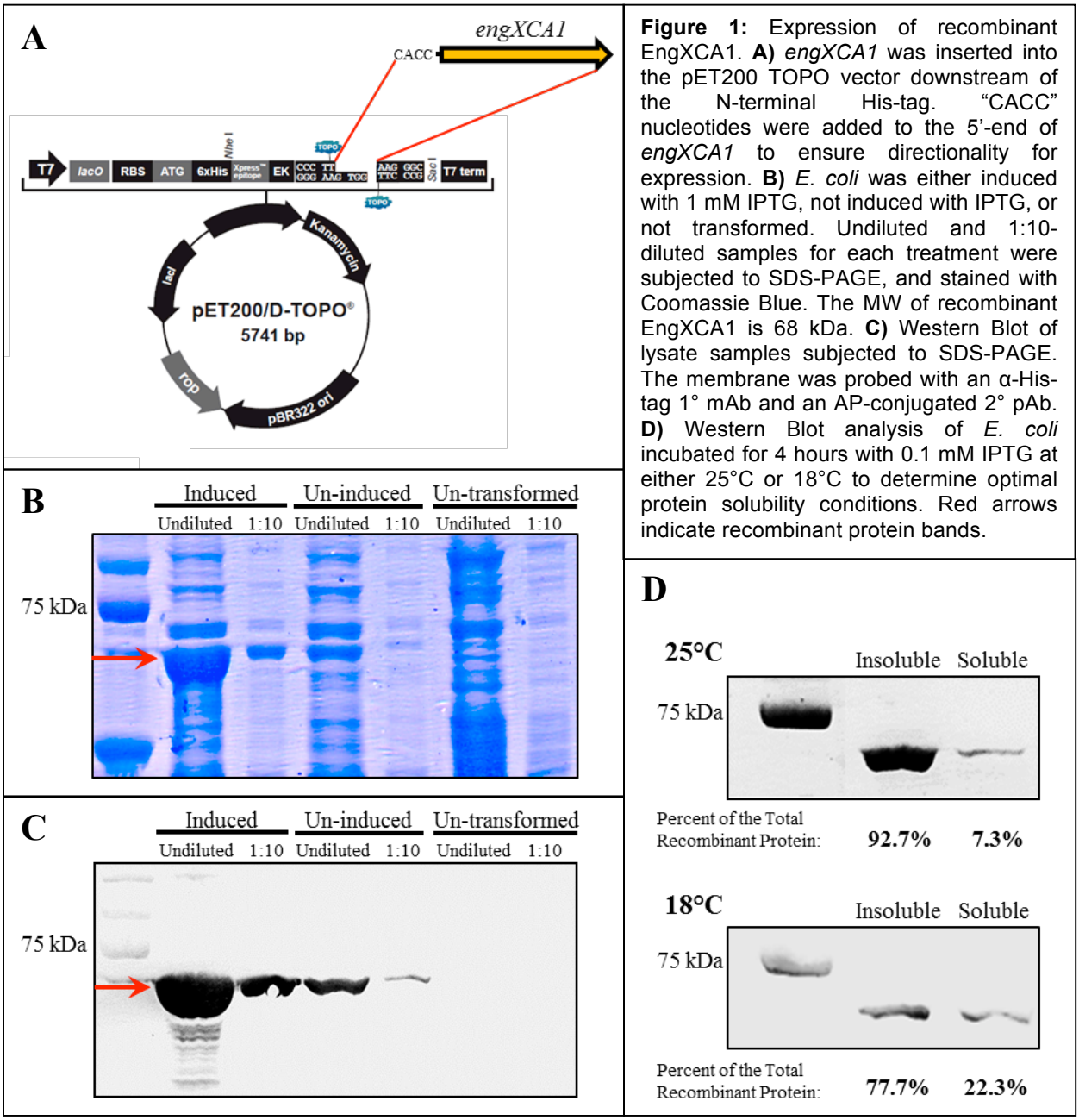

Figure 1: Expression of recombinant EngXCA1. **A)** *engXCA1* was inserted into the pET200 TOPO vector downstream of the N-terminal His-tag. "CACC" nucleotides were added to the 5'-end of *engXCA1* to ensure directionality for expression. **B)** *E. coli* was either induced with 1 mM IPTG, not induced with IPTG, or not transformed. Undiluted and 1:10-diluted samples for each treatment were subjected to SDS-PAGE, and stained with Coomassie Blue. The MW of recombinant EngXCA1 is 68 kDa. **C)** Western Blot of lysate samples subjected to SDS-PAGE. The membrane was probed with an α-His-tag 1° mAb and an AP-conjugated 2° pAb. **D)** Western Blot analysis of *E. coli* incubated for 4 hours with 0.1 mM IPTG at either 25°C or 18°C to determine optimal protein solubility conditions. Red arrows indicate recombinant protein bands.

Using the optimized induction conditions (18°C, 0.1 mM IPTG, 4 hours) and increasing the total volume of bacterial cells from 10 ml to 40 ml allowed for the expression of a sufficient quantity of soluble recombinant EngXCA1 protein to proceed with protein purification. The recombinant protein was purified via column chromatography using Ni-NTA resin (ThermoFisher), following the product instructions. Elution of the protein was monitored by absorbance readings at 280 nm. A sufficient quantity of recombinant protein was eluted from the column and subsequently dialyzed using a Slide-A-Lyzer cassette (ThermoFisher) in PBS. After concentrating the protein, an absorbance reading at 280 nm was taken to determine the concentration, only to discover that no protein remained after dialysis and subsequent concentration. Dialysis membranes are made of regenerated cellulose, thus, it is likely

that the recombinant EGase may be binding to the membrane. Currently, alternative purification and dialysis methods are being pursued.

Previous attempts to determine endoglucanase activity for EngXCA1 using a radial diffusion assay as outlined by Kasana et al. (2008) were inconclusive as the negative controls also showed zones of hydrolysis usually associated with endoglucanase activity. It is possible that the zones of hydrolysis in the negative controls are false positives for reasons outlined by Johnsen and Krause (2014). Due to these difficulties and the fact that radial diffusion assays are not very sensitive, we will be focusing our efforts on determining endoglucanase activity via reducing sugar assays instead. The reducing sugar assay, as outlined by Gross KC (1982), is more sensitive than the radial diffusion assay, and the methodology has already been used to show that another *Xf* endoglucanase, EngXCA2, has endoglucanase activity (Roper, 2006).

Endoglucanase activity of EngXCA1 will be determined using the soluble fraction of the cell lysate in a reducing sugar assay (Gross, 1982). 0.02% CMC or XyG substrate will be dissolved in 0.1 M sodium acetate (pH 5.0). 3 ml of the substrate solution will be incubated with 0.5 ml of the cell lysate containing the recombinant endoglucanase. 0.5 ml aliquots will be taken at time = 0 and every hour for 6 hours, and the reactions will be stopped with 1 ml of 0.1 M sodium borate (pH 10.0). 200 µl of 1% 2-cyanoacetamide will be added and the samples will be boiled for 10 minutes. The absorbance for each sample will be determined spectrophotometrically at 276 nm.

We will also assess expansin activity of the recombinant protein (i.e., its ability to promote the extension of plant tissues that are subjected to stress) in close collaboration with the Cosgrove Laboratory (Penn State University). The cell wall elongation assay will then be performed using an extensometer apparatus as described by Cosgrove, D. J. (1989), and expansin activity will be determined by measuring the extension of wall specimens over a 2-h period.

Assessment of the biological contribution of the *Xf* EGase/Expansin and other *Xf* endoglucanases to pathogenicity and host colonization. To test the role of the *Xf* EGase/expansin *in planta*, we constructed a deletion mutant (Δ*engXCA1*) in the *Xf* Temecula 1 strain using established mutagenesis techniques and confirmed the mutant via PCR (Matsumoto et al., 2009). We mechanically inoculated the Temecula 1 wild-type and the Δ*engXCA1* mutant into grapevine (Cabernet Sauvignon variety) using the pin-prick method (Hill and Purcell, 1995). Grapevines inoculated with 1X phosphate buffered saline (PBS) were used as negative controls. Both the wild-type and the Δ*engXCA1* mutant were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment). All plants in the experiment were randomized in the greenhouse, and disease ratings were normalized to the PBS negative control to account for symptoms caused by environmental conditions.

Disease ratings for all plants were recorded using a scale of 0 – 5 where 0 = healthy, 5 = dead, and 1 – 4 are increasing degrees of leaf scorching as described by Guilhabert and Kirkpatrick (2005). Interestingly, the Δ*engXCA1* mutant strain is less virulent than the wild-type parent strain (Fig. 2A). Statistical analysis using the Wilcoxon rank sum with continuity correction statistical test revealed that this difference in virulence between the wild-type and mutant strains at week 20 was statistically significant ($P = 0.008$). Furthermore, the percentage of plants inoculated with the Δ*engXCA1* mutant strain rating 2 or higher on the disease index was significantly less than the percentage of plants inoculated with wild-type *Xf* rating 2 or higher over a 20-week period (Fig. 2B). This indicates that the onset of disease in plants inoculated with the Δ*engXCA1* mutant is significantly delayed relative to plants inoculated with wild-type *Xf*. We have also constructed the *engXCA1*/*engXCA1*+ complement by inserting the *engXCA1* gene and its native promoter into a neutral site in the *Xf* chromosome in the *Xf* Δ*engXCA1* mutant strain (Matsumoto et al., 2009). Initial *in planta* experiments revealed that the *engXCA1*/*engXCA1*+ complement restored virulence to wild-type levels (*data not shown*), and repeat experiments will be done this year to confirm this result.

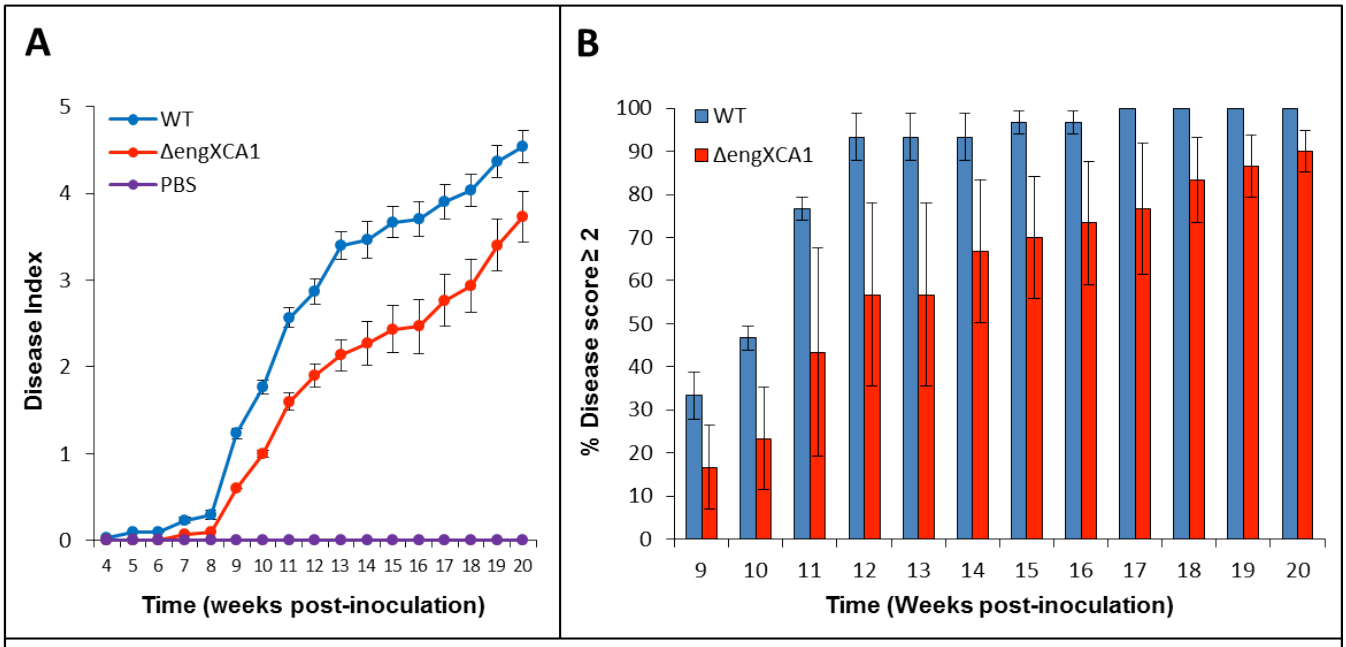

Figure 2: Pierce's disease development in Cabernet Sauvignon grapevines after inoculation with either the Δ*engXCA1* mutant strain or the wild-type strain over 20 weeks. 1X PBS served as the negative control. **A)** The Δ*engXCA1* mutant strain maintains a lower average disease score per week relative to the wild-type strain. **B)** The percentage of plants inoculated with the Δ*engXCA1* mutant strain that rated 2 or greater was consistently lower relative to the percentage of plants inoculated with the wild-type strain. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

A deletion mutant of another *Xf* endoglucanase (Δ*engXCA2*) has been constructed and transformed into the *Xf* Temecula 1 wild-type strain. EngXCA2 has previously been characterized experimentally as an endoglucanase capable of degrading both CM cellulose and xyloglucan (Roper, 2006). It has also been implicated in the degradation of pit membranes when combined with a polygalacturonase, indicating that this endoglucanase may be required for systemic colonization of the grapevine host (Perez-Donoso et al., 2010). Therefore, the *in planta* data should provide concrete evidence for the role of this endoglucanase in systemic colonization. Grapevines (Cabernet Sauvignon) were inoculated using using the pin-prick method (Hill and Purcell, 1995). Grapevines inoculated with 1X PBS were used as negative controls. All treatments were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment). The grapevines were randomized in the greenhouse, and disease ratings were normalized to the PBS negative control to account for symptoms caused by environmental conditions.

Using the 0 – 5 disease rating index for PD symptoms, inoculated grapevines were assessed weekly. Grapevines inoculated with the Δ*engXCA2* mutant strain displayed milder PD symptoms over a 21-week period relative to the grapevines inoculated with the wild-type strain (Fig. 3A). Additionally, the percentage of plants inoculated with the Δ*engXCA2* mutant strain rating 2 or higher on the disease index was notably less than the percentage of plants inoculated with the wild-type strain rating 2 or higher (Fig. 3B). Statistical analysis at week 21 revealed that the difference in PD symptom severity caused by the Δ*engXCA2* mutant strain was significantly lower than that caused by the wild-type strain (P = 0.02), indicating that EngXCA2 plays a significant role in *Xf* virulence.

Both the Δ*engXCA1* and Δ*engXCA2* mutants displayed significantly reduced virulence when assessed *in planta*, but neither displayed a complete loss of virulence like the Δ*pglA* mutant (Roper, et al., 2007). Given that *Xf* maintains several putative EGases, it is possible the loss of one EGase is compensated for by the others. As such, we intend to create double and triple mutants to delete several of the EGase genes at one time. If our redundancy hypothesis is correct, we expect to find that a double mutant will be significantly less virulent than a single mutant, and a triple mutant will likely result in the loss of virulence.

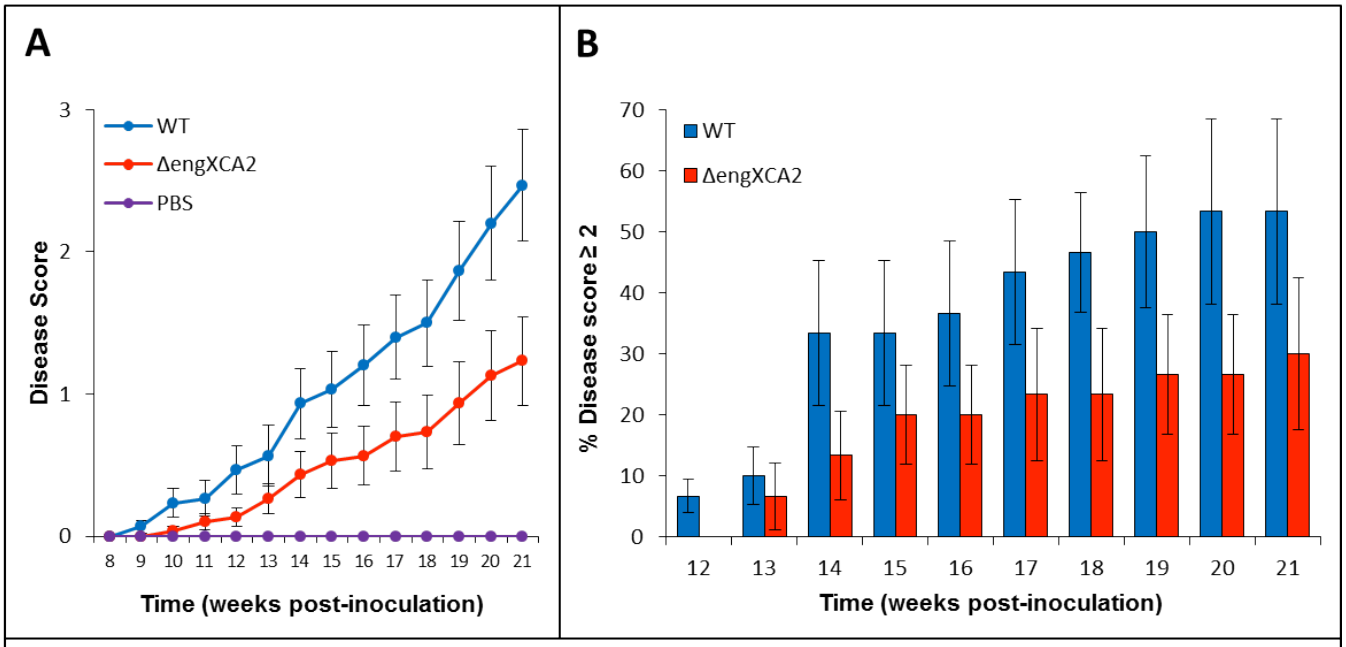

Figure 3: Pierce's disease development in Cabernet Sauvignon grapevines after inoculation with either the Δ*engXCA2* mutant strain or the wild-type strain over 21 weeks. 1X PBS served as the negative control. **A)** The Δ*engXCA2* mutant strain maintains a lower average disease score per week relative to the wild-type strain. **B)** The percentage of plants inoculated with the Δ*engXCA2* mutant strain that rated 2 or greater was consistently lower relative to the percentage of plants inoculated with the wild-type strain. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

We assessed initial colonization of the host by quantifying *Xf* wild-type and Δ*engXCA1* populations *in planta* by isolating these strains from the petioles at the point of inoculation (POI), 11 weeks post-inoculation (Fig. 4A). The Δ*engXCA1* mutant did colonize grapevines, indicating that the mutation is not lethal, but its population size at the POI was significantly lower than the wild-type population (P = 0.001). To assess the ability of the Δ*engXCA1* mutant strain to move within the xylem, *Xf* populations were quantified from petioles at ≥ 37 cm above the POI at 12 weeks post-inoculation (Fig. 4B). Interestingly, the Δ*engXCA1* mutant population was not statistically different from the wild-type population at ≥ 37 cm above the POI (P = 0.078), suggesting that the movement abilities of the Δ*engXCA1* mutant are not impaired despite reduced initial colonization. To determine if the mutant is impaired in traveling further distances in the xylem, the *Xf* wild-type and Δ*engXCA1* mutant strains were isolated at 20 nodes above the POI at 18 weeks post-inoculation (*data not shown*). The wild-type and mutant populations were not statistically different (P = 0.887), confirming that this mutation does not disrupt *Xf* movement in the xylem. Due to the non-parametric nature of the data, all statistical analyses were done using the Wilcoxon rank sum test to make pair-wise comparisons.

The Δ*engXCA2* mutant population at the POI was also quantified and compared to the wild-type Temecula 1 strain at 18 weeks post-inoculation (Fig. 5A). This mutant also displayed significantly lower initial colonization relative to the wild-type (P = 0.009). However, unlike the Δ*engXCA1* mutant, the Δ*engXCA2* mutant population was significantly lower than the wild-type population at 20 nodes above the POI (P = 0.015) at 18 weeks post-inoculation (Fig. 5B). This suggests that the ability for the Δ*engXCA2* mutant to move within the xylem is greatly reduced, and supports the findings made by Perez-Donoso et al. (2010) where the synergistic action of both EngXCA2 and a polygalacturonase was necessary for pit membrane degradation in grapevine stem explants. Interestingly, the Δ*engXCA2* mutant strain only displayed a reduction in movement and not the complete loss of mobility manifested by the Δ*pglA* mutant (Roper, 2007). This reduction in movement rather than complete impairment could be the result of over-compensation by the remaining two EGases, and a triple mutant strain devoid of all three EGases would likely mimic the phenotype displayed by the Δ*pglA* mutant.

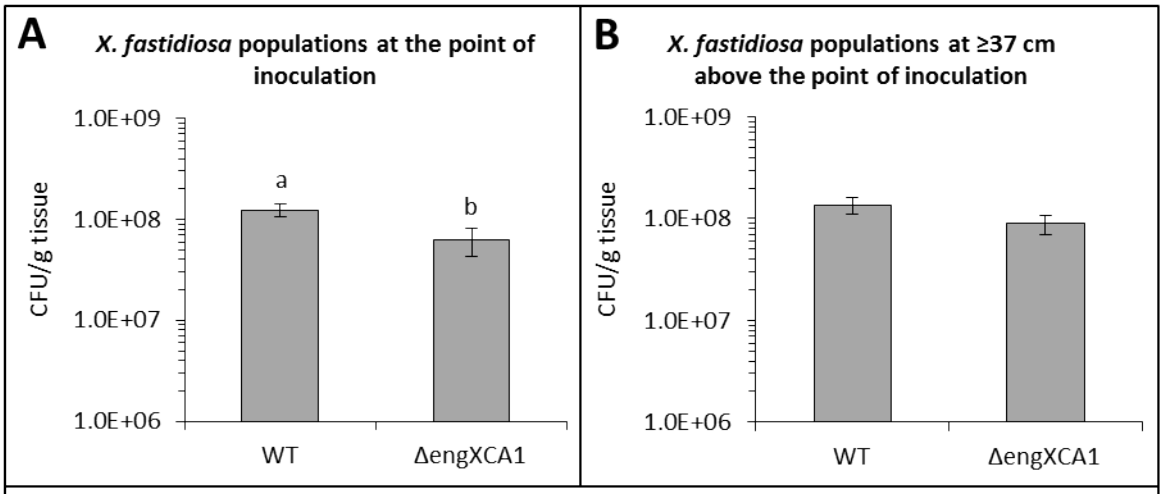

Figure 4: *In planta* populations of the Δ*engXCA1* mutant strain relative to the Temecula 1 wild-type strain in Cabernet Sauvignon grapevines. **A)** The point of inoculation (POI), 11 weeks post-inoculation: the Δ*engXCA1* mutant population is significantly lower than the wild-type population. **B)** ≥ 37 cm above the POI, 12 weeks post-inoculation: the Δ*engXCA1* mutant population is not significantly different from the wild-type population. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

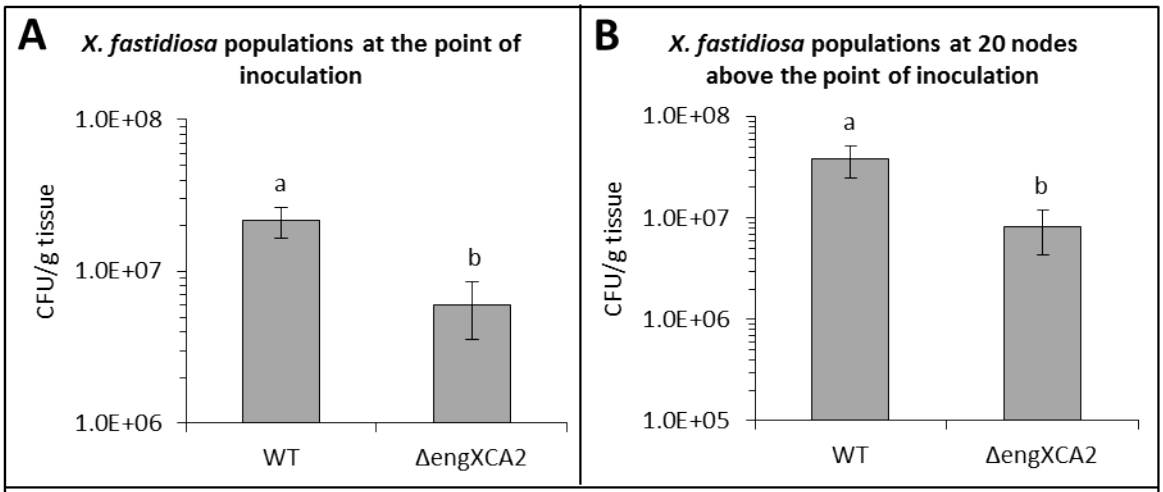

Figure 5: *In planta* populations of the Δ*engXCA2* mutant strain relative to the Temecula 1 wild-type strain in Cabernet Sauvignon grapevines. **A)** The Δ*engXCA2* mutant population is significantly lower than the wild-type population at the point of inoculation (POI) at 18 weeks post-inoculation. **B)** The Δ*engXCA2* mutant population is also significantly lower than the wild-type population at 20 nodes above the POI at 18 weeks post-inoculation. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

Objective 2: Inhibition of *Xf* endoglucanases and the endoglucanase/expansin using endoglucanase inhibiting proteins. Because the combined action of a PG and an *Xf* EGase was required to digest pit membranes, both are logical targets for inhibition. PG is a major pathogenicity factor for *Xf* and grapevines expressing a pear PGIP were more tolerant to *Xf* infection (Aguero et al., 2005). Several plant proteins have also been identified and characterized as xyloglucan-specific EGase inhibiting proteins (XEGIPs) that could potentially inhibit *Xf* EGases. These include XEGIPs from

tomato and tobacco (Naqvi et al., 2005, Qin et al., 2003). We propose to assess the ability of the tobacco and tomato XEGIPs to inhibit the degradative ability of the *Xf* EGases and the EGase/expansin.

Currently, we are working on expressing and purifying these *Xf* EGases and assessing their activity as stated in Objective 1. Once these studies have been completed, we will test for inhibition using a radial diffusion assay performed in agarose containing either CMC or XyG as a substrate with increasing concentrations of each XEGIP. In addition, we will quantify the generation of reducing sugars produced by the *Xf* EGases or EGase/expansin alone or in combination with each of the XEGIPs (Naqvi et al., 2005).

Objective 3: Characterization of the *Xf* Type II secretion system. The T2SS is composed of twelve-fifteen different proteins, involved either structurally or mechanistically in the function of the T2SS, depending on the species that is being examined. These proteins are encoded in a single operon and the *Xf* genome contains a similar operon strongly suggesting a functional T2SS (Jha et al., 2005). The T2SS can be divided into four different subassemblies that are 1) the pseudopilus; 2) the outer membrane complex; 3) the inner membrane platform and 4) the secretion ATPase. The pseudopilus is composed primarily of the major pseudopilin protein, G (XpsG), and also contains the minor pseudopilins, S, H, I, J and K (XpsH, I, J and K). The XpsE ATPase harnesses the energy that drives secretion through the T2SS via hydrolysis of ATP. Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Slonczewski, 2014). The T2SS then uses a pilus-like piston to push proteins through the T2 channel. This piston action is a function of the cyclic assembly and disassembly of pilin subunits (primarily XpsG).

We have created a mutation in the *xpsE* gene, encoding the putative ATPase that powers the T2SS. Grapevines inoculated with the *xpsE* mutant never developed PD symptoms and remained healthy, a phenotype similar to the *Xf pglA* mutant (Fig. 6). Thus, we have compelling preliminary data indicating that *Xf* has a functional T2SS system and the proteins secreted by T2SS are critical for the infection process.

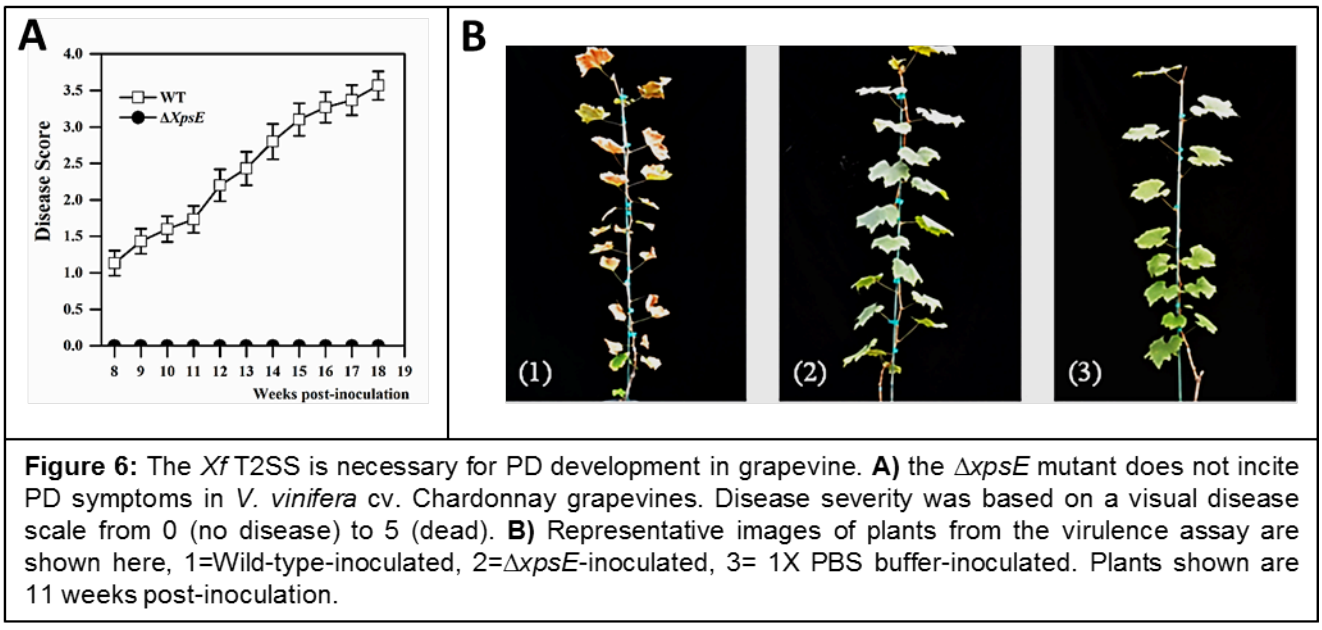

Figure 6: The *Xf* T2SS is necessary for PD development in grapevine. **A)** the Δ*xpsE* mutant does not incite PD symptoms in *V. vinifera* cv. Chardonnay grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). **B)** Representative images of plants from the virulence assay are shown here, 1=Wild-type-inoculated, 2=Δ*xpsE*-inoculated, 3= 1X PBS buffer-inoculated. Plants shown are 11 weeks post-inoculation.

We hypothesize that the non-pathogenic phenotype of the *ΔxpsE* mutant is due largely to the inability to secrete host CWDEs. Indeed, we have indirect experimental evidence that *Xf* utilizes the T2SS to secrete PG. This is based on an assay performed on the defined growth medium, XFM. When XFM is supplemented with pectin as the sole carbon source, this induces production of copious amounts of the

carbohydrate-based exopolysaccharide (EPS) (Killiny & Almeida, 2009). Pectin is a complex carbohydrate comprised in its simplest form of repeating galacturonic acid residues. Therefore, when grown on XFM with pectin as the sole carbon source, we hypothesize that *Xf* must first digest the pectin source utilizing its endo-PG (Roper et al., 2007) and likely other pectin-digesting enzymes that eventually disassemble the pectin polymer into individual galacturonic acid residues that can then feed into various metabolic processes within the bacterium, such as EPS production.

In support of our hypothesis that PG, and potentially other CWDEs, are secreted through the T2SS, we demonstrate that the Δ*xpsE* mutant produces visibly less EPS on XFM+pectin medium resulting in a much less mucoid phenotype (Fig. 7A). Furthermore, when wild type *Xf* and Δ*xpsE* are grown on XFM+galacturonic acid (i.e., the monomeric sugar that makes up the pectin polymer) or on XFM+glucose, both strains produce similar amounts of EPS (Fig. 7B, C). We infer from this that, indeed, breakdown of the pectin substrate is necessary to produce EPS and when the T2SS is disrupted this prevents secretion of PG and the subsequent breakdown of pectin. To confirm these results, we will be quantitatively determining the amount of EPS produced by both the *Xf* wild-type Temecula 1 strain and the Δ*xpsE* mutant strain via a total carbohydrate assay.

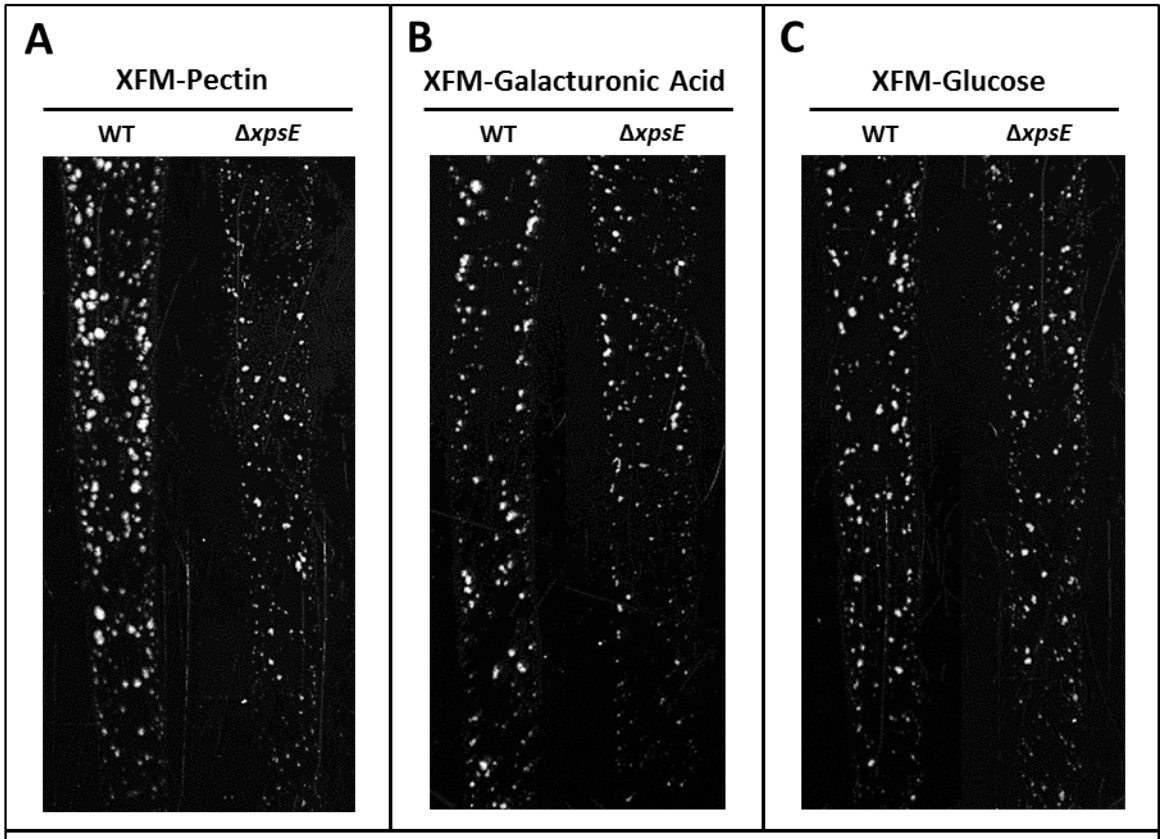

Figure 7: EPS assay with either the *Xf* wild-type Temecula 1 strain or the *Xf* Δ*xpsE* mutant strain grown on XFM minimal media containing a single carbon source. **A)** XFM containing pectin as the single carbon source. The *Xf* wild-type strain is able to produce more EPS than the Δ*xpsE* mutant strain. **B)** XFM containing galacturonic acid as the single carbon source, and **C)** XFM containing glucose as the single carbon source. No discernable differences in growth of the *Xf* wild-type strain and the *Xf* Δ*xpsE* mutant strain were detected on either of these media types.

To determine if the T2SS definitively secretes CWDEs, a whole secretome analysis of the wild-type and Δ*xpsE* mutant strains was performed. Proteins secreted into the media by either the wild-type strain or the Δ*xpsE* strain were precipitated and run on an SDS-polyacrylamide gel. Additionally, proteins secreted, but remaining at the bacterial cell surface, were also analyzed for each strain. The proteins

were transferred to a nitrocellulose membrane via Western Blotting and the membrane was probed with an antibody targeting the EngXCA2 EGase (Fig. 8). After Western Blot development, bands at approximately 62 and 68 kDa were present in the wild-type media fraction, but not in the Δ*xpsE* media fraction (Lanes 2 – 5). Additionally, a band of approximately 63 kDa was present in the wild-type cell surface protein fraction, but not in the Δ*xpsE* cell surface protein fraction (Lanes 6 – 9). The protein targeted by the antibody (EngXCA2) is 62.05 kDa and is likely the lower band present in the wild-type media fraction. The presence of other bands on the Western Blot can be explained by the fact that the anti-EngXCA2 antibody targets the whole protein. Two other proteins have similar primary and secondary structure to EngXCA2: the endoglucanase EngXCA1 and the cellobiohydrolase, CbhA. Due to these similarities, it is entirely possible that the antibody is cross-reacting with these other proteins. Indeed, CbhA is 67.82 kDa and could account for the upper band in the wild-type media fraction. EngXCA1 is 63.49 kDa and could account for the band in the wild-type cell surface protein fraction. Several large bands were present on the Coomassie stained gel (*data not shown*) that were not present on the Western Blot, indicating that the antibody was not binding non-specifically. To confirm these results, a mass spectrometry analysis of the media fractions and cell surface protein fractions will be conducted for both the wild-type and the Δ*xpsE* strains. It is important to note that this antibody targets an *Xf* EGase (EngXCA2), which was definitively present in the wild-type fractions and not in the Δ*xpsE* fractions. These results provide clear evidence that this EGase is secreted via the T2SS.

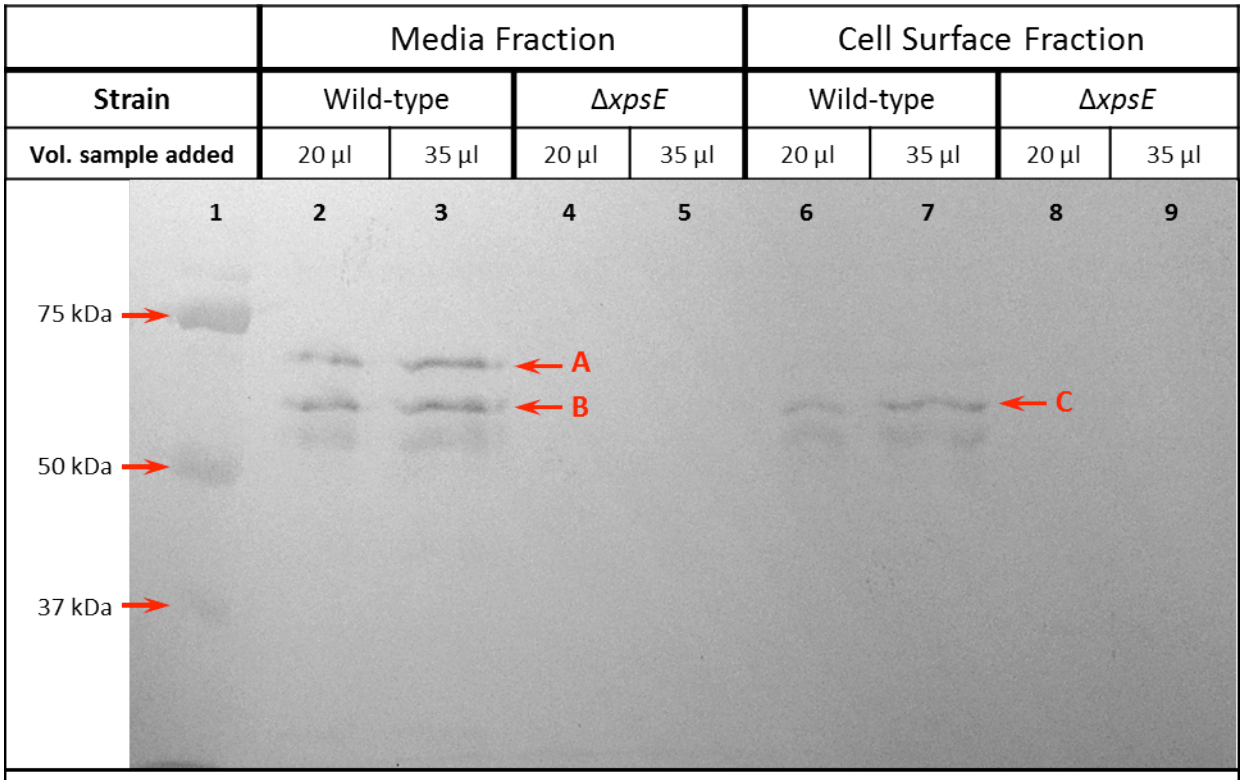

Figure 8: Western Blot analysis of the *Xf* secretome. The membrane was probed with an anti-EngXCA2 antibody followed by a goat anti-rabbit antibody conjugated with alkaline phosphatase. Lane 1, molecular weight standards. Lanes 2 and 3, Proteins secreted into the media by the wild-type Temecula 1 strain. Lanes 4 and 5, proteins secreted into the media by the Δ*xpsE* strain. Lanes 6 and 7: wild-type-secreted proteins remaining at the bacterial cell surface. Lanes 8 and 9: Δ*xpsE*-secreted proteins remaining at the bacterial cell surface. Band A = 68 kDa; Band B = 62 kDa; Band C = 63 kDa.

Objective 4: Inhibition of the *Xf* Type II secretion system.

Proteins destined for secretion by the T2SS are first exported to the periplasm by the Sec or Tat pathways. *Xf* appears to only possess the Sec-dependent secretion pathway. Disruption of the T2SS by small molecule inhibitors was demonstrated in *Pseudomonas aeruginosa* and *Burkholderia*

pseudomallei, and could be used to inhibit the *Xf* Sec-dependent pathway (Moir et al., 2011). A chemical compound library will be screened for Sec-inhibitory molecules, including those compounds used by Moir et al. (2011). Inhibition of the Sec-dependent pathway will be confirmed by monitoring the secretion of a CWDE using a polyclonal antibody raised against EngXCA2 and analyzed via Western Blot and ELISA.

PUBLICATIONS PRODUCED AND PRESENTATIONS MADE

Publications

Roper, C., Kirkpatrick, B., Labavitch, J. and Cosgrove, D. 2014. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery. California Department of Food and Agriculture. p.74-81. Proceedings of the Pierce's Disease Research Symposium, Sacramento, CA.

Presentations

Roper, C. 2014. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery. Presented as an Oral Presentation at the Annual Pierce's Disease Symposium, Sacramento, CA.

Ingel, B., Kirkpatrick, B., Labavitch, J., Cosgrove, D. and Roper, C. 2014. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery. Presented as a Poster at the Annual Pierce's Disease Symposium, Sacramento, CA.

RESEARCH RELEVANCE STATEMENT

Our goal is to first understand the roles each of the EGases produced by *Xf* has in pit membrane degradation, as well as the role of the T2SS in secreting these CWDEs. Ultimately, we speculate that inhibition of the EGases and/or the T2SS will significantly reduce the ability of *Xf* to systemically colonize its grapevine host. Preliminary results indicate that the EGase/expansin hybrid protein (EngXCA1) retains endoglucanase activity. These experiments will be repeated with additional negative controls, followed by reducing sugar assays to confirm these findings. EngXCA1 also plays a role in virulence, and could possibly be an elicitor of the host defense response. These studies will be repeated with the addition of the *engXCA1/engXCA1+* complement strain to confirm these results. In addition, an *Xf* strain with a deficient T2SS (Δ*xpsE*) displayed reduced virulence than unmodified *Xf*, lending credence to the hypothesis that the T2SS secretes CWDEs such as PG and EngXCA2 that are necessary for systemic colonization. We speculate that further characterization of these EGases and the T2SS will elucidate significant targets for controlling Pierce's Disease.

LAYPERSON SUMMARY

Xylella fastidiosa relies on degradation of the plant cell wall to move within the grapevine. This is accomplished by the cooperation of at least two classes of enzymes that target different components of the complex scaffold of the plant cell wall. A major goal of this research is to further elucidate the factors that lead to disassembly of the plant cell wall, thereby, allowing the bacteria to systemically colonize the plant. Systemic colonization is highly correlated with Pierce's Disease development and preventing movement of the bacteria is critical to devising successful control strategies. We propose that characterizing and inhibiting *Xf* enzymes that facilitate movement throughout the plant and/or the secretion machinery responsible for delivering those *Xf* enzymes into the grapevine's water pipes will provide a comprehensive approach to restriction of disease development.

STATUS OF FUNDS

The funding for this project is largely going towards supporting a Ph.D. graduate student, Mr. Brian Ingel. This project is the main focus of his Ph.D. dissertation. We anticipate spending the remainder of the salary, supply, services and greenhouse recharge money associated with this project as it progresses.

INTELLECTUAL PROPERTY STATEMENT
Thus far, there is no Intellectual Property associated with this project.

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