CHARACTERIZATION OF THE LIPOPOLYSACCHARIDE-MEDIATED RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION IN GRAPEVINE.

REPORT TYPE: Final Report for CDFA agreement number 11-0148-SA

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REPORTING PERIOD: The results reported here are from work conducted July 1, 2011 to June 30, 2015

ABSTRACT

Xylella fastidiosa (*Xf*) is a gram-negative, xylem-limited bacterium that causes serious diseases in economically important crops, such as Pierce's Disease (PD) of grapevine. Lipopolysaccharide (LPS) is the dominant macromolecule displayed on the bacterial cell surface. LPS acts as a selective barrier, preventing entry of toxic substances into the cell, and as an anchor for superficial structures. Finally, LPS is a well-described pathogen-associated molecular pattern (PAMP) and is known to elicit host basal defense responses in model plant systems. LPS is composed of a conserved lipid A-core oligosaccharide component and a variable O-antigen. Through mutations made in *wzy* (XP0836), which encodes an O-antigen polymerase, we have demonstrated that the *Xf* O-antigen contributes to plant and insect colonization, and depletion of the O-antigen causes a severe reduction in overall virulence *in planta*. The goal of this project was to determine the role of the *Xf* O-antigen in modulation of basal defense responses in grapevine. Specifically, we demonstrated that specific alterations to the LPS structure caused a change in the elicitation of the grapevine response to *Xf*, therefore affecting critical, early stages of *Xf* establishment *in planta*. We also demonstrated that *Xf* LPS and O-antigen structural variants have the potential to be used as a preventative treatment for the control of PD.

LAYPERSON SUMMARY: *Xylella fastidiosa* (*Xf*), a bacterial pathogen, is the causal agent of Pierce's disease (PD) of grapevine and poses a serious threat to the viticulture industry. We have demonstrated that truncation of the O-antigen portion of the lipopolysaccharide (LPS) entity alters the adhesive properties of the cell, leading to a defect in mature biofilm formation. Furthermore, depletion of the O-antigen results in a significantly less virulent pathogen that is severely compromised in host colonization**.** Additionally, LPS is a Pathogen-Associated Molecular Pattern (PAMP) that potentially triggers the basal immune response. We hypothesize that the long chain O-antigen also allows *Xf* to circumvent the innate immune system by masking the conserved core and lipid A portions of the LPS chain from the host immune system. The goal of the proposed work is to further explore the role of LPS, specifically focusing on the O-antigen moiety, in the interaction between *Xf* and the grapevine host and to use this information to develop and evaluate an environmentally sound preventative application for PD.

INTRODUCTION: *Xylella fastidiosa (Xf)*, a gram-negative, fastidious bacterium, is the causal agent of Pierce's Disease (PD) of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee *et al.*, 2008; Varela, 2001). PD has devastated some viticulture areas in California, and there are currently no effective control measures available to growers targeted towards the bacterium itself. Lipopolysaccharide (LPS) is a tripartite glycolipid molecule that is an integral part of the Gram-negative bacterial outer membrane. It is prominently displayed on the outer surface of the cell, thereby mediating interactions between the bacterial cell wall and its environment. LPS plays diverse roles for the bacterial cell. It provides structural integrity to the cells and can act as a permeability barrier to toxic antimicrobial substances (Raetz & Whitfield, 2002). Because of is location in the outer membrane, it is also a key contributor to the initial adhesion to a surface or host cell (Walker *et al.*, 2004). We have been exploring the roles of LPS in the Pierce's Disease cycle and in the Plant-Microbe-Insect (PMI) interactions of *Xf*. We targeted our studies towards the outermost exposed region of the LPS molecule, the O-antigen. By mutating a key O-antigen polymerase, *wzy* (XP0836), in the Temecula1 (Pierce's

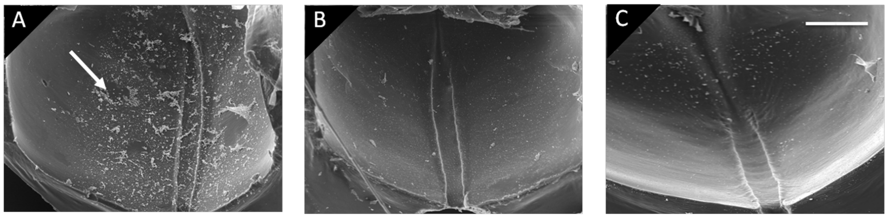

Figure 1. Truncation of the O-antigen compromises colonization of the cibarium. The *wzy* mutant was notably reduced in the ability to attach to the cibarium. Scanning electron micrographs of the hypopharyngeal surface of the cibarium of BGSS fed on artificial diets containing wild type *X. fastidiosa (*A)*, wzy* mutant (B), or artificial diet only (C) for 6 hours. Images are oriented so that the stylet food canal would be at the bottom and the pharynx at the top; thus ingested fluid would flow inward from bottom to top. Images are a representation of 30 total replicates per treatment. Arrow indicates bacterial aggregates. Diet-fed insects represent negative controls. Bar = 20µm.

Disease) isolate, we have demonstrated that severe truncation of the O-antigen alters the adhesive and aggregative properties of the cell considerably, thus causing a marked defect in biofilm formation. Furthermore, the resulting mutation of the O-antigen caused increased sensitivity of the bacterium to hydrogen peroxide stress *in vitro* and resulted in a significantly less virulent pathogen that is severely impaired in host colonization (Clifford et al, 2013). It has long been speculated that *Xf* surface polysaccharides play a role in the host-pathogen interaction with grapevine, and our ongoing studies confirm that LPS is a major virulence factor for this important agricultural pathogen. We have also determined that the O-antigen plays an important role in the acquisition of *Xf* by an efficient insect vector, the Blue-Green Sharpshooter (BGSS) (*Graphocephala atropunctata*). In collaboration with Dr. Thomas Perring (University of California-Riverside, Dept. of Entomology) and Dr. Elaine Backus (United States Department of Agriculture, Agricultural Research Service, Parlier, CA) we have conducted intricate acquisition assays and determined that alterations in O-antigen structure and composition, indeed, result in a change in the adhesive properties of *Xf* within the insect as well (**Fig 1,2**).

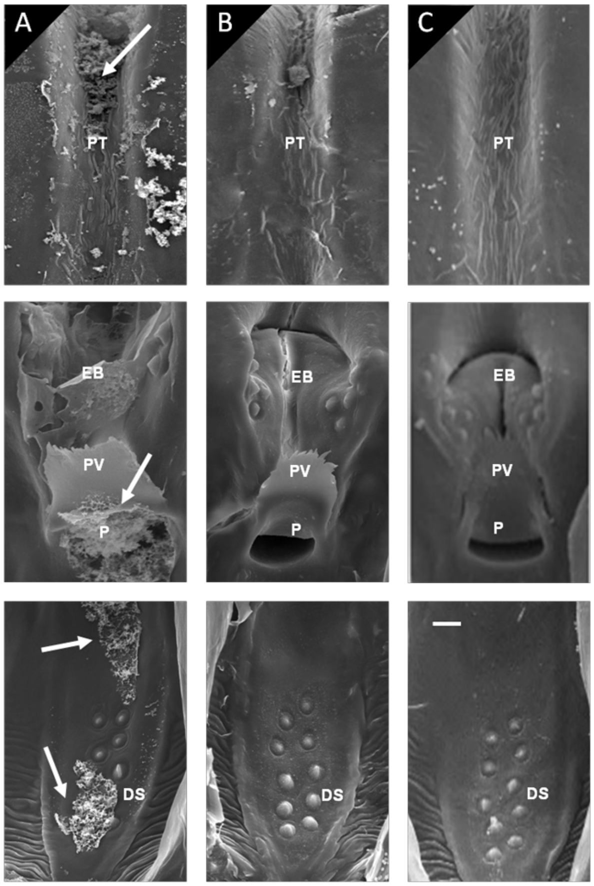

Figure 2. Truncation of the O-antigen compromises colonization of the precibarium. The *wzy* mutant was notably reduced in the ability to attach to all structures within the precibarium, including the distal sensilla, pit, precibarial valve, epipharyngeal basin, and the precibarial trough. Scanning electron micrographs of the epipharyngeal surface of the precibarium of BGSS fed on artificial diets containing wild type *X. fastidiosa* (A)*, wzy* mutant (B), or artificial diet only (C) for 6 hours. The stylet food canal is oriented towards the bottom and the pharynx at the top. Therefore, the ingested fluid would flow inward from bottom to top. Images are a representation of 30 total replicates per treatment. Arrows indicate bacterial aggregates. Diet-fed insects represent negative controls. DS = distal sensilla; P = pit; PV = precibarial valve; EB = epipharyngeal basin; PT = precibarial trough. Bar = 5µm.

Contrary to the role of LPS in promoting bacterial survival *in planta*, the immune systems of plants have also evolved to recognize the LPS structure and mount a basal defense response to counteract bacterial invasion (Dow *et al.*, 2000; Newman *et al.*, 2000). LPS is considered a Pathogen Associated Molecular Pattern (PAMP). PAMPs, also known as Microbial Associated Molecular Patterns (MAMPs), are conserved molecular signatures that are often structural components of the pathogen (ie. LPS, flagellin, etc.). These PAMPs are recognized by the host as "non-self" and can be potent elicitors of the basal defense response. This line of defense against invading pathogens is referred to as PAMP triggered immunity (PTI) and represents the initial layer of defense against pathogen ingress (Nicaise *et al.*, 2009). PTI is well studied in both mammalian and plant hosts. However, little is known about the mechanisms involved in perception of the LPS PAMP in grapevine, particularly the *Xf* LPS PAMP. *Xf* is introduced by its insect vector directly into the xylem, a non-living tissue, which cannot mount a defense response on its own. However, in other systems, profound changes do occur in the adjacent living parenchyma cells upon infection suggesting that these cells communicate with the xylem and are capable of recognizing the presence of a pathogen (Hilaire *et al.*, 2001). The plant immune system can recognize several regions of the LPS structure, including the conserved lipid A and core polysaccharide components (Newman *et al.*, 2007; Silipo *et al.*, 2005). Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule. Specifically, bacteria can display different O-antigen profiles by varying the extent of polymerization or by completely abolishing synthesis of the O-antigen depending on the environment and developmental phase of the cell (Bergman *et al.*, 2006; Guerry *et al.*, 2002; Lerouge & Vanderleyden, 2002). We speculate that during the interaction between *Xf* and a susceptible grapevine host, the bacterium's long chain, rhamnose-rich O-antigen shields the conserved lipid A and core-oligosaccharide regions of the LPS molecule from being recognized by the grapevine immune system, providing an opportunity for it to subvert the basal defense response and establish itself in the host. A similar scenario occurs in *E. coli*, where truncation of the O-antigen caused an increased sensitivity to serum suggesting the full length O-antigen provides a masking effect towards the host immune system (Duerr *et al.*, 2009; Guo *et al.*, 2005).

Our main aim is to further explore the role of LPS, specifically focusing on the O-antigen moiety, in the interaction between *Xf* and the grapevine host and to use this information to develop and evaluate an environmentally sound preventative application for PD. In addition to its contribution to biofilm formation, we hypothesized that the LPS molecule also modulates the host's perception of *Xf* infection. The *Xf* O-antigen mutant we currently have provides a unique platform designed to test this hypothesis. The fundamental goal is to elucidate the mechanism(s) that *Xf* uses to infect the grapevine host and exploit this knowledge to evaluate the use of LPS structural variants as a preventative treatment for control of PD.

OBJECTIVES OF PROPOSED RESEARCH

Objective 1: Characterization of *Xf* LPS mutants *in vitro* and *in planta*.
Objective 2: Examination of the LPS-mediated response to *Xf* infection.
Objective 3: Evaluation of structural variants of LPS as a preventative treatment for PD.

RESULTS AND DISCUSSION

Objective 1: Characterization of *Xf* LPS mutants *in vitro* and *in planta*.

We have determined that the wild-type *Xf* O-antigen is composed primarily of 2-linked rhamnose with smaller amounts of glucose, ribose, xylose and mannose (Clifford et al, 2013). Most importantly, we demonstrated that mutation of the O-antigen polymerase, Wzy, results in a severely truncated O-antigen resulting from a depletion of the majority of the 2-linked rhamnose. This change was confirmed both electrophoretically and biochemically utilizing gas chromatography and mass spectrometry (GC/MS) techniques in collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia. Notably, the depletion of rhamnose led to a marked reduction in virulence and host colonization (Clifford et al, 2013). This indicates that the process of rhamnose biosynthesis and its incorporation into the O-antigen is a vulnerable step in the *Xf* LPS biosynthetic pathway that could be exploited for disease control. Therefore, in this objective we are building on our current knowledge and continuing our studies by focusing on the process of rhamnose biosynthesis in *Xf*. We are presently creating mutants that we hypothesize will be unable to synthesize rhamnose, and we will structurally characterize the O-antigen from these mutants in collaboration with the CCRC. Following this, we will define the biological impact of these mutations by conducting virulence and colonization studies in grapevine. We will also determine the effect of these mutations *in vitro* using substrate attachment, cell-cell aggregation, and visualized biofilm studies that reflect host colonization behaviors.

Using comparative genomics, we have identified five genes with high homology to those involved in rhamnose biosynthesis in other bacterial systems. The genes are designated *rmlB*$_1$ (XP0208), *rmlA* (XP0209), *rmlC* (XP0210) and *rmlD* (XP0211) (in map order) that encode proteins involved in the conversion of glucose-1-phosphate into dTDP-rhamnose (Jiang *et al.*, 1991; Koplin *et al.*, 1993; Rahim *et al.*, 2001). The first step is catalyzed by RmlA (dTDP-glucose synthase). Subsequent reactions are catalyzed by RmlB (dTDP-D-glucose-4,6-dehydratase), RmlC (dTDP-L-rhamnose synthetase) and RmlD (dTDP-4-dehydrorhamnose reductase) (Koplin *et al.*, 1993). The resulting dTDP-L-rhamnose is then incorporated into the O-antigen. The *rml* genes are usually clustered within a single locus and our *in silico* analysis demonstrates the presence of a *rml* locus in *Xf*. We also identified an additional, unlinked copy of *rmlB*, designated *rmlB*$_2$ (XP1617).

Mutant construction: We have constructed a Δ*rmlAB*$_1$*CD* mutant. Our next step is to make a *rmlAB*$_1$*CD*/*rmlB*$_2$ double mutant. In the event that deletion of the entire locus has pleiotropic effects (ie. affects exopolysaccharide production), we will knock out each gene individually. We will begin with *rmlA,* which encodes the enzyme that catalyzes the first step in pathway of rhamnose biosynthesis. If needed, we will then sequentially knock out *rmlB*$_1$, *rmlC* and *rmlD* and also create a Δ*rmlB*$_2$ mutant. All mutants constructed in this study will be complemented with the wild type copy of the gene, including the native promoter, using the complementation vectors now available for *Xf* developed by the Igo lab at UC-Davis (Matsumoto *et al.*, 2009). Once we have obtained all of these mutants and their complements, we will begin biochemically characterizing their O-antigen. We predict that the O-antigen in the *rml* mutants will be completely devoid of rhamnose. We will confirm this by conducting glycosyl composition and linkage analyses in collaboration with the Complex Carbohydrate Research Center (CCRC) (University of Georgia). This Center routinely performs carbohydrate linkage analysis studies on a recharge basis.

LPS isolation and O-antigen purification: In order to supply LPS in amounts sufficient for NMR analysis, the CCRC requested that we send cell pellets of both *Xf* wild type and O-antigen mutant strains (5g per strain), instead of purified and lyophilized LPS. Wild type and *wzy* mutant cell pellets were shipped in the fall of 2014. Prior to shipment, cell pellets were prepared in the following manner: bacterial strains were grown on solid PD3 medium (minus starch) for 7 days at 28°C. Agar squares, containing *Xf* colonies, were then cut from the plates and added to 250mL liquid PD3 (minus starch) and placed onto a 28°C shaker at 180rpm for an additional 7 days. Following incubation, cells were spun down, treated with 1% phenol to kill cells, and then washed three times with 1xPBS to remove any residual media or phenol. Pellets were stored at -80°C until shipping to the CCRC in Georgia. We are in regular communication with the CCRC, and due to the extensive analytical analyses required for the *Xf* LPS, we anticipate receiving the final structural results for the wild type and *wzy* O-antigen in the next few months.

Surface attachment, aggregation and biofilm studies: We have begun linking *Xf* LPS structure to function using *in vitro* assays. Attachment to a surface is the first step in successful biofilm formation, and because of the location and abundance of LPS in the outer membrane, we hypothesized that LPS plays a key role in mediating initial attachment to the cellulose and chitin substrates *Xf* encounters in the plant and insect, respectively. We previously demonstrated that a mutant in the Wzy polymerase was deficient in cell-cell aggregation and hyperattached to surfaces, which led to a defect in biofilm formation (Clifford et al., 2013). We recently tested the Δ*rmlAB*$_1$*CD* mutant for these behaviors to determine if the inability to synthesize rhamnose results in defective biofilm formation. We found that, similar to the *wzy* mutant phenotype, the Δ*rmlAB*$_1$*CD* mutant strain hyperattached to a glass surface (**Figure 3A**) but was significantly impaired in cell-cell aggregation (**Figure 3B**). In addition, preliminary biofilm studies of the mutant strain (in which biofilm formation at the air-liquid interface was visually analyzed) indicated malformation of a mature biofilm (**Figure 4**).

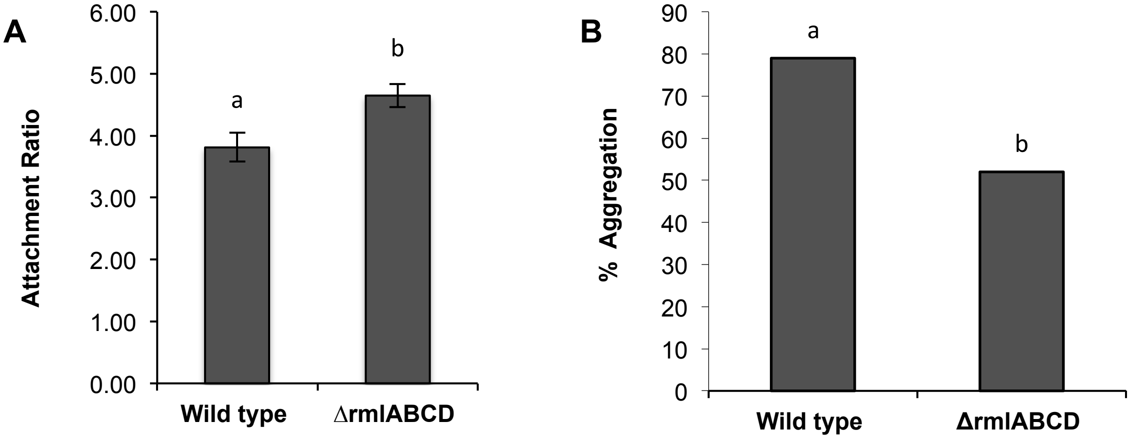

Figure 3. *In vitro* attachment and aggregation assays. Attachment ratio (A) and % aggregation (B) of *X. fastidiosa* wild type and Δ*rmlAB*$_1$*CD* mutant strains. The Δ*rmlAB*$_1$*CD* mutant strain hyperattached to a glass surface ($P < 0.05$) but was significantly impaired in cell-cell aggregation ($P < 0.0001$), compared with wild type. Graphs represent the mean of 9 samples per treatment ± standard error of the mean. Treatments with different letters on bars are statistically different.

<u>Virulence and host colonization assays</u>: In July, we mechanically inoculated *Vitis vinifera* 'Cabernet Sauvignon' vines with *ΔrmlAB*$_1$*CD* using the pin-prick method (Hill & Purcell, 1995). Each plant was inoculated twice with a 20µL drop of a 10^8 CFU/mL suspension of either wild type *Xf* or the *rml* mutant. We inoculated 7 plants/mutant and repeated each experiment 3 times. Plants inoculated with 1x PBS served as negative controls. As symptoms develop, all plants will be rated on a disease scale of 0-5 with 0 being healthy and 5 being dead (Guilhabert & Kirkpatrick, 2005). We will also assess the *Xf* populations in the plants by isolating cells from the petioles at the point of inoculation, and 25 cm above the point of inoculation, to assess the ability of the *rml* mutant to systemically colonize the host. Isolations will be performed at six and fourteen weeks post-inoculation. Petioles will be surface sterilized and ground in 2 mL of sterile 1x PBS. The resulting suspension will be diluted and plated on solid PD3 medium and colonies will be counted and normalized to tissue weight.

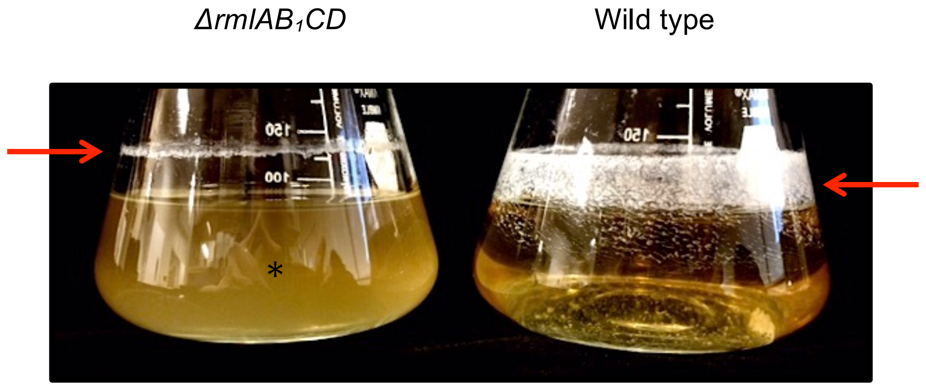

Figure 4. *In vitro* biofilm formation assay. The Δ*rmlAB*$_1$*CD* mutant strain was severely impaired in building a mature biofilm. Image represents biofilm formation at 4dpi. Arrows point to the formation of biofilm at the air liquid interface. *Turbidity of the media displays the defect in aggregation seen in the Δ*rmlAB*$_1$*CD* mutant.

Objective 2: Examination of the LPS-mediated response to *Xf* infection.

In grapevine, recognition of PAMPs other than LPS, such as the *Botrytis cinerea* endopolygacturonase BcPG1and β-glucans, trigger a cascade of signaling events including calcium ion influxes, reactive oxygen radical accumulations, and activation of protein kinases, that coordinate the transcriptional activation of defense genes (Aziz *et al.*, 2003; Aziz *et al.*, 2007; Poinssot *et al.*, 2003). The LPS PAMP can induce similar responses in other plant species, but these studies have been performed primarily in model systems, such as *Arabidopsis thaliana* or tobacco (Desaki *et al.*, 2006; Zeidler *et al.*, 2004). There is limited knowledge about the grapevine response to the LPS PAMP, particularly on the transcriptional level. However, one study demonstrated that a rhamnolipid MAMP from *P. aeruginosa* could induce defense-related responses in grapevine cell suspensions (Varnier *et al.*, 2009).

The defense reactions activated upon PAMP recognition involve intricate networks of transcriptional regulators and phytohormone signaling. Genome-wide transcriptional profiling is a logical starting point to begin understanding this complex process in the *Xf*-grape pathosystem (Jones & Dangl, 2006). We speculated that mutated *Xf* LPS (deplete of O-antigen) recognition elicits a transcriptional response that results in the deployment of specific defense reactions in grape that results in less disease and host colonization. We hypothesized that the grapevine is recognizing the conserved core/lipid A portions of the *Xf* LPS molecule and that the long chain O-antigen serves to camouflage the rest of the LPS PAMP (the core-lipid A complex) from being recognized by the host innate immune system. Thus, we expected an increase in expression of defense-related genes in plants inoculated with the O-antigen mutants, that are deplete of O-antigen, as compared to wild type *Xf.* The studies detailed below were designed to test our hypothesis that loss of the rhamnose-rich O-antigen allows the grapevine to more readily perceive the *Xf* LPS molecule and that this recognition leads to elicitation of a specific transcriptional response associated with defense.

LPS-induced oxidative burst in grapevine: To explore the role of LPS as an elicitor of basal defense responses in grapevine, we first investigated (Reactive Oxygen Species) ROS production *ex vivo* using a luminol assay. Both wild type and *wzy* mutant LPS induced an oxidative burst in grapevine leaf disks. However, the burst induced by *wzy* mutant LPS was stronger and more prolonged than wild type, peaking around 5 minutes and returning to near-basal levels around 32 minutes post-elicitation (**Fig. 5**). We hypothesized that this was due to the increased exposure of the Lipid A-Core Oligossacharide region of the *wzy* mutant.

ROS production in response to live *Xf* cells: Once we established that *X. fastidiosa* LPS induced an oxidative burst in *V. vinifera* 'Cabernet Sauvignon' leaf disks, we then turned our attention to ROS produced in response to living *X. fastidiosa* wild type and *wzy* mutant live culture. The *wzy* mutant culture induced a strong response from grapevine leaf disks. ROS production peaked at around 12 minutes and lasted nearly 100 minutes. Wild type culture failed to produce a sharp peak, as compared with the *wzy* mutant, and ROS production plateaued around 60 minutes.

Transcriptome profiling: The application of transcriptome profiling approaches using next generation RNA sequencing (RNA-seq) will allow us to monitor the activation or suppression of specific defense pathways at the genome scale. In early July of 2014, individual vines of *V. vinifera* 'Cabernet Sauvignon' were inoculated with *Xf* wild type or *wzy*::*kan* live culture. We inoculated 9 vines for each treatment. Vines inoculated with 1xPBS buffer alone served as the negative controls for the experiment. Using the pin-prick method described previously, each vine was inoculated 2x with a 20 µL drop of a 10^8 CFU/mL suspension of either wild type *Xf* or the *wzy* mutant. The inoculum was immediately drawn into the petiole due to the negative pressure in the xylem. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow *et al.*, 2000; Tao *et al.*, 2003). Thus, petioles were harvested at the following four time points: 0,1,8, and 24 hours post-inoculation. To stabilize transcripts, petioles were submerged into liquid Nitrogen immediately after harvesting and stored at -80°C until RNA extraction.

Sequencing libraries were generated from the polyadenylated plant messenger RNA and sequenced using an Illumina HiSeq 2500 platform. The sequencing of four HiSeq lanes generated a total of 763 million 50 bp single-end reads. Reads were trimmed and filtered to retain high-quality sequence information only (Q > 30). An average of 24.5 ± 3 millions of reads per sample (87.6±1.7% of the total) were unambiguously mapped on the reference PN40024 transcriptome. Counts were normalized to control for technical variation using DESeq2, which was also used for statistical testing.

Our analysis focused on specific patterns of transcriptional regulation to identify genes specifically responsive to the *wzy* mutant and not to wild-type *Xf*. 112 genes, which showed consistent up-regulation (fold change > 1.4, $P < 0.05$) in response to the *wzy* mutant at 8hrs post inoculation, were either unchanged in the wounded or wild-type inoculated plants or were differentially regulated only at the later time point. The 112 genes included chitinases, endoglucanases and other known antimicrobial peptides, typically transcriptionally induced during incompatible interactions. Enrichment analysis of these 112 genes showed an interesting over-representation of immune response functions in these sets of genes transcriptionally responsive to *wzy* mutant (e.g. defense responses, response to stress, oxidation-reduction process). At 24 hrs, 53 genes were significantly up-regulated specifically in response to *wzy*. Overall, the RNA-seq data suggest that grapevines are activating specific defense responses (namely those involved in oxidative stress) upon recognition of *wzy* mutant infection (**Fig. 6**). RT-qPCR will be performed to validate the expression of the most interesting genes. Investigation into the differential expression of genes during later stages of the *X. fastidiosa* infection process is also currently in progress. Beginning in June 2015, individual vines of *V. vinifera* 'Cabernet Sauvignon' were inoculated with *Xf* wild type or *wzy*::*kan* live culture. We inoculated 9 vines for each treatment. Vines inoculated with 1xPBS buffer alone served as the negative controls for the experiment. Using the pin-prick method described previously, each vine was inoculated 2x with a 20 µL drop of a 10^8 CFU/mL suspension of either wild type *Xf* or the *wzy* mutant. Petioles were harvested at 0, 48hr, 1 week, and 4 weeks post-inoculation with live culture. To stabilize transcripts, petioles were submerged into liquid nitrogen immediately after harvesting and stored at -80°C until RNA extraction. All petioles were shipped to the Cantu lab at UC Davis for RNA extraction and sequencing.

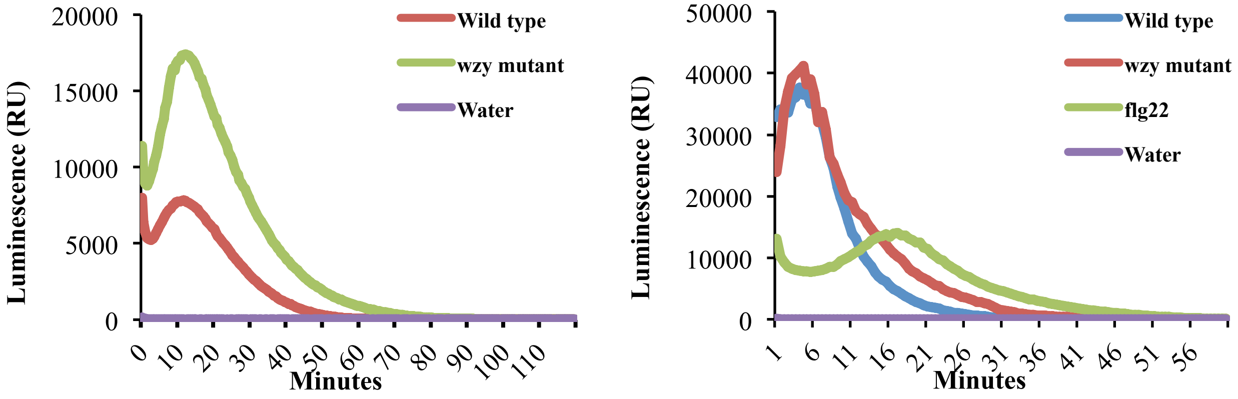

Figure 5. Purified LPS-induced ROS production in grapevine (left). Presence of an oxidative burst was determined through the chemiluminescence of luminol. The *wzy* mutant LPS elicits a stronger, more prolonged response in grapevine leaf disks, compared with *Xf* wild type LPS. **ROS production in response to live *Xf* cells (right).** Suspensions of wild type or *wzy* mutant culture were added to grapevine leaf disks, and ROS production was monitored through the chemiluminescence of luminol. The O-antigen mutant culture induced a strong response from grapevine leaf disks, peaking at around 12 minutes and plateauing around 100 minutes. Wild type culture failed to produce a sharp peak, compared with *wzy* mutant cells, and ROS production plateaued at around 60 minutes. Data are means of three independent assays with 8 replications per treatment.

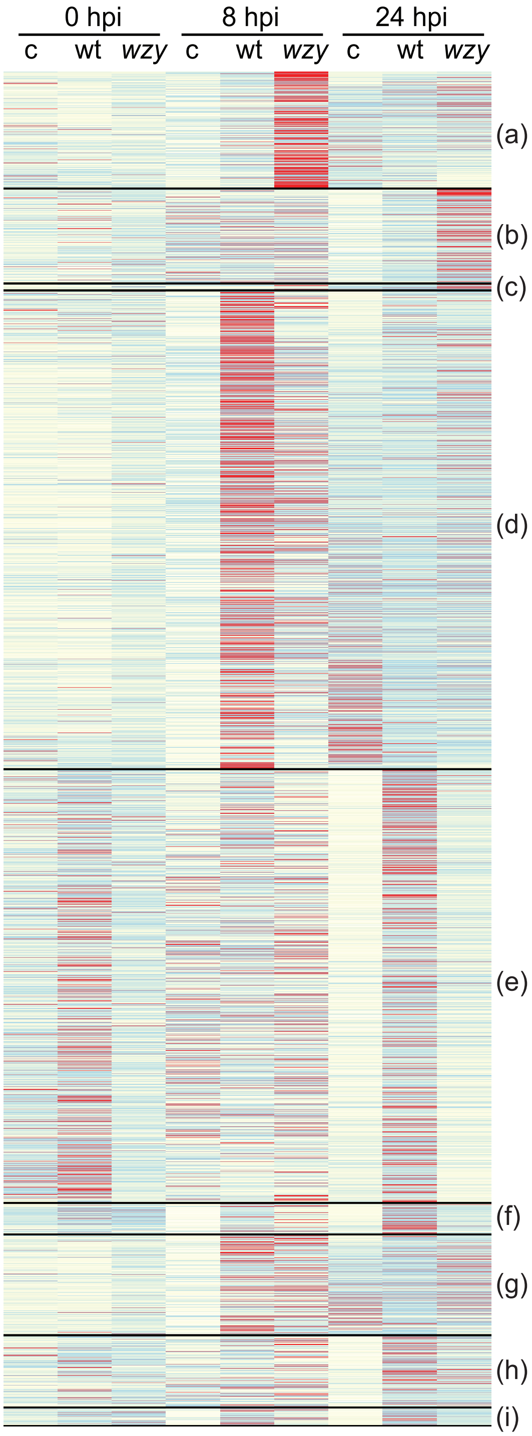

Figure 6. Heat map of the LPS-mediated response to *Xf* infection in grapevine. Genes were grouped based on their expression pattern relative to the wounded control "c". At 8hr post-inoculation, plants inoculated with the *wzy* mutant are expressing a large number of genes in group (a), which consists of genes specifically involved in immune responses (i.e. "response to oxidative stress"). At the same time point, plants inoculated with wild type are expressing a large number of genes in group (d), which consists of genes involved in plant metabolism. This indicates that plants are mounting an immune response to the *wzy* mutant, but the wild type cells remain undetected by the host immune system. The genes up-regulated in response to wild type *Xf* are related to plant metabolic functions suggesting that the wild type *Xf.* Successfully subverted the basal defense response and is causing general stress to the plant as it colonizes the xylem.

Objective 3: Evaluation of structural variants of LPS as a preventative treatment for PD. In some systems, treatment with LPS alone does not induce a measurable difference in gene expression. However, it does potentiate a more robust and measurable defense response following challenge with a pathogen. Pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent pathogen attack. This defense-related "memory" is called priming and stimulates the plant to initiate a faster and/or stronger response against future invading pathogens (Conrath, 2011). Priming often results in rapid and robust activation of defense responses such as the oxidative burst, nitric oxide synthesis and expression of defense-related genes (Erbs & Newman, 2003; Newman *et al.*, 2000). The LPS PAMP has been specifically implicated in priming in the *X. campestris* pv. vesicatoria pathosystem. Pepper leaves pre-treated with LPS isolated from incompatible (non-virulent) xanthomonads had enhanced expression of several PR proteins after being challenged with virulent *X. campestris* pv. vesicatoria (Newman *et al.*, 2000). In this objective, we hypothesized that pre-treatment with

LPS isolated from *Xf* O-antigen mutants would result in a difference in the grapevine's tolerance to *Xf* by stimulating the host basal defense response. To determine if the primed state affects the development of PD symptoms, we documented disease progress in plants that were pre-treated with either wild type or *wzy* mutant LPS and then challenged with *Xf* either 4 or 24 hours later. Notably, we observed a decrease in PD severity in vines pre-treated with *Xf* LPS and then challenged with *Xf* (**Fig. 7**).

Grapevine petioles were pre-treated with 40µL of either wild type or *wzy* mutant LPS (50µg/mL). 1xPBS served as the negative control. After we mechanically inoculated the vines with LPS, we challenged with an inoculation of live wild type *Xf* cells (40µL of a $1x10^8$ CFU/mL suspension). These inoculations were performed at 4 and 24 hours after the original inoculation with the LPS. These time points were established based on previously described assays (Newman *et al.*, 2002). We inoculated 24 vines/treatment/LPS concentration/time point. Plants were rated 12 weeks later with 0 being healthy and 5 being dead or dying (Guilhabert & Kirkpatrick, 2005). As shown in **Fig. 7**, plants pre-treated with either the wild type or *wzy* mutant LPS were delayed in PD symptom development when challenged with *Xf* 4 hours later, compared to those plants that received no LPS pre-treatment. This indicates that treatment with either form of LPS (wild type or *wzy* mutant) does elicit a defense response against *Xf* within a 4-hour time window. We reason that the purified forms of wild type LPS and *wzy* LPS both elicit a priming response because all portions of the LPS molecule, including the conserved lipid A + core LPS, which are most often associated with activity of the LPS as a PAMP are exposed and available for recognition by the grapevine immune system. Whereas, in intact cells, the majority of the LPS molecule (lipid A and some of the core LPS) is embedded in the bacterial outer membrane and shielded from perception by the host immune system. Interestingly, two-way ANOVA analysis indicates that there is a significant interaction between time of inoculation and type of LPS applied supporting our hypothesis that we will see large differences in the long term defense responses elicited by *wzy* mutant LPS vs. wild type LPS at later time points than what we have previously tested.

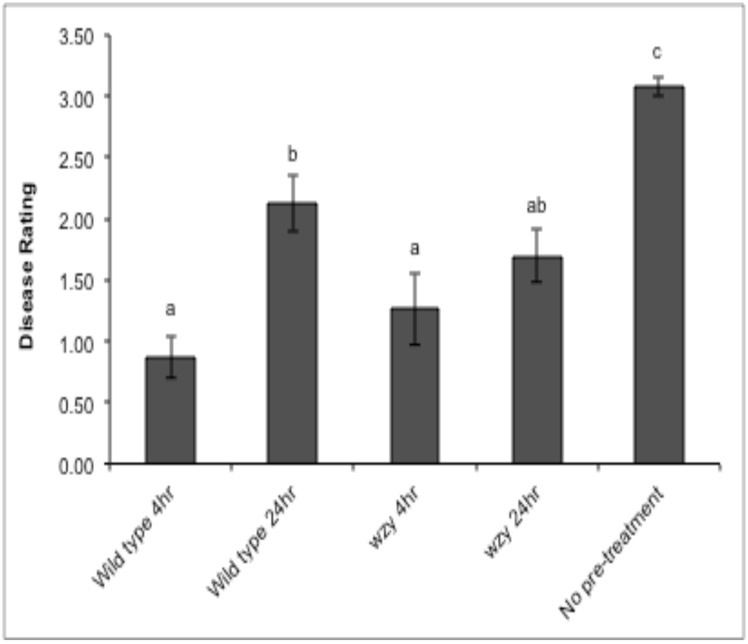

Figure 7. Pierce's disease ratings of LPS pre-treated plants. Mean disease ratings of Cabernet Sauvignon grapevines pre-treated with wild type or *wzy* mutant LPS (50µg/mL), then challenged at 4 or 24hr post LPS treatment. We inoculated 24 vines/treatment/time point. Disease ratings were taken at 12 weeks post-challenge. The LPS pre-treated plants are significantly delayed in symptom development, compared with plants that did not receive pre-treatment. Bars represent standard error of the mean.

Most importantly, now that we have established that we can directly elicit an LPS-mediated defense response and also induce the primed state in grapevine, it will be important to assess how long the temporal window of the heightened defense response and primed state lasts by increasing the amount of time between the inoculation with the LPS and the challenge with live *Xf* cells. These experiments are currently in progress. *Vitis vinifera* 'Cabernet Sauvignon' vines were pre-treated with either wild type or *wzy* mutant LPS and then challenged with *Xf* either 48hr, 1 week, or 4 weeks post-inoculation with LPS. Our plan is to isolate *Xf* populations at 4 weeks post-inoculation with *Xf* in both local tissue (at the point of inoculation), to determine if primed plants show a decrease in bacterial growth, and systemic tissue (5 petioles above point of inoculation), to asses movement within the plant. *Xf* is a slow growing organism, which is why we chose these long time points. Our working hypothesis is that for plants treated with *wzy* mutant LPS, whose conserved portions are more exposed, plant basal defense responses will be faster and more aggressive than for plants treated with wild type LPS, therefore resulting in overall reduced bacterial titer *in planta*.

CONCLUSIONS

This project has addressed key aspects of the interaction of *Xf* with its grapevine host. In addition, the results have provided important knowledge about basal resistance to disease in grapevines and plant hosts in general. Notably, we have also tested a potential preventative measure for PD. Information gleaned from this project could help guide traditional breeding programs aimed at disease resistance by identifying potential resistance markers. The overall outcomes have resulted in a foundation of fundamental knowledge about PD at the molecular level that we will utilize to develop innovative and environmentally sound approaches to controlling this disease.

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FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

ACKNOWLEDGMENTS
The grapevine cuttings used in this study were graciously provided by Foundation Plant Services, University of California, Davis.