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PROJECT TITLE: Characterization of *Xylella fastidiosa* lipopolysaccharide and its role in key steps of the disease cycle in grapevine.

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INTRODUCTION

Xylella fastidiosa (Xf) is a Gram-negative bacterium comprised of subspecies that cause disease on several different economically important crops, such as grapevine, almond, and oleander. We are currently exploring the role of lipopolysaccharide (LPS) as both a virulence factor in grapevine and host specificity determinant for this pathogen. LPS is a structural component of the Gram-negative bacterial outer membrane and is primarily displayed on the outer surface of the cell, thereby mediating interactions between the bacterial cell wall and its environment. LPS is a tripartite glycolipid composed of Lipid A, oligosaccharide core and O-antigen polysaccharide (**Figure 1**) (32). Both Lipid A and the oligosaccharide core are highly conserved among all Gram-negative bacteria, whereas, the O-antigen can be varied even among subspecies. LPS has been implicated as a major virulence factor in both plant and animal pathogens, such as *Escherichia coli*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* (8, 12, 22). LPS can also contribute to the initial adhesion of the bacterial cell to a surface or host cell (10, 24). Additionally, host perception of LPS is well documented and occurs in both plants and animals (25). Host immune receptors can recognize several regions of the LPS structure and mount a defense response following bacterial invasion based on this recognition. Bacteria can also circumvent the host's immune system by altering the structure of the O-antigen moiety or by masking it with capsular or exopolysaccharides.

In this study, we are working with three sequences strains of *Xf* originally isolated from three different hosts, Temecula1 (grape), M12 (almond) Ann-1 (oleander). We identified two genes, *waaL* (PD0077) and *wzy* (PD0814) in the genomes of the three *Xf* isolates included in this study based on previously reported investigations of O-antigen biosynthesis in other bacterial systems (15, 30). These genes are predicted to encode proteins required for production of a full O-antigen moiety in *Xf*. Wzy is a putative O-antigen polymerase that plays a role in chain length determination of the O-antigen. WaaL is a putative O-antigen ligase that ligates the preformed O-antigen onto the oligosaccharide core. Therefore, we originally predicted that mutants in *wzy* would produce a truncated O-antigen and that mutants in *waaL* would be completely lacking the O-antigen (**Figure 1**). Based on these alterations in the O-antigen, we hypothesized that we would see profound differences in virulence, surface attachment, aggregation, and insect transmission.

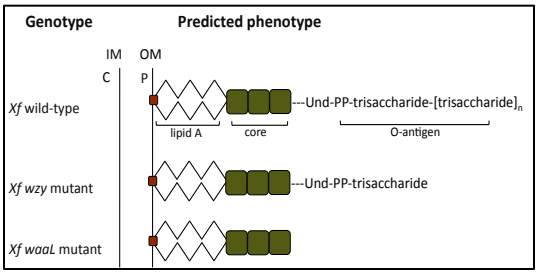


Figure 1. *Xf* strains lacking a functional Wzy (polymerase) are predicted to have truncated O-antigen with only the initiating polysaccharide unit. Strains lacking a functional WaaL (ligase) are predicted to lack the O-antigen. Und-PP = undecaprenylphosphate; IM = inner membrane; OM = outer membrane; C = cytoplasm; P = periplasm.

Thus far, we have demonstrated that mutations in *wzy* and *waaL* do affect O-antigen biosynthesis and cause a visible change in the O-antigen profiles as seen in **Figure 2**. LPS was extracted from Temecula1 wild-type, *wzy,* and *waaL* using a hot-phenol extraction method (31) and then subjected to Tris-Tricine polyacrylamide gel electrophoresis (PAGE). Both the *waaL* and *wzy* mutants had a significant reduction in the amount of O-antigen (**Figure 2**) (33). We had expected the *waaL* mutant to be devoid of O-antigen, which is not the case. This suggests to us that there may be a protein(s) that shares a redundant function with WaaL. Both mutant phenotypes were restored by introducing *waaL* or *wzy* into their respective mutant genomic backgrounds using the chromosomal complementation vector, pAX1Cm (19) (**Figure 2**) (33).

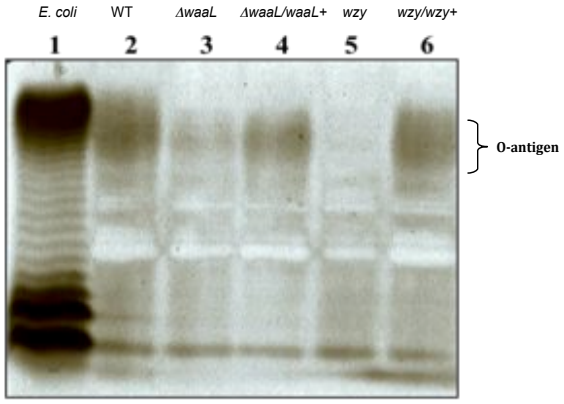


Figure 2. Mutations in key enzymes of the *Xf* LPS biosynthetic pathway result in reduction or abolishment of O-antigen. Both *waaL* (lane 3) and *wzy* (lane 5) mutant strains had less O-antigen than the wild-type (lane 2). Complementation of these mutants restored O-antigen quantity to near that of the wild-type parent (lanes 4 and 6). LPS was extracted from cells normalized to cell density using a modified hot phenol method and analyzed on a 4, 12% discontinuous Tricine-PAGE gel and silver stained (26). (1) *E. coli* standard, (2) Temecula1 wild-type, (3) *waaL*, (4) *waaL*/*waaL*+, (5) *wzy*, (6) *wzy*/*wzy*+.

Following the confirmation of the change in the O-antigen profiles for the *waaL* and *wzy* mutants, we then tested these strains for differences in virulence *in planta* (33). Temecula1 O-antigen mutant strains were inoculated into grapevine, cv. 'Thompson Seedless' according to the method of Hill and Purcell (1995) (13). Plants were rated weekly on a disease index scale of 0-5, with 0 being healthy and 5 being dead or dying (11). PD symptoms were detected 4 weeks after inoculation, similar to other greenhouse PD virulence studies conducted in Riverside County. The Temecula1 *wzy* mutant was significantly delayed in causing PD symptoms, and after 11 weeks of incubation, it did not cause the extensive PD symptoms observed in plants inoculated with wild-type (as determined by an ANOVA analysis with Tukey's pairwise comparisons) (**Figure 3**) (33). **This indicates that depletion of the O-antigen correlates with a profound defect in *Xf* virulence.**
We observed no difference in disease progress between the Temecula1 *waaL* mutant and the wild-type strain. Plants inoculated with 1X PBS buffer control did not develop any PD symptoms.

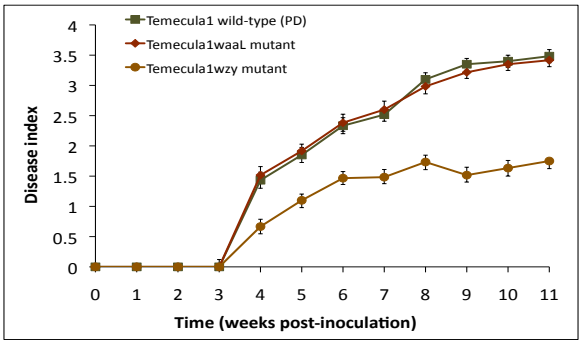


Figure 3. Disease progress of *Xf* Temecula1 wild-type and O-antigen mutant strains on grapevine cv. 'Thompson Seedless'. The *wzy* mutant strain lags behind the wild-type in causing leaf scorching symptoms and does not cause wild-type levels of PD throughout the disease progress curve. The *waaL* mutant is as virulent as wild type. Data are means of three independent assays with 10 replications each. Bars represent standard error.

In addition, we quantified bacterial population levels to assess any colonization differences between Temecula1 wild-type and the O-antigen mutant strains that may be occurring *in planta*. We isolated bacteria from surface-sterilized petioles at 13 weeks post-inoculation and found that plants inoculated with the *wzy* mutant harbored significantly less bacteria than plants inoculated with wild-type Temecula1 (**Figure 4**)(33). Populations were quantified by plate counts and the data were analyzed using a Wilcoxon rank test. The *waaL* mutants colonized the plants to similar levels as wild type *Xf*.

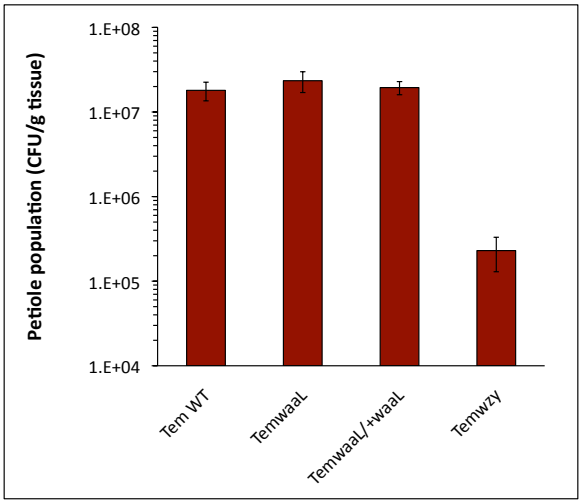


Figure 4. *In planta* populations of the *wzy* mutant after inoculation of grapevine petioles are significantly less than those of the wild-type parent. Populations were quantified by plate counts. Five petioles from three independent inoculations. Bars represent the standard error of the mean.

Currently, *wzy* and *waaL* mutans are being made in the M12 (ALS) and Ann-1 (OLS) strains of *Xf*. LPS will be extracted, and their O-antigen profiles will be analyzed. Pathogenicity assays using these mutants will be conducted this summer.

LPS is considered to be a Pathogen Associated Molecular Pattern (PAMP) that is recognized by the immune system of both plants and mammals causing the host to mount a defense response. One strategy that pathogens use to evade the host defense response is to mask the conserved portions of the LPS molecule (core and Lipid A) with a varying O-antigen or even exopolysaccharide. We speculate that the high molecular weight O-antigen chain in the *Xf* LPS molecule may serve to mask the rest of the LPS molecule from the host to avoid triggering a defense response, and the removal of this protective O-antigen may trigger the host defense response, resulting in reduced colonization and reduced disease symptoms as observed for the *wzy* mutant.

OBJECTIVES

Objective 1: Expand the characterization of the LPS profiles from the grape, almond, and oleander strains of *Xf*.
Objective 2: Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants to the biologically relevant substrates, chitin and cellulose.
Objective 3: Test O-antigen mutants for insect transmissibility.
Objective 4: Test O-antigen mutants for increased vulnerability to environmental stress and antimicrobial compounds.

SUMMARY OF ACCOMPLISHMENTS AND RESULTS

Objective 1: Expand the characterization of the LPS profiles from the grape, almond, and oleander strains of *Xf*. A bacterium with only lipid A and core oligosaccharide is said to have “rough” LPS and one with lipid A, core oligosaccharide, and O-antigen is said to have “smooth” LPS. There can be both smooth and rough variants of the same bacterial species. We compared the LPS profiles of three *Xf* strains (Temecula1 (grape), M12 (almond), and Ann-1 (oleander)) by Tris-Tricine polyacrylamide gel electrophoresis (PAGE). This work is based on the hypothesis that there are strain specific differences among the variable O-antigen portion of the LPS molecules that contribute to the host specificity of these three isolates. We isolated LPS from all three *Xf* isolates that were grown on solid PW medium using a hot-phenol extraction method (31). The extracted LPS preparations were then subjected to Tris-Tricine PAGE. These analyses have confirmed that all three strains possess smooth LPS (ie. O-antigen), which was previously unknown (**Figure 5**). The gels also revealed small shifts in the molecular weights of the

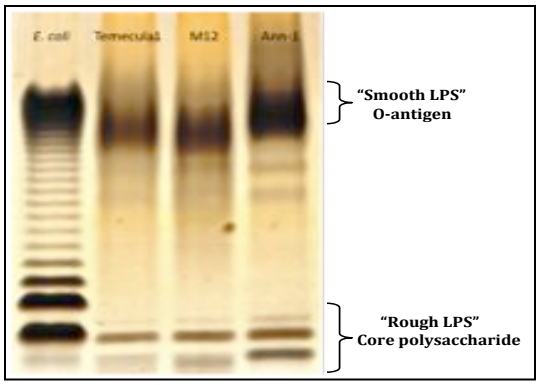


Figure 5. Tris-Tricine SDS-PAGE gel indicating the presence of both smooth (**O-antigen**) and rough (core polysaccharide) LPS in **1)** Temecula1 (PD), **2)** M12 (ALS), and **3)** Ann-1 (OLS) strains of *Xf*. LPS was extracted by the hot-phenol extraction method.

smooth LPS for each strain, indicating a fundamental difference among the O-antigen chain length or composition.

We recently conducted a more detailed carbohydrate composition and linkage analysis of *Xf* LPS using Gas chromatography/Mass Spectrometry analysis in collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens. Our studies included the Temecula1 strain and its respective *wzy* mutant. We isolated total LPS from 100 mg of starting cell mass from wild-type Temecula1 and Temecula *wzy*. The CCRC performed O-antigen purification using a mild acid hydrolysis technique and performed carbohydrate composition and glycosyl linkage analysis. Results of the glycosyl composition analysis revealed that *Xf* O-antigen is a heteropolymer consisting mostly of rhamnose and glucose, with smaller amounts of ribose, xylose and mannose (Table 1). The Temecula *wzy* mutant O-antigen was similar in carbohydrate composition to wild-type with regard to the residues present in the polysaccharide. However, there was a striking depletion in the percentage of rhamnose in the *wzy* mutant as compared to the WT, which decreased from 68.2% in the wild-type to 9.4% in the *wzy* mutant. In addition, the percentage of glucose increased from 19% in the wild-type to 84.6% in the *wzy* mutant (Table 2) (33). Deletion of *wzy* in *Xf* resulted in an O-antigen composed primarily of glucose with much of the rhamnose absent. This suggests that the initial O-unit linked to the core LPS is composed primarily of glucose and the remainder of the polymer that extends out into the environment is a rhamnose-rich repeat. We are currently coordinating a series of experiments with the CCRC that are based on NMR spectroscopy. These experiments will include the wild-type Temecula1, M12 and Ann-1 isolates and will elucidate the absolute structure of the *Xf* O-antigen in all of these isolates.

O-antigen sugars (Mol %)					
	Rib	Rha	Xyl	Man	Glc
Strain					
Wild type	6.5	**68.2**	5.5	0.8	19.0
wzy	4.9	9.4	0.5	0.6	**84.6**

Table 1. Glycosyl composition analysis of O-antigen isolated from wild type or the *wzy* mutant. Values are expressed as mole percent of total carbohydrate.

Host specificity plant assays. While there are likely several factors that contribute to host specificity of *Xf*, we would like to investigate if O-antigen is involved. *Xf* Temecula1 can colonize and cause disease in grape and, to some extent, in almond. *Xf* M12, an ALS strain, causes disease in almond and elicits some symptoms in grape (2). Moreover, *Xf* Ann-1 cannot cause disease in grape or almond and neither Temecula 1 nor can M12 cause disease in oleander. We speculated that removal or truncation of the O-antigen would affect the ability of Temecula1 to infect (and elicit scorch symptoms) on grape, almond, and oleander. Furthermore, perhaps changes in O-antigen may enable Ann-1 to become a pathogen of grape or almond while M12 and Temecula1 strains become pathogens of oleander.

We inoculated Temecula1 WT and the Temecula O-antigen mutant strains into host (grape) and non-host (almond and oleander) plants. We observed that the grape isolate (Temecula1 wild-type) can colonize and cause disease in almond, albeit, to a lesser extent than the wild-type Almond Leaf Scorch (ALS) isolate (M12), which is consistent with what other research groups have observed (1). Interestingly, the Temecula1 *waaL* mutant strain is **more virulent** in almond than its Temecula1 wild-type parent and is similarly virulent to the wild-type ALS isolate (M12). This suggests that an alteration in the O-antigen moiety of the LPS molecule does affect host specificity. The Temecula1 *wzy* mutant is similarly less virulent in almond as in grape. As expected, *Xf* Ann-1, the OLS isolate, does not cause scorch symptoms on almond (**Figure 6**). Symptoms of OLS develop much later than those in grape and almond (ten months post-inoculation). We observed leaf scorch symptoms in the oleander plants that were inoculated in June of 2011, and we rated plants for OLS development. However, results for both the disease progress and detection of *Xf* in oleander plants using immunocapture PCR were inconclusive. We repeated oleander inoculations in August of 2012 and we are currently waiting to harvest the data from these experiments. We will be testing those plants over the following months for the presence of *Xf* using more sensitive methods, including ELISA and quantitative PCR.

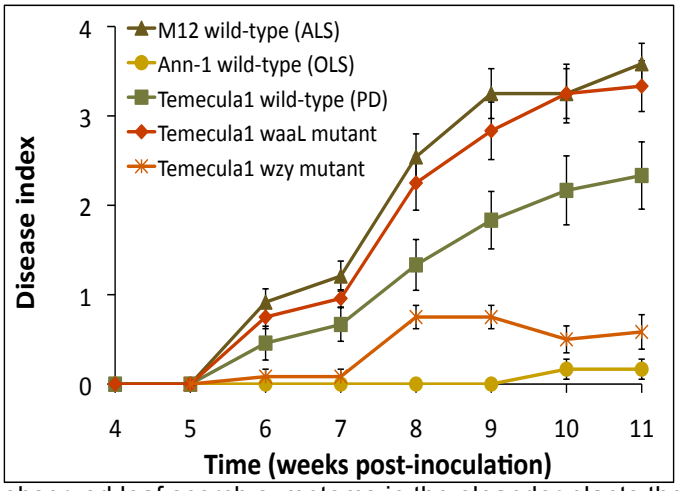


Fig. 6. Disease progress of M12 (ALS), Ann-1 (OLS) Ann-1, Temecula1 (PD) isolates, and Temecula1 O-antigen mutants in almond cv. 'Sonora'. Loss of WaaL increases virulence of a PD isolate of *Xf* in almond. Data are means of two independent assays with 6 replications each. Bars represent standard error.

Objective 2: Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants to the biologically relevant substrates, chitin and cellulose. Biofilm formation is an important component of the plant-microbe and plant-insect interaction. To test the role of LPS in *Xf* biofilm formation, we quantified the Temecula1 *waaL* and *wzy* mutant strains in two biofilm related behaviors: 1) surface attachment and 2) cell-cell aggregation. Both of these phenotypes are critical early steps in the formation of a mature biofilm. We hypothesized that LPS may contribute to these behaviors because of its location and abundance in the outer membrane. Glass is a hydrophilic surface and the polysaccharide components (ie. pectin, cellulose, hemicellulose) that make up the xylem primary cell wall are also highly hydrophilic. This similarity allows us to extrapolate our *in vitro* results to what might be occurring in portions of the plant xylem tissue that have an abundance of exposed primary cell wall polysaccharides, such as xylem pit membranes. Interestingly, when grown in glass tubes, the Temecula1 *wzy* mutant aggregated less, but attached more to a glass surface (**Figure 7**). The *wzy* mutant was also significantly less virulent *in planta* which may be a result of its hyperattaching phenotype causing it to adhere more strongly to the xylem primary cell wall, which does not allow it to move as efficiently throughout the plant.

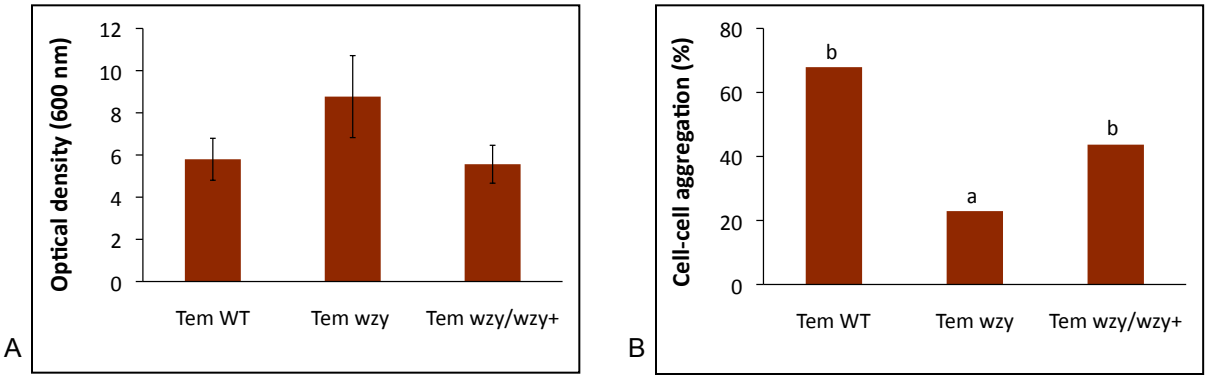


Figure 7. Surface attachment and cell-cell aggregation of the O-antigen mutant, *wzy*. A) The Temecula1*wzy* mutant cells attach to a solid surface to a greater extent than the wild-type parent. Attachment assays involved crystal violet staining of cells attached to a glass surface at the medium/air interface (2) after 7 days of incubation at 28°C, 100 rpm. **B)** The Temecula1*wzy* mutant cells are reduced in the ability to aggregate to each other compared to the wild-type. Aggregation assays reflect the proportion of the total cell population that remains in culture after 10 days of static incubation (3). At least three independent assays were performed in triplicate. Bars represent standard error of the mean.

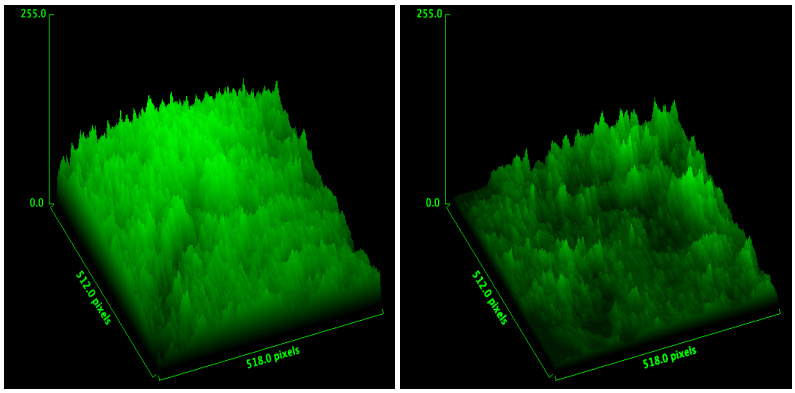
We performed a series of zeta potential measurements to determine if depletion of O-antigen correlated with a change in the net charge on the surface of the bacterial cell, which in turn could account for the differences in attachment and aggregation that we observed. Zeta potential is calculated based on the electrophoretic mobility of particles in a given solution. These values provide information about the net charge on the surface of a particle, in this case, a bacterial cell. The zeta potential was quantified for both the wild-type, *wzy* and *wzy/wzy+* mutant strains grown on solid PD3 medium. The surface of the *wzy* mutant was more negatively charged as indicated by an average zeta potential measurement of -27.1 mV compared to the zeta potential of WT at -10.5 mV (**Table 2**) (33). The ionic strength of PD3 medium was estimated to be 85 mM and the zeta potential of a glass microscope slide submerged in PD3 medium was estimated to be approximately -12mV. Therefore, under the growth conditions tested here, it is logical that the more negatively-charged *wzy* mutant would adhere more strongly to the glass surface than the more positively-charged wild-type strain.

Particles with lower zeta potentials tend to flocculate or aggregate, which explains the high capacity for *Xf* to aggregate in culture (7). However, if particles have a large negative or positive zeta potential this causes high repulsion among the particles, and will cause them to resist flocculation or aggregation. The *wzy* mutant has a significantly large negative zeta potential as compared to wild-type, which explains the inability of this strain to aggregate *in vitro*. We are currently testing the *waaL* mutant for attachment to glass and cell-cell aggregation and will also measure its zeta potential. In addition, we are expanding the attachment studies to the more biologically relevant substrates, chitin and cellulose.

Strain	zeta potential (mV)
Wild type	-10.5±2.2
wzy	-27.12±1.91
wzy/wzy+	-10.9±0.97

Table 2. Zeta potential measurements of *Xf* Temecula1 wild type, *wzy* mutant, and *wzy/wzy+* complemented strain. The *wzy* mutant bacterial cells exhibit a significantly more negative zeta potential measurement than the wild-type cells, which likely account for its hyperattaching and non-aggregative phenotype. *Xf* strains were harvested and resuspended in 10mM KCl. Cell density was adjusted to OD_{600} = 0.2. Data are means of three independent assays with 5 replications each.

In addition, we tested the ability of the *wzy* mutant to form 3-dimensional biofilms *in vitro*. Because the *wzy* mutant is impaired in cell-cell aggregation and hyperattaches to surfaces, we hypothesized that it would be impaired in biofilm formation. Both the wild-type and the *wzy* mutant were grown in liquid culture at 28°C for 2,4,6, or 8 days with constant shaking at 180rpm. A glass microscope slide was placed vertically into each tube, allowing biofilms to form at the air-liquid interface as previously described (27). Slides were removed at each time point, gently heat fixed, and stained with the fluorescent dye Syto 9. Specimens were mounted in Slow Fade mounting fluid and observed using a Zeiss 510 Confocal Laser Scanning Microscope. As shown in **Figure 9**, *wzy* mutant biofilms were impaired in building the typical three-dimensional biofilm architecture (33). The *wzy* mutant was capable of attaching to the glass surface but was unable to build the towers characteristic of a wild type biofilm.



Temecula1 wild- type *wzy* mutant

Figure 9. *In vitro* biofilm assay of the wild-type and the *wzy* mutant. Images are representations of three independent assays with three replications each.

Moreover, the *wzy* biofilms were, on average, significantly thinner (120µm) than the wild-type biofilm (145µm), as determined by a student's t-test ($P<0.05$). Biofilm heterogeneity of the *wzy* mutant was also further examined using roughness coefficients. Biofilm surface roughness characterizes the variation in mean thickness. Higher roughness coefficients indicate a rough (or irregular) biofilm, while smaller coefficients indicate a smoother (or uniform) biofilm. Roughness coefficients were calculated according to the methods of Murga et al. (34) and revealed that the wild-type biofilms have a significantly smaller roughness coefficient (0.019) than the *wzy* mutant biofilms (0.133), further supporting that the *wzy* mutant is impaired in biofilm formation.

Objective 3: Test O-antigen mutants for insect transmissibility. In this objective, we are comparing sharpshooter transmission rates for wild type versus either the *waaL* or *wzy* mutant. We are using the artificial feeding sachet technique developed by Killiny and Almeida (16). The artificial sachet technique is extremely useful because it allows us to normalize all strains to the same starting cell density in the individual feeding sachets, thereby avoiding any *in planta* multiplication differences. We initially began these studies using the highly abundant Glassy-Winged Sharpshooter (GWSS) (*Homalodisca vitripennis*) species in Riverside County. In collaboration with Dr. Matt Daugherty (UCR, Dept. of Entomology), we successfully reared a clean (*Xf*-free) GWSS colony and conducted preliminary sharpshooter transmission studies. However, due to the inherently low *Xf* transmission rates for the GWSS, we have opted to proceed with the Blue-Green Sharpshooter (BGSS) (*Graphocephala atropunctata*) which has a much higher rate of transmission than the GWSS. This will allow us to more accurately quantify differences in acquisition and transmission rates between the WT and either mutant.

In collaboration with Dr. Thomas Perring (UCR, Dept. of Entomology), we have initiated a BGSS colony. We conducted insect acquisition studies using the artificial sachet technique, according to the methods of Killiny and Almeida (17). In brief, we grew *Xf* Temecula1 wild type and mutant strains on PD3 media for 7 days at 28°C. Cells were harvested and resuspended in liquid XFM media. 30µl aliquots were

striped onto XFM plates supplemented with pectin and incubated for an additional 4-6 days at 28°C. Cells were harvested in liquid diet solution and adjusted to an OD_{600} of 0.25 (1 x 10^8 CFU/mL). Insects were fed 35µl aliquots of bacterial suspension or diet solution only (negative control) and given an acquisition access period of 6 hours. Following the acquisition access period, insects were removed from the feeding sachets and placed onto clean basil plants for a clearing and multiplication period of 48 hours. Following this period, half of the insects were stored at -20°C for quantitative PCR (qPCR), and the other half were placed into glutaraldehyde fixative (4%) for examination of *Xf* colonization within the insect foreguts using scanning electron microscopy (SEM).

We are currently in the process of quantifying the bacterial cells within the sharpshooter foreguts using qPCR. Concomitant transmission studies will be performed this spring by limiting fed sharpshooters onto healthy grapevine seedlings for an inoculation access period of 4 days. Seedlings will be observed for symptom development over the following months, and bacterial populations within infected plants will also be quantified using qPCR.

We have collected preliminary images of the insect foreguts using SEM. In brief, in collaboration with Dr. Elaine Backus (USDA-ARS, Parlier), insects were prepped for SEM examination using a series of dehydrations, followed by critical point drying. Insect foreguts were dissected out from the heads and mounted onto copper tape attached to aluminum stubs. Stubs were sputter-coated in gold/palladium prior to analysis, and insects were imaged using the Hitachi TM-1000 SEM. As shown in **Figure 11**, the *wzy* mutant was significantly impaired in colonization within sharpshooter foreguts, while the Temecula1 wild type readily attached and formed microcolonies.

The remaining insects from the assay will be critical point dried and imaged this summer, using a higher-powered SEM to more closely examine *Xf* attachment to the insect cuticle.

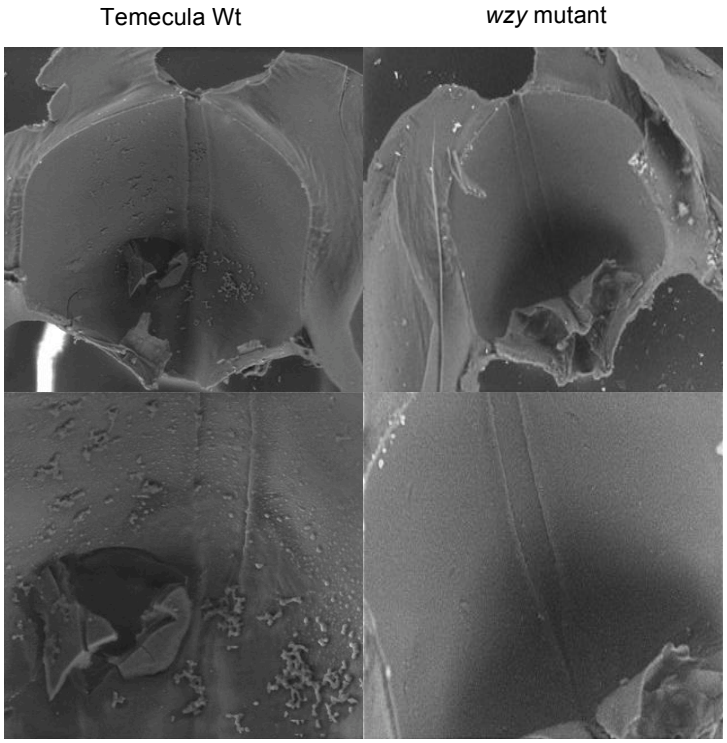


Figure 11. SEM examination of blue-green sharpshooter foreguts fed on *Xf* Tem1 wild-type or *wzy* mutant. Overview of blue-green sharpshooter hypopharynx (**A**) and close-up of hypopharynx (**B**).

Objective 4: Test O-antigen mutants for increased vulnerability to environmental stress and antimicrobial compounds. The outer membrane of a bacterial cell is strongly influenced by its environment, and variation in the O-antigen portion of the LPS molecule can aid in the adaptation or tolerance to different environmental stresses such as oxidative stress, temperature, and resistance to

antimicrobial peptides (3, 4, 23, 28). We are investigating if the absence or truncation of the O-antigen affects tolerance to environmental stress and antimicrobial compounds by testing the wild-type Temecula1, *waaL,* and *wzy* mutants for increased susceptibility to three environmental stresses: 1) oxidative stress, 2) cold temperature, and 3) treatment with antimicrobial peptides.

We rationalized that LPS is likely involved in resistance to oxidative stress because of its physical location in the outer leaflet of the bacterial cell membrane. During the plant infection process, bacteria encounter oxidative stress in the form of reactive oxygen species (ROS). ROS can be a product of the elicitation of the host defense response or a by-product of normal plant metabolism and development (25). In any case, oxidative stress is detrimental to the bacterial cell, and the cells must have a mechanism to cope with this environmental insult. We hypothesize that LPS, in particular the high molecular weight O-antigen chain, provides some protective effect to the cells and that the *waaL* and *wzy* mutants will be more vulnerable to ROS than the wild-type Temecula1 strain. We performed a simple disc inhibition assay protocol as previously described (20) to test sensitivity to peroxide. In brief, the wild-type Temecula1, *wzy* and complemented strains were plated in PD3 top agar. A Whatman paper disk impregnated with 100 µM H_2O_2 was overlaid onto the top agar. Plates were incubated for 7 days at 28ºC and observed for zones of inhibition around the disk containing the H_2O_2. The diameter of the zone of inhibition correlates with sensitivity to H_2O_2. We observed that the *wzy* mutant was more sensitive to peroxide treatment as compared to the Temecula1 wild-type (**Figure 12**). We are currently testing the *waaL* mutant for sensitivity to peroxide.

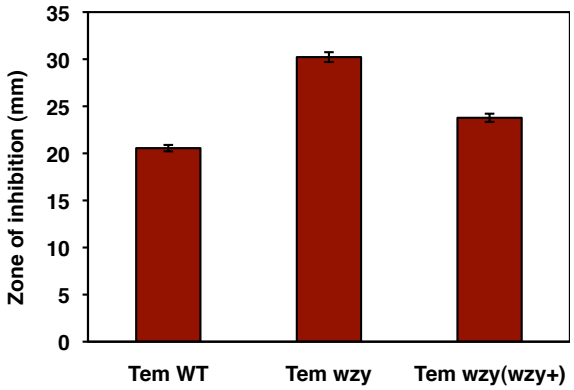


Figure 12. The role of O-antigen in the protection against oxidative stress. In the disk inhibition assay, the *Xf* Temecula1 *wzy* mutant strain was less tolerant of hydrogen peroxide stress than the wild-type parent or the complemented mutant strain. Three independent assays were performed in triplicate. Error bars represent the standard error of the mean.

In addition, we are testing the *waaL* and *wzy* mutants for increased susceptibility to cold temperature and treatment with antimicrobial peptides. We chose these two treatments based on current strategies being evaluated for control of PD (5, 18). Antimicrobial peptides (AMPs) are a unique and diverse group of molecules that have potent antimicrobial effects even in very small doses (21). They are short peptide sequences composed of no more than 50 amino acids and can display broad-spectrum antimicrobial activity. There are some AMPs, such as polymyxin B, that have been shown to bind to LPS with high affinity (28).

We have chosen to test cecropin B because it has previously been identified to have antimicrobial activity against *Xf* (6). We have also chosen polymyxin B because its mode of action is to bind directly to LPS (28). Both peptides are readily available from Sigma-Aldrich, Inc. Antibacterial activity will be expressed as Minimum Inhibitory Concentrations (MIC), the concentration at which no growth is observed. We are initially conducting these experiments in liquid cultures, but the results will be confirmed by testing on solid PD3 medium overlaid with a Whatman paper disk containing the different concentrations of the AMP (cecropin B or polymyxin B) being tested. The MICs will be calculated by measuring the diameter of the inhibition zones around the disc containing the AMP. In all cases, the cultures will be incubated at 28°C and monitored for growth over the course of 7-10 days.

Cold temperatures have been associated with the geographic distribution of Pierce's disease (24). The effects of cold temperature-mediated therapy of *Xf*-infected grapevines is an ongoing area of research, and certain aspects of this phenomenon are currently being exploited as possible control strategies for PD (18, 26). Bacteria can modify their LPS in response to temperature (3, 23). These temperature-induced modifications of LPS are generally regarded as an adaptive response to the cells surrounding environment.

We are basing our assay on previously established protocols (9). We anticipate that both the antimicrobial peptide and cold temperature studies will be completed this summer.

PUBLICATIONS PRODUCED AND PRESENTATIONS MADE

Publications

Clifford, J., Rapicavoli, J.N. and Roper, M.C. 2013. A rhamnose-rich O-antigen mediates adhesion, virulence and host colonization for the xylem-limited phytopathogen, *Xylella fastidiosa*. *Molecular Plant Microbe Interactions*. (Epub ahead of print) http://dx.doi.org/10.1094/MPMI-12-12-0283-R.

Roper, M. 2011. Characterization of *Xylella fastidiosa* lipopolysaccharide and its role in key steps of the disease cycle in grapevine. California Department of Food and Agriculture. p.139-145. Pierce's Disease Research Symposium, Sacramento, CA.

Clifford, J. and Roper, M.C. 2011.The role of lipopolysaccharide in virulence, biofilm formation, and host specificity of *Xylella fastidiosa. Phytopathology,* 101:S38.

Roper, M. 2011. Characterization of *Xylella fastidiosa* lipopolysaccharide and its role in key steps of the disease cycle in grapevine. California Department of Food and Agriculture. p.139-145. Pierce's Disease Research Symposium, Sacramento, CA.

Roper, M. 2010. The role of lipolysaccharide in virulence, biofilm formation, and host specificity of *Xylella fastidiosa*. California Department of Food and Agriculture. p.139-145. Pierce's Disease Research Symposium, San Diego, CA.

Roper, M. 2009. The role of lipolysaccharides in virulence, biofilm formation, and host specificity of *Xylella fastidiosa*. California Department of Food and Agriculture. p.106-111. Pierce's Disease Research Symposium. Sacramento, CA.

Presentations

Roper, M.C., Rapicavoli, J. and Clifford, J.C. 2012. The role of the cell surface lipopolysaccharide molecule in *Xylella fastidiosa* biofilm formation and virulence in the grapevine host. Invited presentation at the Annual meeting of the American Phytopathological Society, Providence, Rhode Island.

Clifford, J. and Roper, M.C. 2011. Characterization of *Xylella fastidiosa* lipopolysaccharide and its role in key steps of the disease cycle in grapevine. Presented as a poster at the Annual Pierce's Disease Research Symposium, Sacramento, CA.

Clifford, J. and Roper, M.C. 2011. The role of lipopolysaccharide in virulence, biofilm formation, and host specificity of *Xylella fastidiosa.* Presented as a poster at the Annual Meeting of the American Phytopathological Society, Honolulu, HI.

Clifford, J. and Roper, M.C. 2010. The role of lipopolysaccharide in virulence and host specificity of *Xylella fastidiosa*. Presented as a poster at the Annual Pierce's Disease Research Symposium, Sacramento, CA.

Clifford, J. and Roper, M.C. 2009. The role of lipopolysaccharide in virulence and host specificity of *Xylella fastidiosa*. Presented as a poster at the Annual Pierce's Disease Research Symposium, San Diego, CA.

RESEARCH RELEVANCE STATEMENT

The focus of this project is to further understand the molecular mechanisms governing *Xf* virulence. We are working toward understanding the role of the O-antigen component of LPS in contributing to virulence and those behaviors associated with xylem colonization, such as host cell wall attachment and cell-cell aggregation required for biofilm formation. In addition, the broad host range, but stringent host specificity, of *Xf* provides an opportunity to study the molecular mechanisms underlying the essential traits that lead to host specificity observed for this pathogen. There are likely numerous bacterial traits that contribute to host specificity of *Xf*, and we hypothesize that O-antigen plays a role. Most importantly, we have demonstrated that O-antigen in an important factor in PD development and host colonization and because of its abundance in the outer membrane, LPS may provide a target for disease control.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is a bacterium capable of colonizing many different plant hosts. It is the causal agent of Pierce's Disease of grapevine (PD), which has caused major losses for the California grape

industry. *Xf* also causes disease in other economically important crops, such as almond, citrus, and oleander. While all identified *Xf* isolates belong to the same species, some isolates can cause disease in one host, but not the other. For example, oleander isolates do not incite symptom development in grapevine and vice versa. One major goal of this project is to understand the bacterial characteristics that dictate host specificity. This research is particularly focused on elucidating the role of the *Xf* cell surface component, lipopolysaccharide (LPS), in the pathogenic interaction between the grapevine, almond, and oleander hosts. Because LPS plays an important role in several host-pathogen interactions, we are investigating the contribution of LPS in allowing *Xf* to colonize its host. LPS imparts traits that may contribute to pathogenesis, such as the ability to attach to host cell walls. Thus far, we have shown that certain components of the LPS molecule are essential for the *Xf* interaction with its host, which together with its abundance on the bacterium's cell surface makes it a logical target for disease control. Antimicrobial compounds that disrupt or retard LPS biosynthesis exist which make bacteria more susceptible to other stresses. Potentially, these compounds could be used alone or in combination with other anti-*Xf* compounds to control disease.

STATUS OF FUNDS

The funding for this project is largely going towards supporting a Ph.D. graduate student, Mrs. Jeannette Rapicavoli. This project is the main focus of her Ph.D. dissertation. In addition, a portion of the *in vitro* assays described in Objectives 2 and 4 are being conducted by another Ph.D. student, Ms. Lindsey Burbank. Therefore, a small portion of her salary has fallen under the umbrella of this grant fund. We anticipate spending the remainder of the supply, services and greenhouse recharge money associated with this project as it progresses.

INTELLECTUAL PROPERTY

Thus far, there is no intellectual property associated with this project.

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