THE ROLE OF LIPOPOLYSACCHARIDE IN VIRULENCE, BIOFILM FORMATION, AND HOST SPECIFICITY OF *XYLELLA FASTIDIOSA*

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ABSTRACT
This project aims to further elucidate the molecular mechanisms *Xylella fastidiosa* (*Xf*) employs during the infection of its host. We are focusing on the lipopolysaccharide (LPS) component of the outer membrane of *Xf*. LPS contains a conserved lipid A and core portion and a variable O-antigen portion. In particular, we are examining the variable portion of the LPS molecule, the O-antigen. O-antigen as been implicated in virulence in many bacterial species and we hypothesize it may also be involved in *Xf* virulence. More specifically, we are investigating if this particular portion of the LPS molecule contributes to *Xf* surface attachment and biofilm formation, two critical steps for successful infection of the xylem of the host. Additionally, we will determine if LPS contributes to the high level of host specificity observed for this pathogen.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is a bacterium that has the ability to infect and colonize many different plant species, causing significant damage in some. In grapevine, this disease is known as Pierce's disease (PD), which has caused major losses to the California grape industry. *Xf* also infects other economically important crops such as almond, oleander and citrus. Interestingly, while all *Xf* isolates belong to the same group or species, some isolates can cause disease in one host but not another. For example, oleander isolates cannot cause disease in grapevine and vice versa. One major goal of this project is to understand the bacterial mechanisms that dictate this high level of host specificity. This research project is specifically focused on understanding the role of the *Xf* cell surface component lipopolysaccharide (LPS) in the pathogenic interaction between the grapevine, almond, and oleander hosts. LPS plays an important role in virulence for many bacterial pathogens. We are investigating the involvement of LPS in *Xf* colonization of its host and other key aspects of the disease process, like attachment to the plant cell wall. Most importantly, should LPS prove to be an important factor during *Xf* plant infection, its abundance on the bacterial cell surface makes it a logical target for disease control. Furthermore, antimicrobial compounds exist that disrupt LPS synthesis or weaken the LPS layer, making the bacterium more sensitive to other stresses. Therefore, compounds targeted towards LPS synthesis could increase the efficacy of other anti-*Xf* compounds currently being developed by other researchers when both are used in conjunction.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a gram negative, xylem-limited bacterium with a broad host range. *Xf* causes disease in economically important hosts such as grape, almond, citrus, coffee, peach, plum and alfalfa as well as several tree and ornamental hosts such as oleander. Additionally, *Xf* colonizes many plant species that never develop any visible symptoms or stresses. (Hopkins and Purcell, 2002). The molecular mechanisms that determine this host specificity are poorly understood. We are currently exploring the role of lipopolysaccharides (LPS) as both a virulence factor and host specificity determinant of *Xf*. We are focusing on the O-antigen portion of the LPS molecule in 3 isolates of *Xf* that colonize different hosts: Fetzer, a Pierce's Disease (PD) isolate; Dixon, an almond leaf scorch (ALS) isolate; and Ann-1, an oleander leaf scorch (OLS) isolate. While the grape and almond isolates are considered to be separate subspecies or pathovars, both ALS and PD isolates can cause disease in grapevine (Almeida and Purcell, 2003). However, *Xf* isolated from grapevines cannot cause disease in almonds indicating a fundamental difference between ALS and PD isolates. Furthermore, the oleander strain cannot infect grape or almond and both the almond and grape strains cannot infect oleander. This provides an opportunity to study the role of the O-antigen moiety of the LPS molecule as a potential host specificity determinant for *Xf*.

LPS is primarily displayed on the cell surface, thereby mediating interactions between the bacterial cell and its environment by way of initial adhesion of the bacterial cell to a surface or host cell (Genevaux *et al*. 1999, Nesper *et al*. 2001). LPS (sometimes called "endotoxin") has also been implicated as a major virulence factor in animal and plant and pathogens, such as *Escherichia coli*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* (Muhldhorfer and Hacker 1994, Dow *et al*. 1995; Hendrick *et al*. 1984). Additionally, host perception of LPS is well documented and occurs in both plants and animals (Newman *et al*. 2000). The immune system can recognize several regions of the LPS structure and can mount a defense response against bacterial invasion based on this recognition. Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule or by masking it with capsular or exopolysaccharides.

LPS is composed of 3 parts: 1) lipid A, 2) core oligosaccharide and 3) O-antigen polysaccharide (see **Figure 1**). Lipid A is anchored in the membrane and the core oligosaccharides are attached on the preformed lipid A molecule. O-antigen is

assembled in the cytoplasm and subsequently ligated onto the core oligosaccharide-lipid A complex. Both lipid A and core oligosaccharide are relatively conserved among bacterial species while the O-antigen is highly variable even amongst strains of the same species, thus contributing to the serotype designation of different strains within the same species. To our knowledge, O-antigen is not required for bacterial viability but is often implicated in virulence and host specificity where even small changes in the type and order of the sugars comprising the O-antigen can result in major changes in virulence.

The lifestyle of *Xf* requires attachment to diverse carbohydrate-based substrates such as the plant xylem wall and chitin in the mouthparts and foregut of the sharpshooter insect vector. In both environments, *Xf* forms biofilms or biofilm-like structures. Previous studies show that *Xf* produces an extracellular exopolysaccharide (EPS) that is present in small quantities during initial surface attachment and early biofilm formation. However, in mature biofilms this EPS (termed fastidian gum) is a major component of the 3-dimensional *Xf* biofilm both *in vitro* and *in planta* (Roper *et al*. 2008). Other studies have demonstrated that proteinacous adhesins such as type I pili and hemagluttinins contribute to surface adhesion and cell-cell aggregation (Li *et al*. 2007, Guilhabert and Kirkpatrick, 2005). Because of the location and abundance of LPS in the outer membrane we hypothesize that LPS also plays a key role in mediating initial attachment to the carbohydrate substrates *Xf* encounters in the plant and insect as well as the development of a mature biofilm. To accomplish our objectives, we are utilizing mutants that are disrupted in the O-antigen biosynthesis pathway to better understand the role of LPS in virulence, host-specificity, and biofilm formation.

OBJECTIVES

1a. Characterization and comparison of the LPS profiles from the grape, almond and oleander strains of *Xf* .
1b. Investigate the possibility of phase variation in *Xf* LPS.
2. Construct *Xf* mutants in O-antigen biosynthetic genes.
3. Test virulence and host specificity of the O-antigen mutants *in planta*.
4. Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants.

RESULTS AND DISCUSSION

Objective 1a. Characterization and comparison of the LPS profiles from the grape, almond and oleander strains of Xf. LPS is a tripartite molecule consisting of lipid A, an oligosaccharide core, and O-antigen. LPS variants can be classified as "rough" (those lacking the O-antigen) and "smooth" (those with all three components). A bacterial species can possess both rough and smooth variants and this difference is easily discerned by electrophoretic analysis. Three experimental isolates: *Xf* Fetzer (grape), Dixon (almond), and Ann-1 (oleander) were grown in PW broth or on solid PW medium. Cells were harvested and LPS was extracted using the hot-phenol method described by Westphal and Jann (1965) followed by electrophoresis on sodium deoxycholate-PAGE and Tricine-SDS PAGE gels. Preliminary results suggest that LPS extracted from the grape strain Temecula 1 grown on solid media contains both rough and smooth forms. Sodium deoxycholate gels is used to visualize the general forms of LPS (i.e., rough vs. smooth), while Tricine-SDS PAGE gels provide enhanced resolution of individual LPS bands, allowing the visualization of subtle differences in LPS profiles between the three isolates, a method we are also using when characterizing the LPS mutants (Δ*waaL* and Δ*wzy*) generated in this study.
In other systems, it has been shown that the rate of LPS biosynthesis differs with respect to growth phase and that, generally, LPS is synthesized in greater quantities when bacterial populations have reached stationary phase. Therefore, we are currently determining detailed growth curves for the three *Xf* isolates to ensure that we harvest LPS from similar points in their growth phase (mid-log and stationary) before comparing LPS profiles. We have conducted detailed growth curves by enumerating cell density (OD_{600nm}) coupled with dilution plating and colony counts and found that for the Fetzer and Dixon isolates, exponential growth occurs between ~2-5 days of incubation and stationary phase is reached at ~7-8 days of incubation. Growth curves are still in progress for the slower growing Ann-1 isolate. At this time, both solid and liquid cultures are being utilized for the hot phenol LPS extraction and subsequent visualization by sodium deoxycholate-PAGE and Tricine-SDS PAGE analyses.

Objective 1b. Investigate the possibility of phase variation in Xf LPS. Phase variation is the process by which Gram-negative bacteria undergo changes in antigenic properties in response to shifts in environmental conditions. Such changes are documented to occur in the extracellular and membrane-bound polysaccharide portions of the cell surface, including exopolysaccharide, capsular polysaccharide, and LPS (Bergman *et al*. 2006; Lerouge and Vanderleyden, 2002). We expect that the O-antigen moiety of LPS may differ depending on the types of carbohydrate available. We are investigating the possibility of phase variation in *Xf* Fetzer LPS by examining if there is a change in the LPS profile in cells grown in different culture conditions: i) PW, ii) PW amended with grapevine xylem sap, and iii) grapevine xylem sap alone.
Xylem sap (springtime bleeding sap) from 15 year-old 'Cabernet Sauvignon' grapevines was kindly provided by Dr. Philippe Rolshausen (Dept. of Plant Pathology & Microbiology, UCR). The collected fluid was filter-sterilized and assayed for carbohydrate content. Analysis of carbohydrate content (Dygert *et al*. 1965) indicated a reducing sugar content of ~1mg per mL of xylem sap. This will provide us with a means of normalizing the xylem sap based on carbohydrate content before adding it to the PW growth medium and ensure that equivalent concentrations of xylem sap constituents are added to each culture tube. Characterized xylem sap was aliquoted into10 mL volumes and stored at -80 C. *Xf* cells were successfully grown in PD3 amended with either 50% or 90% xylem sap using a protocol previously described by Zaini *et al*. 2009. LPS has been isolated from these cultures and is awaiting electrophoretic characterization.

Objective 2. Construct mutants in Xf with altered LPS profiles.
Genes targeted for mutation in the LPS biosynthetic pathway encode proteins necessary for the completion of a fully functional O-antigen moiety. We have identified two genes (*waaL* and *wzy*) putatively involved in *Xf* O-antigen biosynthesis. Both genes occur in single copy in all three *Xf* genomes used in this study. *waaL* (PD0077) encodes an O-antigen ligase that is responsible for attaching the completed O-antigen onto the assembled lipid A/core component of the LPS. Mutations in the *waaL* homologue of *Escherichia coli* prevent the ligation of O-antigen (Perez *et al*. 2008). Therefore, we predict that mutation of *waaL* will result in *Xf* strains that lack O-antigen ("rough" LPS mutants) (**Figure 1**). The second gene of interest is *wzy* (PD0814). *wzy* encodes an O-antigen polymerase protein that plays a role in chain length determination of the O-antigen, prior to its ligation onto the core component of LPS. Mutations in *wzy* in *E. coli* and *Shigella flexneri* resulted in severely truncated O-antigen (Carter *et al*. 2007; Cheng *et al*. 2007; Daniels *et al*. 1998). We predict that a *wzy* deletion will result in *Xf* strains that carry a truncated O-antigen ("semi-rough" mutants) (**Figure 1**).

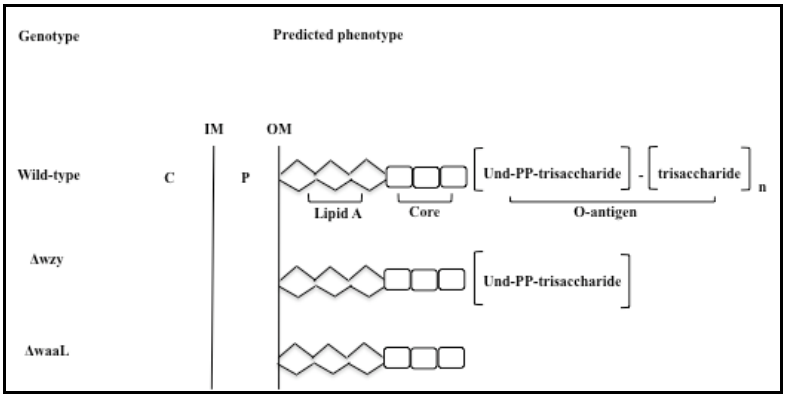

Figure 1. Models of a wild type *Xf* LPS molecule containing all three components (lipid A, core polysaccharide, and O-antigen), a hypothetical Δ*wzy Xf* LPS molecule with truncated O-antigen (i.e., consists only of undecaprenyl pyrophosphate linked to a single trisaccharide unit), and a hypothetical Δ*waaL Xf* LPS molecule completely devoid of O-antigen.
IM=Inner membrane; OM= Outer membrane; C= cytoplasm; P=periplasm

Prior to designing our mutant construction strategies, we conducted protein alignment and domain analyses using NCBI (www.ncbi.nlm.nih.gov), Wellcome Trust SangerInstitute Pfam (pfam.sanger.ac.uk), and Integrated Microbial Genomes (www.img.jgi.doe.gov/cgi-bin/pub/main.cgi) software. These analyses ensure that when creating the *waaL* and *wzy* mutants, we delete the proper catalytic domains of both the WaaL and Wzy proteins rendering them non-functional. The protein alignment results indicate that WaaL and Wzy are highly conserved among all the *Xf* strains used in this study (**Figure 2**). Additionally, protein domain analysis identified a Wzy_C catalytic domain in both WaaL and Wzy, providing further evidence that these proteins are involved in LPS biosynthesis (Wzy_C domain is enclosed in the box in **Figure 2**A and 2B). Wzy_C domains are found in the family of proteins containing O-antigen ligase (including the well characterized *E. coli* O-antigen ligase, RfaL). This domain contains the necessary amino acid residues for O-antigen ligase activity (Perez *et al*. 2008). There is considerable variation in primary amino acid sequence among O-antigen ligases and O-antigen polymerases, even between closely related bacterial species (Raetz *et al*. 2007; Schnaitman and Klena, 1993). When analyzed by blastx, WaaL homologues in *Xf* Dixon and Ann-1 have 80 and 81% identity, respectively, compared to Temecula 1 PD0077. Similarly, Wzy homologues in *Xf* Dixon and Ann-1 have 80 and 79% identity compared to PD0814.

Furthermore, we analyzed the gene neighborhood surrounding the *waaL* and *wzy* genes in each of the 3 *Xf* genomes using the *Xf* Comparative Genome Project site (www.xylella.lncc.br) as well as Integrated Microbial Genomes (www.img.jgi.doe.gov/cgibin/ pub/main.cgi). Understanding the genomic context within which *waaL* and *wzy* lie provides further insight into the *Xf* LPS biosynthetic pathway. Not unexpectedly, these analyses demonstrated that both *waaL* and *wzy* are located in similar gene neighborhoods in all three strains. Function prediction for proteins encoded by genes neighboring *waaL* and *wzy* include those known to be important in LPS biosynthesis in enteric bacteria (Schnaitman and Klena, 1993). Representation of the genomic context of *waaL* and *wzy* is shown in **Figure 3**.

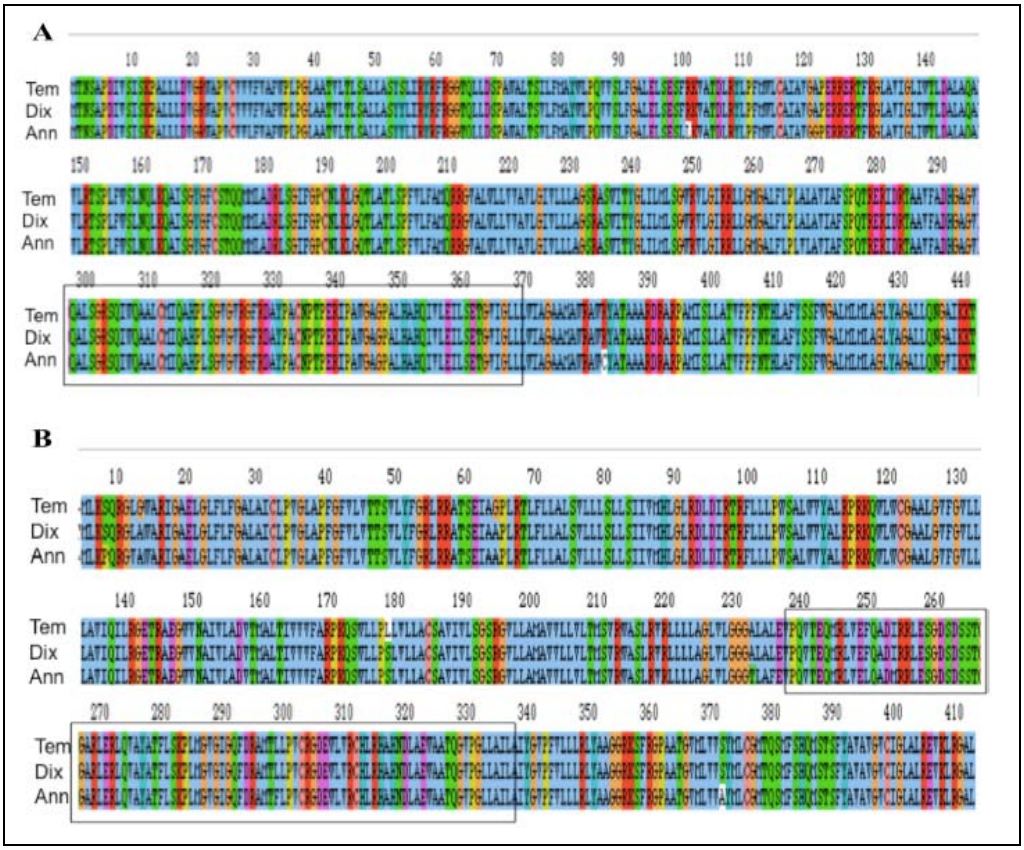

Figure 2. Protein Alignment and evidence for a Wzy_C domain in **A,** WaaL and **B,** Wzy of *Xf* strains Temecula1 (grape), Dixon (almond), and Ann-1 (oleander). The Wzy_C domain belongs to Pfam 04932, a family of proteins that includes the O- antigen ligases. The predicted Wzy-C domain is enclosed in the box in both A and B.

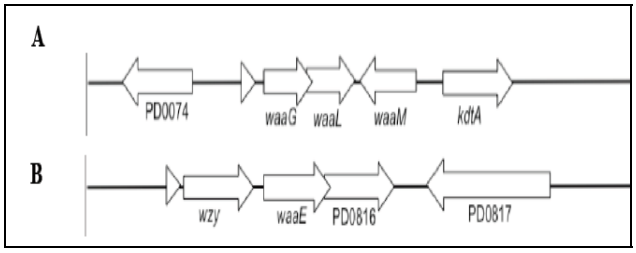

Figure 3. Representation of the genomic context of *waaL* and *wzy* in *Xf*. The gene arrangement within all 3 genomes of interest is highly similar. **A,** *waaL* lies within a cluster of several genes involved in LPS biosynthesis: *waaG* encodes glycerol transferase which transfers sugars to the core component; *waaM* encodes lauroyl acyltransferase, an enzyme responsible for transferring activated myristate or laurate to the lipid A moiety; *kdtA* encodes 3-deoxy-D-manno-octulosonic acid transferase, the enzyme that initiates the synthesis of core oligosaccharides to lipid A. **B,** *wzy* is located directly upstream of *waaE*, the gene that encodes a glycosyl transferase. PD0816 and PD0817 are currently annotated as genes belonging to protein families involved in teichoic acid and riboflavin biosynthesis, respectively.

A mutagenesis construct to make the *waaL* mutants in all three strains (due to high nucleotide sequence similarity between strains, the same construct can be used for all three strains) has been completed (**Figure 4**). Briefly, Fetzer *waaL* was PCR amplified and cloned into pCR8/GW/TOPO (Invitrogen). Restriction digest with *Age*I and *Eco*NI removed a 1383 bp fragment from the *waaL* amplified region and was replaced by the 1239 bp *Eco*RI Kan-2 (kanamycin resistance) fragment from pUC18 (Guilhabert *et al*, 2001) by blunt-end cloning, resulting in pJC3 (pJC*waaL*::*kan-2*). pJC3 was electroporated into *Xf* Fetzer, Dixon, and Ann-1 competent cells as previously described (Matsumoto *et al*, 2009) where a double recombination event would replace the full length *waaL* ORF with the selective marker, kan-2. The genomic context of the

waaL deletion mutant is shown in **Figure 5**.

A *waaL* deletion mutant in Ann-1 has been confirmed by PCR amplification of the *waaL* region with *waaL* primers and kan-2 primers as well as being resistant to 5µg/mL kanamycin (**Figure 4**). Further confirmation using primers designed to the flanking region at the point of the Kan-2 cassette insertion is underway. Candidate mutant strains of *waaL* deletion mutants in Fetzer and Dixon are currently being evaluated. All strains are confirmed at the genus-species as well as strain level by PCR and restriction digest analysis (Minisavage *et al*. 1994, Chen *et al*. 2005, Huang, 2009). A similar strategy is currently underway to create pJC4 (pJC*wzy*::*kan-2*). Preliminary data indicate that the Ann-1 Δ*waaL* mutant is devoid of smooth LPS (ie. O-antigen) as predicted. We are currently analyzing the LPS of this mutant more closely and have begun characterization of the Fetzer Δ*waaL* mutant.

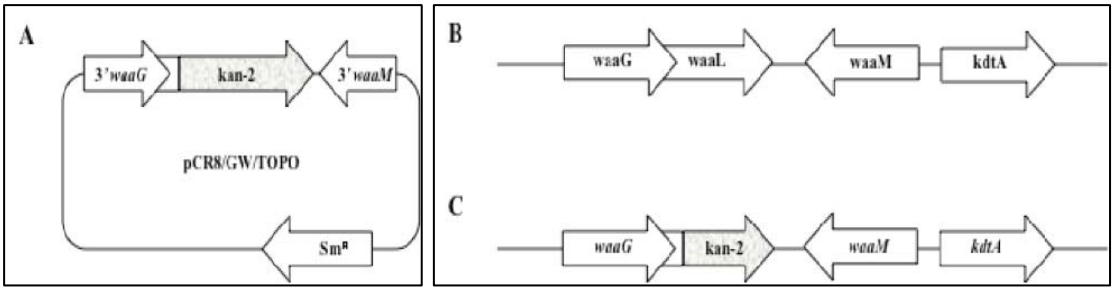

Figure 4. The construction of a Δ*waaL* mutant. **A,** mutagenesis construct pJC3 (pJC*waaL*::*kan-2*). After cloning the wild-type fragment into pCR8/GW/TOPO, 1384 bp of the *waaL* ORF was replaced by *kan-2*, a gene encoding for kanamycin resistance. The left flanking region consists of the 3[1] end of the PD0076 ORF (*waaG*), which has partial overlap with *waaL*, the 5[1]end of which remains intact in this construct. The stippled arrow represents the kan-2 cassette. The white box to the right of kan-2 represents the right flanking region (3' *waaM*). Note, figure not drawn to scale. **B,** *waaL* in the wild-type genomic context and **C,** *waaL* in the deletion mutant where *waaL* is replaced with kan-2. The 5' region of *waaL* remains intact to ensure a fully functional WaaG**.**

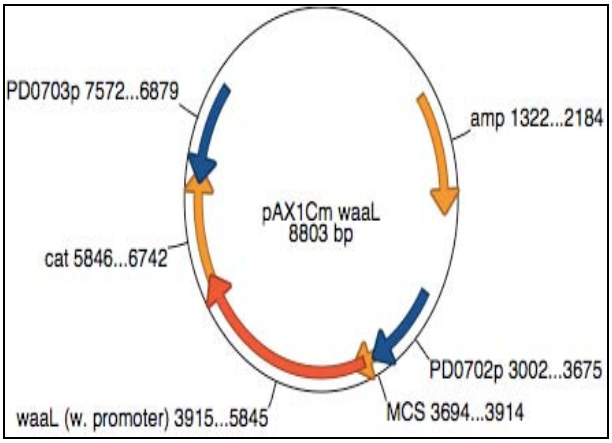

Figure 5. The *waaL* ORF with its promoter was amplified from *Xf* strains Fetzer, Dixon, and Ann-1 using primers containing *Xba*I ends and was cloned into the *Xba*I site of the multiple cloning region upstream of the chloramphenicol resistance gene (cat) in pAX1Cm. pAX1Cm plasmid courtesy of Dr. Michelle Igo (UCD).

Using the method of Matsumoto *et al* (2009), we have constructed complementation vectors with amplified Δ*waaL* and its promoter from each of the *Xf* strains of interest (Fetzer, Dixon, and Ann-1) (represented in **Figure 5**). Because of the divergence in amino acid sequence, we are interested to see if *waaL* from one strain can complement the Δ*waaL* mutant phenotype in other strains, as well as its own. For example, we will introduce the wild-type *waaL* from Fetzer back into the Fetzer Δ*waaL* mutant strain and also into the Dixon and Ann-1 Δ*waaL* mutant strains (**Table 1**). We will analyze the LPS isolated from the cross complemented mutants by running electrophoretic gels as described above. The same course of action will be taken with *wzy* mutants and complemented strains.

Objective 3. Test virulence and host specificity of the O-antigen mutants in planta.
As stated in Objective 2, mutant strains complemented with the wild-type locus will also be used for *in planta* studies. This approach will provide insight into whether LPS plays a role in virulence as well as host specificity observed in these three isolates. This can be determined by inoculating plants with each wild type, mutant, and complemented strain into their hosts as well as their non-hosts (**Table 1**). Inoculating with the complemented mutant strains will confirm any role of O-antigen in the ability to promote disease on grapevine, almond, and oleander.

Plant experiments are currently underway to evaluate the *Xf* Ann-1Δ*waaL* and *Xf* wild-type in the oleander host as well as almond and grapevine. The plants are six weeks post-inoculation and we are awaiting symptom development (ie. usually occurs 12 weeks post-inoculation).

Table 1. Virulence and host specificity assays.

	Host inoculation – for LPS profile and disease progress		
***Xf* strain**[a]	**Grape**	**Almond**	**Oleander**
WT Fetzer	host	+	+
Δ*waaL* Fetzer	host	+	+
Δ*waaL*/*waaL*+. Fetzer	host	+	+
Δ*wzy* Fetzer	host	+	+
Δ*wzy*/*waaL*+. Fetzer	host	+	+
WT Dixon	+	host	+
Δ*waaL* Dixon	+	host	+
Δ*waaL*/*waaL*+	+	host	+
Δ*wzy* Dixon	+	host	+
Δ*wzy*/*wzy*+ Dixon	+	host	+
WT Ann-1	+	+	host
Δ*waaL* Ann-1	+	+	host
Δ*waaL*/*waaL* Ann-1	+	+	host
Δ*wzy* Ann-1	+	+	host
Δ*wzy*/*wzy*+ Ann-1	+	+	host

[a] WT=wild-type, *waaL* and *wzy* mutant strains are or will be constructed as deletions, complemented strains are mutant strains with a wild-type copy of the gene of interest introduced into the genome via homologous recombination from the pAX1Cm vector (provided courtesy of Dr. Michele Igo; UCD). + represents inoculation of a non-host.

Objective 4. Test attachment and biofilm formation phenotypes of Xf O-antigen mutants.
No activity for this reporting period.

CONCLUSIONS
The primary goal of this project is to further understand the molecular mechanisms of *Xf* virulence and host specificity *in planta*. We are investigating if the O-antigen component of the LPS molecule plays a key factor in behaviors associated with xylem colonization, such as attachment to the plant cell wall and biofilm formation. While there are likely several factors that contribute to host specificity of *Xf*, we hypothesize that O-antigen presence and composition plays a major role in host specificity. We feel that the wide host range but stringent host specificity of different *Xf* isolates affords a unique opportunity to study the molecular mechanisms underlying the host specificity observed for this pathogen. Should LPS prove to be an important factor during *Xf* plant infection, its abundance in the bacterial outer membrane makes it a rational target for disease control.

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