I. Project Title: The role of lipopolysaccharides in virulence, biofilm formation, and host specificity of *Xylella fastidiosa.*

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III and IV. Research Objectives and Results

Objective 1. Characterize the lipopolysaccharide (LPS) profiles from the grape, almond, and oleander strains of *Xylella fastidiosa* (*Xf*) grown either in Periwinkle Wilt (PW) broth or on PW solid medium. We will also investigate if the *Xf* grape strain LPS molecule undergoes phase variation by comparing the LPS profiles of cells grown in PW broth, PW amended with xylem sap or xylem sap alone.

Objective 1a. To compare LPS profiles from *Xf* grown *in vitro*. 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. All experimental strains (*Xf* Fetzer (grape), Dixon (almond), and Ann-1 (oleander)) will be grown in PW broth or solid medium. Cells will be harvested and LPS will be 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 will be used to visualize the forms of LPS (i.e., rough vs. smooth). Tricine-SDS PAGE gels will provide enhanced resolution of individual LPS bands allowing the visualization of subtle differences in LPS profiles between the three strains, a method we will also use when characterizing the LPS mutants (Δ*waaL* and Δ*wzy*) generated in this study.

Progress to date: We have established optimal media and incubation conditions for growing the 3 *Xf* strains. This has been somewhat of a challenge due to the different growth rates of the 3 strains (Fetzer, 7 days; Dixon, 10 days; Ann-1, 21 days on solid medium). We are currently determining detailed growth curves for these strains to ensure that we harvest LPS from each strain at similar points in their growth phase (mid-log) before comparing their LPS profiles. Currently, both solid and liquid cultures are being prepared for the hot phenol LPS extraction and subsequent visualization by SDS-PAGE and Tricine SDS PAGE analyses.

Objective 1b. To compare the LPS profile of *Xf* Fetzer when grown in PW medium versus grape xylem sap. 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.

Progress to date: We are currently collecting xylem sap (springtime bleeding sap) from grapevine stems being grown in the field and in the greenhouse. Field-grown grapevines (cvs. 'Cabernet Sauvignon' and 'Chardonnay') are located at the Agricultural Operations site on the University of California-Riverside campus. Grapevines maintained in the greenhouse were kindly provided by Thomas Miller (Dept. of Entomology, UCR). The collected fluid will be filter-sterilized and assayed for carbohydrate content (both total sugar and reducing sugars). Both values will provide us with a means of normalizing the xylem sap based on carbohydrate content before adding it to the PW growth medium. This will ensure that equivalent concentrations of xylem sap constituents are added to each culture tube. Characterized xylem sap will be aliquoted into 10 mL volumes and stored at -80 C until needed.

Objective 2. To construct mutants in *Xf* that have 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 3 *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 1B). 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 shortened 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 1C).

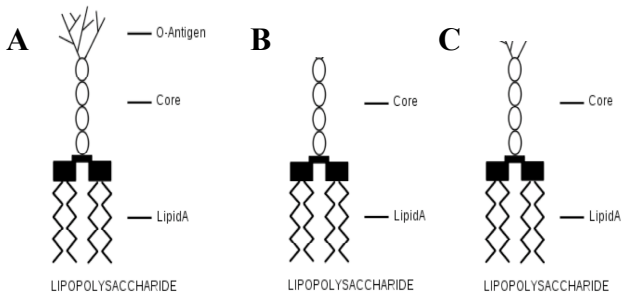

Figure 1. A. Model of wild type *Xf* LPS molecule containing all three components: lipid A, core polysaccharide, and O-antigen **B**. Model of hypothetical Δ*waaL Xf* LPS molecule containing no O-antigen. **C.** Model of hypothetical Δ*wzy Xf* LPS molecule with truncated O-antigen. Images adapted from http://www.wikipedia.org/.

Progress to date: Prior to designing our mutant construction strategies, we conducted protein alignment and domain analyses using NCBI (www.ncbi.nlm.nih.gov), Wellcome Trust Sanger Institute Pfam (pfam.sanger.ac.uk), and Integrated Microbial Genomes (www.img.jgi.doe.gov/cgi-bin/pub/main.cgi) software. These analyses will 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 2A 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 of primary amino acid sequence among O-antigen ligase and O-antigen polymerase, even between closely related bacterial species (Raetz et al. 2007; Schnaitman and Klena, 1993). When analyzed by blastx, WaaL homologues in *X. fastidiosa* Dixon and Ann-1 have 80 and 81% identity, respectively, compared to Temecula 1 PD0077. Similarly, Wzy homologues in *X. fastidiosa* Dixon and Ann-1 have 80 and 79% identity compared to PD0814.

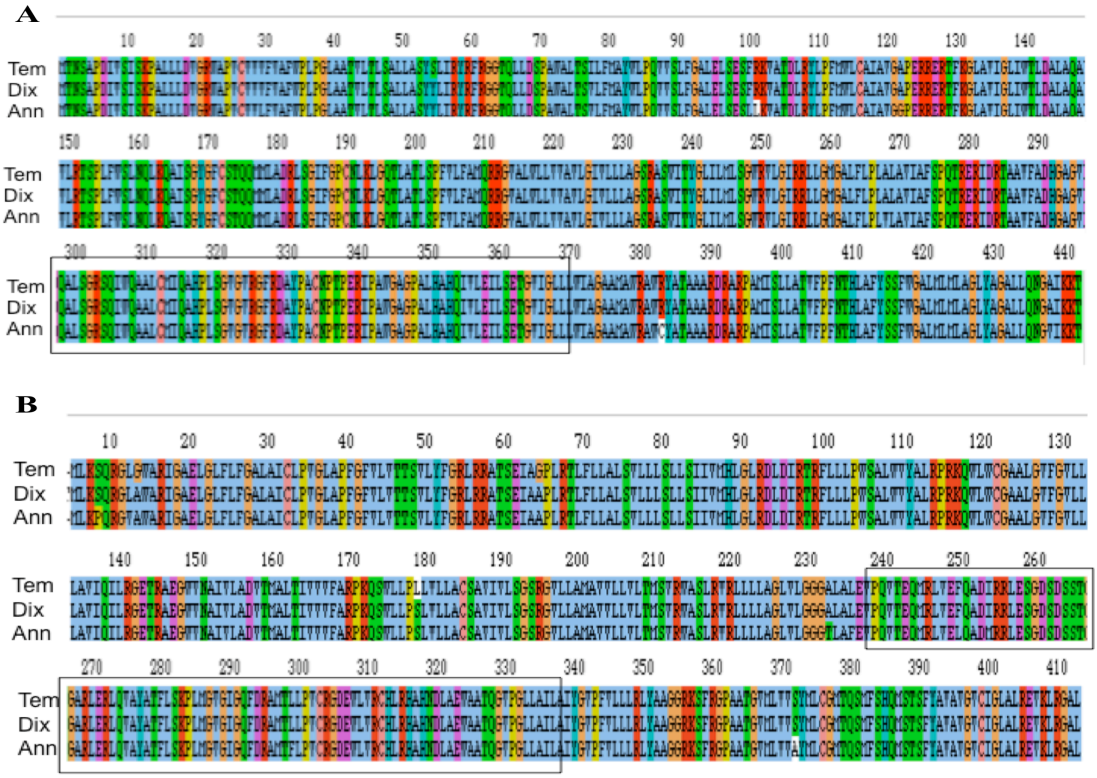

Figure 2. Protein Alignment and evidence for a Wzy_C domain in **A,** WaaL and **B,** Wzy of *Xf* strains Temecula 1 (grape), Dixon (almond), and Ann-1 (oleander). The Wzy_C catalytic domain belongs to Pfam 04932, a family of proteins that includes the O- antigen ligases. The Wzy-C domain is enclosed in the box in both A and B. Data obtained from NCBI (www.ncbi.nlm.nih.gov), Wellcome Trust Sanger Institute Pfam (pfam.sanger.ac.uk), and Integrated Microbial Genomes (www.img.jgi.doe.gov/cgi-

Furthermore, we analyzed the gene neighborhood surrounding the *waaL* and *wzy* genes in each of the 3 *Xf* genomes using the *Xylella fastidiosa* Comparative Genome Project site (www.xylella.lncc.br) as well as Integrated Microbial Genomes (www.img.jgi.doe.gov/cgi-bin/pub/main.cgi). Understanding the genomic context within which *waaL* and *wzy* lie may

provide 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 3 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.

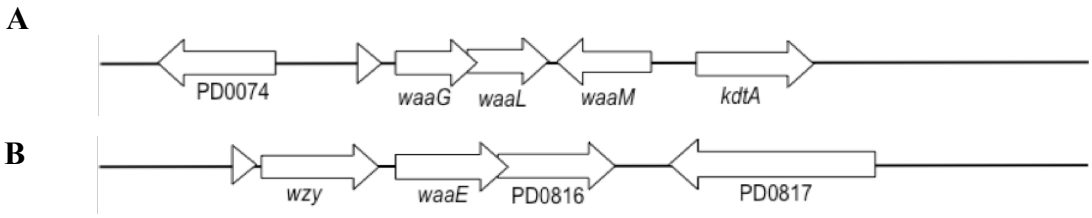

Figure 3. Representation of the genomic context of *waaL* and *wzy* in *Xf*. The gene arrangement within all 3 genomes of interest is highly similar. **A,** *waaL* lies within a cluster of several genes involved in LPS biosynthesis: *waaG* encodes glycerol transferase which transfers sugars to the core 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. Data derived from Integrated Microbial Genomes (www.img.jgi.doe.gov/cgi-bin/pub/main.cgi) and the *Xylella fastidiosa* Comparative Genome Project (www.xylella.lncc.br/).

Mutagenesis constructs have been completed to make the *waaL* mutants in all 3 strains (due to high nucleotide sequence similarity between strains, the same construct can be used for all 3 strains). *waaL* was PCR amplified and cloned into pCR8/GW/TOPO. Restriction digest with *Age*I and *Eco*NI removed 1229 bp from the *waaL* ORF and was replaced by the 1239 bp *Eco*RI Kan-2 (kanamycin resistance) fragment from pUC18(9) by blunt-end cloning. This resulted in pJC3 (pJC*waaL*::*kan-2*) (Figure 4). A similar strategy is currently underway to create pJC4 (pJC*wzy*::*kan-2*). We are preparing *Xf* (Fetzer) electrocompetent cells in order to introduce the pJC3 and pJC4 constructs.

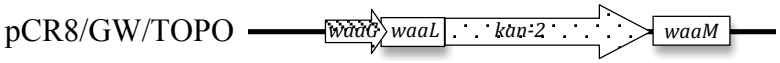

Figure 4. Mutagenesis construct pJC3 (pJC*waaL*::*kan-2*). After cloning the wild-type fragment into pCR8/GW/TOPO, 1229 bp of the *waaL* ORF was replaced by *kan-2*, a gene encoding for kanamycin resistance. The left flanking region consists of the 3[1] end of the PD0076 ORF (*waaG*), which has partial overlap with *waaL*, the 5' 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 (*waaM*). Note, figure not drawn to scale.

Experiments are also underway to construct complementation vectors using a chromosomal-based complementation system described by Matsumoto *et al.* (2009). The pAX1 series of vectors have kindly been provided to us by the Igo lab (UC-Davis). This system should restore any LPS phenotype observed in the mutant strains.

Objective 3. Determine contribution of *Xf* O-antigen to virulence and host specificity. In other bacterial species, including *Salmonella enterica* and the phytopathogen *Erwinia amylovora*, mutants in *waaL* are reduced in their ability to colonize the host (Berry et al. 2009; Carroll et al. 2004). Therefore, deletions of *waaL* and *wzy* in *Xf* are expected to result in a virulence defect in

their hosts. Plant inoculations will be performed using the pin-prick method originally described by Hill and Purcell (1995). We will inoculate 10 plants/mutant and repeat each experiment 3 times. All plants will be rated on a disease scale of 0-5 with 0 being healthy and 5 being dead (10). Each LPS mutant strain will be tested for virulence in their respective susceptible host plants. We will also quantify differences in colonization by determining bacterial populations/gram of plant tissue in petioles near the point of inoculation as well as away from the point of inoculation. The results of these experiments will indicate if the O-antigen portion of the LPS molecule is indeed a virulence factor for *Xf*.

As part of Objective 3, we will also examine the host specificity of each of our LPS mutants. While there are likely several factors that contribute to host specificity of *Xf* we will investigate if O-antigen presence and composition is involved. To test this, each LPS mutant strain will be tested for virulence in their "non-host" plants and determine if an alteration in O-antigen correlates with a shift in host range or specificity. For these experiments, *Xf* Fetzer mutants will be inoculated into almond and oleander. The Ann-1 mutants will be inoculated into grape and almond, and the Dixon mutants will be inoculated into grape and oleander (Table 1). All mutants and wild type strains will also be inoculated in their respective host plants in order to compare symptoms.

Table 1. Cross-inoculation of *Xf* strains on three hosts. WT=wild-type.

	Host Inoculations		
***Xf* mutant**	**Grape**	**Almond**	**Oleander**
WT Fetzer		x	x
Δ*waaL* Fetzer		x	x
Δ*wzy* Fetzer		x	x
WT Dixon	x		x
Δ*waaL* Dixon	x		x
Δwzy Dixon	x		x
WT Ann-1	x	x	
Δ*waaL* Ann-1	x	x	
Δ*wzy* Ann-1	x	x	

Progress to date: We have completed the constructs necessary to create the Δ*waaL* mutant in all 3 strains and have begun preparing the electrocompent *Xf* cells. Several commercial sources of grapevine, almond and oleander have been identified and we expect to order these plants in the coming weeks. Most importantly, adequate greenhouse space has been secured by the Roper lab to accommodate the plants for the pathogenicity and host specificity experiments outlined above.

Objective 4: Test surface attachment and biofilm formation of *Xf* (Fetzer) O-antigen mutants. We are further characterizing the *Xf* Fetzer Δ*waaL* and Δ*wzy* O-antigen mutant strains by assaying for surface attachment and biofilm formation. In order to quantify surface attachment, mutant strains will be grown in PD3 or PW medium in 10 mL borosilicate glass tubes and incubated at 28ºC in a vertical position without shaking for 10 days. Attachment on the surface walls of the tubes will be assessed by a crystal violet staining method (Espinos-Urgel et al., 2000). To assess the capability of the *Xf* Fetzer Δ*waaL* and Δ*wzy* O-antigen mutant strains to form biofilms on glass surfaces, we will perform *in vitro* assays biofilm assays as previously

described by Roper *et al*. 2007. Biofilms will be imaged using confocal laser scanning microscopy available in the UCR core microscopy facility.

Progress to date: The postdoctoral researcher working on this project attended a rigorous training workshop on the use of the Leica confocal microscope housed in the UCR Core Microscopy facility. The remaining experiments are currently at the planning stage and are pending construction of Δ*waaL*, and Δ*wzy* mutants in the *Xf* grape strain, Fetzer.

V. Publication/Reports: Funding for this project began on November 1st, 2009. Postdoctoral Scholar, Dr. Jennifer Clifford arrived to the Roper lab at UCR on December 1, 2009 and commenced working on this project shortly thereafter. We recently reported our preliminary findings in the Pierce's Disease Research Symposium Proceedings (Roper, 2009). There are currently no scientific journal publications resulting from this project.

VI. Presentations on research: A poster outlining this project was presented at the 2009 Pierce's Disease Research Symposium held in Sacramento, CA .

VII. Research relevance statement: This project focuses on the lipopolysaccharide (LPS) component of the outer membrane of *Xf*. In particular, we are investigating if the O-antigen portion of this molecule contributes to *Xf* surface attachment and biofilm formation. More importantly, by targeting genes involved in O-antigen biosynthesis, we will determine if LPS is an important virulence factor for *Xf* infection of grape. Additionally, we will determine if LPS contributes to the high level of host specificity observed for this pathogen. The discovery of a new virulence factor for *Xf* will provide a novel target for the potential mitigation of PD. The LPS molecule makes up 70% of the bacterial outer membrane and is required for bacterial viability, making it both an abundant and vulnerable target for potential antimicrobial compounds. Indeed, there are several antimicrobial peptides (AMPs) that either disrupt or inhibit LPS synthesis in other bacterial systems (Ding *et al*. 2008; Farnaud et al., 2007; Srinivas et al. 2010) Additionally, when used in conjunction with other antimicrobial strategies, treatment with an LPS-targeting AMP may make the cells more susceptible to other antimicrobial compounds being developed by other researchers, thereby increasing the efficacy of that particular product. The findings from this study will provide the framework and generate the necessary tools (*Xf* LPS mutants) for future studies investigating LPS-targeting antimicrobials. Once indentified, these compounds could either be applied directly to plants or produced by transgenic grapevines with the hopes of a successfully mediating PD in a field setting.

VIII. Lay summary of current year's results:

Xylella fastidiosa is a bacterium that has the ability to infect many different plant species. In some plants, this bacterium can cause serious disease. In grapevine, this disease is known as Pierce's disease and has caused millions of dollars of damage to the California grape industry alone. *Xf* species also infect 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 strains 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. The lipopolysacharide (LPS) molecule is a key component of the bacterial outer membrane. This molecule is involved in virulence for

many bacterial pathogens. We are investigating how the LPS molecule is related to *Xf* virulence and other key aspects of the disease process, like attachment to the plant cell wall. This molecule makes up more than 70% of the bacterial membrane and if LPS does prove to be an important factor during *Xf* plant infection, its abundance in the bacterial cell membrane makes it a logical target for disease control. Furthermore, antimicrobial compounds exist that disrupt LPS synthesis or function. There are also others that weaken the LPS molecule, making the bacterium more sensitive to other stresses. Therefore, compounds targeted towards LPS synthesis could increase the efficacy of other anti-Xylella compounds currently being developed by other researchers when both are used in conjunction. To accomplish our objectives, we are well on our way to constructing mutants in the LPS pathway that will aid us in ascertaining the role of LPS in the disease process of *Xf*. We have also begun the characterization of the *Xf* LPS molecule using biochemical and computer based methods.

IX. Status of funds: This is the 1st year of a 2-year project. We have approximately $50,000 remaining on this grant, which has an 8/31/10 end date. We anticipate using all of the awarded funds for the first year and pending approval by the board would like to continue this project for the 2nd year.

X. Summary and status of intellectual property: There is currently no intellectual property associated with this project.

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