I. Project Title

The oxidative stress response: Identifying proteins critical for *Xylella fastidiosa* survival in grapevines.

II. Principal Investigators and Cooperators

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III. List of objectives and description of activities conducted to accomplish each objective.

Overview

Xyllela fastidiosa (*Xf*) is exposed to reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions, at potentially two points in the infection cycle. First, ROS are a normal part of both the plant defense response and certain developmental processes (Bolwell and Daudi, 2009). In xylem tissue, there are two primary sources of ROS (Barcelo, 2005). One is developmentally related and comes from the process of lignifying xylem. The second source is the living xylem parenchyma cells, which can diffuse H_2O_2 to the adjacent xylem vessels. *Xf* may also be exposed to the ROS produced by the immune systems of the insect vector (Vallet-Gely et al., 2008). Since elevated levels of ROS are highly toxic and can disrupt many cellular processes through their oxidation of lipids, modification of proteins and damage to DNA, most pathogenic bacteria have developed strategies to overcome this toxicity.

The immediate detoxification of ROS is accomplished in accomplished, in part, by scavenging enzymes designed to cope with specific oxidative stresses (for review, see (Imlay, 2008)). Comparative genomics suggests that many of these enzymes are present in *Xf* (Table 1):

Table 1. Predicted *Xf* enzymes and regulators

Enzyme	***Xf* gene(s)**	**Oxidative signal**	**Regulator**
Alkyl hydroperoxide reductase	*ahpC, ahpF*	H_2O_2, organic peroxides	OxyR
Catalase	*cpeB*	H_2O_2.	OxyR
Superoxide dismutase	*sodA, sodM*	superoxides	unknown
Flavodoxin $NADP^+$ reductase	*fpr*	superoxides	unknown
Thiol-dependent peroxidase	*ohr*	organic peroxides	unknown

The goal of this project is to understand how *Xf* responds to the different ROS it encounters in the xylem and to characterize the enzymes and regulatory proteins induced in this response. Understanding the vunerability of *Xf* to oxidative stress could lead to targeted strategies for mitigating the devastating symptoms of Pierce's Disease in grapevines.

Objectives:

Objective 1: Determine the key components in the response of *Xf* to ROS and the contribution of OxyR to this regulation.

Objective 2: Determine the role of the scavenging enzymes designed to cope with specific oxidative stresses in *Xf* cell physiology and virulence.

Objective 3: Determine the role of the transcription factor OxyR in oxidative stress sensing, biofilm formation, and virulence.

Objective 4: Test mutants generated in Objectives 2 and 3 for virulence in grapevines and for sharpshooter transmission.

Objective 5: Develop a bioluminescent (Lux) reporter system for *Xf*.

IV. Summary of major research accomplishments and results for each objective. (Includes a description of the activities conducted to accomplish each objective).

Many bacteria have evolved distinct sensing mechanisms to detect different forms of oxidative stress and to induce the synthesis of a particular set of scavenging enzymes. In Gram-negative bacteria, much of this regulation occurs at the transcriptional level through regulatory proteins such as OxyR, SoxRS, and Ohr (Imlay, 2008). Examination of the *Xf* genome revealed a potential homolog to OxyR. However, unlike other Xanthomonads, *Xf* is missing both the homolog to Ohr, which regulates the response to organic peroxides and the SoxRS system, which regulates the response to superoxide stress. In this project, we have been examining how *Xf* responds to different types of ROS, whether or not these responses are regulated, and the importance of OxyR in these responses.

Objective 1: Determine the key components in the response of *Xf* to ROS and the contribution of OxyR to this regulation. As shown in Table 1, *Xf* is predicted to contain multiple scavenging enzymes that respond to different ROS. During the past year, we have focused primarily on the response of *Xf* to H_2O_2 and the role of OxyR in the regulation of this response. We first examined how different exposure times and concentrations of H_2O_2 impacted *Xf* transcription, growth, and viability. For transcriptional analysis, we chose sublethal H_2O_2 conditions that did not impact RNA quality. Liquid cultures of *Xf* Temecula1 (WT) and the *Xf oxyR* mutant (*oxyR*) were grown in PD3 for three days and the cultures were split. One sample was exposed to H_2O_2 (0.5 mM final concentration) for 10 minutes; the other sample served as the untreated control. Total RNA was then extracted from all four cultures with TRIZOL reagent (Invitrogen) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). The cDNA samples were then subjected to qRT-PCR analysis using SsoFast EvaGreen Supermix Kit (BioRad) and primers pairs exhibiting homology to *oxyR* and the seven genes listed in Table 1. The results from this analysis are shown in Figure 1.

As expected, transcription of four chosen target genes was induced in WT cells following the addition of H_2O_2: *aphC, aphF, oxyR,* and *cpeB*. The *aphC* and *aphF* genes encode alkyl hydroperoxide reductase, whereas the *cpeB* gene encodes catalase. These enzymes are known to play a critical role in the detoxification of H_2O_2. Given its location in the same operon as *aphC* and *aphF,* the induction of *oxyR* was also expected. Furthermore, induction of these genes does not occur in the *oxyR* mutant, confirming the role of OxyR in their regulation. In contrast, transcription of three genes encoding scavenging enzymes for superoxides and organic peroxides (*sodM, fpr* and *ohr*) were not induced to a significant level by H_2O_2 or by the absence OxyR transcription factor.

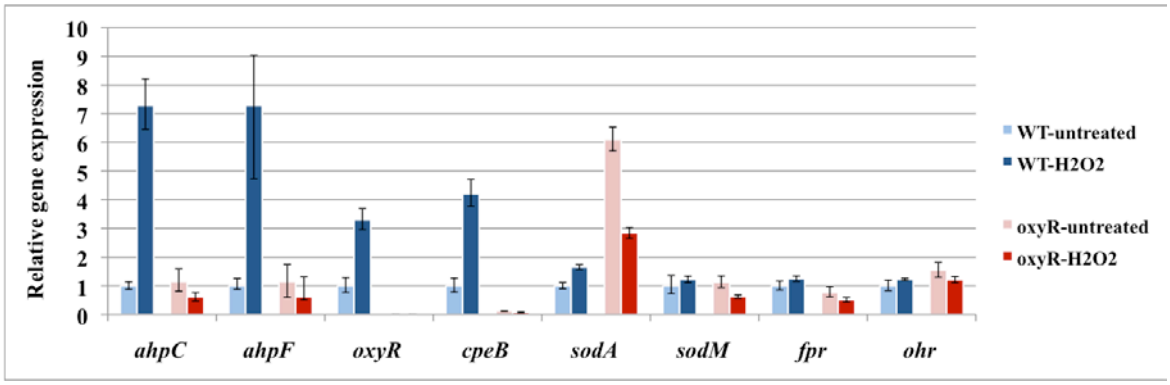


Figure 1: Impact of H_2O_2 and OxyR on transcription of oxidative stress genes.

The most intriguing result came from our analysis of *sodA*, which encodes the manganese-containing superoxide dismutase. In *E. coli*, *sodA* transcription is regulated by the superoxide-sensing transcription factor SoxR (Imlay, 2008). However, not all bacteria that respond to superoxide stress contain SoxR. Hence, they have developed different strategies for regulating superoxide detoxifying enzymes. For example, in the obligate anaerobe *Porphyromonas gingivalis*, *sodA* is positively regulated by OxyR (Ohara et al., 2006). It has been proposed that *P. gingivalis* OxyR is functioning as an intracellular redox sensor rather than as a peroxide sensor, which triggers *sodA* transcription. Like *P. gingivalis*, the *Xf* genome does not contain a homolog to SoxR. Instead, as shown in Figure 1, transcription of *Xf sodA* appears to be negatively regulated by OxyR. Whether this regulation occurs through a direct interaction between OxyR and the *Xf sodA* regulatory region or some indirect effect remains to be determined. However, this unusual regulatory pattern for *sodA* suggests that *Xf* may have developed novel methods for coping with oxidative stress.

<u>Objective 2: Determine the role of the scavenging enzymes designed to cope with specific oxidative stresses in *Xf* cell physiology and virulence</u>. Scavenging enzymes play a critical role in the response of bacteria to oxidative stress. In an earlier study, we established that deletion of the catalase-encoding *cpeB* gene (Δ*cpeB*) resulted in increased sensitivity to H_2O_2 on solid media (Matsumoto et al., 2009). Moreover, the Δ*cpeB* mutant exhibits a lower survival rate compared to WT following treatment of a culture with 0.5 mM H_2O_2. As shown in Figure 2A, a dramatic decrease in *Xf* survival is observed following even a 1 minute exposure to 0.5 mM H_2O_2. Similar results were obtained when the strains were compared using the LIVE/DEAD cells staining method following treatment with 0.5 mM H_2O_2 or the superoxide generator, paraquat (PQ) for 1 hr (Figure 2B).

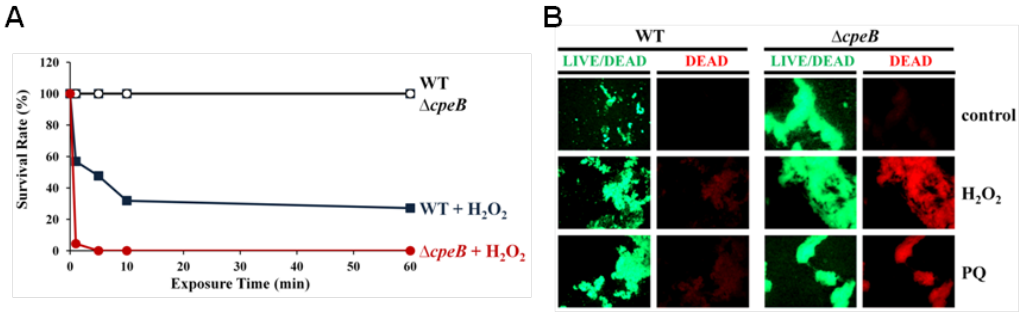


Figure 2: Importance of catalase for *Xf* survival following exposure to oxidative stress.

Objective 3: Determine the role of the transcription factor OxyR in oxidative stress sensing, biofilm formation and virulence. A bacterial biofilm is an aggregation of bacterial cells, which adheres to living or non-living surfaces (Hall-Stoodley et al., 2004). These adhered bacterial communities are usually embedded in a protective self-produced matrix exopolysaccharide (EPS). The stages of biofilm development generally include: (1) initial attachment, (2) microcolony formation, (3) maturation, and (4) dispersion. Recent studies have shown that OxyR is involved in biofilm formation by regulating fimbrial gene expression, a key aspect of the early attachment stage of biofilm formation (Shanks et al., 2007). The Roper laboratory has also observed that OxyR also control EPS production in the xylem-dwelling phytopathogen, *Pantoea stewartii* (*unpublished data*). We hypothesize that OxyR may also play a similar role in regulating genes involved in biofilm formation in *Xf*.

To test this hypothesis, Peng Wang (Roper laboratory) created a null mutation in the *oxyR* gene by site-directed mutagenesis and established that the resulting *oxyR* mutant exhibits a greater sensitivity to H_2O_2 on solid media than WT. Moreover, Dr. Yunho Lee (Igo laboratory) compared the relative transcription levels of eight oxidative stress genes in the *oxyR* mutant to WT (Figure 1). This analysis indicated that OxyR is required for the transcriptional regulation of *ahpC, ahpF, oxyR*, *cpeB,* and possibly *sodA* in response to peroxide stress. We also examined how the absence of OxyR impacts two biofilm related behaviors: surface attachment and cell-cell aggregation. These studies revealed that the *Xf oxyR* mutant is severely compromised in both behaviors. Surface attachment to three different surfaces (polystyrene, glass and polyprolylene) was assessed using crystal violet (Espinosa-Urgel et al., 2000). As shown in Figure 3A, the tubes containing WT *Xf* had clear purple rings at the air-medium interface indicating a large number of attached cells. Notably, there was less of a purple ring observed for the *Xf oxyR* mutant, indicating that the *oxyR* mutant did not attach as well as the WT parent to any surface tested.

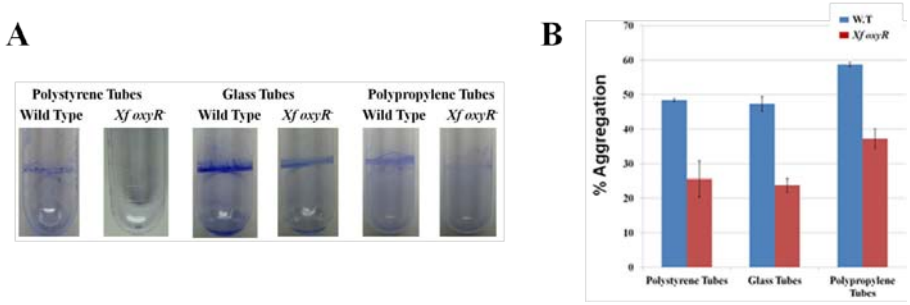


Figure 3: The *oxyR* mutation impacts attachment to solid surfaces and autoaggregation.

The second phase of biofilm formation involves cell-cell aggregation, an important aspect of microcolony formation. We first observed the marked decrease in the aggregative ability of the *oxyR* mutant simply by growing them in liquid cultures and visually comparing them to WT liquid cultures. The decrease in the ability of the *oxyR* mutant to aggregate was visible to the naked eye. We then quantified cell-cell aggregation using an established protocol (Guilhabert and Kirkpatrick, 2005) . Briefly, WT and *oxyR* mutant cells were grown in PD3 for 10 days without agitation. Then, *Xf* cell cultures were gently agitated and allowed to settle for 20 minutes. The OD_{540} of upper culture (ODs) was measured and returned to the original tube. The aggregated cells were dispersed by briefly vortexing. The OD_{540} of total cell culture (OD_t) was measured. The percentage of cell aggregation was calculated as below: aggregated cells percentage=

$100(OD_t-OD_s)/OD_t$. The results indicate that a mutation in *oxyR* greatly affects cell-cell aggregation (Figure 3B).

Another important component of the maturing biofilm is exopolysaccharide (EPS) matrix production. In other bacterial systems, *oxyR* mutants have a marked decrease in EPS production (Roper, *unpublished data*). To determine if the *Xf oxyR* mutant is similarly affected, we are quantifying EPS production by the *oxyR* mutant using a Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS ELISA) method (Roper et al., 2007). This assay is very sensitive and even small amounts of EPS can be detected (detection limit is 1µg). We speculate that EPS production will be decreased in EPS production based on findings in other bacterial systems in the Roper laboratory. We anticipate completion of this study by May 2012. Finally, we are visualizing *Xf* biofilm architecture of both the *oxyR* mutant and WT *Xf* using the confocal microscope available in the UCR core microscope facility. Based on the decreased ability of the *oxyR* mutant to attach and self-aggregate, we suspect the *oxyR* mutant will form only a monolayer of cells and will be unable to achieve the 3-dimensional architecture of a WT biofilm.

Objective 4: Test mutants generated in Objectives 2 and 3 for virulence in grapevines and for sharpshooter transmission. The *cpeB* mutant (Objective 2) and the *oxyR* mutant (Objective 3) are important for *Xf* survival under laboratory conditions. Furthermore, the *oxyR* mutant is defective in biofilm formation, a property known to be important for *Xf* virulence. The next step in our analysis has been to examine how these mutations affect *Xf* colonization in host plants and transmission in insect vector.

The experiments on the *oxyR* mutant were carried out at UC Riverside. *Vitis vinifera* cv. Thompson seedless grapevines were pin-prick inoculated using a 20-gauge syringe needle as described by Hill and Purcell (1995). The populations of *Xf* wild type and the *oxyR* mutant were quantified from petioles harvested 11 weeks and 14 weeks post-inoculation as previously described (Roper et al., 2007). Although the results indicate that the *oxyR* mutant does not colonize grapevines as efficiently as wild type (Table 2), we had anticipated a larger decrease based on the apparent role of OxyR in the early steps of biofilm formation. Therefore, we will be reexamining the properties of the *oxyR* mutant this Spring and including earlier time points in our analysis (ie. 4 weeks post-inoculation).

Table 2. The *oxyR* mutant does not colonize grapevines as efficiently as *Xf* wild type.

Time post-inoculation	*Xf* wild type (CFU)	*Xf oxyR* (CFU)
11 weeks	$(28.65\pm7.92)\times10^6$	$(11.39\pm4.3)\times10^5$
14 weeks	$(1.20\pm0.19)\times10^7$	$(6.10\pm3.60)\times10^6$

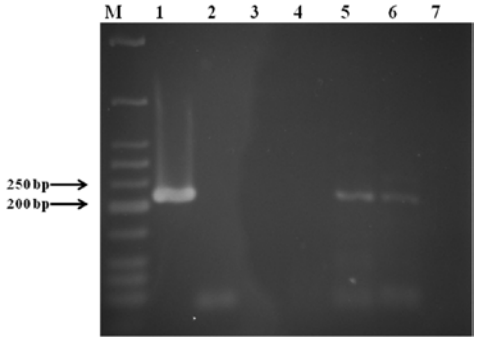
The populations of *Xf* wild type and *oxyR* mutant were determine in the leaf petioles at (11 weeks post-inoculation) or near (14 weeks post-inoculation) the point of inoculation (POI) in grapevines (Average CFU/g ±SE) and the results analyzed using a Wilcoxon rank sums test.

The greenhouse experiments on the *cpeB* mutant were initiated at UC Davis last year using our standard protocol (Matsumoto et al., 2012). Unfortunately, we ran into two problems. First, the grapevines obtained for the experiment were already infected with a virus. Second, there was a major problem with insects in the greenhouse. As a result, no data was obtained from last year's grapevine experiments. Other UC Davis researchers using the same grapevine supplier and greenhouse ran into similar problems. To minimize these issues in the future experiments, we are using a different grapevine supplier and will be rooting our own vines with the assistance of

members of the Kirkpatrick laboratory (UCD Department of Plant Pathology). This should increase the probability that the starting vines will be healthy. We will also use a different greenhouse that is closer to the lab. This will allow daily monitoring of the vines by the scientists conducting the experiments. With these changes, we anticipate that we will be able to complete the experiments concerning the importance of catalase (CpeB) for *Xf* survival in grapevines by Fall 2012.

During the past year, we also initiated the insect transmission studies for the *oxyR* mutant at UC Riverside. In cooperation with Dr. Matt Daugherty (UCR Department of Entomology), we conducted insect acquisition and transmission studies using the Glassy-winged sharpshooters (GWSS) (*Homalodisca vitripennis*) that were collected and reared at the UCR Agricultural Operations facility. For this analysis, we are using the artificial feeding sachet technique developed by Killiny and Almeida (Killiny and Almeida, 2009), 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. Briefly, we confined individual sharpshooter adults in a feeding apparatus for 8 hours, which contained the artificial diet solution alone or a solution inoculated with either wild-type *Xf* or *Xf oxyR*. The sharpshooters were then transferred to healthy grape seedlings for 4 days. Insect acquisition rates were assessed by monitoring the presence or absence of *Xf* in sharpshooter heads using conventional PCR and the *Xf*-specific detection primers HL-5 and HL-6 (Francis et al., 2006). As shown in Figure 4, *Xf* was detected in sharpshooters fed with either WT or *oxyR* mutant.

Figure 4. Using PCR to detect the presence of *Xf* in sharpshooter heads.



The PCR products were separated by electrophoresis in a 1.7% agarose gel: NEB Low Molecular Weight DNA ladder (Lane M); Positive control-WT Temecula genomic DNA (Lane 1); Negative control- water (Lane 2); Insect fed with diet solution only (Lane 3); Insects fed with WT Xf (Lanes 4 &5); Insects fed with *Xf oxyR* mutant (Lanes 6 & 7)

We are currently testing the remainder of the insect samples (10 total samples each for WT and *oxyR* fed sharphooters) and will then move on to quantify the bacterial titer in the heads using qRT-PCR. We have also begun to look at the impact of the *oxyR* mutation of *Xf* transmission by the sharpshooter. At 8 and 9 weeks, we were not able to detect WT or *oxyR* in the grapevine seedlings that the GWSS fed upon. We are currently incubating the plants longer in the growth chamber, which may allow *Xf* titer to reach a detectable level.

We are planning to repeat this pilot study this coming year and have all the necessary insect rearing equipment in place in which to do so. However, due to the inherently low rate of *Xf* transmission by the GWSS, we are opting to move to a vector with a higher *Xf* transmission rate, the Blue green sharpshooter (BGSS) (*Graphocephala atropunctata*). In cooperation with Dr. Tom Perring (UCR, Department of Entomology), we have initiated a BGSS colony here at UCR and will use these for future insect acquisition and transmission studies. This will allow us to more accurately quantify differences in acquisition and transmission rates between the WT and the *oxyR* mutant.

Objective 5: Develop a bioluminescent (Lux) reporter system for *Xf.*

Fusions to luciferase are excellent tools for tagging bacteria for *in vivo* studies and for monitoring dynamic changes in transcript or protein abundance both *in vitro* and *in vivo* (Gheysens and Mottaghy, 2009). The advantage of using the Lux system is that organisms produce light without the need of an exogenous substrate. We have constructed plasmids that carry various *Xf* promoters upstream of the Lux operon. However, when introduced into *Xf,* none of these consults result in bioluminescence. The next step will be to determine if the *lux* operon is transcribed from the selected *Xf* promoters by conducting qRT-PCR analysis.

V. Publications or reports resulting from the project.

Igo M. and Roper, C. 2010. The oxidative stress response: Identifying proteins critical for *Xylella fastidiosa* survival in grapevines. Proceedings, 2010 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA. pp. 103-106.

VI. Presentations on research.

Poster presentations at the 2010 Pierce's Disease Research Symposium (December 15-17).
Poster presentations at the 2011 Pierce's Disease Research Symposium (December 13-15).

VII. Research Relevance Statement

This project is designed to uncover the vulnerabilities of *Xylella fastidiosa* (*Xf*) during the infection process. We are focusing on the impact of reactive oxygen species (ROS) during the initial stages of infection and during the later stages when the bacteria are protected by a biofilm. Once we identify when *Xf* is most susceptible to oxidative stress, these vulnerabilities could be exploited at precise stages of infection for disease control. Another goal is to genetically engineer a bioluminescent *Xf* strain. This strain would ill allow researchers to monitor *Xf* 's response to specific prophylactic or curative measures for PD in living tissues and facilitate the tracking of *Xf* in the xylem tissue, which would be especially useful in the evaluation of control strategies targeted toward restricting the movement of *Xf* as a way of mitigating PD.

VIII. Layperson Summary

One of the immediate responses of plants to invading microorganisms is the release of reactive oxygen species (ROS), such as hydrogen peroxide. ROS are thought to serve as antimicrobial agents and as signals to activate further plant defense reactions. We have generated a strain lacking OxyR, a key regulatory protein that acts as a bacterial oxidative stress sensor. Using this mutant, we have established that OxyR is required for the production of enzymes important for ROS detoxification and for formation of a robust biofilm, two important survival strategies. Our grapevine studies suggest that OxyR may be important for the initial colonization of the grapevine xylem. This would suggest that the survival of *Xf* in grapevines is dependent on its ability to successfully mount an effective oxidative stress response. Studies are currently underway to determine whether *Xf* is also exposed to ROS in the insect vector.

IX. Status of funds

Although the performance period for this grant is for 3 years (7/1/10-6/30/13), the budget in the original application was based on the funds required to pay personnel and purchase research materials for only two years (7/1/10-6/30/12). The funds from this grant have been distributed between the PI (UC Davis) and the Co-PI (UC Riverside). There will be no funds remaining in the UC Davis account after 7/1/12. At UC Riverside, there will be $40,000 remaining 6/30/12.

Although Peng Wang has been working on the project since 7/1/10, he was awarded TAships for Fall of 2011 and Spring of 2012, which covered his salary. The remaining funds will be used to pay Mr. Wang for the summer of 2012 and for the following academic year when we will complete the key virulence and insect transmission studies.

X. Summary and Status of Intellectual Property: During the period under review, this research lead to materials of use to the PD research community and did not lead to the development of materials or procedures that were subject to intellectual property restrictions.

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