I. Project Title

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

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

Overview

The initial plant response to bacterial infection includes the rapid production and accumulation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anions. These elevated levels of ROS are highly toxic to the bacterium and can disrupt many cellular processes through their oxidation of lipids, modification of proteins and damage to DNA. Therefore, most pathogens have evolved a variety of enzymes capable of detoxifying ROS. The goal of this project is to understand how *Xylella fastidiosa* (*Xf*) responds to different types of ROS and to characterize the enzymes and regulatory proteins induced in this response.

Specific Objectives

1) Determine the key components in the response of *Xf* to ROS and the contribution of OxyR to this regulation.
2) Determine the role of the scavenging enzymes designed to cope with specific oxidative stresses in *Xf* cell physiology and virulence.
3) Determine the role of the transcription factor OxyR in oxidative stress sensing, biofilm formation, and virulence.
4) Test mutants generated in Objectives 2 and 3 for virulence in grapevines and for sharpshooter transmission.
5) Develop a bioluminescent (Lux) reporter system for *Xf*.

Reporting Period and Key Personnel

Funding for this project was received on October 21, 2010. The work at UC Davis has been conducted by Dr. Yunho Lee, a post-doctoral fellow who joined the Igo laboratory on November 24, 2010. The work at UC Riverside has been conducted by Peng Wang, a graduate student in the laboratory of Dr. Caroline Roper.

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

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

An important stress for plant pathogens are reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$). ROS are a key component of the initial plant defense response (often termed the "oxidative burst") and are produced by the plant at the point of invasion during the first 5 minutes after exposure to a potential pathogen (1, 3). Therefore, it seems likely that the immediate detoxification of ROS is critical for bacterial survival in the plant xylem. In other bacteria, this detoxification is accomplished, in part, by scavenging enzymes designed to cope with a specific oxidative stress (11). Comparative genomics suggests that many of these enzymes are present in *Xf* (Table 1).

Table 1. Predicted *Xf* enzymes

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
Thiol-dependent peroxidase	*ohr*	organic peroxides	unknown

The goal of Objective 1 is to confirm that the *Xf* genes listed in Table 1 are induced by oxidative stress and to determine which oxidative signal(s) is responsible for their induction. We are still in the early stages of this analysis and have worked out many of the technical details for the successful completion of this objective. Specifically, we are able to produce high quality RNA from *Xf* cultures grown in PD3 (6) and PW (5), two complex, rich media that are commonly used for growing *Xf*. Their inclusion will allow us to compare our results with previously published studies. We also plan to isolate RNA from XFM-pectin medium (12), and a nutrient poor, xylem chemistry-based medium developed in the Igo laboratory named PIM-6. Based on work of Killiny and Almeida (12), growth in XFM-pectin should favor conditions associated with the insect acquisition phase. In contrast, growth in PIM-6 should favor conditions associated with the plant colonization phase. Although *Xf* grown in PIM-6 have doubling times similar to those observed in PD3, the cells enter stationary phase at a much lower density and when grown with aeration do not aggregate or produce much biofilm (unpublished result).

The next step will be to assess how exposure to the different ROS signals influences the RNA levels of the genes listed in Table 1. We have already designed a series of primers for the analysis of our RNA samples using qRT-PCR. We are still in the process of determining the appropriate ROS concentrations for this analysis. (The ROS under consideration are H_2O_2, the superoxide generator menadione, and organic hydroperoxides such as cumene hydroperoxide (CuOOH), tert-butyl hydroperoxide (t-BOOH) and linoleic acid hydroperoxide (LOOH) (4, 15, 17)). Once the inducing conditions are identified for a specific scavenging enzyme, we will use the condition and the appropriate primers to determine the contribution of OxyR to its regulation using the *oxyR* null mutant and the *oxyR* complementation strain described under Objective 3. We will also use the conditions and primers to characterize the mutants generated in Objective 2.

Finally, in addition to assessing the impact of ROS on RNA levels, it is also possible to examine their impact on the activity of the individual enzymes. Therefore, we have been adapting various enzyme protocols for use in *Xf*. Figure 1 shows a superoxide dismutase activity gel for *Xf* grown in PD3 or PW media in the absence of ROS induction. The next step will be to repeat this analysis after exposure of the cultures to different ROS.

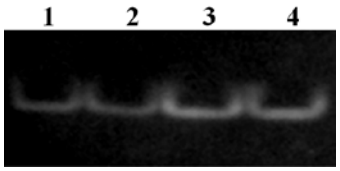


Figure 1. Detection of superoxide dismutase activity in wild-type *Xf*. Duplicate cultures of *Xf* were grown in the indicated medium for 8 days. The proteins were harvested and analyzed on a 12% nondenaturing polyacrylamide gel. SOD activity was detected using the Nitro blue tetrazolium (NBT)-gel assay (2). Lanes 1 & 2: *Xf* in PD3; Lanes 3 & 4: *Xf* in PW.

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

We are taking a genetic approach to determine the role of the individual scavenging enzymes in the response of *Xf* to oxidative stress. The specific scavenging enzymes under investigation are listed in Table 1. Our strategy will be to generate strains carrying a null mutation in the individual gene using site-directed gene disruption (8) and the corresponding complementation strains using our chromosome-based complementation system (14). We have successfully used both methods to study the *cpeB* gene (14) and the *oxyR* gene (see Objective 3). Some of the plasmids required to generate the null mutants have been constructed and introduced into *Xf* by electroporation. We are currently in the process of analyzing some of the resulting *Xf* transformants by PCR to confirm the presence of the desired mutation. Finally, we have also constructed some of the plasmids necessary to generate the complementation strains.

The next step will be to use the mutants and complementation strains to examine how the absence of a particular scavenging enzyme influences the response of *Xf* to oxidative stress. The precise tests will depend on the specific mutation and the results of our analysis in Objective 1. For example, our characterization of *sodA* mutants will include a NBT-gel assay (Figure 1) and exposure to the specific ROS identified in Objective 1 that influences *sodA* gene expression. We will also determine if the absence of SodA influences *Xf* survival in either grapevines or insects.

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

In most bacteria, the response to hydrogen peroxide stress is regulated by the transcription factor OxyR. The resulting regulatory network allows differential expression of H_2O_2-induced genes in terms of growth phase, cell density, and other environmental signals. Moreover, there is emerging evidence that OxyR is involved in biofilm formation. Therefore, the OxyR-mediated oxidative stress response pathway helps bacteria survive the initial exposure to ROS through induction of scavenging enzymes and later exposure through its involvement in biofilm formation. Analysis of the *Xf* genome revealed the presence of an OxyR ortholog encoded by PD0747. In order to answer the questions in this objective, we first constructed an *oxyR* null mutant and the corresponding complementation strain.

Construction and complementation of the Xf oxyR null mutant:
The *Xf oxyR* null mutant was generated by site-directed mutagenesis. In brief, the *oxyR* gene was cloned into pUC19 and then disrupted with the Tn5 <kan> cassette using the Epicentre *in vitro* Transposome kit. The *oxyR*::*kan* construct was electroporated into wild-type *Xf* (Temecula1). Kanamycin-resistant colonies were selected and then screened to identify transformants in which the *oxyR* gene had been disrupted and replaced by the kanamycin cassette.

To generate the *Xf oxyR*::*kan* complementation strain, the *oxyR* open reading frame with its native promoter region was cloned into vector pAX1Cm (14). As a result of this construction, the wild-type *oxyR* gene becomes linked to a chloramphenicol cassette and flanked by sequences homologous to a neutral site (NS1) in the *Xf* chromosome. The pAX1Cm vector carrying the wild-type *oxyR* gene was introduced into *oxyR::kan* mutant electrocompetent cells. Chloramphenicol-resistant transformants were selected and PCR was used to confirm the presence of wild-type *oxyR* at NS1 and the *oxyR*::*kan* construct at the normal *oxyR* locus.

Growth curve analysis:
To determine the role of OxyR on *Xf* cell physiology, we examined the impact of the *oxyR::kan* mutation on growth. For this experiment, the growth of *oxyR::kan* mutant in PD3 medium supplemented with kanamycin (5 µg/ml) was compared to the growth of *Xf* wild type in PD3 medium. The cells were first grown on agar plates at 28°C for seven days. The resulting colonies were resuspended in 1 ml of 1X PBS and the concentration of the cells was adjusted to OD_{600}=0.0125 in PD3 liquid. Aliquots of the wild type and *oxyR::kan* mutant cell suspensions were then incubated at 28°C with constant 100 rpm shaking and the absorbance was measured at OD_{600} every 24 hours. As shown in Figure 2, although it had a longer lag phase, the *oxyR::kan* mutant exhibits a faster growth rate and achieves a higher final OD_{600} as compared to wild type. To confirm that these results are due to the absence of the OxyR protein, we are currently repeating this experiment with the *oxyR* complementation strain.

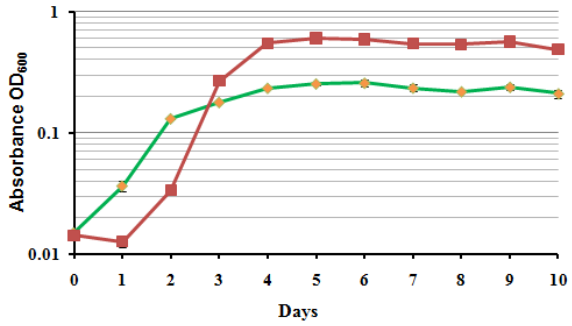
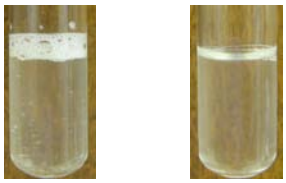


Figure 2. Graph comparing the growth of wild type and the *oxyR* mutant in PD3 liquid. The absorbance of the cultures was measured at OD_{600} every 24 hours. The log of the absorbance was then plotted as a function of time.

Catalase activity test:
Catalase is an enzyme that can convert hydrogen peroxide into water and gaseous oxygen. When hydrogen peroxide is added to a catalase-positive culture, oxygen gas bubbles form immediately, which can be easily detected *in vitro*. If no bubbles appear, the organism is considered to be catalase-negative. OxyR is known to regulate ROS detoxifying genes such as those encoding catalase in other bacterial systems. Therefore, we hypothesized that the *oxyR::kan* mutant would produce less catalase. As shown in Figure 3, wild-type *Xf* produced more bubbles than the *oxyR::kan* mutant supporting our hypothesis that the induction of catalase is regulated by OxyR.



WT *oxyR::kan*

Figure 3. Hydrogen peroxide degradation assay. A drop of 3% H_2O_2 solution was put into liquid cultures of wild-type *Xf* and the *oxyR::kan* mutant. Bubbles indicate the presence of gaseous oxygen resulting from the breakdown of H_2O_2.

H_2O_2 sensitivity test (Disk Diffusion Method):

Once we had established that the *oxyR::kan* mutant was deficient in the ability to breakdown H_2O_2, we wanted to investigate if it also had an increased sensitivity to H_2O_2. For this experiment, we measured the ability of the *oxyR::kan* mutant to grow in the presence of H_2O_2 using a disk diffusion assay. As shown in Figure 4, the diameter of the zone of inhibition for the *oxyR::kan* mutant was higher than *Xf* wild type, which correlates to an increase in sensitivity to H_2O_2. This would suggest the *oxyR::kan* mutant is compromised in its ability to mount an effective oxidative stress response.

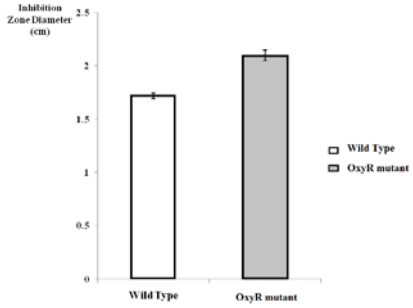


Figure 4. Graph showing the difference in the diameter of the zone of inhibition between wild type and *oxyR::kan* mutant. Bacteria embedded in PD3 top agar were exposed to a Whatman disk paper treated with 10 µl of 100 mM H_2O_2. Zones of growth inhibition were measured after 7 days of incubation. Three replications were performed. The error bars show one standard deviation.

Cell attachment assays:

OxyR may also play an important role in regulating gene products involved in the attachment of *Xf* to surfaces and biofilm formation. Because *Xf* readily adheres to surfaces, this phenotype is relatively easy to measure *in vitro*. For this analysis, a cell suspension of wild-type *Xf* or the *oxyR::kan* mutant was added to PD3 liquid in polystyrene or glass tubes, and grown for 7 days at 28°C, without agitation. Surface adherence was assayed by adding 100 µl 0.1% crystal violet to the tubes and then rinsing them with distilled water. The presence of cell attachment was visualized as a purple ring on the tube side wall, usually at the air-medium interface. For *Xf* wild type, purple rings are present on polystyrene tubes and glass tubes. In contrast, no purple rings are observed for the *oxyR::kan* mutant, indicating the absence of cell attachment (Figure 5A). We also eluted the stained cells with 95% ethanol and measured the OD_{600} (7). The absorbance of the eluted crystal violet solution showed that *oxyR::kan* mutant was significantly reduced in its ability to attach to both polystyrene tubes and glass tubes (Figure 5A & 5B).

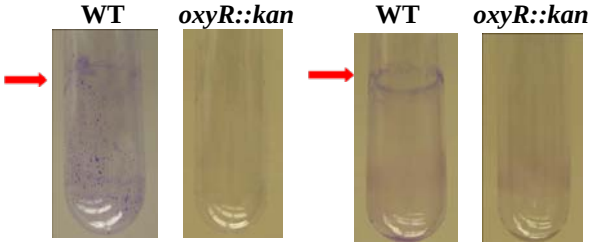


Figure 5A. Surface attachment of the *oxyR::kan* mutant and wild-type *Xf* on polystyrene tubes and glass tubes surfaces. Cells were grown for 7 days (stationary phase culture) and surface adherent cells were stained with crystal violet. The arrows point to the purple ring of attached cells.

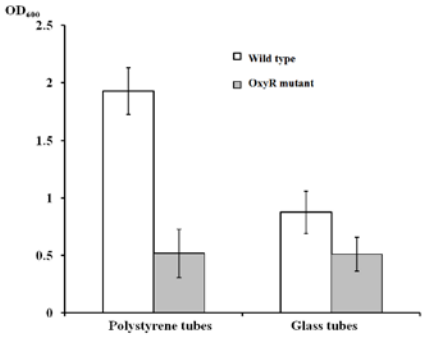


Figure 5B. Cell attachment analysis of *Xf* wild type and *oxyR::kan* mutant on polystyrene tubes and glass tubes surfaces by the crystal violet method. Attached cells were stained with crystal violet, and then dissolved with ethanol; the OD_{600} of the eluted solution was measured. The error bars represent the standard deviation.

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

We hypothesized that OxyR plays a role in the immediate detoxification of ROS when the bacteria are first introduced into the xylem and, therefore, the *oxyR::kan* mutant would be compromised in its ability to do so. To test this premise, we have initiated studies to assess the ability of the *oxyR::kan* mutant to colonize and cause disease in grapevines. Thus far, we have gathered *in planta* colonization data, which are reported below.

Colonization assay for Xf oxyR mutant in grapevines:

Vitis vinifera cv. Thompson seedless grapevines were pin-prick inoculated using a 20-gauge syringe needle. Grapevines (15 per treatment) were inoculated with 20 µl drops of a 10^8 colony forming unit (CFU)/ml solution of wild-type *Xf*, the *oxyR::kan* mutant, or 1x Phosphate Buffered Saline (PBS). Each of the plants was inoculated twice on the stem (10). The populations of *Xf* wild type and the *oxyR::kan* mutant were quantified from petioles sampled at the point of the inoculation (POI). Petioles were weighed and surface-sterilized and homogenized in mesh sample bags (Agdia, Inc., Elkhart, IN, U.S.A) containing 2 ml of 1xPBS. Serial dilutions of the crushed petiole slurry were spread onto PD3 medium or PD3 medium with 5 µg/ml of kanamycin. The colonies were counted after 10 days incubation at 28°C (16). The CFU/g petiole tissue was calculated and analyzed using a Wilcoxon rank-sum test. The results indicate that at 14 weeks post-inoculation, the *oxyR::kan* mutant does not colonize the POI as efficiently as the wild type. However, when measured at a later time point (17 weeks post-inoculation), the *oxyR::kan* mutant and *Xf* wild type have colonized the plant tissue to similar levels. Based on these results, it seems likely that OxyR plays a role in detoxifying peroxides during the initial phase of infection when *Xf* is establishing itself in the xylem.

Table 2. The populations of *Xf* wild type and the *oxyR::kan* mutant in the leaf petioles at the point of inoculation (POI) and 37 cm above POI in grapevines (Average CFU/g ±SE).

Distance	Time post-inoculation	*Xf* wild type (CFU)	*Xf oxyR* (CFU)
POI	14 weeks	$(1.20\pm0.19)\times10^7$	$(6.10\pm3.60)\times10^{6}$ *
37 cm	17 weeks	$(1.96\pm0.45)\times10^7$	$(1.07\pm0.28)\times10^7$

*The Cfu/g tissue data were analyzed using a Wilcoxon rank-sum test. Note the lower number of CFU/g obtained at the POI for grapevines infected with the *oxyR::kan* mutant.

The above experiments were done using the mechanical needle inoculation technique, which introduces an artificially large number of bacteria into the plant at the POI. These high numbers could mask the important contribution of OxyR during the early stages of infection. To test this

hypothesis, we will be conducting sharpshooter transmission assays in Spring 2011, which will introduce a more biologically relevant number of bacteria into the plant. These assays will be performed in collaboration with Dr. Matt Daugherty (UCR). We predict that there will be much less colonization of the grapevine xylem by the *oxyR::kan* mutant using this assay.

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* (9). The advantage of using the Lux system is that organisms produce light without the need of an exogenous substrate. We are currently constructing derivatives of pBBR1MCS-5 (13) that carry various *Xf* promoters upstream of the Lux operon. Once the construction of these plasmids is complete, they will be introduced into *Xf* by electroporation in order to determine if any of the constructs result in bioluminescence. The results of these initial experiments will determine the direction of subsequent experiments.

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 presentation at the 2010 Pierce's Disease Research Symposium (December 15-17).

VII. Research Relevance Statement

This project is designed to uncover the vulnerabilities of *Xylella fastidiosa* (*Xf*) to reactive oxygen species (ROS) during the initial stages of infection and during the later stages when the bacteria are protected by a biofilm. These vulnerabilities could be exploited for disease control. Another goal is to genetically engineer a bioluminescent *Xf* strain that will allow researchers to monitor *Xf* 's response to specific prophylactic or curative measures for PD in living tissues. This strain would facilitate many different types of research, thereby expediting the development of treatments for 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. During the past 4.5 months, we have adapted a number of different protocols for studying the ROS response to *X. fastidiosa*. We have also generated a strain lacking OxyR, the key regulatory protein in this response and have preliminary evidence indicating that OxyR is important for the initial colonization of the grapevine xylem. This would suggest that the survival of *X. fastidiosa* in grapevines is dependent on its ability to successfully mount an effective oxidative stress response.

IX. Status of funds

The funding for this project was obtained on 10/21/2010. As a result, we have only used a portion of the funds allocated for 2010-2011. However, we anticipate that the remaining funds will be used by the end of the 2010-2011 budget year.

X. Summary and Status of Intellectual Property: Not applicable. The goal of this project is to produce materials and procedures that will help uncover the underlying mechanisms of *Xf* virulence. This information will be made available to other researchers interested in finding a solution to PD. As anticipated, during the period under review, this research did not lead to the development of materials or procedures that were subject to intellectual property restrictions.

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