THE OXIDATIVE STRESS RESPONSE: IDENTIFYING PROTEINS CRITICAL FOR *XYLELLA FASTIDIOSA* SURVIVAL IN GRAPEVINES

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ABSTRACT
A key component of the initial plant response to bacterial infection is the rapid production and accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions. These elevated levels ROS are highly toxic to the bacteria 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. Our initial studies have focused on OxyR, a regulatory protein involved in the response to hydrogen peroxide. Strains carrying a null mutation in *oxyR* are more sensitive to killing by hydrogen peroxide and are impaired in their ability to attach to solid surfaces. Experiments are underway to determine the impact of the *oxyR* null mutation on survival and symptom development in grapevines.

LAYPERSON SUMMARY
One of the immediate responses of plants to invading microorganisms is the release 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. 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 Pierce's disease (PD) in living tissues. This strain would facilitate many different types of research, thereby expediting the development of treatments for mitigating PD.

INTRODUCTION
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 five minutes after exposure to a potential pathogen (Apel and Hirt, 2004; Bolwell and Daudi, 2009). This initial response is often followed by a second response, which involves more prolonged production of ROS (1.5-3 hours after invasion) (Apel and Hirt, 2004). The plant defense response typically requires contact between the pathogen and metabolically active cells. Since xylem tissue, for the most part, is not living, the introduction of a pathogen into the xylem might not result in significant ROS production by the xylem tissue itself. However, pathogens in the xylem make contact with the adjacent living parenchyma cells via the pit membranes, which could result in higher ROS levels in the xylem. Another source of ROS comes from differentiating thin-walled xylem cells and particular non-lignifying xylem parenchyma cells, which are capable of sustained H_2O_2 production (Barcelo, 2005). This H_2O_2 is important for the cross-linking that occurs during the lignification process of developing xylem elements. Since the H_2O_2 produced by the xylem parenchyma can diffuse widely between neighboring xylem cells, bacteria present in the xylem are likely exposed to H_2O_2, especially when introduced into the tips of growing shoots where the majority of xylem lignification is occurring. Therefore, it seems likely that the immediate detoxification of ROS is critical for bacterial survival in the plant xylem.

The immediate detoxification of ROS is accomplished, in part, by scavenging enzymes designed to cope with specific oxidative stresses. Comparative genomics suggests that many of these enzymes are present in *Xylella fastidiosa (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

One predicted *Xf* scavenger enzyme is the alkyl hydroperoxide reductase AhpCF, a two-component NADH peroxidase. This enzyme, which is required for optimal resistance to both hydrogen and organic peroxides, is the predominant scavenger at low concentrations of H_2O_2 (Imlay, 2008). In contrast, at high H_2O_2 concentrations, catalases are induced and become the primary scavenging enzymes. We have already established that *Xf* catalase is encoded by the *cpeB* gene (Matsumoto et al., 2009). The primary scavenger enzymes of superoxides are the superoxide dismutases (SODs), which convert $O_2^{\bullet-}$ to H_2O_2. *Xf* is predicted to encode two members of the iron/manganese superoxide dismutase family (SodA and SodM). Finally, studies of *Xf*-CVC identified a thiol-dependent peroxidase, encoded by the *ohr* gene (Cussiol et al., 2003). Unlike the other scavenging enzymes, Ohr belongs to a family of proteins that are only present in bacteria. This property led Cussiol et al. (Cussiol et al., 2003) to suggest that Ohr might be a promising target for drug development in medicine and agriculture. The goal of Objective 2 is to characterize these key scavenger enzymes.

Not surprisingly, since peroxide and superoxide stresses do not always occur simultaneously, 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 most bacteria, the response to hydrogen peroxide stress is regulated by the transcription factor OxyR. The *Xf* ortholog of this protein is encoded by PD0747. Our screening of the *Xf* genome for potential OxyR binding sites suggests that both catalase (CpeB) and alkyl hydroperoxide reductase (AhpCF) are controlled at the transcriptional level by OxyR. OxyR-like systems are widespread among bacteria (Imlay, 2008), including many plant pathogens. The resulting transcription regulatory network allows differential expression of H_2O_2-induced genes in terms of growth phase, cell density, and biofilm formation (Imlay, 2008; Shanks et al., 2007). Therefore, the OxyR-mediated oxidative stress response pathway helps bacteria survive the initial exposure to ROS through induction of scavenger enzymes and later exposure from ROS that may be present during biofilm formation. Based on this link between oxidative stress and biofilm formation, we hypothesize that OxyR participates in the signaling that triggers *Xf* to enter and maintain the biofilm state within the plant. The resulting biofilm, in turn, affects plant colonization, virulence and acquisition by the insect. We will test this hypothesis in the series of experiments in Objective 1, 3, and 4.

The goal of objective 5 is to develop a sensitive reporter system for detecting the response of *Xf* to ROS with the plant xylem. Our strategy will be to generate luciferase fusions using the Lux reporter system from *Photorhabdus luminescens* (Meighen, 1993). The bioluminescent reaction is catalyzed by bacterial luciferase (LuxAB) that oxidizes $FMNH_2$ and a long-chain fatty-acid aldehyde the presence of molecular oxygen. The fatty-acid aldehyde is synthesized by LuxC, LuxD, and LuxE. The advantage of this system over other luciferase reporter systems is that it does not require an exogenous substrate. The development of a LUX reporter system will allow Pierce's disease (PD) researchers to monitor the response of their gene of interest in a noninvasive manner in the host insect or plant. It might also be used to monitor the early responses of *Xf* to various treatments, including treatments involving transgenic plants and for some high throughput screening applications.

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*.

PRELIMINARY RESULTS

The Igo laboratory has already described their characterization of a *Xf* strain containing a null mutation *cpeB*, the gene encoding catalase (Matsumoto et al., 2009). This mutant is more sensitive to hydrogen peroxide than a wild-type strain. Moreover, our preliminary experiments suggest that a functional catalase is required for virulence in grapevines.

The Roper laboratory has generated a null mutation in the *oxyR* gene by site-directed mutagenesis and has begun to examine how this mutant responds to oxidative stress. The results from the hydrogen peroxide degradation assay are shown in **Figure 1**.

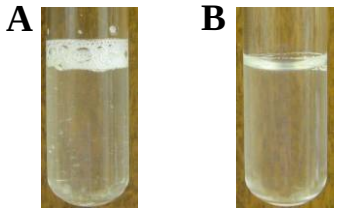

Figure 1. Hydrogen peroxide degradation assay.
Bubbles indicate the presence of gaseous oxygen resulting from the breakdown of H_2O_2.
A *Xf* wild type. (**B**) *Xf oxyR* null mutant.

In this assay, a 3% H_2O_2 solution was added to *Xf* wild type and *oxyR* null mutant liquid cultures. Within 30 seconds, bubbles formed in the tube containing the wild-type culture. In contrast, few bubbles were present in the *oxyR* null mutant culture, indicating that this mutant is significantly impaired in its ability to degrade H_2O_2 (**Figure 1**). Furthermore, the *oxyR* null mutant exhibited greater sensitivity to H_2O_2 than wild type in a disk diffusion assay. For this analysis, *Xf* wild type and the *oxyR* null mutant was grown in liquid medium to an OD_{600}=0.1. Inocula from these cultures (300 µL) were added to 3 mls of PD3 top agar, which was poured onto PD3 plates. A paper disk treated with 10 µL of 100 mM H_2O_2 was then placed on the center of the top agar and zones of growth inhibition were measured after seven days of incubation. Three replications were performed. The inhibition zone diameter of the *oxyR* null mutant was higher than wild type indicating that the *oxyR* null mutant was significantly more sensitive to H_2O_2 than wild type (**Figure 2**). Taken together, these results suggest that the *oxyR* null mutant is likely compromised in its ability to mount an effective oxidative stress response.

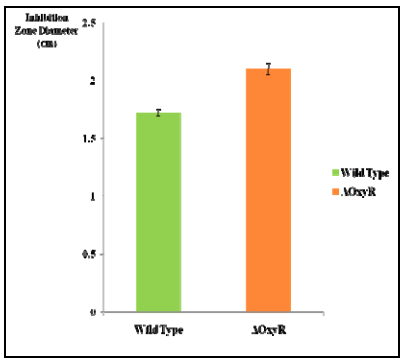

Figure 2. Measurements of the diameter of the zone of inhibition surrounding a paper disc containing 10 µL of 100 mM H_2O_2. Results are shown for both *Xf* wild type and the *oxyR* null mutant. The larger zone of inhibition correlates to an increase in sensitivity to H_2O_2. The error bars indicate the standard deviation.

The OxyR protein may also play an important role in regulating gene products (at the transcriptional level) involved in the attachment of *Xf* to surfaces and biofilm formation. **Figure 3** shows the results from experiments examining the impact of the *oxyR* null mutation on surface attachment to both polystyrene and glass tubes, which was assessed based on a protocol described in (Espinosa-Urgel et al., 2000). Cultures of wild type *Xf* or the *oxyR* null mutant were grown in polystyrene tubes and glass tubes at 28°C, upright without agitation. After seven days, 100 µL of 0.1% crystal violet was added to the culture medium and incubated for 20 min. The presence of attached cells was visualized as a purple ring on the tube side wall. Cultures containing *Xf* wild type had clear purple rings at the air-medium interface in both polystyrene and glass tubes. Notably, there was no purple ring observed for the *oxyR* null mutant, indicating the absence of attachment by the Δ*oxyR* mutant. Cell attachment was then quantified by measuring the absorbance of the eluted crystal violet solution. These data support the conclusion that the *Xf oxyR* null mutant is significantly reduced in its ability to attach to the solid surfaces (**Figure 3**).

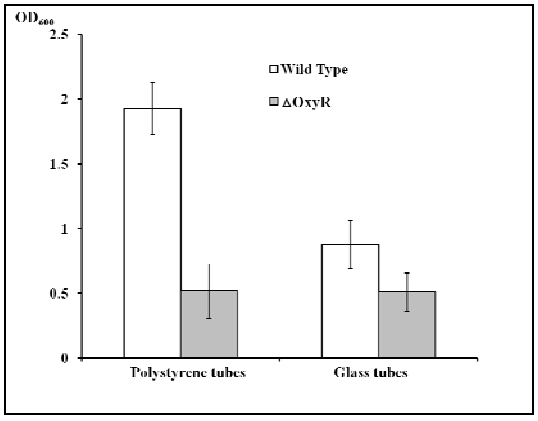

Figure 3. Quantification of surface attached cells for *Xf* wild type and *oxyR* null mutant for polystyrene and glass tubes surfaces. Attached cells were stained with crystal violet, and then eluted with ethanol; the OD_{600} of the eluted solution was measured. The error bars indicate the standard deviation.

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