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Characterization of the Oxidative Stress Response and the Type II Secretion System for the Phytopathogen, *Xylella fastidiosa*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Pathology

by

Peng Wang

March 2014

Dissertation Committee:
Dr. M. Caroline Roper, Chairperson
Dr. Wenbo Ma
Dr. Katherine Borkovich

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The Dissertation of Peng Wang is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Characterization of the Oxidative Stress Response and the Type II Secretion System for the Phytopathogen, *Xylella fastidiosa*

by

Peng Wang

Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, March 2014
Dr. M. Caroline Roper, Chairperson

Xylella fastidiosa (*Xf*), a xylem-limited fastidious bacterium, is the causal agent of Pierce's Disease (PD) of grapevine. *Xf* has a very broad host range, including grapevine, citrus, almond, oleander, peach and maple. PD is a lethal disease of grapevine and understanding the disease from the perspective of molecular interactions between *Xf* and grapevine is very important. Pathogens often encounter reactive oxygen species (ROS) from a variety of sources during the infection process. These ROS can be toxic to the pathogen and correspondingly, the pathogen has evolved several tightly regulated mechanisms to cope with this stress. OxyR is a redox-responsive transcription factor that regulates expression of antioxidant enzymes. Interestingly. OxyR is the only known oxidative stress regulator in the *Xf* genome leading us to speculate that it plays a vital role in adaptation to the host environment. We constructed an *oxyR* mutant and found it was significantly more sensitive to H_2O_2 than wild type *Xf*. In addition, we found that the *Xf* *oxyR* mutant was reduced in surface attachment, cell-cell aggregation and mature biofilm

formation. *In planta* tests indicated that the *oxyR* mutant was significantly compromised in the ability to colonize the host xylem, but, interestingly, no difference in virulence was observed when compared with wild type *Xf*.

The Type II secretion system (T2SS) is an important protein secretion system in plant pathogenic bacteria. The repertoire of proteins secreted by this system are largely involved in nutrition and include plant cell wall degrading enzymes (CWDEs). *Xf* employs CWDEs to degrade xylem pit membranes to facilitate systematic movement within the xylem. The majority of the demonstrated and putative CWDEs are predicted to be secreted by the T2SS. By knocking out a T2SS structure gene *xpsE*, which encodes a putative ATPase that provides the energy that drives the T2SS, I demonstrated that *Xf* requires XpsE for full virulence and establishment in the xylem vessels indicating that the T2SS is an important factor employed during the infection process.

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1. Chapter 1 Introduction

Pierce's disease (PD) of grapevine is a lethal disease of grapevines. PD was originally discovered and described by a pioneer plant pathologist Newton Pierce (Pierce, 1892) for whom the disease was named. Typical PD symptoms include leaf chlorosis, leaf scorch, leaf marginal necrosis, leaf blade drop, irregular vine lignification, cordon dieback and vine death (Varela, 2001). Due to the inability to culture the causal agent and its similarities to viral diseases (systemic infection, inability to be observed under a light microscope), PD was originally considered to be caused by a virus (Purcell, 2013). However, it was later discovered that PD was caused by a bacterium when the organism was successfully cultured in 1978 (Davis et al., 1978). This bacterium was classified as belonging to the Xanthomonadaceae and was named *Xylella fastidiosa* (*Xf*) (Wells et al., 1987; Brenner D.J., 2005). *Xf* has a broad host range. In addition to grapevine, *Xf* can also colonize many other plants, such as citrus, almond, oleander, peach and maple (Hopkins, 1977; Hopkins, 1989; Chatterjee et al., 2008a). *Xf* is transmitted from one plant to another by xylem-feeding insect vectors (Hopkins, 1989). *Xf* is a Gram-negative, rod-shaped, non-flagellated bacterium. Its diameter is from 0.25 to 0.50 μm, and its length ranges from 1.1 to 2.3 μm (Davis et al., 1978). It is catalase positive, oxidase negative with an optimal growth temperature ranging from 26 ℃ to 28 ℃ (Wells et al., 1987). Based on phylogenetic analysis, four subspecies have been identified within the *Xf* species. These are *X. fastidiosa* subspecies *fastidiosa*, subsp. *sandyi*, subsp. *multiplex* and subsp. *pauca* (Nunney et al., 2012).

In 2000, the CVC (Citrus Variegated Chlorosis) strain of *Xf* (*X. fastidiosa* subsp. *pauca*) was sequenced (Simpson et al., 2000), and was notably the first plant pathogenic

bacterium to be fully sequenced. The complete genome sequence of the PD strain (*X. fastidiosa* subsp. *fastidiosa*) followed (Van Sluys et al., 2003). Two strains (M12 and M23) of *Xf*, which cause Almond Leaf Scorch (ALS) in California, were also completely sequenced (Chen et al., 2010). M12 strain can only cause ALS, it belongs to *X. fastidiosa* subsp. *multiplex*. M23 strain can cause both ALS and PD, it belongs to *X. fastidiosa* subsp. *fastidiosa* (Chen et al., 2010). The whole genome of *X. fastidiosa* subsp. *fastidiosa* strain GB514 (isolated from a grapevine in Texas) was also completely sequenced (Schreiber et al., 2010). The draft genome sequences of *X. fastidiosa* subsp. *multiplex* strain Dixon (isolated from almond) (Bhattacharyya et al., 2002), *X. fastidiosa* subsp. *sandyi* strain Ann-1 (isolated from oleander) (Bhattacharyya et al., 2002), *X. fastidiosa* subsp. *multiplex* strain Griffin-1 (isolated from a red oak tree in Georgia) (Chen et al., 2013), biocontrol strain EB92-1(isolated from elderberry) (Zhang et al., 2011), a mulberry strain Mul-MD (isolated from a mulberry tree in Beltsville, MD) (Guan et al., 2014) and two coffee strains *Xf*6c and *Xf*32 (isolated from coffee plants in Brazil) (Alencar et al., 2014) also became available. The availability of these genome sequences has greatly accelerated research in elucidating the basic biology of *Xf*.

Using genome sequences, several critical virulence factors of *Xf* have been identified (Table 1.1). Polygalacturonase (PG), a pectin degrading enzyme, was the first pathogenicity factor identified in *Xf* (Roper et al., 2007b). Mutants in PG were unable to achieve systemic colonization and were non-pathogenic in grapevines (Roper et al, 2007). In addition, TolC, which is a component of the multidrug resistance (MDR) efflux pumps and involved in Type I secretion system, is required for *Xf* resistance to

phytochemicals, and, hence, colonization and pathogenicity in grapevines (Reddy et al., 2007). Another defining feature of the infection process for *Xf* is the formation of biofilms within the xylem tissue and several factors have been implicated in different developmental phases of this process including surface attachment, cell-cell aggregation, exopolysaccharide (EPS) production and mature biofilm formation. Specifically, afimbrial adhesins XadA and HxfB contribute to initial cell attachment to a surface (Feil et al., 2007), while fimbrial adhesins FimA and FimF were found to contribute to cell-cell aggregation and biofilm formation (Feil et al., 2007). All of these adhesin mutants (*xadA*, *hxfB*, *fimA* and *fimF*) showed significantly reduced virulence in grapevines compared with the wild type strain (Feil et al., 2007). In the *Xf* subsp. *pauca* CVC strain, western blot quantification and immunofluorescence study of the afimbrial adhesins Xad1 and Xad2 shows that their abundance and spatial pattern are different during the various phases of *in vitro* biofilm formation (Caserta et al., 2010). Particularly, immunofluorescence analysis indicates that the presence of XadA2 in the biofilm becomes very obvious in the microcolony formation stage, which implies this afimbrial protein is involved in forming the biofilm architecture of *Xf* (Caserta et al., 2010). *In vivo* assays indicated that they are present in the tested host plant (citrus, periwinkle, and hibiscus), and most of Xad1 and Xad2 are localized in the extracellular space but in the proximity to the outer membrane (Caserta et al., 2010). In another study, mutations in hemagglutinin adhesins genes *hxfA* (PD2118) and *hxfB* (PD1792) result in a significantly reduced cell-cell aggregation and biofilm maturation. Mutants in *hxfA* and *hxfB* achieved higher populations and were hypervirulent in grapevines. In addition, they systemically

colonized plants more rapidly than wild type strain, which suggests that *Xf* movement ability is positively correlated with its virulence (Guilhabert and Kirkpatrick, 2005). HxfA and HxfB act as attenuators of cell-cell aggregation and biofilm formation, thus contribute to *Xf* movement and virulence *in planta* (Guilhabert and Kirkpatrick, 2005). *Xf* O-antigen, a component of lipopolysaccharide, acts as an attenuator of surface adhesion and as an enhancer of cell-cell adhesion (Clifford et al., 2013). A mutation in the O-antigen biosynthetic pathway that leads to truncation of the O-antigen results in cells that are hyper-adherent to a surface but do not adhere to each another. As a result, the course of biofilm formation is disrupted and the mutant is significantly impaired in colonization of the xylem and in virulence in grapevine (Clifford et al., 2013). XatA, an autotransporter, was determined to contribute to *Xf* cell-cell aggregation, biofilm formation and virulence in grapevines (Matsumoto et al., 2012). TonB, a proton motive force in a TonB complex for cellular processing and transportation, contributes to *Xf* twitching motility, biofilm formation and virulence in grapevines (Cursino et al., 2009). The *Xf gumD* and *gumH* mutants, unable to produce two important enzymes in the EPS synthesis pathway, were deficient in biofilm formation, vector transmission and virulence in grapevines (Killiny et al., 2013).

From a regulatory perspective, an alternate sigma factor AlgU, and a transcription regulator GacA that is putatively a member of a two component regulatory system in *Xf*, both play a role in regulating cell attachment, cell-cell aggregation, biofilm formation *in vitro* and virulence in grapevine (Shi et al., 2007, 2009). XhpT, a response regulator in another predicted two component regulatory system, contributes to *Xf* surface attachment,

cell-cell aggregation *in vitro* and virulence in grapevines, but negatively regulates EPS production (Voegel et al., 2013). However, where these regulators fit into the hierarchy of overall gene regulation in *Xf* have not been deciphered. In addition, a chemosensory operon Pil-Chp was identified as a regulator of *Xf* twitching motility, biofilm formation and virulence in grapevines (Cursino et al., 2011).

Xf possesses an interesting cell-cell communication system termed the *rpf* (regulation of pathogenicity factors) signaling system (Dow and Daniels, 2000; da Silva et al., 2001; Chatterjee et al., 2008a). Comparative genome analysis shows that the *Xf rpf* gene cluster organization is highly similar to that in *Xanthomonas* species, except the that *Xf* is missing *rpfH*, *rpfD* and *rpfI* (Simpson et al., 2000; da Silva et al., 2002; Van Sluys et al., 2003; Lee et al., 2005; Chatterjee et al., 2008a). In addition, *rpfA* and *rpfB* are separate from the main *rpf* cluster. Through the production and perception of a diffusible signaling factor (DSF), *Xf* controls the expression of genes belonging to the Rpf regulon in a cell density-dependent manner (Newman et al., 2004). A mutation in the *Xf rpfF* gene, which encodes a putative enoyl-CoA hydratase (a DSF synthase), results in a deficiency of DSF production, insect transmissibility, colonization and biofilm formation in insects. However, the *rpfF* mutant was hypervirulent in grapevines (Newman et al., 2004). This study indicates RpfF is required for DSF production and DSF is involved in regulation of *Xf* virulence and vector transmission (Newman et al., 2004). Further DNA microarray analysis revealed that RpfF is involved in regulating genes related to cell attachment and biofilm formation, such as hemagglutinin, Type IV pili and *gum* genes (Wang et al., 2012). Furthermore, RpfF is not only required for DSF synthesis (Newman

et al., 2004), but also for the response to DSF, such as DSF induced gene expression (Ionescu et al., 2013). RpfC is a putative *Xf* cell-cell signaling sensor. *Xf rpfC* mutant exhibits almost contrary phenotypes of *rpfF* mutant. *Xf rpfC* mutant displays overexpression of *rpfF* and adhesin genes (*fimA*, *hxfA* and *hxfB*), *in vitro* hyperattachment ability, overproduction of DSF, deficiency of migration in the xylem, significantly reduced virulence in grapevines, proficiency in insect colonization but deficiency in the insect transmission (Chatterjee et al., 2008c). In *rpfC* mutant, it is proposed that overproduced DSF could bind to another putative intracellular sensor and induce autophosphorylation and relay the phosphate to a response regulator, such as RpfG. The activated regulator can promote the expression of cell attachment and biofilm formation related genes (*gumJ*, *fimA*, *hxfA* and *hxfB*), leading to the hyperattachment phenotype. This increased attachment ability can result in the deficiency in migration, reduced virulence *in planta* and proficient colonization in insect vector, but deficient insect transmission (Chatterjee et al., 2008c). The *Xf rpfB* mutant exhibits a different profile of fatty acid, reduced colonization and transmission in vectors and straggled PD development, which indicates that RpfB contributes to *Xf* DSF processing, insect transmission and virulence in grapevine (Almeida et al., 2012). *Xf* DSF has been isolated and characterized as a 2(Z)-tetradecenoic acid (Beaulieu et al., 2013). It can attenuate *Xf* movement and colonization in grapevines (Chatterjee et al., 2008c) and suppress the factors contributing to *Xf* attachment to insect vectors and transmission (Baccari et al., 2014). Surprisingly, degradation of DSF results in reduced *Xf* virulence in grapevines

(Newman et al., 2008), not an expected hypervirulent phenotype, which was observed from a DSF deficient strain-*rpfF* mutant (Newman et al., 2004).

Xf has two lifestyles, living in the host plant and residence in the insect vector (Chatterjee et al., 2008a). *Xf* is vectored by xylem feeding insect primarily, sharpshooter leafhoppers and spittlebugs (Houston et al., 1947; Severin, 1950). *Xf* transmission by sharpshooters has several interesting features. The bacterium is persistent but noncirculative and it lacks a latent period (Almeida et al., 2005). *Xf* is not transtadially or transovarially transmitted (Almeida et al., 2005). The blue-green sharpshooter (BGSS) (*Graphocephala atropunctata*), green sharpshooter (*Draeculacephala minerva*) and red-headed sharpshooter *Xyphon* (formerly *Carneocephala*) *fulgida* are vectors of *Xf* that are native to California (Hewitt, 1949; Purcell, 1975). To understand the epidemiology of PD in the context of these native vectors, one must discuss their feeding and flying behaviors. The native vectors reside in riparian habitats or grasslands. When these areas neighbor vineyards, the insects can fly into the vineyards. They prefer to feed on lush new growth so transmit the bacteria into the tips of growing shoots. If inoculation occurs early in the growing season the bacteria have time to move to the cordons and the trunk of the vine, resulting in chronic infections. If inoculation occurs late in the season, the bacteria generally do not have time to reach the cordon or trunk and the infection is pruned off. Native sharpshooters are not strong flyers so tend to fly only into the first few rows of vineyards adjacent to the riparian areas, this creates the edge effect seen in vineyards with PD in growing areas where these native sharpshooters are the primary transmitters of *Xf* (Almeida et al., 2005). Thus, transmission mediated by native California vectors is

considered primary transmission and results in a monocyclic disease (Purcell, 1975; Almeida et al., 2005).

The epidemiology of PD in California drastically changed following the introduction of the glassy winged sharpshooter (*Homalodisca vitripennis*, GWSS) in Southern California (Blua et al., 1999). The feeding behavior of GWSS is dramatically different from the other native vectors. GWSS can feed on dormant grapevines during the winter, which introduces the bacterium directly into the woody tissues resulting in chronic infections (Hopkins and Purcell, 2002). In addition, the GWSS is a much stronger flier and can fly further into vineyards than native sharpshooters, thereby spreading the disease further into the vineyards. Another defining feature of the GWSS is that it can reproduce to high numbers on plants, such as citrus. And many citrus orchards neighbor vineyards in Southern California. This unfortunate situation has resulted in a shift in the epidemiology and an increase in disease incidence that resulted in a severe PD epidemic (Perring et al., 2001). GWSS is endemic to the southeastern United States and for this reason, it is not profitable for these regions to grow susceptible varieties of grapes, such as *Vitis vinifera* L. (European type) and *Vitis labrusca* L. (American type) (Hopkins, 1977). The GWSS was likely introduced from southeastern regions of U.S. into California as egg masses on nursery stock. In 1996-1997, GWSS presence in vineyards and its surrounding regions was first observed in the Temecula Valley, a major area for grape production in Southern California (Hopkins and Purcell, 2002). Since then, it has invaded many regions of Southern California, including San Diego, Orange, Riverside, San Bernardino, Los Angeles, Ventura and Santa Barbara counties (Blua et al., 1999). PD

has become a widespread disease and the possibility of the movement of the GWSS to the Central and Northern parts of the California State remain a major threat for grapevine growers in these regions (Committee on California Agriculture and Natural Resources, 2004). Presently, there is no cure for PD besides severe pruning in some cases. Different PD control strategies are being investigated, including host resistance, physical control, biological control and chemical control. However, current control of PD still largely relies on insecticide application to minimize vector populations.

To develop PD resistant or tolerant grapevine varieties, many studies have been carried out. In order to develop a PD resistant high quality *Vitis vinifera* cultivar, a major quantitative trait locus (QTL) was identified and mapped between markers VMCNg3h8 and VVIN64 in the linkage group 14 of a male parent F8909-17. This locus is denoted as Pierce's disease resistance 1 (*PdR1*), which controls the PD resistance of grapevine. It is very promising to introgress *PdR1* resistance allele into *Vitis vinifera* (Krivanek et al., 2006; Riaz et al., 2006). In order to develop transgenic grapevine with PD tolerance, *Vitis vinifera* cv. Thompson Seedless and Chardonnay were transformed to express pear polygalacturonase-inhibiting proteins (PGIPs) designed to inhibit the major virulence factor, PG, produced by *Xf* (Agüero et al., 2005). Compared with untransformed controls, one Thompson Seedless line (with high PGIP activity) and two Chardonnay lines (with moderate and high PGIP activity) exhibited less PD symptoms in eleven tested transgenic lines (Agüero et al., 2005). In the study of developing the transgenic PD resistant grapevine, the transgenic *Vitis vinifera* cv. Thompson seedless grapevines expressing a chimera in xylem exhibited significantly reduced PD symptoms. This chimera contains

two important domains, an elastase targeting *Xf* outer membrane protein MopB and a lytic peptide (cecropin B) targeting lipid moieties and outer membrane of *Xf* (Dandekar et al., 2012).

Cold temperature was also found to have "curing effects" for *Xf* infected grapevines. Bases on this phenomenon, a mathematical model was developed for *Vitis vinifera* cv. Pinot Noir and Cabernet Sauvignon grapevines in California. This model predicts temperature dependent PD development, which includes two essential elements: "killing index" and "curing effect" (Lieth et al., 2011).

For the biological control study of PD, the benign *Xf* strain, EB92-1, (isolated from elderberry) was found to be effective in controlling PD in *Vitis vinifera* cv. Carignane under greenhouse conditions. It is a very promising biological control agent for managing PD and is currently being tested in the field. Interestingly, it was observed that only avirulent or weakly virulent strains with normal systemic colonization and multiplication ability in grapevines have the protective effects (Hopkins, 2005).

To manage the GWSS populations, several studies have been conducted. In a physical control study, coating grapevines with a white particle film (Surround WP) was able to protect them from GWSS nymphs and adults in field studies (Puterka et al., 2003). This white film has the same efficiency as insecticides to reduce GWSS population. A color preference study shows white is the least attractive color for GWSS, which may explain its protective mechanism (Puterka et al., 2003). For the biological control study of GWSS, parasitic wasps *Gonatocerus ashmeadi*, *G. triguttatus*, *G. morrilli* and *G.*

fasciatus from southeastern United States were able to parasitize within GWSS eggs and they were already introduced into California to control GWSS (Pilkington et al., 2005). In studies aimed at identifying additional chemicals to control GWSS, immature stages of GWSS were found to be susceptible to ten selected insecticides using a petri dish or uptake technique under laboratory conditions. Acetamiprid (neonicotinoid) and bifenthrin (pyrethroid) were found to be the most toxic ones (Prabhaker et al., 2006). Two insect growth regulators buprofezin and pyriproxyfen were also investigated for their toxicity towards GWSS (Prabhaker and Toscano, 2007). The chitin synthesis inhibitor-buprofezin was most effective for first and second instars of GWSS (Prabhaker and Toscano, 2007), A juvenile hormone analog-pyriproxyfen was lethal to GWSS eggs with one or two days old (Prabhaker and Toscano, 2007). The insecticides pyrethroids (bifenthrin, fenpropathrin and cyfluthrin) and the neonicotinoids (thiamethoxam, acetarniprid, thiacloprid and imidacloprid) also have exhibited strong control effects for GWSS in field trials (Akey et al., 2001).

Studies aimed at chemical control of the bacterium itself, indicate that some antibiotics, antimicrobial peptides and phenolic compounds can inhibit *Xf in vitro* growth. In the twelve tested antibiotics, gentamicin, tetracycline, ampicillin, kanamycin, novobiocin, chloramphenicol and rifampin have the lowest minimal inhibitory concentration for *Xf* growth (Kuzina et al., 2006). In the 18 tested antimicrobial peptides, Magainin 2, Indolicidin, PGQ, and Dermaseptin showed toxicity to all ten strains of *Xf* (Kuzina et al., 2006). Twelve phenolic compounds (plant secondary metabolites) also

showed *in vitro* inhibition effects for *Xf* growth; specifically, catechol, caffeic acid and resveratrol exhibited high inhibitory effects (Maddox et al., 2010).

PD is a complex pathosystem that includes *Xf* (pathogen), grapevine (host), sharpshooter (vector) and environmental factors that favor the disease. To develop effective integrated management strategies for PD, understanding the mechanisms involved in the disease process is critical. In the following chapters, I report my studies that explored the molecular interactions between *Xf* and its grapevine host.

In Chapter 2, I studied the role of the transcription regulator, OxyR, in coping with oxidative stress and biofilm formation. OxyR is the only known oxidative stress regulator in the *Xf* Temecula1 genome (Van Sluys et al., 2003). As a xylem-limited pathogen, *Xf* may experience oxidative stress from a variety of sources in the plant. Detoxification of oxidative species is likely an important strategy for successful colonization in the host xylem. Through my investigations, I found *Xf* requires OxyR for survival under H_2O_2 stress as well as for behaviors involved in biofilm formation, an important aspect of the colonization process in grapevine.

In Chapter 3, I studied the role of a putative type II secretion system (T2SS) ATPase (XpsE) for protein secretion, colonization and virulence in the grapevine. *Xf* possesses all the essential genes encoding a functional T2SS (Van Sluys et al., 2003), that is similar to the one described in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Jha et al., 2005). We hypothesize that *Xf* secretes the confirmed and putative cell wall degrading enzymes (CWDEs) that are essential for bacterial movement within the xylem through the T2SS

(Roper et al., 2007b; Pérez-Donoso et al., 2010). By characterizing an *xpsE* deletion mutant, I found that *Xf* requires XpsE for colonization and full virulence in grapevine. We speculate that this is due to the inability to secrete CWDEs and possibly yet to be described virulence factors.

Literature Cited

Agüero, C.B., Uratsu, S.L., Greve, C., Powell, A.L., Labavitch, J.M., Meredith, C.P., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pathol. 6:43-51.

Akey, D.H., Henneberry, T.J., and Toscano, N.C. 2001. Insecticides sought to control adult glassy-winged sharpshooter. Calif. Agric. 55:22-27.

Alencar, V.C., Barbosa, D., Santos, D.S., Oliveira, A.C.F., de Oliveira, R.C., and Nunes, L.R. 2014. Genomic sequencing of two coffee-infecting strains of *Xylella fastidiosa* isolated from Brazil. Genome Announc. 2:e01190-01113.

Almeida, R.P., Killiny, N., Newman, K.L., Chatterjee, S., Ionescu, M., and Lindow, S.E. 2012. Contribution of *rpfB* to cell-to-cell signal synthesis, virulence, and vector transmission of *Xylella fastidiosa*. Mol. Plant-Microbe Interact. 25:453-462.

Almeida, R.P.P., Blua, M.J., Lopes, J.R.S., and Purcell, A.H. 2005. Vector transmission of *Xylella fastidiosa*: applying fundamental knowledge to generate disease management strategies. Ann. Entomol. Soc. Am. 98:775-786.

Baccari, C., Killiny, N., Ionescu, M., Almeida, R.P.P., and Lindow, S.E. 2014. Diffusible signal factor-repressed extracellular traits enable attachment of *Xylella fastidiosa* to insect vectors and transmission. Phytopathology 104:27-33.

Beaulieu, E.D., Ionescu, M., Chatterjee, S., Yokota, K., Trauner, D., and Lindow, S. 2013. Characterization of a diffusible signaling factor from *Xylella fastidiosa*. mBio 4:e00539-00512.

Bhattacharyya, A., Stilwagen, S., Reznik, G., Feil, H., Feil, W.S., Anderson, I., Bernal, A., D'Souza, M., Ivanova, N., Kapatral, V., Larsen, N., Los, T., Lykidis, A., Selkov, E., Jr., Walunas, T.L., Purcell, A., Edwards, R.A., Hawkins, T., Haselkorn, R., Overbeek, R., Kyrpides, N.C., and Predki, P.F. 2002. Draft sequencing and comparative genomics of *Xylella fastidiosa* strains reveal novel biological insights. Genome Res. 12:1556-1563.

Blua, M.J., Phillips, P.A., and Redak, R.A. 1999. A new sharpshooter threatens both crops and ornamentals. Calif. Agric. 53:22-25.

Brenner D.J., K.N.R., Staley J.T., Garrity G.M. . 2005. The Proteobacteria. In Bergey's manual of systematic bacteriology. Springer, New York.

Caserta, R., Takita, M., Targon, M., Rosselli-Murai, L., de Souza, A., Peroni, L., Stach-Machado, D., Andrade, A., Labate, C., and Kitajima, E. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. Appl. Environ. Microbiol. 76:4250-4259.

Chatterjee, S., Almeida, R.P.P., and Lindow, S. 2008a. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. Annu. Rev. Phytopathol. 46:243-271.

Chatterjee, S., Newman, K.L., and Lindow, S.E. 2008b. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. Mol. Plant-Microbe Interact. 21:1309-1315.

Chatterjee, S., Wistrom, C., and Lindow, S.E. 2008c. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. Proc. Natl. Acad. Sci. USA 105:2670-2675.

Chen, J., Huang, H., Chang, C.J., and Stenger, D.C. 2013. Draft Genome Sequence of *Xylella fastidiosa* subsp. *multiplex* Strain Griffin-1 from *Quercus rubra* in Georgia. Genome Announc. 1.

Chen, J., Xie, G., Han, S., Chertkov, O., Sims, D., and Civerolo, E.L. 2010. Whole genome sequences of two *Xylella fastidiosa* strains (M12 and M23) causing almond leaf scorch disease in California. J. Bacteriol. 192:4534.

Clifford, J.C., Rapicavoli, J.N., and Roper, M.C. 2013. A rhamnose-rich O-antigen mediates adhesion, virulence, and host colonization for the xylem-limited phytopathogen *Xylella fastidiosa*. Mol. Plant-Microbe Interact. 26:676-685.

Committee on California Agriculture and Natural Resources, N.R.C. 2004. California agricultural research priorities: Pierce's disease. The National Academies Press.

Cursino, L., Li, Y.X., Zaini, P.A., Fuente, L.d.l., Hoch, H.C., Burr, T.J., and de la Fuente, L. 2009. Twitching motility and biofilm formation are associated with *tonB1* in *Xylella fastidiosa*. FEMS Microbiol. Lett. 299:193-199.

Cursino, L., Galvani, C.D., Athinuwat, D., Zaini, P.A., Li, Y.X., Fuente, L.d.l., Hoch, H.C., Burr, T.J., Mowery, P., and de la Fuente, L. 2011. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. Mol. Plant-Microbe Interact. 24:1198-1206.

da Silva, A.C., Ferro, J.A., Reinach, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B., Monteiro-Vitorello, C.B., Van Sluys, M.A., Almeida, N.F., Alves, L.M., do Amaral, A.M., Bertolini, M.C., Camargo, L.E., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L.P., Cicarelli, R.M., Coutinho, L.L., Cursino-Santos, J.R., El-Dorry, H., Faria, J.B., Ferreira, A.J., Ferreira, R.C.,

Ferro, M.I., Formighieri, E.F., Franco, M.C., Greggio, C.C., Gruber, A., Katsuyama, A.M., Kishi, L.T., Leite, R.P., Lemos, E.G., Lemos, M.V., Locali, E.C., Machado, M.A., Madeira, A.M., Martinez-Rossi, N.M., Martins, E.C., Meidanis, J., Menck, C.F., Miyaki, C.Y., Moon, D.H., Moreira, L.M., Novo, M.T., Okura, V.K., Oliveira, M.C., Oliveira, V.R., Pereira, H.A., Rossi, A., Sena, J.A., Silva, C., de Souza, R.F., Spinola, L.A., Takita, M.A., Tamura, R.E., Teixeira, E.C., Tezza, R.I., Trindade dos Santos, M., Truffi, D., Tsai, S.M., White, F.F., Setubal, J.C., and Kitajima, J.P. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature 417:459-463.

da Silva, F.R., Vettore, A.L., Kemper, E.L., Leite, A., and Arruda, P. 2001. Fastidian gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. FEMS Microbiol. Lett. 203:165-171.

Dandekar, A.M., Gouran, H., Ibanez, A.M., Uratsu, S.L., Aguero, C.B., McFarland, S., Borhani, Y., Feldstein, P.A., Bruening, G., Nascimento, R., Goulart, L.R., Pardington, P.E., Chaudhary, A., Norvell, M., Civerolo, E., and Gupta, G. 2012. An engineered innate immune defense protects grapevines from Pierce disease. Proc. Natl. Acad. Sci. USA 109:3721-3725.

Davis, M.J., Purcell, A.H., and Thomson, S.V. 1978. Pierce's disease of grapevines: isolation of the causal bacterium. Science 199:75-77.

Dow, J., and Daniels, M. 2000. *Xylella* genomics and bacterial pathogenicity to plants. Yeast 17:263-271.

Feil, H., Feil, W.S., and Lindow, S.E. 2007. Contribution of fimbrial and afimbrial adhesins of *Xylella fastidiosa* to attachment to surfaces and virulence to grape. Phytopathology 97:318-324.

Guan, W., Shao, J., Zhao, T., and Huang, Q. 2014. Genome sequence of a *Xylella fastidiosa* strain causing Mulberry Leaf Scorch Disease in Maryland. Genome Announc. 2:e00916-00913.

Guilhabert, M.R., and Kirkpatrick, B.C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute a biofilm maturation to *X. fastidiosa* and colonization and attenuate virulence. Mol. Plant-Microbe Interact. 18:856-868.

Hewitt, W.B., Frazier, N.W., and J.H. Freitag, and A. J. Winkler. 1949. Pierce's disease investigations. Hilgardia 19:207-264.

Hopkins, D.L. 1977. Diseases caused by leafhopper-borne, rickettsia-like bacteria. Annu. Rev. Phytopathol. 15:277-294.

Hopkins, D.L. 1989. *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.

Hopkins, D.L. 2005. Biological control of Pierce's disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine. Plant Dis. 89:1348-1352.

Hopkins, D.L., and Purcell, A.H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis. 86:1056-1066.

Houston, B., Esau, K., and Hewitt, W.B. 1947. The mode of vector feeding and the tissues involved in the transmission of Pierce's disease virus in grape and alfalfa. Phytopathology 37:247-253.

Ionescu, M., Baccari, C., Da Silva, A.M., Garcia, A., Yokota, K., and Lindow, S.E. 2013. Diffusible signal factor (DSF) synthase RpfF of *Xylella fastidiosa* is a multifunction protein also required for response to DSF. J. Bacteriol. 195:5273-5284.

Jha, G., Rajeshwari, R., and Sonti, R.V. 2005. Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. Mol. Plant-Microbe Interact. 18:891-898.

Killiny, N., Martinez, R.H., Dumenyo, C.K., Cooksey, D.A., and Almeida, R.P.P. 2013. The exopolysaccharide of *Xylella fastidiosa* is essential for biofilm formation, plant virulence, and vector transmission. Mol. Plant-Microbe Interact. 26:1044-1053.

Krivanek, A.F., Riaz, S., and Walker, M.A. 2006. Identification and molecular mapping of *PdR1*, a primary resistance gene to Pierce's disease in *Vitis*. Theor. Appl. Genet. 112:1125-1131.

Kuzina, L.V., Miller, T.A., and Cooksey, D.A. 2006. *In vitro* activities of antibiotics and antimicrobial peptides against the plant pathogenic bacterium *Xylella fastidiosa*. Lett. Appl. Microbiol. 42:514-520.

Lee, B.M., Park, Y.J., Park, D.S., Kang, H.W., Kim, J.G., Song, E.S., Park, I.C., Yoon, U.H., Hahn, J.H., Koo, B.S., Lee, G.B., Kim, H., Park, H.S., Yoon, K.O., Kim, J.H., Jung, C.H., Koh, N.H., Seo, J.S., and Go, S.J. 2005. The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. Nucleic Acids Res. 33:577-586.

Lieth, J.H., Meyer, M.M., Yeo, K.H., and Kirkpatrick, B.C. 2011. Modeling cold curing of Pierce's disease in *Vitis vinifera* 'Pinot Noir' and 'Cabernet Sauvignon' grapevines in California. Phytopathology 101:1492-1500.

Maddox, C.E., Laur, L.M., and Tian, L. 2010. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. Curr. Microbiol. 60:53-58.

Matsumoto, A., Huston, S.L., Killiny, N., and Igo, M.M. 2012. XatA, an AT-1 autotransporter important for the virulence of *Xylella fastidiosa* Temecula1. MicrobiologyOpen 1:33-45.

Newman, K.L., Almeida, R.P.P., Purcell, A.H., and Lindow, S.E. 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. Proc. Natl. Acad. Sci. USA 101:1737-1742.

Newman, K.L., Chatterjee, S., Ho, K.A., and Lindow, S.E. 2008. Virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-to-cell signaling factors. Mol. Plant-Microbe Interact. 21:326-334.

Nunney, L., Elfekih, S., and Stouthamer, R. 2012. The importance of multilocus sequence typing: cautionary tales from the bacterium *Xylella fastidiosa*. Phytopathology 102:456-460.

Pérez-Donoso, A.G., Sun, Q., Roper, M.C., Greve, L.C., Kirkpatrick, B., and Labavitch, J.M. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. Plant Physiol. 152:1748-1759.

Perring, T., Farrar, C., and Blua, M. 2001. Proximity to citrus influences Pierce's disease in Temecula Valley vineyards. Calif. Agric. 55:13-18.

Pierce, N.B. 1892. The California vine disease. U.S. Bureau of Agriculture, Division of Vegetable Pathology Bulletin No. 2.

Pilkington, L.J., Irvin, N.A., Boyd, E.A., Hoddle, M.S., Triapitsyn, S.V., Carey, B.G., Jones, W.A., and Morgan, D.J.W. 2005. Introduced parasitic wasps could control glassy-winged sharpshooter. Calif. Agric. 59:223-228.

Prabhaker, N., and Toscano, N.C. 2007. Toxicity of the insect growth regulators, buprofezin and pyriproxyfen, to the glassy-winged sharpshooter, *Homalodisca coagulata* say (Homoptera: Cicadellidae). Crop Protect. 26:495-502.

Prabhaker, N., Castle, S.J., and Toscano, N.C. 2006. Susceptibility of immature stages of *Homalodisca coagulata* (Hemiptera: Cicadellidae) to selected insecticides. J. Econ. Entomol. 99:1805-1812.

Purcell, A. 2013. Paradigms: examples from the bacterium *Xylella fastidiosa*. Annu. Rev. Phytopathol. 51:339-356.

Purcell, A.H. 1975. Role of the blue-green sharpshooter, *Hordnia circellata*, in the epidemiology of Pierce's disease of grapevines. Environ. Entomol. 4:745-752.

Puterka, G.J., Reinke, M., Luvisi, D., Ciomperik, M.A., Bartels, D., Wendel, L., and Glenn, D.M. 2003. Particle film, Surround WP, effects on glassy-winged sharpshooter behavior and its utility as a barrier to sharpshooter infestations in grape. Plant Health Progress doi:10.1094/PHP-2003-0321-01-RS. 10.

Reddy, J.D., Reddy, S.L., Hopkins, D.L., and Gabriel, D.W. 2007. TolC is required for pathogenicity of *Xylella fastidiosa* in *Vitis vinifera* grapevines. Mol. Plant-Microbe Interact. 20:403-410.

Riaz, S., Krivanek, A.F., Xu, K., and Walker, M.A. 2006. Refined mapping of the Pierce's disease resistance locus, *PdR1*, and *Sex* on an extended genetic map of *Vitis rupestris* x *V. arizonica*. Theor. Appl. Genet. 113:1317-1329.

Roper, M.C., Greve, L.C., Warren, J.G., Labavitch, J.M., and Kirkpatrick, B.C. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Mol. Plant-Microbe Interact. 20:411-419.

Schreiber, H.L., Koirala, M., Lara, A., Ojeda, M., Dowd, S.E., Bextine, B., and Morano, L. 2010. Unraveling the first *Xylella fastidiosa* subsp. *fastidiosa* genome from Texas. Southwest. Entomol. 35:479-483.

Severin, H.H.P. 1950. Spittle-insect vectors of Pierce's disease virus. II. Life history and virus transmission. Hilgardia 19:357-376.

Shi, X.Y., Dumenyo, C.K., Hernandez-Martinez, R., Azad, H., and Cooksey, D.A. 2007. Characterization of regulatory pathways in *Xylella fastidiosa*: genes and phenotypes controlled by *algU*. Appl. Environ. Microbiol. 73:6748-6756.

Shi, X.Y., Dumenyo, C.K., Hernandez-Martinez, R., Azad, H., and Cooksey, D.A. 2009. Characterization of regulatory pathways in *Xylella fastidiosa*: genes and phenotypes controlled by *gacA*. Appl. Environ. Microbiol. 75:2275-2283.

Simpson, A.J., Reinach, F.C., Arruda, P., Abreu, F.A., Acencio, M., Alvarenga, R., Alves, L.M., Araya, J.E., Baia, G.S., Baptista, C.S., Barros, M.H., Bonaccorsi, E.D., Bordin, S., Bove, J.M., Briones, M.R., Bueno, M.R., Camargo, A.A., Camargo, L.E., Carraro, D.M., Carrer, H., Colauto, N.B., Colombo, C., Costa, F.F., Costa, M.C., Costa-Neto, C.M., Coutinho, L.L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A.P., Ferreira, A.J., Ferreira, V.C., Ferro, J.A., Fraga, J.S., Franca, S.C., Franco, M.C., Frohme, M., Furlan, L.R., Garnier, M., Goldman, G.H., Goldman, M.H., Gomes, S.L., Gruber, A., Ho, P.L., Hoheisel, J.D., Junqueira, M.L., Kemper, E.L., Kitajima, J.P., Krieger, J.E., Kuramae, E.E., Laigret, F., Lambais, M.R., Leite, L.C., Lemos, E.G., Lemos,

M.V., Lopes, S.A., Lopes, C.R., Machado, J.A., Machado, M.A., Madeira, A.M., Madeira, H.M., Marino, C.L., Marques, M.V., Martins, E.A., Martins, E.M., Matsukuma, A.Y., Menck, C.F., Miracca, E.C., Miyaki, C.Y., Monteriro-Vitorello, C.B., Moon, D.H., Nagai, M.A., Nascimento, A.L., Netto, L.E., Nhani, A., Jr., Nobrega, F.G., Nunes, L.R., Oliveira, M.A., de Oliveira, M.C., de Oliveira, R.C., Palmieri, D.A., Paris, A., Peixoto, B.R., Pereira, G.A., Pereira, H.A., Jr., Pesquero, J.B., Quaggio, R.B., Roberto, P.G., Rodrigues, V., de, M.R.A.J., de Rosa, V.E., Jr., de Sa, R.G., Santelli, R.V., Sawasaki, H.E., da Silva, A.C., da Silva, A.M., da Silva, F.R., da Silva, W.A., Jr., da Silveira, J.F., Silvestri, M.L., Siqueira, W.J., de Souza, A.A., de Souza, A.P., Terenzi, M.F., Truffi, D., Tsai, S.M., Tsuhako, M.H., Vallada, H., Van Sluys, M.A., Verjovski-Almeida, S., Vettore, A.L., Zago, M.A., Zatz, M., Meidanis, J., and Setubal, J.C. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151-159.

Van Sluys, M.A., de Oliveira, M.C., Monteiro-Vitorello, C.B., Miyaki, C.Y., Furlan, L.R., Camargo, L.E., da Silva, A.C., Moon, D.H., Takita, M.A., Lemos, E.G., Machado, M.A., Ferro, M.I., da Silva, F.R., Goldman, M.H., Goldman, G.H., Lemos, M.V., El-Dorry, H., Tsai, S.M., Carrer, H., Carraro, D.M., de Oliveira, R.C., Nunes, L.R., Siqueira, W.J., Coutinho, L.L., Kimura, E.T., Ferro, E.S., Harakava, R., Kuramae, E.E., Marino, C.L., Giglioti, E., Abreu, I.L., Alves, L.M., do Amaral, A.M., Baia, G.S., Blanco, S.R., Brito, M.S., Cannavan, F.S., Celestino, A.V., da Cunha, A.F., Fenille, R.C., Ferro, J.A., Formighieri, E.F., Kishi, L.T., Leoni, S.G., Oliveira, A.R., Rosa, V.E., Jr., Sassaki, F.T., Sena, J.A., de Souza, A.A., Truffi, D., Tsukumo, F., Yanai, G.M., Zaros, L.G., Civerolo, E.L., Simpson, A.J., Almeida, N.F., Jr., Setubal, J.C., and Kitajima, J.P. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185:1018-1026.

Varela, L., Smith, R., Phillips, P. and Varela, L. G. . 2001. Pierce's disease. UCANR Publications.

Voegel, T.M., Doddapaneni, H., Cheng, D.W., Lin, H., Stenger, D.C., Kirkpatrick, B.C., and Roper, M.C. 2013. Identification of a response regulator involved in surface attachment, cell–cell aggregation, exopolysaccharide production and virulence in the plant pathogen *Xylella fastidiosa*. Mol. Plant Pathol. 14:256-264.

Wang, N., Li, J.L., and Lindow, S.E. 2012. RpfF-dependent regulon of *Xylella fastidiosa*. Phytopathology 102:1045-1053.

Wells, J.M., Raju, B.C., Hung, H.Y., Weisburg, W.G., Mandelco-Paul, L., and Brenner, D.J. 1987. *Xylella fastidiosa* gen. nov., sp. nov: gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. 37:136-143.

Zhang, S., Flores-Cruz, Z., Kumar, D., Chakrabarty, P., Hopkins, D.L., and Gabriel, D.W. 2011. The *Xylella fastidiosa* biocontrol strain EB92-1 genome is very similar and syntenic to Pierce's disease strains. J. Bacteriol. 193:5576-5577.

Locus Tag	Gene	Protein	Reference
PD1485	*pglA*	Polygalacturonase precursor	Roper et al., 2007b
PD1964	*tolC*	Outer membrane export factor	Reddy et al., 2007
PD0731	*xadA*	Outer membrane protein XadA	Feil et al., 2007
PD1792	*hxfB*	Hemagglutinin-like protein	Feil et al., 2007; Guilhabert and Kirkpatrick, 2005
PD0062	*fimA*	Fimbrial subunit precursor	Feil et al., 2007
PD0058	*fimF*	Fimbrial adhesin precursor	Feil et al., 2007
PD2118	*hxfA*	Hemagglutinin-like secreted protein	Guilhabert and Kirkpatrick, 2005
PD0814	*wzy*	Wzy polymerase	Clifford et al., 2013
PD0528	*xatA*	Autotransporter protein XatA	Matsumoto et al., 2012
PD0843	*tonB*	TonB protein	Cursino et al., 2009
PD1394	*gumD*	GumD protein	Killiny et al., 2013
PD1391	*gumH*	GumH protein	Killiny et al., 2013
PD1284	*algU*	Alternate sigma factor	Shi et al., 2007
PD1984	*gacA*	Transcriptional regulator GacA	Shi et al., 2009
PD1386	*xhpT*	Response regulator XhpT	Voegel et al., 2013
XP0874	*pilL*	Chemotaxis histidine kinase	Cursino et al., 2011
PD0407	*rpfF*	Regulation of pathogenicity factor F	Newman et al., 2004
PD0406	*rpfC*	Regulation of pathogenicity factor C	Chatterjee et al., 2008c
PD0233	*rpfB*	Regulation of pathogenicity factor B	Almeida et al., 2012

Table 1.1 List of known *Xf* virulence factors.

2. Chapter 2

The role of the transcriptional regulator, OxyR, in biofilm formation and coping with oxidative stress for the bacterial phytopathogen, *Xylella fastidiosa*

Abstract

Plant pathogens often encounter reactive oxygen species (ROS) from a variety of sources during host invasion. *Xylella fastidiosa* (*Xf*) is xylem-limited and obligately vectored by xylem feeding insects, and thus, has limited contact with living cells in the plant. However, ROS are present in the xylem as byproducts of xylem lignification processes and may also be derived from adjacent living xylem parenchyma cells as part of the host defense response. Therefore, *Xf* likely experiences ROS stress in the grapevine xylem from different sources. Interestingly, OxyR is the only known oxidative stress regulator found in the *Xf* Temecula1 genome, which belongs to LysR-type family of transcription regulators (LTTRs). In *Escherichia coli* and other species, OxyR can sense oxidative stress and activate the expression of antioxidant genes. An *Xf oxyR* null mutant was significantly more sensitive to H_2O_2, demonstrating its importance as a component of the oxidative stress response. In addition, we found that the *Xf oxyR* mutant was reduced in surface attachment and cell-cell aggregation indicating that OxyR is a contributor to the temporal regulation of biofilm initiation, a critical facet of the xylem colonization process.

Introduction

Xylella fastidiosa (*Xf*), the causal agent of Pierce's disease (PD) of grapevines (*Vitis vinifera*), is a gram-negative, xylem-limited bacterium that is transmitted by xylem-feeding insect vectors, mainly sharpshooters (Hopkins, 1985). The typical symptoms of PD include leaf marginal necrosis, leaf blade drop, leaf scorch, cordon dieback, stunting and vine death (Varela, 2001). In addition to grapevine, *Xf* has many other plant hosts, such as citrus, maple, alfalfa, peach, plum, sycamore, elm, almond, coffee and oleander (Hopkins, 1989; Hopkins and Purcell, 2002). *Xf* cells are directly delivered into the xylem of their hosts by insect vectors that introduce the bacterium during feeding (Chatterjee et al., 2008a). Following this, *Xf* multiplies and moves into adjacent xylem vessels (Newman et al., 2003; Chatterjee et al., 2008a; Pérez-Donoso et al., 2010). This process requires enzymatic degradation of the primary plant cell wall material that comprises the pit membranes that separate xylem vessels from one another (Roper et al., 2007c; Pérez-Donoso et al., 2010). This movement results in systemic colonization of the plant, which is accompanied by prolific tylose production in response to pathogen invasion (Sun et al., 2013).

In the xylem tissue, there are two primary sources of reactive oxygen species (ROS). The first is developmentally related and comes from lignifying xylem, which can produce H_2O_2 as a byproduct of lignification process (Ros Barcelo, 1998). The second comes from non-lignifying xylem parenchyma cells, which can diffuse H_2O_2 necessary for the lignification of adjacent differentiating xylem vessels (Ros Barcelo, 2005). Thus, *Xf* likely encounters transient amounts of ROS in concentrations high enough to induce

oxidative stress. In addition, in an effort to defend themselves against invading pathogens, plants often produce ROS, such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$) as part of the plant defense response (Bolwell and Wojtaszek, 1997). Following invasion of the plant host by bacterial pathogens, there is a rapid and transient accumulation of the ROS within the first 5 minutes (phase I). There can also be a second, more prolonged production of ROS between 3 to 6 hours after inoculation (phase II) (Lamb and Dixon, 1997). It is unclear if living xylem parenchyma cells initiate an oxidative burst in response to pathogen infection of the xylem. However. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), primarily a xylem-dwelling pathogen, elicits rice defense responses from adjacent living parenchyma cells that includes secondary xylem cell wall thickening and accumulation of a pathogen-induced peroxidase (Hilaire et al., 2001). It is speculated that peroxidases produce toxic ROS intermediates that are detrimental to the pathogen and aimed at preventing pathogen ingress (Nicholson and Hammerschmidt, 1992). Regardless of the source, we speculate that *Xf* encounters ROS in the xylem during the infection process.

ROS are toxic to bacteria, because they can damage DNA, RNA, protein and lipids (Storz and Imlay, 1999; Cabiscol et al., 2000). In order to survive under oxidative stress, bacteria use scavenging enzymes to detoxify ROS. For instance, oxidative stress can induce catalase, alkyl hydroperoxide reductase and superoxide dismutase (SOD) production in *Escherichia coli*. Catalase detoxifies H_2O_2 into water and gaseous oxygen. Alkyl hydroperoxide reductase reduces various organic hydroperoxides. SOD can catalyze the dismutation reaction, which converts superoxide (O_2^-) into oxygen and H_2O_2

(Storz et al., 1989; Farr and Kogoma, 1991). These enzymes are tightly regulated by redox sensing transcription factors, which sense ROS and initiate components of the bacterial oxidative stress response. In *E. coli*, the redox-responsive OxyR transcription factor is activated through the formation of a disulfide bond between two cysteines in response to H_2O_2, and oxidized OxyR can bind to promoter regions of antioxidant genes and activate their transcription (Storz et al., 1990; Zheng et al., 1998; Zheng et al., 2001). OxyR-regulated antioxidant genes expression has been widely studied in several plant pathogenic bacteria. When *Ralstonia solanacearum* is treated with H_2O_2, alkyl hydroperoxide reductase subunit gene *ahpC1*, catalase genes, *katG* and *katE*, are positively regulated by OxyR (Flores-Cruz and Allen, 2011). In *Xanthomonas campestris* pv. *phaseoli* and *Agrobacterium tumefaciens*, the expression of the major catalase gene *katA* is regulated by OxyR following exposure to H_2O_2 (Nakjarung et al., 2003; Chauvatcharin et al., 2005). When *Pantoea stewartii* subsp. *stewartii* is challenged with H_2O_2, induced expression of alkyl hydroperoxide reductase subunit gene *ahpC* is also OxyR dependent (Burbank and Roper, 2014). The *Xf* Temecula1 genome contains a single copy of an *oxyR* ortholog (PD0747) and appears to be the only gene encoding a redox-sensing regulatory protein in *Xf* (Van Sluys et al., 2003). In addition, further sequence analyses predicted that several antioxidant enzymes exist in *Xf*. These include alkyl hydroperoxide reductase encoded by *ahpC* and *ahpF*, catalase encoded by *cpeB*, superoxide dismutase encoded by *sodA* and *sodM*, and a thiol-dependent peroxidase encoded by *ohr* (Van Sluys et al., 2003). CpeB has been characterized in *Xf* and is a

functional catalase that contributes to tolerance to exogenous H_2O_2 (Matsumoto et al., 2009).

OxyR was originally described as part of the oxidative stress response and recently has been implicated in bacterial biofilm formation (Shanks et al., 2007; Hennequin and Forestier, 2009; Honma et al., 2009). A biofilm is defined as an aggregate of bacterial cells, which adheres to living or non-living surfaces and is encased in a self-produced matrix exopolysaccharide (EPS) (Hall-Stoodley et al., 2004). The ability to form a biofilm is strongly correlated to pathogen fitness and colonization within host plants (Bogino et al., 2013). A *Serratia marcescens oxyR* mutant was severely impaired in biofilm formation, and transmission electron microscopy showed that it lacked fimbria-like surface structures, indicating that OxyR plays an important role in the early stages of *S. marcescens* biofilm formation by regulating fimbrial gene expression (Shanks et al., 2007). *Tannerella forsythia* and *Klebsiella pneumoniae* also require OxyR for biofilm formation (Hennequin and Forestier, 2009; Honma et al., 2009).

Little is known about the specific stresses *Xf* encounters during the xylem colonization process and how *Xf* copes with these stresses. In this study, we investigated the role of the redox-sensing transcriptional regulator, OxyR, in coping with oxidative stress *in vitro* and assessed the behavior of an *Xf oxyR* mutant *in planta*. In addition, we demonstrate that OxyR plays a role in biofilm formation for *Xf*.

Materials and Methods

Bacterial strains, plasmids and PCR primers. All bacterial strains, plasmids and PCR primers employed in this study are listed in Table 2.1.

Media and growth conditions for bacterial strains. *Xf* was grown in solid or liquid PD3 medium (Davis et al., 1981) at 28 ℃. *E. coli* strains were cultured at 37 ℃ in Luria-Bertani (LB) media. For the selection of transformants, antibiotics were supplemented in the media at following final concentrations: 5μg/mL (selection for *Xf* transformants), or 50μg/mL kanamycin (selection for *E. coli* transformants), 100μg/mL ampicillin (selection for *E. coli* transformants) or 5μg/mL chloramphenicol (selection for *Xf* transformants).

DNA manipulations and *in silico* analysis of protein sequence. *Xf* genomic DNA was extracted using the DNeasy tissue kit according to manufacturer's instructions (Qiagen, Chatsworth, CA). Electroporation of *Xf* competent cells was performed as previously described (Matsumoto et al., 2009). Plasmid DNA was isolated from *E. coli* using the Zyppy plasmid miniprep kit according to manufacturer's instructions (Zymo Research, Irvine, CA). Restriction enzyme digestions, ligations, and *E. coli* transformation were performed by standard procedures. *Xf* Temecula1, *X. campestris* pv. *campestris* and *E. coli* OxyR proteins sequence were obtained from the website of National Center for Biotechnology Information. OxyR proteins sequence alignment analysis was carried out using the Clustal Omega protein alignment tool (Sievers et al., 2011).

Construction of the *oxyR*::*kan* mutant. PCR primers OxyRfwdEcoRI and OxyRrevHindIII were used to amplify the WT *oxyR* gene (PD0747) open reading frame (ORF) with its flanking regions from *Xf* genomic DNA to yield a 1.3kb amplicon. The amplicon was double digested with EcoRI and HindIII. The resulting EcoRI/HindIII fragment was ligated to pUC19 that had been linearized with EcoRI and HindIII to create pPW01. Construct pPW01 was mutagenized using the EZ-Tn5 <KAN-2> insertion kit (Epicentre Technologies, Madison, WI) and transformed into *E. coli* DH10β cells and selected on LB solid medium containing 50μg/mL kanamycin. The candidate constructs were extracted from *E. coli* transformants and sequenced bidirectionally using two transposon specific primers, KAN-2 FP-1 and KAN-2 RP-1. These two primers are designed to anneal to the two ends of transposon and sequence their flanking regions from the transposon insertion site. Based on the sequencing results, a construct with the transposon inserted into the *oxyR* ORF was selected and named pPW02. In construct pPW02 (5234bp), the transposon (1221bp) was mapped at 1236-2456bp.

Xf Temecula1 (Guilhabert et al., 2001) electrocompetent cells that were prepared as previously described (Matsumoto et al., 2009) were electroporated with 200 ng of the mutagenesis construct pPW02 and transformants were selected on PD3 solid medium containing 5μg/mL kanamycin.

Replacement of the WT *oxyR* gene (936 bp) with the *oxyR*::*kan*-2 (Figure 2.1) was confirmed by PCR, using primers OxyRoutfwd and OxyRoutrev (Figure 2.2.A), which were designed to anneal to the regions flanking the double cross-over event (Figure 2.1). The resulting amplicons were sequenced to confirm the gene replacement

event. The *Xf oxyR* mutant was also confirmed by *Xf* specific detection primers RST-31 and RST-33 through PCR (Minsavage et al., 1994) (Figure 2.2.B). The *Xf oxyR* mutant strain is named PW31 (Table 2.1).

Complementation of the *oxyR*::*kan* mutant. The *oxyR* operon (Figure 2.3) containing the *Xf oxyR* gene was amplified using primers Operon OxyR fwd XhoI and Operon OxyR rev XhoI by PCR. The resulting PCR product and complementation vector, pAX1Cm, were both digested with XhoI and then ligated to create the construct pAX1Cm::*oxyR* operon (pPW11). This complementation construct was electroporated into electrocompetent *oxyR* mutant cells. Transformants were selected on PD3 solid medium containing 5µg/mL chloramphenicol. Integration into the neutral site between PD0702 and PD0703 in the *oxyR* mutant chromosome was confirmed by PCR using primers PW-For and PW-Rev. This *oxyR* complemented strain is named as PW32 (Table 2.1).

H_2O_2 sensitivity assay. A disk diffusion method was employed to test H_2O_2 sensitivity as previously described with minor modifications (Matsumoto et al., 2009). The WT, *oxyR* mutant and PW32 cells were grown for seven days on PD3 solid medium. Following this, the cells were harvested, resuspended into PD3 liquid medium and adjusted to OD_{600}=0.1. 500µL of the cell suspension was mixed with five milliliters of PD3 top agar (0.8% agar), gently vortexed and poured onto the PD3 solid medium. An autoclaved Whatman disk paper was placed in the center of the plate. Ten microliters of 100mM H_2O_2 was dispensed to the paper to provide the H_2O_2 stress source. The inhibition zone

diameter surrounding the disc was measured following seven days incubation at 28 ℃. Three replications were performed for each strain.

H_2O_2 survival assay. Bacterial survival in the presence of H_2O_2 stress was evaluated using a modified version of a previously established protocol (Pericone et al., 2003). Bacterial strains were harvested from PD3 solid medium and resuspended in phosphate buffered saline (PBS) after seven days incubation at 28 ℃. The harvested cells were adjusted to OD_{600}=0.25 in PBS. 500µL of the cell culture and 500µL PBS were mixed in a 50mL Falcon tube (BD Biosciences, San Jose, CA) and five microliters of 100mM H_2O_2 was added to the mixture. Cells without H_2O_2 treatment served as negative controls. The tubes were shaken at 100 rpm, 28 ℃ for 30 minutes and then incubated on ice. The cell cultures were serially diluted by PBS and 20µL of each dilution of the H_2O_2-treated or untreated cells were striped onto PD3 solid medium and incubated at 28 ℃ for ten days. The number of colony forming unit (CFU) was enumerated after incubation. The survival percentage of each strain was calculated by the following formula: 100(H_2O_2 treated cells CFU/mL)/(non-treated cells CFU/mL).

Cell attachment assay. A crystal violet staining method was used to compare cell attachment between WT and *oxyR* mutant as previously described (Espinosa-Urgel et al., 2000). Cells were grown for seven days on PD3 solid medium, harvested and adjusted to OD_{600}=0.1 in PD3 liquid medium. The cell suspension was diluted 1:10 in PD3 liquid medium in glass tubes. After seven days of static incubation at 28 ℃, 100 µL of 1%

crystal violet solution was added to the tube and incubated for 20 minutes. The supernatant was removed and the tube was washed three times with diH_2O. The remaining stained cells were eluted in one milliliter of 95% ethanol and the OD_{600} was measured in a spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Twelve replications were performed for each strain.

Cell-cell aggregation assay. Cell-cell aggregation was compared between *Xf* WT and the *oxyR* mutant using a previously described protocol (Burdman et al., 2000; Leite et al., 2004; Guilhabert and Kirkpatrick, 2005). Each strain was grown for seven days on solid PD3 medium, harvested and resuspended in PD3 liquid medium and adjusted to OD_{600}=0.1. All strains were diluted 1:10 in one milliliter PD3 liquid medium in glass tubes. Following ten days static incubation at 28 ℃, cultures were agitated slightly and allowed to settle for 20 minutes. The OD_{540} (ODs) of the supernatant was determined and returned to the original tube. Then, the aggregated cells were vortexed briefly to disperse them. Then the total cell culture OD_{540} (ODt) was also measured. Finally, the cell aggregation percentage was expressed by the following formula: aggregated cells percentage=100(ODt-ODs)/ODt.

Biofilm formation. Biofilm formation was evaluated on two surfaces, a glass flask and a 50mL polypropylene Falcon tube (BD Biosciences, San Jose, CA). WT, *oxyR* mutant, PW32 and Δ*ahpCF* cells were grown on PD3 solid medium for seven days, then harvested and adjusted to OD_{600}=0.25 in PD3 liquid medium. For the biofilm formation comparison among WT, *oxyR* mutant and PW32, 50 milliliter of PD3 liquid was

inoculated with two milliliter of bacterial culture for each strain in a 125mL glass flask and incubated at 28 ℃ with 180 rpm shaking. For the biofilm formation comparison among WT, *oxyR* mutant and Δ*ahpCF*, one milliliter bacterial culture was diluted in 50mL PD3 liquid in a 250mL glass flask and incubated at 28 ℃ with 180 rpm shaking. Biofilm formation was observed after four days.

For biofilm formation in the polypropylene tubes, WT, *oxyR* mutant and PW32 cells were grown on PD3 solid medium for seven days, then harvested and adjusted to OD_{600}=0.1 in PD3 liquid medium. 200 µL of bacterial culture was diluted in 20mL PD3 liquid in 50mL Falcon polypropylene tubes (BD Biosciences, San Jose, CA). The culture was incubated at 28 ℃ with 180 rpm shaking. Biofilm formation was observed after six days.

The kinetics of three dimensional biofilm formation was evaluated by confocal laser scanning microscope (CLSM) using a previously established protocol (Roper et al., 2007a). In brief, *Xf* WT and *oxyR* mutant cells were grown on solid PD3 medium for seven days, harvested and adjusted to OD_{600}=0.25 in PD3 liquid medium. Two hundred microliter cell suspensions were inoculated into 20mL of PD3 liquid in 50mL Falcon tubes (BD Biosciences, San Jose, CA). A sterilized glass microscope slide was placed vertically into each tube. The tubes were incubated upright at 28 ℃ with 180 rpm shaking to facilitate biofilm formation at the air-liquid interface. Following two, four, six or eight days incubation, the glass slides were removed and gently heat fixed. The fixed biofilm was stained with 20 µM Syto9 (Invitrogen, Carlsbad, CA) for 15 minutes, then rinsed with PBS and mounted in Slowfade mounting fluid (Invitrogen, Carlsbad, CA). Biofilm

images were obtained using a Zeiss 510 confocal laser scanning microscope fitted with a Plan Neofluar 25× water immersion objective (numerical aperture=0.8) (Zeiss, Germany). Green fluorescence was excited with a 488nm laser. Three replications were performed for each strain at each time point. Ten images were captured for each replicate along the z-axis with a 0.4μm constant interval. Orthogonal views were obtained using Image J software (Schneider et al., 2012). Imaris software (Bitplane USA, South Windsor, CT) was used to create three-dimensional view of the biofilm and calculate its thickness.

Virulence assay. *Xf* WT and *oxyR* mutant cells were grown on solid PD3 medium for seven days, harvested in PBS and adjusted to OD_{600}=0.25. *Vitis vinifera* cv. Thompson seedless grapevines were pin-prick inoculated with 20 μL of bacterial solution with a 20-gauge syringe needle as previously described (Hill and Purcell, 1995). Grapevines were inoculated twice on two opposite sides of the stem with either *Xf* WT, the *oxyR* mutant, or PBS. Three biological replications were performed, and ten technical replications were included for each biological replication. Plants were rated weekly for disease severity. The PD symptom severity scale is from 0 to 5 as described below, where 0 = no PD symptoms (healthy), 1=one or two leaves appearing marginal necrosis, 2 = two or three leaves showing heavy marginal necrosis, 3=fifty percent or even more of leaves displaying marginal necrosis and a few match sticks (attached petioles whose leaf blade had abscised), 4=all the leaves displaying significant scorch symptoms and numerous matchsticks, and 5=a dead vine (Hopkins, 1985; Guilhabert and Kirkpatrick, 2005; Roper et al., 2007c).

Host colonization study. WT and *oxyR* mutant populations were quantified from petioles collected from the point of inoculation (POI). For each biological replication of each strain, one petiole per one technical replication plant was collected (in total five petioles collected from five plants in each biological replication). Three biological replications were included in the colonization study. In total, 15 petioles were collected for each strain. Petioles were weighed, sequentially surface-sterilized with 95% ethanol (1 min) and 20% bleach (2 min), then rinsed two times in sterilized distilled water (2 min) and placed in 2 mL PBS buffer in mesh sample bags (Agdia, Inc., Elkhart, IN). Samples were crushed with a hammer. Serial dilutions of the crushed petiole slurry were placed on PD3 solid medium or PD3 solid medium containing 5 μg/mL of kanamycin. The numbers of CFU were enumerated after 10 days incubation at 28 ℃ (Roper et al., 2007c).

Results

Analysis of the *Xf* OxyR protein sequence. *Xf* Temecula1, *Xanthomonas campestris* pv. *campestris* and *E. coli* OxyR proteins were aligned using the online program, Clustal Omega (Sievers et al., 2011). *Xf* Temecula1 OxyR protein shares 80% identity with the *X. campestris* pv. *campestris* OxyR protein, but only 46% identity with the *E. coli* OxyR protein. The redox switch in the OxyR protein (two cysteines at position 199 and 208) (Zheng et al., 1998), was also present in the *Xf* Temecula1 OxyR protein sequence (Figure 2.4). In the *E. coli* response to hydrogen peroxide, OxyR regulated antioxidant signaling is activated by forming a disulfide bond between two cysteines in the OxyR protein (Zheng et al., 1998).

OxyR is involved in protection against hydrogen peroxide stress. Sensitivity to H_2O_2 was compared among WT, *oxyR* mutant and the complemented strain PW32 using a disc diffusion method. The diameter of the zone of inhibition around the H_2O_2 infused disc for the *Xf oxyR* mutant was significantly larger than WT and PW32 ($p<0.05$) (Figure 2.5), indicating that *oxyR* mutant was more sensitive to H_2O_2 than WT. In addition, H_2O_2 tolerance was restored in the complemented strain PW32. The results were analyzed by a Tukey's test following one-way analysis of variance (ANOVA).

Survival in the presence of H_2O_2 was also compared between WT and *oxyR* mutant. The survival percentage of the *oxyR* mutant was significantly lower than the WT ($p<0.05$) (Figure 2.6), indicating that its survival rate under H_2O_2 stress was greatly reduced.

A mutation in *oxyR* affects surface attachment and cell-cell aggregation. The ability to attach to a solid surface was examined using the crystal violet method where attached cells were visualized as a purple ring on the tube side-wall (Espinosa-Urgel et al., 2000). Qualitatively, the observed purple ring was thinner for the *oxyR* mutant as compared to the WT, indicating the *oxyR* mutant was reduced in attachment to a glass surface (Figure 2.7. A and B). Quantitative measurements of attachment confirmed that the *oxyR* mutant was compromised in surface attachment ($P<0.05$) (Figure 2.7.C). Cell-cell aggregation was also evaluated for WT and the *oxyR* mutant in the glass tubes. Compared with WT, the *oxyR* mutant cells were significantly reduced in the ability to aggregate ($p<0.05$) (Figure 2.8).

OxyR contributes to biofilm formation. Biofilm formation was assessed on two different surfaces: (1) a glass flask and (2) a polypropylene tube surface. Unlike the WT strain, the *oxyR* mutant was greatly reduced in the ability to form a biofilm while the complemented strain PW32 had a restored biofilm phenotype (Figure 2.9 and Figure 2.10). The *Xf* Δ*ahpCF* strain forms a biofilm similar to WT, indicating that AhpC and AhpF, two alkyl hydroperoxide reductase subunits, do not have an apparent role in biofilm formation under the conditions tested here (Figure 2.11).

We complemented the *Xf oxyR* mutant with the entire *oxyR* operon, which contains *ahpC*, *ahpF* and *oxyR* in a map order. *ahpC* (PD0745) and *ahpF* (PD0746) are predicted to encode the two subunits of alkyl hydroperoxide reductase. Reverse transcription PCR (RT-PCR) data, confirmed that *ahpC*, *ahpF* and *oxyR* are indeed organized as an operon (personal communication, Dr. Igo Michele, UC Davis). In our attempts to complement the *oxyR* mutant, we initially supplied a wild type copy of *oxyR* driven either by the putative *oxyR* operon promoter or the constitutive *nptII* promoter that is known to drive high expression levels of transcription in *Xf*. However, neither of these constructs fully restored hydrogen peroxide resistance, surface attachment, cell-cell aggregation or biofilm formation for the *oxyR* mutant. Interestingly, complementation with the entire *ahpC*-*ahpF*-*oxyR* operon was the only scenario that restored the reduction in biofilm formation to near wild type levels. This suggests that proper expression of *oxyR* may rely on expression not only from the promoter driving expression of the operon, but also a poorly defined internal promoter. Moreover, the study in *E. coli* has shown that OxyR is self-regulated (Zheng et al., 1998).

In order to rule out the possible contribution of AhpF and AhpC in restoring biofilm formation in the complemented strain PW32, we assessed the biofilm phenotype of the *Xf* Δ*ahpCF* strain (generously provided by Dr. Igo Michele, UC Davis). *Xf* Δ*ahpCF* forms a biofilm in the glass flask similar to the WT strain, suggesting that AhpC and AhpF are not involved in biofilm formation. As a result, we reason that in our complemented strain, PW32, the restoration of biofilm phenotype is due to supplementation of a wild type copy of *oxyR* and not *ahpC* or *ahpF*.

The kinetics of three-dimensional biofilm formation was also evaluated by CLSM over a two, four, six and eight days time course. These data demonstrate that OxyR is required during the initial steps of biofilm formation (Figure 2.12), which was expected based on the significant reduction in surface attachment and aggregation. The *oxyR* mutant was sparsely attached cells to the glass slide and unable to build a mature three-dimensional WT biofilm even after eight days of growth (Figure 2.13).

OxyR contributes to host colonization, but does not affect overall virulence. *In planta* bacterial populations for both WT and the *oxyR* mutant were evaluated at eleven weeks post-inoculation at the point of inoculation (POI). The *oxyR* mutant was recovered from significantly fewer plants as compared to the WT strain. Specifically, the WT strain was recovered from 87% of the collected sample petioles whereas the *oxyR* mutant was recovered from only 33% of the plants. Furthermore, the overall numbers of the *oxyR* mutant 3.80×10^5 CFU/g tissue were significantly lower than that of WT strain with an average population of 2.48×10^7 CFU/g tissue (Figure 2.14).

Interestingly, the disease progress curves indicate that there was no significant difference in virulence observed between the WT and *oxyR* mutant (Figure 2.15). This suggests that while a mutation in *oxyR* does impact the ability of the bacterium to colonize the host, it is still able to achieve the threshold numbers of bacteria necessary to incite WT levels of PD.

Discussion

OxyR is the only annotated redox-sensing transcription factor in the *Xf* Temecula1 genome (Van Sluys et al., 2003), which led us to speculate that it plays a critical role in modulating the oxidative stress response in *Xf*. Indeed, our *in vitro* assays indicate that OxyR is necessary for the tolerance of exogenous ROS in the form of H_2O_2. These results are consistent with previous *in vitro* studies for OxyR in other bacteria, including *X. campestris* pv. *phaseoli*, *X. campestris* pv. *campestris*, *P. stewartii* subsp. *stewartii*, *R. solanacearum*, *S. marcescens*, *K. pneumoniae*, *T. forsythia* and *Burkholderia pseudomallei* (Mongkolsuk et al., 1998; Loprasert et al., 2002; Shanks et al., 2007; Hennequin and Forestier, 2009; Honma et al., 2009; Charoenlap et al., 2011; Flores-Cruz and Allen, 2011; Burbank and Roper, 2014). It is likely that the expression of *cpeB* (catalase), *ahpC* and *ahpF* (alkyl hydroperoxide reductase) are reduced in the *Xf oxyR* mutant under H_2O_2 stress.

In planta, the *Xf oxyR* mutant was unable to reach the same population levels as the WT, suggesting that coping with ROS in the xylem is an important aspect of the colonization process and that the grapevine xylem represents an oxidative environment.

Likewise, *R. solanacearum,* another xylem-dwelling bacterium also experiences an oxidative environment in the xylem (Flores-Cruz and Allen, 2009). ROS can be present in the xylem in association with the lignification process in the xylem that occurs during plant development (Ros Barcelo, 1998, 2005). Sharpshooters prefer to feed on new growth of grapevine shoots (Redak et al., 2004), which is developing and lignifying rapidly. Thus, there may be pronounced high concentrations of developmentally related ROS in these tissues that *Xf* needs to detoxify immediately upon entry into the xylem of young green shoots.

The oxidative burst, another major source of ROS in plant tissue, usually occurs rapidly (within minutes to hours) following infection of the pathogen (Lamb and Dixon, 1997). Thus, the eleven weeks post-inoculation time point we used to quantify bacterial populations in the plant is a late time point to assess if any reduction in population numbers observed for the *oxyR* mutant was due to an oxidative burst. Elicitation of the host defense response relies on interaction with metabolically active cells. The xylem is composed primarily of non-living tissue, but adjacent to the vessel lumen are living parenchyma cells that communicate with the xylem through pit membranes that are composed of porous primary plant cell wall material. It is presently unknown if *Xf* communicates with and elicits an oxidative burst from parenchyma cells that are adjacent to the xylem lumen. However, in PD-infected grapevines, abundant tyloses form in the xylem in response to infection with *Xf* (Sun et al., 2013). Tyloses are outgrowths of the parenchyma cells from pit cavities into vessels that are designed to block pathogen movement within the xylem among other things (Esau, 1977). Tyloses formation

indicates that grapevines are able to sense and respond to *Xf* invasion (Sun et al., 2013). We attempted to quantify the bacterial population at an earlier time point (24 hours post-inoculation), but we were not successful in recovering *Xf* at this time point. *Xf* is a slow colonizer and the bacterial numbers may have been below the detection threshold of the isolation method used in this study. Therefore, it is difficult to assess if *Xf* elicits an oxidative burst in the xylem and if the *oxyR* mutant was vulnerable to the ROS associated with this stage of the infection process.

OxyR also has a described role in bacterial biofilm formation (Hennequin and Forestier, 2009; Honma et al., 2009). In this study, we observed that the *Xf oxyR* mutant was strikingly reduced in its ability to attach to a solid surface and to form cell-cell aggregates. Cell attachment and cell-cell aggregation are initial steps for building a mature biofilm (O'Toole et al., 2000). Thus, the *Xf oxyR* mutant is deficient in initiating the early steps of biofilm formation and fails to form a typical mature WT biofilm as observed by CLSM. Similarly, a *S. marcescens oxyR* mutant was also deficient in primary cell attachment and biofilm formation (Shanks et al., 2007). Furthermore, OxyR contributes to *T. forsythia* auto-aggregation and forming mixed biofilms with *Fusobacterium nucleatum* (Honma et al., 2009). Biofilm formation is also an important mechanism for bacterial survival and colonization in its host (Bogino et al., 2013) and we speculate that response to ROS associated with entering and colonizing the host environment via OxyR may serve as a mechanism that signals *Xf* to enter the biofilm state. Specifically, we speculate that OxyR may be involved in regulating the transcription of genes encoding adhesins that function during initial steps of *Xf* biofilm

formation. Indeed, in *E. coli*, surface antigen, Ag43, is regulated by OxyR and has been implicated in cell-cell aggregation and biofilm formation (Danese et al., 2000; Schembri et al., 2003). Furthermore, OxyR contributes to biofilm formation through regulating the expression of specific cell adhesion genes in *K. pneumoniae* (Hennequin and Forestier, 2009).

Despite a colonization defect *in planta*, there is no significant difference in virulence observed between WT and the *Xf oxyR* mutant. This is consistent with soft rot virulence study of *oxyR* mutant in *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) (Miguel et al., 2000), but contrary to a wilt virulence study of *oxyR* mutant in *R. solanacearum* (Flores-Cruz and Allen, 2011) and black rot virulence study of *oxyR* mutant in *X. campestris* pv. *campestris* (Charoenlap et al., 2011). These contradictory roles of OxyR in virulence may reflect that different bacteria found in diverse niches utilize different survival strategies under oxidative stress. Indeed, study in *P. stewartii* subsp. *stewartii* has shown that OxyR-dependent regulation is critical for xylem colonization induced wilting symptom, but not involved in the apoplast colonization induced water-soaked lesion in the corn seedlings (Burbank and Roper, 2014). It is also plausible that *Xf* possesses a novel regulator of the oxidative stress response, which was not revealed during annotation of the *Xf* genome. This regulator may have a similar function as OxyR or cross-talk with the OxyR regulon.

Literature Cited

Bogino, P.C., Oliva Mde, L., Sorroche, F.G., and Giordano, W. 2013. The role of bacterial biofilms and surface components in plant-bacterial associations. Int. J. Mol. Sci. 14:15838-15859.

Bolwell, G.P., and Wojtaszek, P. 1997. Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. Physiol. Mol. Plant Pathol. 51:347-366.

Burbank, L.P., and Roper, M.C. 2014. OxyR and SoxR modulate the inducible oxidative stress response and are implicated during different stages of infection for the bacterial phytopathogen, *Pantoea stewartii* subsp. *stewartii*. Mol. Plant-Microbe Interact. (in press).

Burdman, S., Jurkevitch, E., Soria-Diaz, M.E., Serrano, A.M.G., and Okon, Y. 2000. Extracellular polysaccharide composition of *Azospirillum brasilense* and its relation with cell aggregation. FEMS Microbiol. Lett. 189:259-264.

Cabiscol, E., Tamarit, J., and Ros, J. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. 3:3-8.

Charoenlap, N., Buranajitpakorn, S., Duang-Nkern, J., Namchaiw, P., Vattanaviboon, P., and Mongkolsuk, S. 2011. Evaluation of the virulence of *Xanthomonas campestris* pv. *campestris* mutant strains lacking functional genes in the OxyR regulon. Curr. Microbiol. 63:232-237.

Chatterjee, S., Almeida, R.P.P., and Lindow, S. 2008. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. Annu. Rev. Phytopathol. 46:243-271.

Chauvatcharin, N., Atichartpongkul, S., Utamapongchai, S., Whangsuk, W., Vattanaviboon, P., and Mongkolsuk, S. 2005. Genetic and physiological analysis of the major OxyR-regulated *katA* from *Xanthomonas campestris* pv. *phaseoli*. Microbiology 151:597-605.

Danese, P.N., Pratt, L.A., Dove, S.L., and Kolter, R. 2000. The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. Mol. Microbiol. 37:424-432.

Davis, M.J., French, W.J., and Schaad, N.W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6:309-314.

Esau, K. 1977. Anatomy of seed plants, Ed 2. John Wiley and Sons, Inc. New York.

Espinosa-Urgel, M., Salido, A., and Ramos, J.L. 2000. Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. J. Bacteriol. 182:2363-2369.

Farr, S.B., and Kogoma, T. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55:561-585.

Flores-Cruz, Z., and Allen, C. 2009. *Ralstonia solanacearum* encounters an oxidative environment during tomato infection. Mol. Plant-Microbe Interact. 22:773-782.

Flores-Cruz, Z., and Allen, C. 2011. Necessity of OxyR for the hydrogen peroxide stress response and full virulence in *Ralstonia solanacearum*. Appl. Environ. Microbiol. 77:6426-6432.

Guilhabert, M.R., and Kirkpatrick, B.C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute a biofilm maturation to *X. fastidiosa* and colonization and attenuate virulence. Mol. Plant-Microbe Interact. 18:856-868.

Guilhabert, M.R., Hoffman, L.M., Mills, D.A., and Kirkpatrick, B.C. 2001. Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mol. Plant-Microbe Interact. 14:701-706.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2:95-108.

Hennequin, C., and Forestier, C. 2009. *oxyR*, a LysR-type regulator involved in *Klebsiella pneumoniae* mucosal and abiotic colonization. Infect. Immun. 77:5449-5457.

Hilaire, E., Young, S.A., Willard, L.H., McGee, J.D., Sweat, T., Chittoor, J.M., Guikema, J.A., and Leach, J.E. 2001. Vascular defense responses in rice: peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. Mol. Plant-Microbe Interact. 14:1411-1419.

Hill, B.L., and Purcell, A.H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopathology 85:1368-1372.

Honma, K., Mishima, E., Inagaki, S., and Sharma, A. 2009. The OxyR homologue in *Tannerella forsythia* regulates expression of oxidative stress responses and biofilm formation. Microbiology 155:1912-1922.

Hopkins, D.L. 1985. Physiological and pathological characteristics of virulent and avirulent strains of the bacterium that causes Pierce's disease of grapevine. Phytopathology 75:713-717.

Hopkins, D.L. 1989. *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.

Hopkins, D.L., and Purcell, A.H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis. 86:1056-1066.

Lamb, C., and Dixon, R.A. 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:251-275.

Leite, B., Andersen, P.C., and Ishida, M.L. 2004. Colony aggregation and biofilm formation in xylem chemistry-based media for *Xylella fastidiosa*. FEMS Microbiol. Lett. 230:283-290.

Loprasert, S., Sallabhan, R., Whangsuk, W., and Mongkolsuk, S. 2002. The *Burkholderia pseudomallei oxyR* gene: expression analysis and mutant characterization. Gene 296:161-169.

Matsumoto, A., Young, G.M., and Igo, M.M. 2009. Chromosome-based genetic complementation system for *Xylella fastidiosa*. Appl. Environ. Microbiol. 75:1679-1687.

Miguel, E., Poza-Carrion, C., Lopez-Solanilla, E., Aguilar, I., Llama-Palacios, A., Garcia-Olmedo, F., and Rodriguez-Palenzuela, P. 2000. Evidence against a direct antimicrobial role of H_2O_2 in the infection of plants by *Erwinia chrysanthemi*. Mol. Plant-Microbe Interact. 13:421-429.

Minsavage, G.V., Thompson, C.M., Hopkins, D.L., Leite, R.M.V.B.C., and Stall, R.E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84:456-461.

Mongkolsuk, S., Sukchawalit, R., Loprasert, S., Praituan, W., and Upaichit, A. 1998. Construction and physiological analysis of a *Xanthomonas* mutant to examine the role of the *oxyR* gene in oxidant-induced protection against peroxide killing. J. Bacteriol. 180:3988-3991.

Nakjarung, K., Mongkolsuk, S., and Vattanaviboon, P. 2003. The *oxyR* from *Agrobacterium tumefaciens*: evaluation of its role in the regulation of catalase and peroxide responses. Biochem. Biophys. Res. Commun. 304:41-47.

Newman, K.L., Almeida, R.P., Purcell, A.H., and Lindow, S.E. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. Appl. Environ. Microbiol. 69:7319-7327.

Nicholson, R.L., and Hammerschmidt, R. 1992. Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. 30:369-389.

O'Toole, G., Kaplan, H.B., and Kolter, R. 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. 54:49-79.

Pérez-Donoso, A.G., Sun, Q., Roper, M.C., Greve, L.C., Kirkpatrick, B., and Labavitch, J.M. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. Plant Physiol. 152:1748-1759.

Pericone, C.D., Park, S., Imlay, J.A., and Weiser, J.N. 2003. Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. J. Bacteriol. 185:6815-6825.

Redak, R.A., Purcell, A.H., Lopes, J.R.S., Blua, M.J., Mizell, R.F., III, and Andersen, P.C. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu. Rev. Entomol. 49:243-270.

Roper, M.C., Greve, L.C., Labavitch, J.M., and Kirkpatrick, B.C. 2007a. Detection and visualization of an exopolysaccharide produced by *Xylella fastidiosa* in vitro and in planta. Appl. Environ. Microbiol. 73:7252-7258.

Roper, M.C., Greve, L.C., Warren, J.G., Labavitch, J.M., and Kirkpatrick, B.C. 2007b. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Molecular plant-microbe interactions : 20:411-419.

Ros Barcelo, A. 1998. Hydrogen peroxide production is a general property of the lignifying xylem from vascular plants. Ann. Bot. 82:97-103.

Ros Barcelo, A. 2005. Xylem parenchyma cells deliver the H_2O_2 necessary for lignification in differentiating xylem vessels. Planta 220:747-756.

Schembri, M.A., Hjerrild, L., Gjermansen, M., and Klemm, P. 2003. Differential expression of the *Escherichia coli* autoaggregation factor antigen 43. J. Bacteriol. 185:2236-2242.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671-675.

Shanks, R.M., Stella, N.A., Kalivoda, E.J., Doe, M.R., O'Dee, D.M., Lathrop, K.L., Guo, F.L., and Nau, G.J. 2007. A *Serratia marcescens* OxyR homolog mediates surface attachment and biofilm formation. J. Bacteriol. 189:7262-7272.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W.Z., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., and Higgins, D.G.

2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:539.

Storz, G., and Imlay, J.A. 1999. Oxidative stress. Curr. Opin. Microbiol. 2:188-194.

Storz, G., Tartaglia, L.A., and Ames, B.N. 1990. The OxyR regulon. Antonie Van Leeuwenhoek 58:157-161.

Storz, G., Jacobson, F.S., Tartaglia, L.A., Morgan, R.W., Silveira, L.A., and Ames, B.N. 1989. An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of ahp. J. Bacteriol. 171:2049-2055.

Sun, Q., Sun, Y.L., Walker, M.A., and Labavitch, J.M. 2013. Vascular occlusions in grapevines with Pierce's disease make disease symptom development worse. Plant Physiol. 161:1529-1541.

Van Sluys, M.A., de Oliveira, M.C., Monteiro-Vitorello, C.B., Miyaki, C.Y., Furlan, L.R., Camargo, L.E., da Silva, A.C., Moon, D.H., Takita, M.A., Lemos, E.G., Machado, M.A., Ferro, M.I., da Silva, F.R., Goldman, M.H., Goldman, G.H., Lemos, M.V., El-Dorry, H., Tsai, S.M., Carrer, H., Carraro, D.M., de Oliveira, R.C., Nunes, L.R., Siqueira, W.J., Coutinho, L.L., Kimura, E.T., Ferro, E.S., Harakava, R., Kuramae, E.E., Marino, C.L., Giglioti, E., Abreu, I.L., Alves, L.M., do Amaral, A.M., Baia, G.S., Blanco, S.R., Brito, M.S., Cannavan, F.S., Celestino, A.V., da Cunha, A.F., Fenille, R.C., Ferro, J.A., Formighieri, E.F., Kishi, L.T., Leoni, S.G., Oliveira, A.R., Rosa, V.E., Jr., Sassaki, F.T., Sena, J.A., de Souza, A.A., Truffi, D., Tsukumo, F., Yanai, G.M., Zaros, L.G., Civerolo, E.L., Simpson, A.J., Almeida, N.F., Jr., Setubal, J.C., and Kitajima, J.P. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185:1018-1026.

Varela, L., Smith, R., Phillips, P. and Varela, L. G. . 2001. Pierce's disease. UCANR Publications.

Zheng, M., Aslund, F., and Storz, G. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science 279:1718-1721.

Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., LaRossa, R.A., and Storz, G. 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J. Bacteriol. 183:4562-4570.

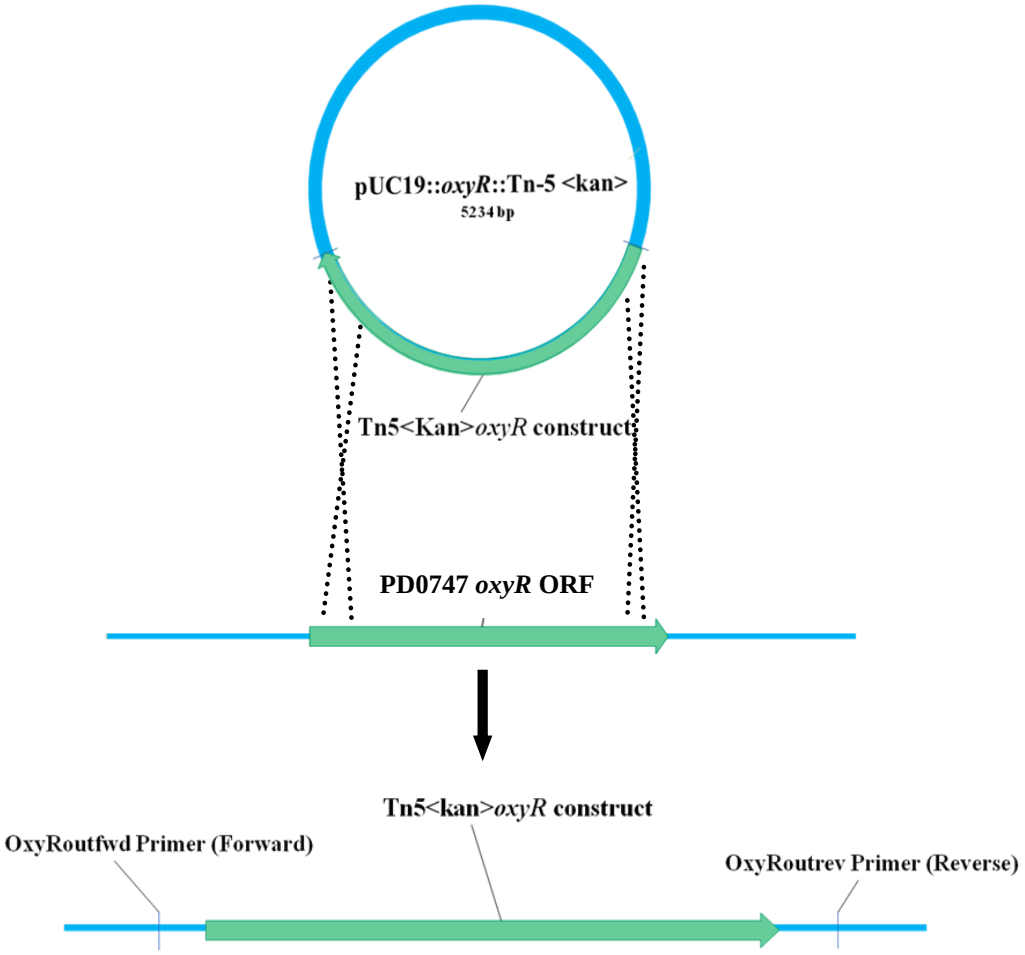


Figure 2.1 Diagram illustrating the site-directed mutagenesis strategy used to obtain the *oxyR* mutant. The wild type *oxyR* gene was replaced by *oxyR*::kan construct by a double crossover event, primers OxyRoutfwd and OxyRoutrev are specific to the regions flanking the *oxyR* gene.

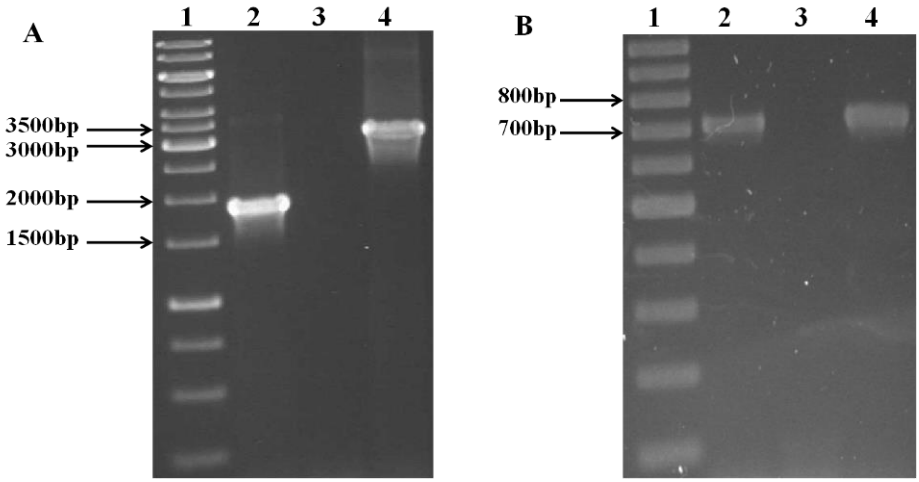


Figure 2.2 PCR confirmation of the *oxyR* mutant. In panel A**,** Lane 1: Gene Ruler^{TM} 1kb DNA ladder; lane 3: negative control; lane 2 and 4: PCR products obtained using primers OxyRoutfwd and OxyRoutrev with DNA template from WT strain and *oxyR* mutant, respectively. The resulting PCR product from WT was 1868bp, and the PCR product from the *oxyR* mutant was 3098bp. In panel B, Lane 1: Gene Ruler^{TM} 100bp DNA ladder; lane 3: negative control; lane 2 and 4: PCR products obtained using primers RST-31 and RST-33 with DNA template from WT or *oxyR* mutant, respectively. The PCR products were 733bp.

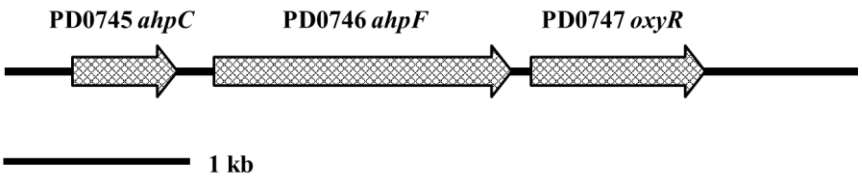
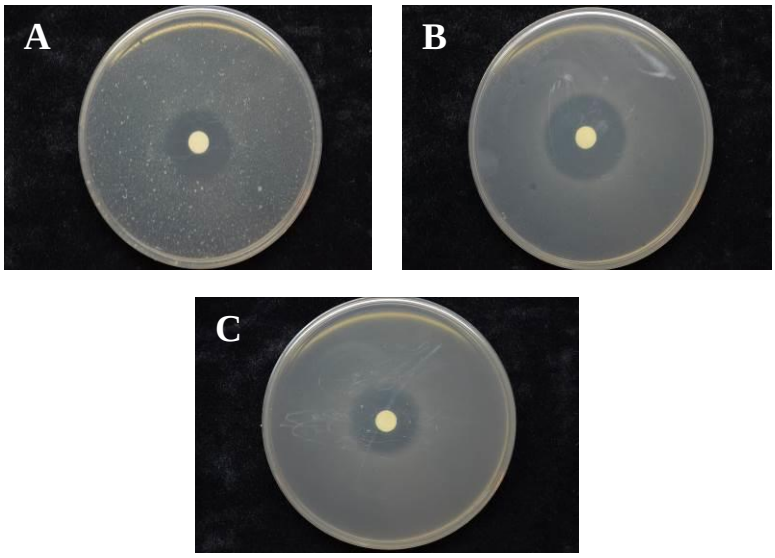


Figure 2.3 Schematic representation of the *oxyR* operon in the *Xf* Temecula1 genome. Based on reverse-transcription PCR data (personal communication, Dr. Michele Igo, UC Davis), *ahpC*, *ahpF* and *oxyR* are organized as an operon. *ahpC* (PD0745) and *ahpF* (PD0746) are predicted to encode subunits C and F of alkyl hydroperoxide reductase. OxyR (PD0747) is annotated as an oxidative stress transcriptional regulator.

```
ECOXYR      MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLFTQA   60
XCOXYR      MNLRDLKYLVALADHKHFGRAATACFVSQPTLSTQIKKLEDELGVPLVERAPRKVMLTPA   60
XFOXYR      MNLRDLKYLIALADYKHFGRAATACFVSQPTLSTQIKKLEGELGVSLVERAPRKVMMTPA   60
            **:***:**:***:::** *** :*.******* **:*** **** *:**: ***::* *

ECOXYR      GMLLVDQARTVLREVKVLKEMASQQGETMSGPLHIGLIPTVGPYLLPHIIPMLHQTFPKL   120
XCOXYR      GREAAMRARSIVAEVEQMKEAARRSQDPEAGTVRLGIFPTLAPYLLPHVVPRIRERFPRL   120
XFOXYR      GREAAIRARSIVAEVEEMKEAARRSRDPEAGAVRLGIFPTLGPYLLPHVVPSIRYRFPQL   120
            *   . :**::: **: :** * :. :  :* :::*::**:.******::* ::  **:*

ECOXYR      EMYLHEAQTHQLLAQLDSGKLDCVILALVKESEAFIEVPLFDEPMLLAIYEDHPWANREC   180
XCOXYR      ELLLIEEKSDQLIHQLREGRMDAALLALPLQDEQLHAEFLFEEPFVLAVPEGHPLSRHDS   180
XFOXYR      ELLLVEEKSDELLAQLREGKLDAALLALPLHDEQLHTEFLFEEPFVLAVPEGHPLATRRE   180
            *: * * ::.:*: ** .*::*..:***  ..* :    **:**::**: * ** : :

ECOXYR      VPMADLAGEKLLMLEDGHCLRDQAMGFCFEAGADEDTHFRATSLETLRNMVAAGSGITLL   240
XCOXYR      MTLDDLSEQRLLLLEDGHCLREQALDVCHLAGALEKSEFQATSLETLRQMVAANVGVTLL   240
XFOXYR      MTMEELADERLLLLQDGHCLREQALDVCHMTGASEKSEFQATSLETLRQMVVANVGITLL   240
            : : :*: ::**:*:******:**: .*. :** *.:.*:********:**.*  *:***

ECOXYR      PALAVPPERKRDGVVYLPC--IKPEPRRTIGLVYRPGSPLRSRYEQLAEAIRARMDGHFD   298
XCOXYR      PMLAVKPPVARSENIRLIRFREDKQPNRRIAMAWRRSSAMTAFLEQLSQIFKELPDSLFT   300
XFOXYR      PLLSVKPPVVCSESIRLINFPLDKQPSRRIAMVWRRSSAMTTFLERFSSMFKELPKELFD   300
            * *:* *    .  : *     . :* * *.:.:* .* : :  *:::. ::   .  *

ECOXYR      KVLKQAV------       305
XCOXYR      LDQPASGPKAVAA       313
XFOXYR      LPQTAVLYKGR--       311
```

Figure 2.4 Alignment of OxyR orthologs using Clustal Omega. Genebank accession number: ECOXYR: *Escherichia coli* K-12 OxyR, AAC76943.1. XCOXYR: *Xanthomonas campestris* pv. *campestris*, NP_636223.1. XFOXYR: *Xylella fastidiosa* Temecula1 OxyR, NP_778967. Asterisks indicates a fully conserved residue in the two OxyR proteins. A colon indicates strong conservation between two groups. A period indicates weak conservation between two groups. The two cysteine residues in the positions of 199 and 208 were highlighted with black color. In *E. coli*, OxyR is activated by forming a disulfide bond between cysteine 199 and 208 (Zheng et al., 1998).



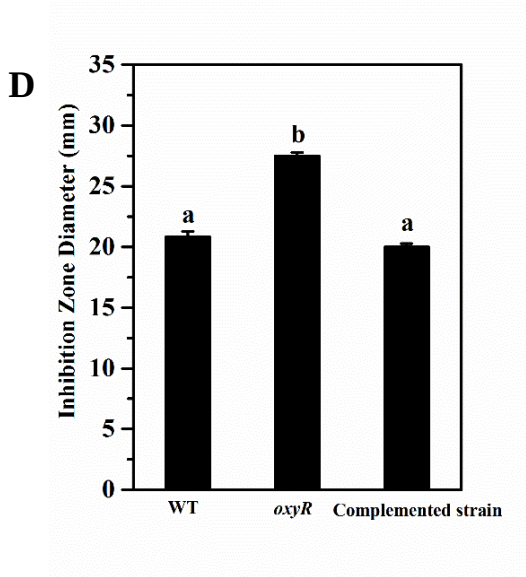


Figure 2.5 OxyR protects *Xf* against H_2O_2 stress. Sensitivity to H_2O_2 was examined using a disk diffusion method as described previously with slight modifications (Matsumoto et al., 2009). Panels A, B and C show inhibition zones surrounding the Whatman paper disk containing H_2O_2. A: WT; B: *oxyR* mutant; C: Complemented strain PW32. Panel D shows the quantitative comparison of the inhibition zone diameters. The error bars represent standard errors of means. Strains with different letters on the bars indicate significant difference. Compared with WT, the *oxyR* mutant is significantly more sensitive to H_2O_2 ($P<0.05$). Three independent biological replications were performed. Three technical replications were included in each biological replication. Results from one representative biological replication were shown here. The results were analyzed by a Tukey's test following one-way analysis of variance (ANOVA).

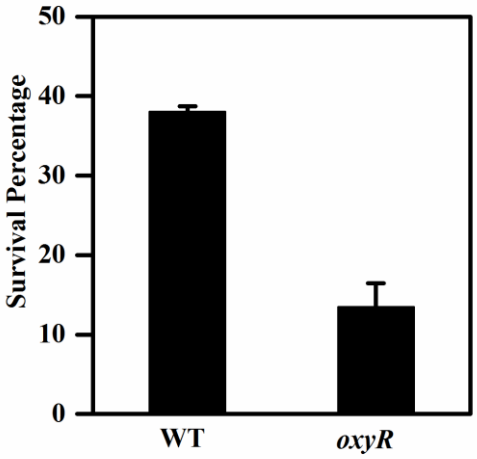
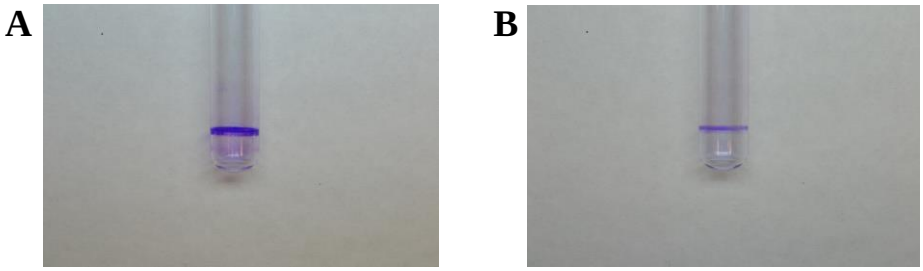


Figure 2.6 *Xf* requires OxyR for survival under H_2O_2 stress. WT and *oxyR* mutant cells were treated with five microliter of 100 µM H_2O_2. The cells without H_2O_2 treatment served as controls. The survival percentage was defined as below: 100(H_2O_2 treated cells CFU/mL)/(non-treated cells CFU/mL). Results indicated that the *oxyR* mutant survival percentage under H_2O_2 stress is significantly lower than WT ($P<0.05$). Error bars indicate the standard errors of the means. Results were analyzed by a two-tailed Student's t test. Two independent biological replications were performed. Three technical replications were included for each biological replication. Results from one representative biological replication were shown here.



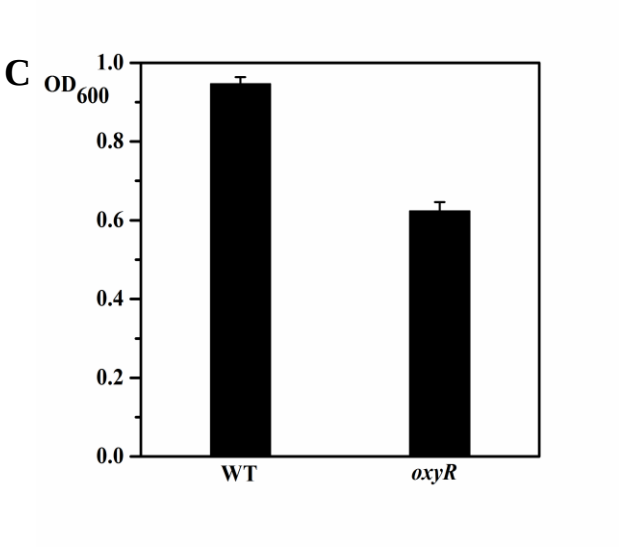


Figure 2.7 OxyR contributes to surface attachment. The ability to attach to a glass surface was examined using a crystal violet staining method as previously described (Espinosa-Urgel et al., 2000). Panels A and B show the crystal violet stained cells on the glass surface, A: WT; B: *oxyR* mutant. Panel C shows the optical density comparison for the crystal violet stained cells. The *oxyR* mutant has less attached cells than WT ($P<0.05$), indicating it is significantly reduced in the ability to attach to the glass surface. Three independent biological replications were performed. Twelve technical replications were included in each biological replication. Results from one representative biological replication were shown here. The error bars indicate the standard errors of means. Results were analyzed by a two-tailed Student's t test.

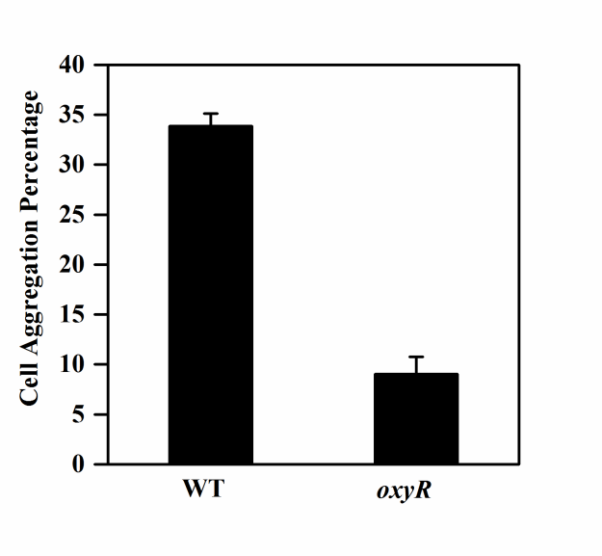


Figure 2.8 OxyR contributes to *Xf* cell-cell aggregation. The cell-cell aggregation percentage was compared between WT and *oxyR* mutant in the glass tubes as previously described (Burdman et al., 2000; Leite et al., 2004; Guilhabert and Kirkpatrick, 2005). Compared with WT, the *oxyR* mutant has a significantly reduced ability to aggregate ($P<0.05$). The error bars indicate the standard errors of means. Four independent biological replications were performed. Three technical replications were included in each biological replication. One representative biological replication results were shown here. Results were analyzed by a two-tailed Student's t test.

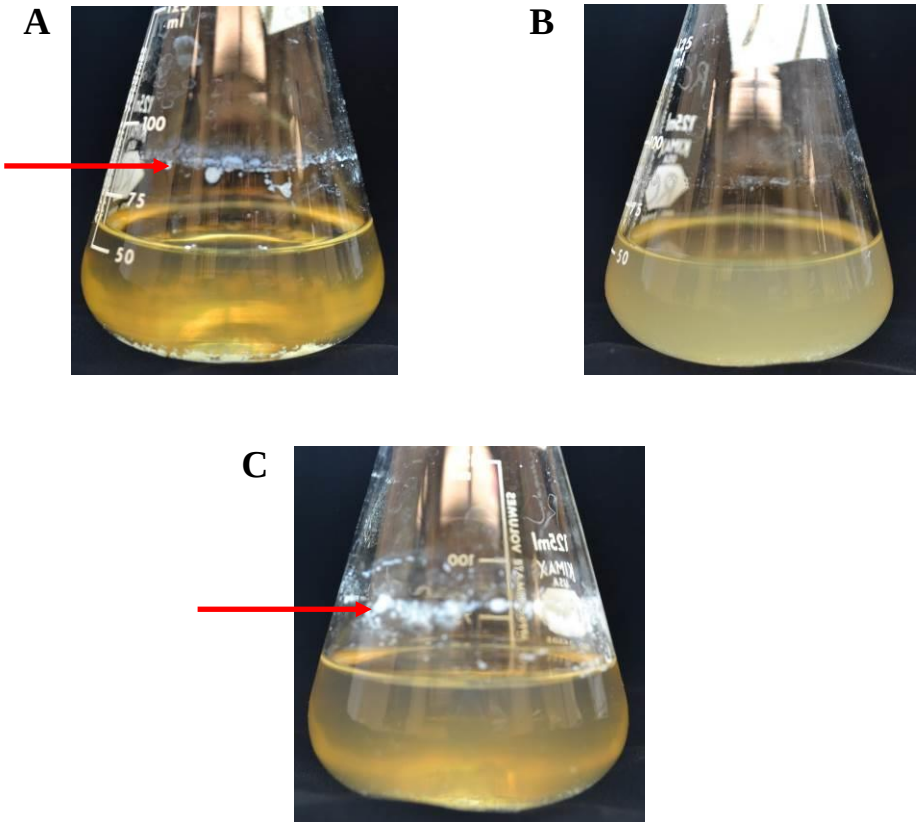


Figure 2.9 OxyR contributes to *Xf* biofilm formation in a glass flask. Seven days old cells were harvested and adjusted to OD_{600}=0.25. Two milliliter of bacterial culture were diluted in 50mL PD3 liquid medium in the 125mL glass flask. Biofilms formed at air-liquid interface were observed after four days incubation at 28 ℃ with 180 rpm shaking. A: WT; B: *oxyR* mutant; C: complemented strain. Red arrows point to the biofilm formed by WT and complemented strain. Compared with WT, *oxyR* mutant has the reduced biofilm formation. Complemented strain restored biofilm formation.

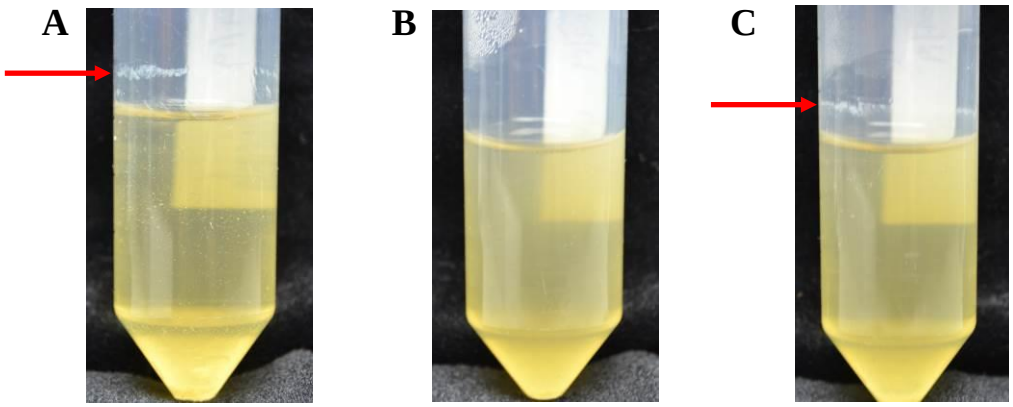


Figure 2.10 OxyR contributes to the *Xf* biofilm formation in polypropylene tubes. Seven day old cells were harvested and adjusted to OD_{600}=0.1. 200 µL of bacterial culture was diluted in 20mL PD3 liquid medium in 50mL polypropylene tubes. Biofilms formed at the air-liquid interface of tubes were observed after six days incubation at 28 ℃ with 180 rpm shaking. A: WT; B: *oxyR* mutant; C: complemented strain. Red arrows point to the biofilm formed by WT and complemented strain. Compared with WT, *oxyR* mutant has reduced biofilm formation. Complemented strain restored biofilm formation.

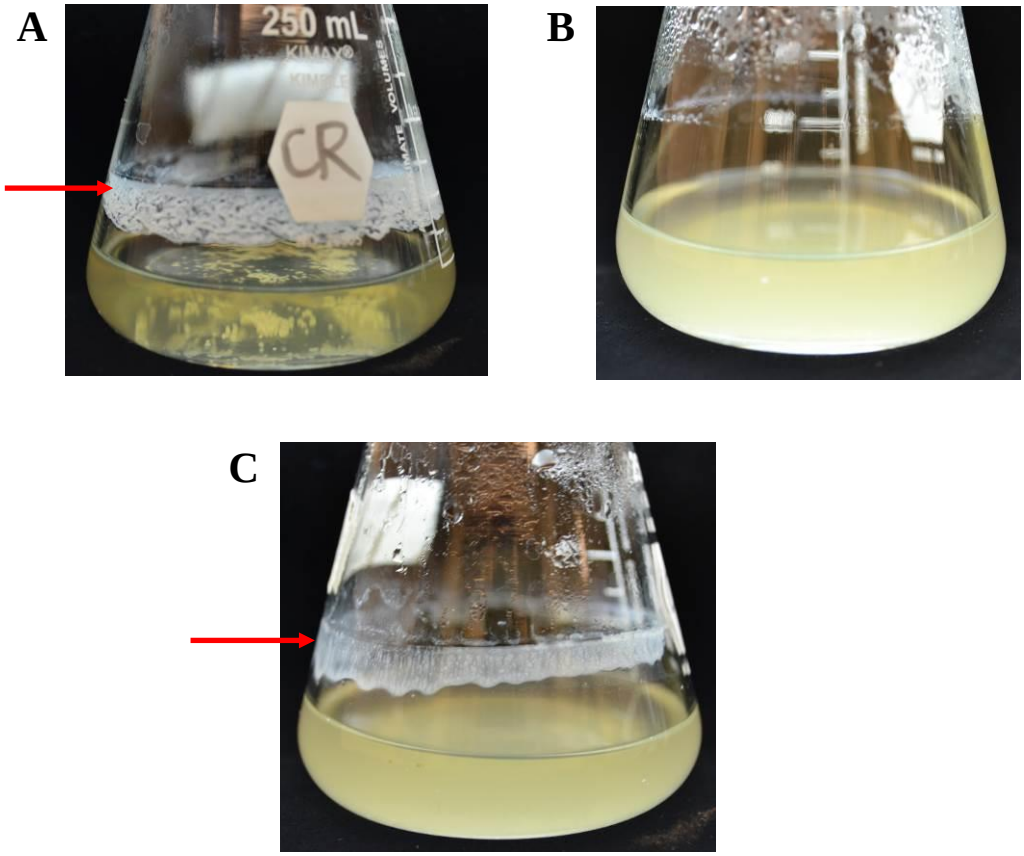


Figure 2.11 AhpC and AhpF are not involved in *Xf* biofilm formation. Seven days old cells were harvested and adjusted to OD_{600}=0.25. One milliliter bacterial culture was diluted in 50mL PD3 liquid medium in the 250mL glass flask. Biofilm formed at the air-liquid interface was observed after four days incubation at 28 ℃ with 180 rpm shaking. A: WT; B: *oxyR* mutant; C: Δ*ahpCF*. Red arrows point to biofilm formed by WT and Δ*ahpCF*. Biofilm formed by Δ*ahpCF* is similar to the WT, indicating AhpC and AhpF are not involved in the biofilm formation.

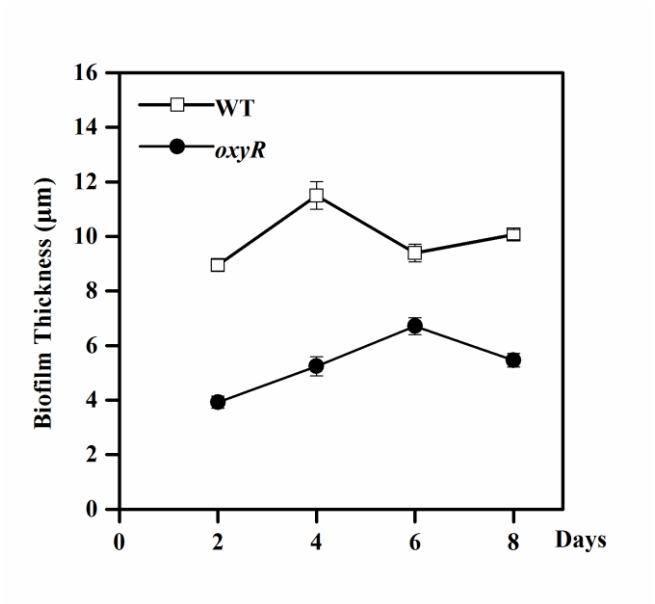


Figure 2.12 OxyR contributes to biofilm formation. The thickness of confocal laser scanning microscopy (CLSM) captured biofilm z-stack was quantified by Imaris software (Bitplane USA, South Windsor, CT). Compared with WT, *oxyR* mutant biofilm has a significantly reduced thickness ($P<0.05$) in the different stages (two, four, six and eight days). The error bars represent standard errors of means. Results were analyzed by a two-tailed Student's t test.

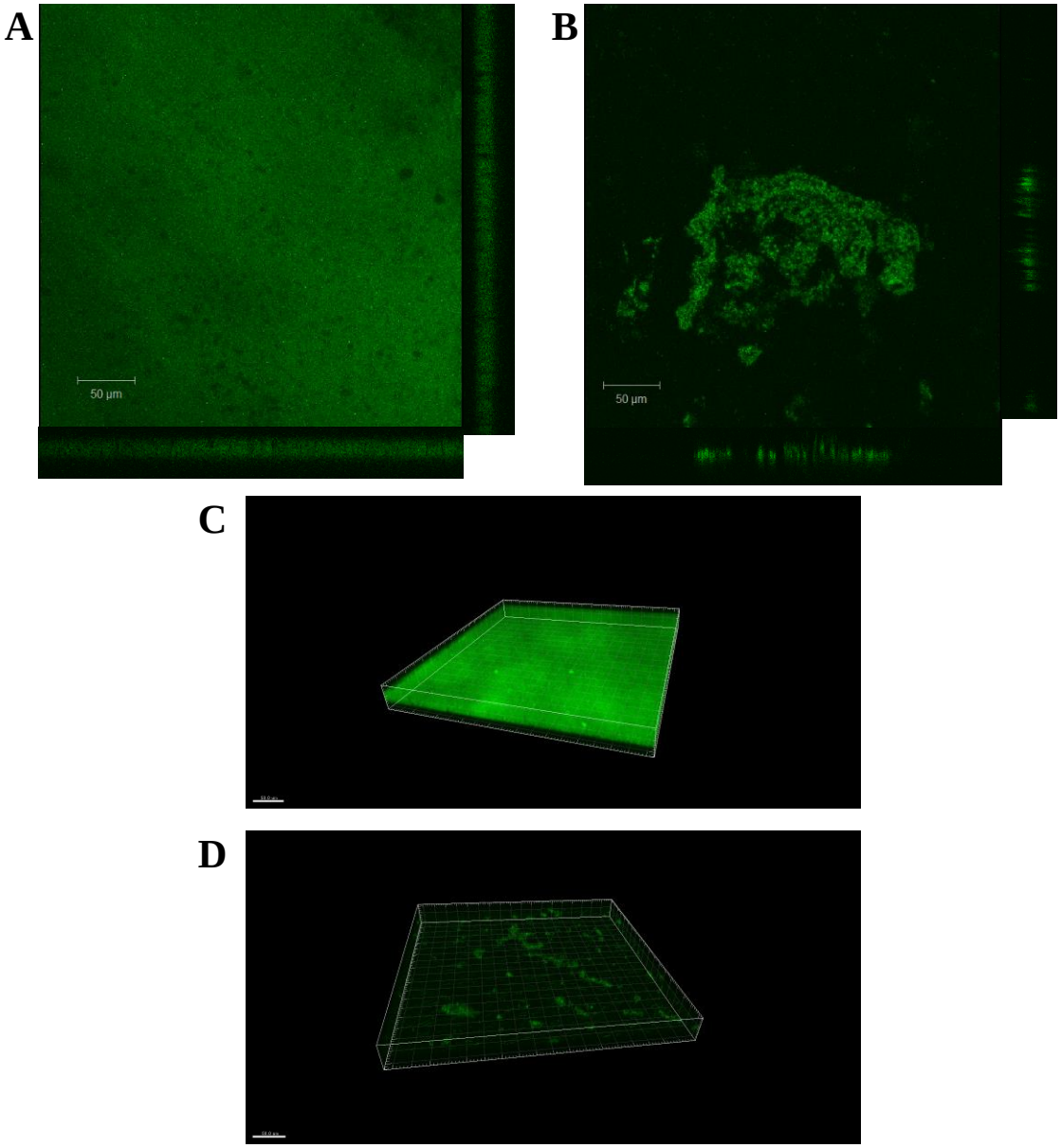


Figure 2.13 OxyR contributes to three dimensional biofilm formation. Three dimensional biofilm formation was compared between WT and the *oxyR* mutant by confocal laser scanning microscopy (CLSM) using a previous protocol (Roper et al., 2007a). A: Orthogonal view for WT formed biofilm after eight days; B: Orthogonal view for *oxyR* mutant formed biofilm after eight days; C: Three dimensional view for WT formed biofilm after eight days; D: Three dimensional view of *oxyR* mutant formed biofilm after eight days. Compared with WT, the *oxyR* mutant has fewer attached cells and was unable to develop into a mature biofilm. Representative pictures were shown here. Scale bars represent 50 µm in all four pictures.

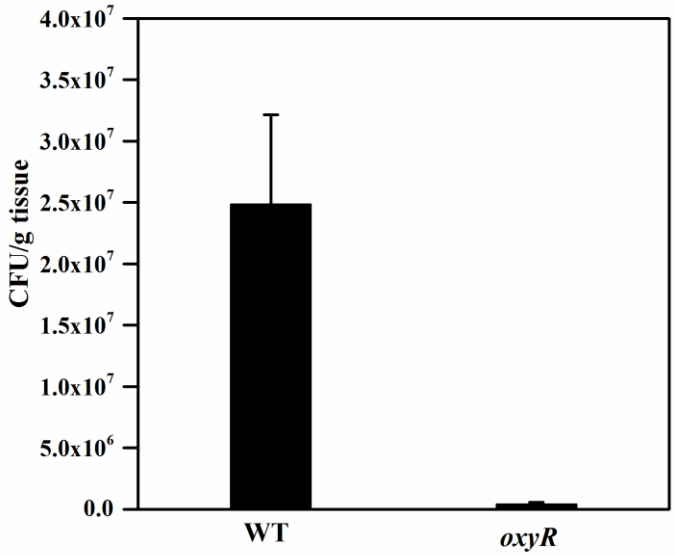


Figure 2.14 OxyR contributes to *Xf* colonization in the grapevines. Following eleven weeks post-inoculation, the populations of WT and *oxyR* mutant were quantified from the leaf petioles at point of inoculation (POI) from Thompson seedless grapevines using a previously described protocol (Roper et al., 2007b). Compared with WT, *oxyR* mutant has a significantly lower population ($P<0.05$), indicating that the *oxyR* mutant was compromised in xylem colonization. Error bars indicate the standard errors of the means. The results were analyzed by a two-tailed Wilcoxon rank-sum test.

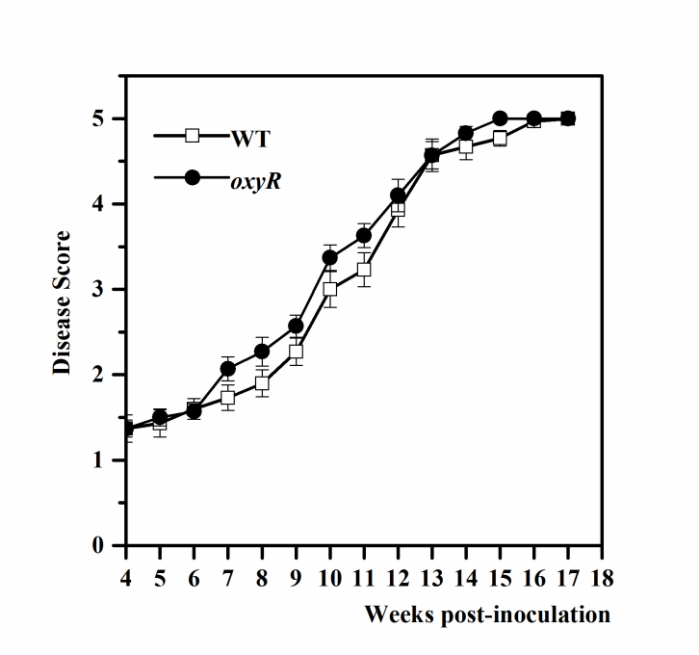


Figure 2.15 OxyR is not involved in *Xf* virulence in grapevines. WT or *oxyR* mutant cells were pin-prick inoculated into Thompson seedless grapevines as previously described (Hill and Purcell, 1995). PD symptoms were rated weekly. The means of three biological replications were shown here. Fifteen technical replications were included in each biological replication. The error bars indicate the standard errors of means. Based on a Tukey's test following one-way analysis of variance with repeated measures (ANOVA-RM), there was no significant virulence difference observed between WT and *oxyR* mutant at the 0.05 level.

Table 2.1 Bacterial strains,plasmids and primers used in this study		
	Characteristics	**Reference**
Bacterial Strains		
Xylella fastidiosa Temecula1	subsp. *fastidiosa* , wild type isolate from grape	(Guilhabert et al. 2001)
Temecula1 *oxyR* mutant (PW31)	EZ-Tn5 transposon inserted in the *oxyR* gene ORF, Km^R*	This study
Complemented *oxyR* mutant (PW32)	*oxyR* operon inserted in the neutral sites of *oxyR* mutant	This study
Temecula1 Δ*ahpCF*	Cm^R	Dr. Igo Michele, UC Davis
Escherichia coli TOP10	F⁻ *mcr* A Δ(*mrr-hsd* RMS-*mcr* BC) φ80*lacZ* ΔM15 Δ*lac* X74*rec* A1	Invitrogen
	ara D139 Δ(*ara-leu*) 7697 *gal* U *gal* K *rps* L (Str^R) *end* A1 *nup* G λ-	
Plasmids		
pUC19	Ap^R	Invitrogen
pAX1Cm	Ap^R, Cm^R	(Matsumoto et al. 2009)
pPW01	Ap^R, pUC19::*oxyR*	This study
pPW02	Ap^R, Km^R, pUC19::Tn5::*oxyR*	This study
pPW11	Ap^R, Cm^R, *oxyR* operon cloned into pAX1Cm	This study
Primers		
OxyRfwdEcoRI	5'-GCAGT**GAATTC**CAGATTGTTATTGCGATG-3'**	This study
OxyRrevHindIII	5'-TCGAC**AAGCTT**CAATTTCAAAACGCTCC-3'	This study
KAN-2 FP-1	5'-ACCTACAACAAAGCTCTCATCAACC-3'	Epicentre
KAN-2 RP-1	5'-GCAATGTAACATCAGAGATTTTGAG-3'	Epicentre
OxyRoutfwd	5'-GTATTGCAACGCAAGCTACGCAGT-3'	This study
OxyRoutrev	5'-ACGTGCAGAGTCGAGATGGGTAAT-3'	This study
Operon OxyR fwd XhoI	5'-ATCTA**CTCGAG**GTGTAGTTTGTGGGGCG-3'	This study
Operon OxyR rev XhoI	5'-CCTGC**CTCGAG**GAAAGAGCAAGAA ACCTTA-3'	This study
RST-31	5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3'	(Minsavage et al, 1994)
RST-33	5'-CACCATTCGTATCCCGGTG-3'	(Minsavage et al, 1994)
PW-For	5'-AGAAGAGCGCGAGATTGAGTTGGA-3'	This study
PW-Rev	5'-AAACAGGCTTCACATGGCTCAACG-3'	This study
*Ap^R,Km^R, Str^R and Cm^R indicate resistance to ampicillin, kanamycin, streptomycin and chloramphenicol		

Table 2.1 Bacterial strains, plasmids and primers used in Chapter 2.

3. Chapter 3

XpsE, a putative Type II secretion system ATPase, is required for host colonization and virulence in grapevines for *Xylella fastidiosa*

Abstract

Xylella fastidiosa (*Xf*), a xylem-limited fastidious bacterium, is the causal agent of Pierce's Disease (PD). To facilitate its systemic spreading in the xylem vessels, *Xf* employs several cell wall degrading enzymes (CWDEs) to degrade pit membranes in xylem. These CWDEs are predicted to be secreted by the Type II secretion system (T2SS). To study the role of *Xf* T2SS in PD development, we constructed an *Xf* Δ*xpsE* mutant. In the *Xf* genome, *xpsE* is predicted to encode a T2SS ATPase, which supplies energy for protein secretion through ATP hydrolysis. Through an *in vitro* secretome analysis, we found a mutation in *xpsE* altered the *Xf* extracellular protein profile. *In planta* results showed that *xpsE* mutant has significantly reduced virulence and colonization ability in grapevines.

Introduction

Xylella fastidiosa (*Xf*), a xylem-limited bacterium, is the causal agent of Pierce's Disease (PD) of grapevine among other economically important diseases, such as almond leaf scorch and citrus variegated chlorosis (Hopkins, 1989). Since the sequencing of *Xf* Temecula1 genome (Van Sluys et al., 2003), significant progress has been made in elucidating the molecular interactions between *Xf* and grapevine. In particular, a *pglA* mutant, which cannot produce the host cell wall degrading enzyme, polygalacturonase (PG), is nonpathogenic and deficient in systemic colonization of grapevines (Roper et al., 2007). Xylem pit membranes are thin walls, which divide two pit cavities in a pit-pair (Esau, 1977). It is composed of the middle lamella and primary cell walls (Esau, 1977). The pit membranes are involved in limiting the pathogen movement in the host plant xylem (Nakaho et al., 2000). The inter-vessel movement of *Xf* through pit membranes was observed and proposed to be a crucial strategy for *Xf* systemic colonization in grapevines (Newman et al., 2003). The *Xf* CWDEs are known to be involved in grapevine primary cell wall degradation and virulence (PG and endo-1,4-β-endoglucanase (EGase)) and the majority of those that have yet to be characterized are predicted to be secreted by the T2SS (Simpson et al., 2000; Roper et al., 2007; Pérez-Donoso et al., 2010). It is hypothesized that *Xf* utilizes T2SS to secrete the CWDEs involved in degrading the pit membranes barriers that separate one xylem vessel from another (Chatterjee et al., 2008). One PG is critical for xylem pit membrane dissolution that is necessary for systemic colonization in grapevine and, subsequently, for pathogenicity (Roper et al., 2007). This indirectly indicates that the PG is secreted extracellularly. In addition, the *Xf* genome

contains several open reading frames (ORFs) predicted to encode additional CWDEs, including a 1,4-β-cellobiosidase and several EGases (Van Sluys et al., 2003). The direct role of EGases in virulence has not been demonstrated. However, a mixture of a purified PG and an *Xf* EGase was able to enlarge pit membrane diameters of healthy grapevine stem explants (Pérez-Donoso et al., 2010). The genome of *Xf* also contains genes encoding several putative proteases (Van Sluys et al., 2003). Indeed, proteome analysis revealed the presence of several proteases in the extracellular space, including a zinc protease and two serine proteases in *Xf* subsp. *pauca* (Citrus Variegated Chlorosis strain) (Smolka et al., 2003). Similarly, we also identified a serine protease in the secretome of wild type *Xf* liquid culture. Protease activity has also been associated with *Xf* grown in liquid culture *Xf*, but the biological role of these enzymes during the infection process has not been demonstrated (Fry et al., 1994; Maria Fedatto et al., 2006).

The T2SS is one of the major protein secretion systems found in gram-negative bacteria. The T2SS apparatus spans the inner cell membrane, periplasm and outer membrane and provides a channel for the secretion of proteins. It is composed of 12-16 different proteins (Table 3.1), depending on the bacterial species. These are designated as Gsp (general secretory pathway), but in *Xf*, the Xps (*Xanthomonas* protein secretion) nomenclature has been adopted (Van Sluys et al., 2003). The genes encoding the T2SS components are organized as an operon and include *gspC-M, gspO, gspAB, gspN,* and *gspS* (Filloux, 2004; Jha et al., 2005). The T2SS is composed of four subassemblies, which include an outer-membrane complex, a pseudopilus, an inner-membrane platform and a secretion ATPase (Korotkov et al., 2012). The outer-membrane complex is mainly

comprised of protein D, which provides a channel for exporting proteins into extracellular space. In some bacterial species, a small outer-membrane lipoprotein S is also present whose role is to anchor protein D in the outer-membrane (Jha et al., 2005; Korotkov et al., 2012). Subunit proteins, G, H, J, I and K make up the pseudopilus, which is a fibrous structure localized in the periplasm (Korotkov et al., 2012). Protein O cleaves and methylates the N-terminus of proteins G, H, I, J and K, which is necessary for grouping these subunits together into a pseudopilus (Campos et al., 2013). The inner-membrane platform is composed of numerous copies of at least four proteins M, L, F and C (Korotkov et al., 2012). Protein E is an ATPase that is localized in the cytoplasm, and provides energy through ATP hydrolysis that drives protein secretion through the T2SS (Filloux, 2004; Korotkov et al., 2012). The function and location of protein N is still unknown (Korotkov et al., 2012). In some bacteria, there are two extra proteins A and B. They can form a complex in the cytoplasmic membrane, and may transfer energy from ATP hydrolysis to the secretion step (Filloux, 2004). Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Pugsley, 1993; Voulhoux et al., 2001). The T2SS then uses a pilus-like piston to push proteins through the T2SS channel (Korotkov et al., 2012). This piston action is a function of the cyclic assembly and disassembly of pilin subunits (primarily XpsG).

The T2SS has been found in many bacteria, including human and plant pathogens (Cianciotto, 2005). Specifically, for plant pathogenic bacteria, the existence of a T2SS has been reported in several genera, including *Erwinia*, *Pseudomonas*, *Xanthomonas* and

Ralstonia (Jha et al., 2005). The primary function of the T2SS is in nutrient acquisition and many of the known T2SS secreted proteins are enzymes involved in degradation of biopolymers including carbohydrates found in plant cell walls. These CWDEs include the PG, protease, endoglucanase and xylanase among others (Cianciotto, 2005; Jha et al., 2005). These enzymes can degrade different components of plant cell wall, which facilitates access to nutrients derived from the host and also contributes to systemic bacterial colonization (Walton, 1994; Jha et al., 2005).

In silico analysis of the *Xf* Temecula1 genome indicates that it possesses all the genes necessary to encode a functional T2SS (Van Sluys et al., 2003). These genes are annotated as *xpsE-xpsN* and *xpsD* in map order (Figure 3.1) and share highest homology to the same locus in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Jha et al., 2005). In the *Xoo* system, the T2SS, specifically XpsF, XpsD and XpsE, are all required full virulence in rice (Ray et al., 2000; Sun et al., 2005), XpsF and XpsD are necessary for xylanase secretion (Ray et al., 2000) and XpsE is required for xylanase and cellulase secretion (Sun et al., 2005). The role of the T2SS in *Xf* virulence is unknown. In this study, I demonstrated that a functional T2SS is, in fact, necessary for *Xf* virulence in grapevine.

Materials and Methods

Bacterial strains, plasmids and PCR primers. All bacterial strains, plasmids and PCR primers used in this study are listed in Table 3.2.

Media and growth conditions for bacterial strains. *Xf* was grown in solid or liquid PD3 medium (Davis et al., 1981) at 28 ℃. *Escherichia coli* strains were cultured at 37 ℃ in Luria-Bertani (LB) medium. For the selection of transformants, antibiotics were supplemented in the media at following final concentrations: 5 μg/ml gentamicin (selection for *Xf* transformants), 10 μg/ml gentamicin (selection for *E. coli* transformants), 100 μg/ml ampicillin (selection for *E. coli* transformants), 5 μg/ml chloramphenicol (selection for *Xf* transformants).

DNA manipulations. *Xf* Temecula1 (Guilhabert et al., 2001) genomic DNA was extracted using the DNeasy tissue kit according to manufacturer's instructions (Qiagen, Chatsworth, CA). Preparation of *Xf* electrocompetent cells and subsequent electroporations were performed as described (Matsumoto et al., 2009). Plasmid DNA was isolated from *E. coli* cells using Zyppy plasmid miniprep kit (Zymo Research, Irvine, CA). Restriction enzyme digestions, ligations, and *E. coli* transformation were all performed by standard procedures.

Prediction of putative *Xf* T2SS secreted proteins. In prokaryotes, many secreted proteins possess an N-terminal signal peptide (SP), which guide the protein across the cytoplasmic membrane (von Heijne, 1990). Usually, this SP is a temporary extension of the N-terminus extension of the proteins, and will be subsequently removed by signal peptidases (von Heijne, 1990). Several putative *Xf* T2SS secreted proteins were predicted to have an N-terminal signal peptide using the program SignalIP (Petersen et al., 2011).

The potential cleavage site was also predicted using SignalIP. However, some reported bacterial secreted proteins are absent of an obvious SP, which are described as leaderless secretory proteins (Harth and Horwitz, 1999; Bendtsen et al., 2005). Thus, *Xf* proteins without an apparent SP were analyzed for the possibility of being leaderless secretory proteins by program SecretomeP (Bendtsen et al., 2005).

Creation of the Δ*xpsE* mutant. *Xf* Temecula1 genomic DNA was used as template for amplifying the flanking regions of the *xpsE* gene (PD0732). The upstream 500bp flanking region of the *xpsE* gene was amplified by PCR using primer pair XpsE LF fwd EcoRI/XpsE LF rev Gm. The downstream 500bp flanking region of the *xpsE* gene was amplified by PCR using primer pair XpsE RF fwd Gm/XpsE RF rev HindIII. To avoid a possible polar mutation for genes downstream of *xpsE* in the predicted *xps* operon, the gentamicin resistance cassette was amplified (without the terminator sequence) using primer pair XpsE Gm fwd/XpsE Gm rev from plasmid pBBR1MCS-5 (Kovach et al., 1995). Following this, these three separate DNA fragments were assembled together using overlapping PCR. In the initial five cycles, three individual PCR products were added to the PCR reaction tube without primers. Then, primer pair XpsE LF fwd EcoRI/XpsE RF rev HindIII designed to amplify the total overlapped product was added to the PCR and the reaction was allowed to run for 25 more cycles. The expected overlapping PCR product and pUC19 vector were both double digested with EcoRI and HindIII, and ligated to create the mutagenesis construct pPW04. The fidelity of pPW04 was confirmed by sequencing.

Xf Temecula1 electrocompetent cells were prepared as previously described (Matsumoto et al., 2009). 200 ng of the mutagenesis construct, pPW04, was electroporated into *Xf* WT competent cells and transformants were selected on PD3 solid medium containing 5µg/ml gentamicin. The *Xf* Δ*xpsE* was confirmed by PCR using primer pair XpsEoutfwd/XpsEoutrev, which is designed to anneal to DNA regions flanking the double cross-over event (Figure 3.2). The amplicon from Δ*xpsE* was sequenced to confirm fidelity.

Colony morphology on defined medium. *Xf* WT and Δ*xpsE* cells were grown on PD3 solid medium for seven days. Following this, the cells were harvested and resuspended in XFM liquid medium (Killiny and Almeida, 2009) and adjusted to OD_{600}=1.0. Thirty microliter of the bacterial culture was striped onto XFM solid medium supplemented with 0.01% pectin (P8471 Sigma-Aldrich) (Killiny and Almeida, 2009) to monitor induction of exopolysaccharide (EPS) production for each strain. Colony morphologies were compared between WT and Δ*xpsE* after 14 days incubation at 28 ℃ by a Leica MZIII Pursuit stereo microscope (Leica Microsystems Inc., Buffalo Grove, IL) with 1X magnification.

Secretome analysis. *Xf* WT and Δ*xpsE* cells were grown on solid PD3 medium for seven days. Following this, the cells were harvested, resuspended in XFM liquid and transferred to XFM-pectin solid medium (Killiny and Almeida, 2009). Following seven days incubation at 28 ℃, cells were harvested and resuspended in XFM liquid. And one

milliliter of this bacterial suspension (OD_{600}=0.3) was diluted into 25mL of modified XFM-pectin liquid medium (without bovine serum albumin (BSA)) and incubated at 28 ℃ while shaking at 180rpm. After 20 days of growth, the liquid culture was centrifuged at 6,000 rpm for 15 minutes to separate the cells from the supernatant. The supernatant was initially filtered through a 0.22 μm polyethersulfone membrane (Millipore, Billerica, MA), then transferred to dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA; molecular cutoffs, 1000) and placed in polyethyleneglycol bisphenol A epichlorohydrin copolymer (P2263, Sigma-Aldrich) to concentrate the supernatant. The concentrated supernatant was then precipitated with two volumes of cold acetone overnight at -20 ℃. Following this, the mixture was centrifuged at 13,000 rpm at 4 ℃ for 15 minutes and the supernatant was removed. The pellet was rinsed twice with acetone and the supernatant was discarded. The protein pellet was air dried and suspended in buffer (Smolka et al., 2003) composed of 10mM trisaminomethane (Tris) (pH 8.8), 0.5% w/v sodium dodecyl sulfate (SDS), 100mM dithiothreitol (DTT), 5mM ethylenediaminetetraacetic acid (EDTA) and 1mM phenylmethanesulfonylfluoride (PMSF), stored in -80 ℃. The protein profile was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), stained with coomassie brilliant blue and subsequent identification was carried out by mass spectrometry in the UC Riverside proteomics facility.

Protein identification by mass spectrometry. The sample for mass spectrometry analysis was prepared using a modified version of an in gel digestion method described

as below (Rosenfeld et al., 1992). Protein samples were subjected to SDS-PAGE until the samples just entered the resolving gel. The gel was then stained by coomassie brilliant blue. The band in the gel was excised and cut into small pieces (about $1mm^2$) and transferred into a 1.5 mL centrifuge tube. Destain solution (50% acetonitrile, 50mM ammonium bicarbonate, pH=8) was added into centrifuge tubes and the gel pieces were destained to colorless by vortexing and subsequently dehydrated with 100% acetonitrile for about 15 minutes until a white color appeared. Following this, the acetonitrile solution residue was discarded from tubes. The gel pieces were fully dried in a speedvac at room temperature for about 20 minutes. The proteins in the gel were digested overnight using trypsin solution (10-20 μg/mL) in a 37 ℃ waterbath. Trypsin (Roche Bioscience, Palo Alto, CA) was dissolved in 50mM ammonium bicarbonate, pH=8. After overnight digestion, the digested product-peptides were sequentially extracted using four different extraction buffers: (1). 5% acetic acid; (2). 5% formic acid; (3). 5% acetic acid, 50% acetonitrile and (4). 5% formic acid, 50% acetonitrile. In each extraction step, the digested sample was mixed with adequate extraction buffer and sonicated in a waterbath sonicator for 30 minutes. Extractions were combined and transferred to a new 1.5 mL tube. The extracted sample was then centrifuged at 14,000 rpm for 30 minutes. The supernatant was transferred to a new 1.5 mL tube and fully dehydrated into a pellet in a speedvac at moderate temperature. The pellet was then fully dissolved in 20 μL of 0.1% trifluoroacetic acid (TFA). After centrifugation at 14,000 rpm for 30 minutes, the resulting supernatant was transferred into a new tube. The proteomic analysis was carried out by a 2D nano-Acquity UPLC and Q-TOF premier tandem mass spectrometer

(Waters, Milford, MA) as previously described (Drakakaki et al., 2012). Initially, a two dimensional liquid chromatography (LC) was employed to fraction, desalt, pre-concentrate and separate peptides samples. In the first dimension LC under high pH reverse phase, solvent A (20 mM ammonium formate, pH=10) and solvent B (100% acetonitrile) were used. Sample peptides were online fractioned by an XBridge BEH130 C18 trap column (8 µm particle, 300 µm i.d., 5 cm long, product number 186003682; Waters) with a gradient percentage of solvent B. Ten fractions and two flushes with a gradient percentage of solvent B were included. In the second dimension of LC under low pH reverse phase, solvent A (0.2% formic acid in water) and a gradient percentage of solvent B (0.2% formic acid in acetonitrile) were used as a mobile phase in a Symmetry C18 trap column (5 µm particle, 180 µm i.d., 20 mm long, product number 186003514; Waters) to desalt and pre-concentrate peptides and in a BEH130 C18 column (1.7 µm particle, 75 µm i.d., 20 cm long, product number 186003544; Waters) for peptide separation.

The settings for the MS instrument were the same as described (Drakakaki et al., 2012), which are listed below: sample cone voltage (42 V), capillary voltage (3 kV), extraction cone voltage (3.0 V), collision energy of MS1 scan (5 V), time of flight (TOF) reflectron (V-mode), source temperature (100 ℃), MS resolution (10000 at FWHM (full width at half maximum) for V-mode), MCP/TDC detector (micro-channel plate/time-to-digital converter) voltage (1950 V). A data-dependent acquisition (DDA) survey method was employed in Nano-LC/MS/MS analysis for peptide samples. Following this, all data were analyzed by PLGS 2.2.5 (ProteinLynx Global Server, Waters). The Mascot 2.3

search engine was used to BLAST the sequences obtained against those in the *Xf* Temecula1 protein database.

Virulence assay. *Xf* WT and Δ*xpsE* cells were grown on solid PD3 medium for seven days, harvested in 1XPBS and adjusted to OD_{600}=0.25. *Vitis vinifera* cv. Merlot (2012), *Vitis vinifera* cv. Cabernet Sauvignon and Chardonnay (2013) were separately pin-prick inoculated with 20 µL of bacterial solution using a 20-gauge syringe needle as previously described (Hill and Purcell, 1995). Grapevines were inoculated twice on two opposite sides of the stem with *Xf* WT, Δ*xpsE*, or 1XPBS. Three biological replications were performed for each strain. Ten technical replications were included in each biological replication. Plants were rated weekly for disease severity on an arbitrary scale of 0-5 where 0 = no PD symptoms (healthy), 1=one or two leaves appearing marginal necrosis, 2 = two or three leaves showing heavy marginal necrosis, 3=fifty percent or even more of leaves displaying marginal necrosis and a few match sticks (attached petioles whose leaf blade had abscised), 4=all the leaves displaying significant scorch symptoms and numerous matchsticks, and 5=a dead vine (Hopkins, 1985; Guilhabert and Kirkpatrick, 2005; Roper et al., 2007).

Host colonization assay. *Xf* WT and Δ*xpsE* populations were quantified from petioles sampled at the point of inoculation (POI) and 25cm above the POI for variety Merlot in year 2012 and at the POI for variety Chardonnay in year 2013. Petioles were weighed, sequentially surface-sterilized with 95% ethanol (1 min) and 20% bleach (2 min), then

rinsed two times in sterilized distilled water (2 min) and placed in 2 mL of 1 ×PBS in mesh sample bags (Agdia, Inc., Elkhart, IN). Samples were then crushed with a hammer. Serial dilutions of the crushed petiole slurry were plated onto PD3 medium or PD3 medium containing 5 µg/mL of gentamicin. The number of colony forming units (CFUs) were enumerated after ten days of incubation at 28 ℃ (Roper et al., 2007).

Results

Confirmation for the *Xf* Δ*xpsE*. Gentamicin-resistant colonies were screened for a double cross over gene replacement event by PCR using primer pair XpsEoutfwd/XpsEoutrev (Figure 3.2). The predicted amplicons were obtained from the WT strain=3067 bp and from the Δ*xpsE*=2101 bp (Figure 3.3.A). The 2101 bp amplicon from Δ*xpsE* was sequenced to confirm the fidelity. The Δ*xpsE* mutant was also confirmed to be *Xf* using *Xf* specific detection primers RST-31 and RST-33 (Minsavage et al., 1994), which amplified a product of the predicted size of 733 bp (Figure 3.3.B).

Prediction of a signal peptide in putative T2SS secreted proteins. Signal peptides and corresponding cleavage sites were found in two endo-1,4-β-endoglucanase (PD1851 and PD2061), one family 3 glycoside hydrolase (PD1829) and one serine protease (PD0313) by the program SignalIP (Petersen et al., 2011) (Table 3.3). There were no apparent signal peptides found in the polygalacturonase (PD1485), extracellular endoglucanase (PD1856) and cellulose 1,4-beta-cellobiosidase (PD0529). These protein sequences were

further checked by the program SecretomeP (Bendtsen et al., 2005). Results indicated they could be leaderless secretory proteins (SecP score>0.5) (Table 3.3).

A mutation in *xpsE* results in a change in colony morphology on EPS inducing medium. Pectin is a major polysaccharide present in the plant primary cell wall that functions as a matrix in which cellulose and cross-linking glycans are embedded (Mohnen, 2008). The degradation of this pectin is necessary for systemic colonization of *Xf* in grape by digesting the pit membrane barrier that separates one xylem vessel from another (Roper et al., 2007). The simplest form of pectin is homogalacturonan, a polymer composed of galacturonic acid residues. Homogalacturonan accounts for approximately 65% of the overall pectin composition (Mohnen, 2008). *In vitro*, pectin degradation results in an increase in EPS production, a virulence factor for *Xf* (Killiny and Almeida, 2009; Killiny et al., 2013).

The WT strain has a very mucoid phenotype on the XFM-pectin medium as a result of an increase in EPS production as compared to PD3 medium where the colonies are dry and opaque (Figure 3.4.A). In contrast, the mucoid phenotypic change in the Δ*xpsE* mutant was not observed on solid XFM-pectin medium, leading us to speculate that the T2SS is secreting PG that is responsible for the pectin degradation that results in induction of EPS production (Figure 3.4.B).

A mutation in *xpsE* alters the extracellular protein profile. Extracellular proteins in the supernatant of liquid cultures of *Xf* grown in XFM-pectin were identified by mass spectrometry (Table 3.4). We opted to use a modified version of the base XFM medium

that did not include bovine serum albumin (BSA), because we found that BSA interfered with analysis of the much less abundant *Xf* proteins. When propagated in XFM-pectin, the outer membrane proteins MopB (PD1709) and OmpW (PD1807) were both present in the secretome of WT and the Δ*xpsE* mutant (Table 3.4). Interestingly, a serine protease PspB (PD0313), a conserved hypothetical protein (PD1063) and TonB-dependent receptor (PD1283) were present in the supernatants of WT cells, but absent in Δ*xpsE* supernatants suggesting that these are either directly secreted by the T2SS or their presence in the supernatant is indirectly affected by the impairment of the T2SS caused by a mutation in *xpsE* (Table 3.4).

***Xf* requires XpsE for full virulence and colonization in grapevine.** Compared with the WT strain, Δ*xpsE* was significantly less virulent in all three grapevine varieties tested ($p<0.05$) (Merlot, Cabernet Sauvignon and Chardonnay) (Figure 3.5, Figure 3.6 and Figure 3.7). Specifically, in Merlot, the average disease rating for WT-inoculated plants was 0.5 as compared to 0.23 for Δ*xpsE*-inoculated plants at twelve weeks post-inoculation ($p<0.05$) (Figure 3.5). Similarly in Cabernet Sauvignon grapevines, *Xf* Δ*xpsE* also showed a significantly decreased virulence as compared with WT, with an average disease rating of 0.6 for WT-inoculated plants and 0.13 for Δ*xpsE*-inoculated plants. This difference was observed at nine weeks post-inoculation ($p<0.05$) (Figure 3.6). Interestingly, Δ*xpsE* did not elicit any PD symptoms in Chardonnay grapevines, which is a variety more susceptible to PD than Cabernet Sauvignon. Thus, the significant virulence difference between WT (average disease rating=1.13) and Δ*xpsE* could be observed even at the beginning of PD symptom development that occurred at eight weeks

post-inoculation ($p<0.05$) (Figure 3.7). The results were analyzed by a Tukey's test following a one way analysis of variance with repeated measures (ANOVA-RM) for each week for each variety.

In year 2012, the *Xf* Δ*xpsE* colony could not be recovered from Merlot grapevines from the point of inoculation (POI) and one petiole above POI at 14 and 21 weeks post-inoculation (Table 3.5). At 14 weeks post-inoculation, the average disease rating is 0.7 for WT and 0.4 for Δ*xpsE*. At 21 weeks post-inoculation, the average disease rating is 1.5 for WT and 1.1 for Δ*xpsE*. Similarly, there were no Δ*xpsE* cells isolated from the POI from inoculated Chardonnay grapevines at thirteen weeks post-inoculation in year 2013 (Table 3.6). At this earlier PD development period, the average disease rating is 2.43 for WT and 0 for Δ*xpsE*. These results indicated that Δ*xpsE* did not effectively colonize grapevines.

Discussion

Movement and multiplication within the xylem is an important step in the development of PD. CWDEs that degraded the plant host cell wall play an important role in this process by enzymatically degrading pit membranes, which serve as a mechanism for *Xf* inter-vessel movement. Many of these CWDEs are predicted to be secreted to the extracellular environment via the T2SS (Chatterjee et al., 2008). The purpose of this study was to explore the function of the T2SS in relation to *Xf* virulence in grapevine. We created a mutant in the putative XpsE ATPase that powers the T2SS and evaluated its behavior *in planta*.

Several T2SS secreted proteins have been characterized in *Xanthomonas* species, close relatives of *Xf* (Jha et al., 2005). Specifically in *Xanthomonas campestris* pv. *campestris* (*Xcc*), α-amylase, extracellular polygalacturonase lyase, and endoglucanase accumulate in the periplasm of a *xpsD* mutant (Hu et al., 1992), the *xpsN* mutant is also deficient in exportation of α-amylase (Lee et al., 2000). Together, these studies indicate *Xcc* secretes α-amylase, extracellular polygalacturonase lyase, and endoglucanase via the T2SS. Similarly, in the *Xoo* system, a mutation in the *xpsF* gene results in accumulation of xylanase in the cytoplasm and periplasm (Ray et al., 2000). In addition, a putative cysteine protease is not detectable in the extracellular space of *Xoo xpsL* mutant (Furutani et al., 2004). Furthermore, a mutation in *xpsE* gene also results in accumulation of xylanase and cellulase in the periplasmic space of *Xoo* (Sun et al., 2005), which leads us to speculate a similar role for XpsE in the *Xf* system. Overall, these results indicate *Xoo* secretes xylanase, cellulase and a putative cysteine protease through the T2SS.

Several proteins identified in the *Xf* genome are also putatively secreted by the T2SS, including PG, several endo-1,4-β-glucanase, 1,4-β-cellobiosidase and numerous proteases (Simpson et al., 2000; Van Sluys et al., 2003). Indeed, we predicted secretion signal peptides for some of these enzymes and predicted some that are apparent leaderless secretory proteins (Table 3.3). The *Xf* PG is an endo-PG that cleaves homogalacturonan, and is required for long distance movement in the xylem (Roper et al., 2007). Furthermore, the *pglA* mutant is non-pathogenic (Roper et al., 2007). Interestingly, it was demonstrated that a PG and a recombinant *Xf* EGase act in concert together to enlarge pit membrane diameters of healthy grapevines to sizes large enough to allow passage of *Xf* from one

xylem vessel to another, which suggests that a consortium of CWDEs are necessary to facilitate movement in the xylem (Pérez-Donoso et al., 2010). Historically, it has been difficult to detect CWDE activity associated with *Xf* grown in culture although protease activity has been reported in *Xf* (Fry et al., 1994; Maria Fedatto et al., 2006). Indeed, our secretome analysis did not reveal the presence of any confirmed or predicted CWDEs except one protease. We speculate that CWDEs are simply not being produced under the culture conditions used in this study and likely rely on specific plant or bacterial factors for induction. In a proposed *Xf* movement model in plant xylem vessels, CWDEs production was predicted to be induced by a diffusible signaling factor (DSF), which mediates quorum sensing of *Xf* cells (Newman et al., 2004). A recent study (Killiny and Almeida, 2009) presents indirect evidence that *Xf* produces PG when propagated on the defined medium XFM supplemented with pectin. EPS production was induced in the WT on XFM-pectin but not for the *pglA* mutant when grown on this medium. However, when the *pglA* mutant was grown on XFM-Na-galacturonate, a monomeric subunit of polygalacturonic acid, EPS production was restored (Killiny and Almeida, 2009). Galacturonic acid is a degradation product of pectin (Mohnen, 2008). Taken together, this suggests pectin degradation is required for induction of EPS production and that at least a small amount of PG is being produced on XFM-pectin (Killiny and Almeida, 2009). This may have been below the detection threshold of our secreted protein purification and secretome analyses, which would explain why we do detect this enzyme or others. Similarly, we observed that *Xf* Δ*xpsE* mutant exhibits a non-mucoid phenotype on XFM-pectin similar to the *pglA* mutant leading us to speculate that PG is being secreted by the T2SS (Figure 3.4).

Interestingly, we found a difference in the composition of the extracellular proteins associated with WT and the Δ*xpsE* mutant that are either directly or indirectly related to a functional T2SS. Interestingly, there is no serine protease PspB (PD0313) found in the supernatants of the Δ*xpsE* mutant as compared to the WT (Table 3.4). Protease activity has been detected in the grapevine and citrus strains of *Xf* (Fry et al., 1994; Maria Fedatto et al., 2006), but the specific role of proteases in virulence and host colonization is unknown. In *Xanthomonas*, proteases are secreted by the T2SS (Hu et al., 1992; Furutani et al., 2004; Jha et al., 2005). Specifically, a protease mutant of *Xcc* shows significantly reduced virulence as it is inoculated into turnip leaves via the endings of cut veins (Dow et al., 1990). Overall, these *in vitro* assay results indicated that a mutation in *xpsE* gene directly or indirectly affects the secretion of multiple proteins in *Xf*.

The *Xf* Δ*xpsE* mutant exhibited significantly reduced virulence in grapevines as compared with the WT strain. Furthermore, we could not isolate any Δ*xpsE* cells from different locations in different varieties of grapevines, which indicates *Xf* Δ*xpsE* is defective in colonization in the grapevine xylem. Further analysis of the *Xf* T2SS components and virulence factors secreted by this system will provide a better understanding for the molecular mechanisms underlying systemic colonization and multiplication process of *Xf* in xylem vessels, which may aid in developing valuable control strategies of PD.

Literature Cited

Bendtsen, J.D., Kiemer, L., Fausboll, A., and Brunak, S. 2005. Non-classical protein secretion in bacteria. BMC Microbiol. 5:58.

Campos, M., Cisneros, D.A., Nivaskumar, M., and Francetic, O. 2013. The type II secretion system-a dynamic fiber assembly nanomachine. Res. Microbiol. 164:545-555.

Chatterjee, S., Almeida, R.P.P., and Lindow, S. 2008. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. Annu. Rev. Phytopathol. 46:243-271.

Cianciotto, N.P. 2005. Type II secretion: a protein secretion system for all seasons. Trends Microbiol. 13:581-588.

Davis, M.J., French, W.J., and Schaad, N.W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6:309-314.

Dow, J.M., Clarke, B.R., Milligan, D.E., Tang, J.L., and Daniels, M.J. 1990. Extracellular proteases from *Xanthomonas campestris* pv. campestris, the black rot pathogen. Appl. Environ. Microbiol. 56:2994-2998.

Drakakaki, G., van de Ven, W., Pan, S., Miao, Y., Wang, J., Keinath, N.F., Weatherly, B., Jiang, L., Schumacher, K., Hicks, G., and Raikhel, N. 2012. Isolation and proteomic analysis of the SYP61 compartment reveal its role in exocytic trafficking in *Arabidopsis*. Cell Res. 22:413-424.

Esau, K. 1977. Anatomy of seed plants, Ed 2. John Wiley and Sons, Inc. New York.

Filloux, A. 2004. The underlying mechanisms of type II protein secretion. Biochim. Biophys. Acta 1694:163-179.

Fry, S.M., Huang, J.S., and Milholland, R.D. 1994. Isolation and preliminary characterization of extracellular proteases produced by strains of *Xylella fastidiosa* from grapevines. Phytopathology 84:357-363.

Furutani, A., Tsuge, S., Ohnishi, K., Hikichi, Y., Oku, T., Tsuno, K., Inoue, Y., Ochiai, H., Kaku, H., and Kubo, Y. 2004. Evidence for HrpXo-dependent expression of type II secretory proteins in *Xanthomonas oryzae* pv. oryzae. J. Bacteriol. 186:1374-1380.

Guilhabert, M.R., and Kirkpatrick, B.C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute a biofilm maturation to *X*.

fastidiosa and colonization and attenuate virulence. Mol. Plant-Microbe Interact. 18:856-868.

Guilhabert, M.R., Hoffman, L.M., Mills, D.A., and Kirkpatrick, B.C. 2001. Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mol. Plant-Microbe Interact. 14:701-706.

Harth, G., and Horwitz, M.A. 1999. Export of recombinant *Mycobacterium tuberculosis* superoxide dismutase is dependent upon both information in the protein and mycobacterial export machinery. A model for studying export of leaderless proteins by pathogenic mycobacteria. J. Biol. Chem. 274:4281-4292.

Hill, B.L., and Purcell, A.H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopathology 85:1368-1372.

Hopkins, D.L. 1985. Physiological and pathological characteristics of virulent and avirulent strains of the bacterium that causes Pierce's disease of grapevine. Phytopathology 75:713-717.

Hopkins, D.L. 1989. *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.

Hu, N.T., Hung, M.N., Chiou, S.J., Tang, F., Chiang, D.C., Huang, H.Y., and Wu, C.Y. 1992. Cloning and characterization of a gene required for the secretion of extracellular enzymes across the outer membrane by *Xanthomonas campestris* pv. campestris. J. Bacteriol. 174:2679-2687.

Jha, G., Rajeshwari, R., and Sonti, R.V. 2005. Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. Mol. Plant-Microbe Interact. 18:891-898.

Killiny, N., and Almeida, R.P. 2009. Host structural carbohydrate induces vector transmission of a bacterial plant pathogen. Proc. Natl. Acad. Sci. USA 106:22416-22420.

Killiny, N., Martinez, R.H., Dumenyo, C.K., Cooksey, D.A., and Almeida, R.P.P. 2013. The exopolysaccharide of *Xylella fastidiosa* is essential for biofilm formation, plant virulence, and vector transmission. Mol. Plant-Microbe Interact. 26:1044-1053.

Korotkov, K.V., Sandkvist, M., and Hol, W.G. 2012. The type II secretion system: biogenesis, molecular architecture and mechanism. Nat. Rev. Microbiol. 10:336-351.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd, and Peterson, K.M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175-176.

Lee, H.M., Wang, K.C., Liu, Y.L., Yew, H.Y., Chen, L.Y., Leu, W.M., Chen, D.C., and Hu, N.T. 2000. Association of the cytoplasmic membrane protein XpsN with the outer membrane protein XpsD in the type II protein secretion apparatus of *Xanthomonas campestris* pv. campestris. J. Bacteriol. 182:1549-1557.

Maria Fedatto, L., Silva-Stenico, M.E., Etchegaray, A., Pacheco, F.T.H., Rodrigues, J.L.M., and Tsai, S.M. 2006. Detection and characterization of protease secreted by the plant pathogen *Xylella fastidiosa*. Microbiol. Res. 161:263-272.

Matsumoto, A., Young, G.M., and Igo, M.M. 2009. Chromosome-based genetic complementation system for *Xylella fastidiosa*. Appl. Environ. Microbiol. 75:1679-1687.

Minsavage, G.V., Thompson, C.M., Hopkins, D.L., Leite, R.M.V.B.C., and Stall, R.E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84:456-461.

Mohnen, D. 2008. Pectin structure and biosynthesis. Curr. Opin. Plant Biol. 11:266-277.

Nakaho, K., Hibino, H., and Miyagawa, H. 2000. Possible mechanisms limiting movement of *Ralstonia solanacearum* in resistant tomato tissues. J. Phytopathol. 148:181-190.

Newman, K.L., Almeida, R.P., Purcell, A.H., and Lindow, S.E. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. Appl. Environ. Microbiol. 69:7319-7327.

Newman, K.L., Almeida, R.P.P., Purcell, A.H., and Lindow, S.E. 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. Proc. Natl. Acad. Sci. USA 101:1737-1742.

Pérez-Donoso, A.G., Sun, Q., Roper, M.C., Greve, L.C., Kirkpatrick, B., and Labavitch, J.M. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. Plant Physiol. 152:1748-1759.

Petersen, T.N., Brunak, S., von Heijne, G., and Nielsen, H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8:785-786.

Pugsley, A.P. 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. 57:50-108.

Ray, S.K., Rajeshwari, R., and Sonti, R.V. 2000. Mutants of *Xanthomonas oryzae* pv. *oryzae* deficient in general secretory pathway are virulence deficient and unable to secrete xylanase. Mol. Plant-Microbe Interact. 13:394-401.

Roper, M.C., Greve, L.C., Warren, J.G., Labavitch, J.M., and Kirkpatrick, B.C. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Mol. Plant-Microbe Interact. 20:411-419.

Rosenfeld, J., Capdevielle, J., Guillemot, J.C., and Ferrara, P. 1992. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal. Biochem. 203:173-179.

Simpson, A.J., Reinach, F.C., Arruda, P., Abreu, F.A., Acencio, M., Alvarenga, R., Alves, L.M., Araya, J.E., Baia, G.S., Baptista, C.S., Barros, M.H., Bonaccorsi, E.D., Bordin, S., Bove, J.M., Briones, M.R., Bueno, M.R., Camargo, A.A., Camargo, L.E., Carraro, D.M., Carrer, H., Colauto, N.B., Colombo, C., Costa, F.F., Costa, M.C., Costa-Neto, C.M., Coutinho, L.L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A.P., Ferreira, A.J., Ferreira, V.C., Ferro, J.A., Fraga, J.S., Franca, S.C., Franco, M.C., Frohme, M., Furlan, L.R., Garnier, M., Goldman, G.H., Goldman, M.H., Gomes, S.L., Gruber, A., Ho, P.L., Hoheisel, J.D., Junqueira, M.L., Kemper, E.L., Kitajima, J.P., Krieger, J.E., Kuramae, E.E., Laigret, F., Lambais, M.R., Leite, L.C., Lemos, E.G., Lemos, M.V., Lopes, S.A., Lopes, C.R., Machado, J.A., Machado, M.A., Madeira, A.M., Madeira, H.M., Marino, C.L., Marques, M.V., Martins, E.A., Martins, E.M., Matsukuma, A.Y., Menck, C.F., Miracca, E.C., Miyaki, C.Y., Monteriro-Vitorello, C.B., Moon, D.H., Nagai, M.A., Nascimento, A.L., Netto, L.E., Nhani, A., Jr., Nobrega, F.G., Nunes, L.R., Oliveira, M.A., de Oliveira, M.C., de Oliveira, R.C., Palmieri, D.A., Paris, A., Peixoto, B.R., Pereira, G.A., Pereira, H.A., Jr., Pesquero, J.B., Quaggio, R.B., Roberto, P.G., Rodrigues, V., de, M.R.A.J., de Rosa, V.E., Jr., de Sa, R.G., Santelli, R.V., Sawasaki, H.E., da Silva, A.C., da Silva, A.M., da Silva, F.R., da Silva, W.A., Jr., da Silveira, J.F., Silvestri, M.L., Siqueira, W.J., de Souza, A.A., de Souza, A.P., Terenzi, M.F., Truffi, D., Tsai, S.M., Tsuhako, M.H., Vallada, H., Van Sluys, M.A., Verjovski-Almeida, S., Vettore, A.L., Zago, M.A., Zatz, M., Meidanis, J., and Setubal, J.C. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151-159.

Smolka, M.B., Martins-de-Souza, D., Winck, F.V., Santoro, C.E., Castellari, R.R., Ferrari, F., Brum, I.J., Galembeck, E., Della Coletta Filho, H., Machado, M.A., Marangoni, S., and Novello, J.C. 2003. Proteome analysis of the plant pathogen *Xylella fastidiosa* reveals major cellular and extracellular proteins and a peculiar codon bias distribution. Proteomics 3:224-237.

Sun, Q.H., Hu, J., Huang, G.X., Ge, C., Fang, R.X., and He, C.Z. 2005. Type-II secretion pathway structural gene *xpsE*, xylanase- and cellulase secretion and virulence in *Xanthomonas oryzae* pv. *oryzae*. Plant Pathol. 54:15-21.

Van Sluys, M.A., de Oliveira, M.C., Monteiro-Vitorello, C.B., Miyaki, C.Y., Furlan, L.R., Camargo, L.E., da Silva, A.C., Moon, D.H., Takita, M.A., Lemos, E.G., Machado, M.A., Ferro, M.I., da Silva, F.R., Goldman, M.H., Goldman, G.H., Lemos, M.V., El-Dorry, H., Tsai, S.M., Carrer, H., Carraro, D.M., de Oliveira, R.C., Nunes, L.R., Siqueira, W.J., Coutinho, L.L., Kimura, E.T., Ferro, E.S., Harakava, R., Kuramae, E.E., Marino, C.L., Giglioti, E., Abreu, I.L., Alves, L.M., do Amaral, A.M., Baia, G.S., Blanco, S.R., Brito, M.S., Cannavan, F.S., Celestino, A.V., da Cunha, A.F., Fenille, R.C., Ferro, J.A., Formighieri, E.F., Kishi, L.T., Leoni, S.G., Oliveira, A.R., Rosa, V.E., Jr., Sassaki, F.T., Sena, J.A., de Souza, A.A., Truffi, D., Tsukumo, F., Yanai, G.M., Zaros, L.G., Civerolo, E.L., Simpson, A.J., Almeida, N.F., Jr., Setubal, J.C., and Kitajima, J.P. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185:1018-1026.

von Heijne, G. 1990. The signal peptide. J. Membr. Biol. 115:195-201.

Voulhoux, R., Ball, G., Ize, B., Vasil, M.L., Lazdunski, A., Wu, L.F., and Filloux, A. 2001. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J. 20:6735-6741.

Walton, J.D. 1994. Deconstructing the Cell Wall. Plant Physiol. 104:1113-1118.

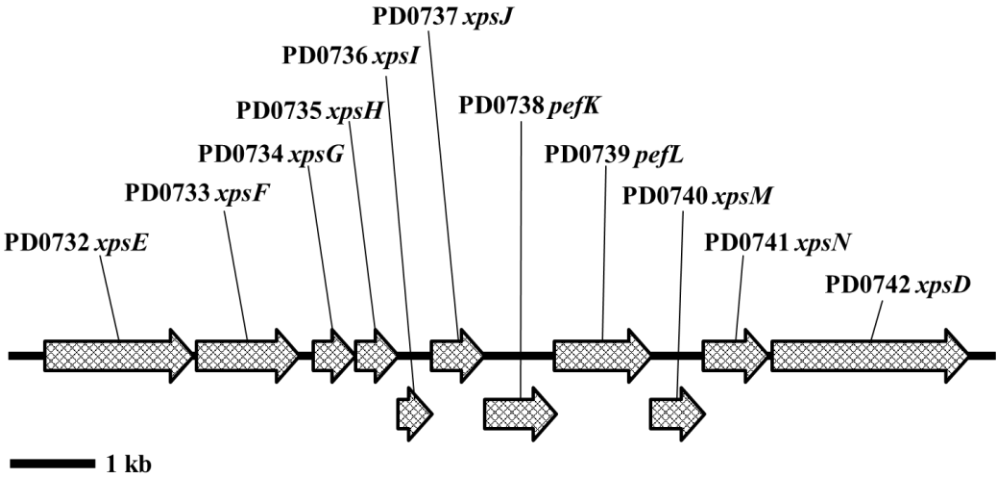


Figure 3.1 Schematic representation of the putative T2SS operon in *Xylella fastidiosa* Temecula1 genome.

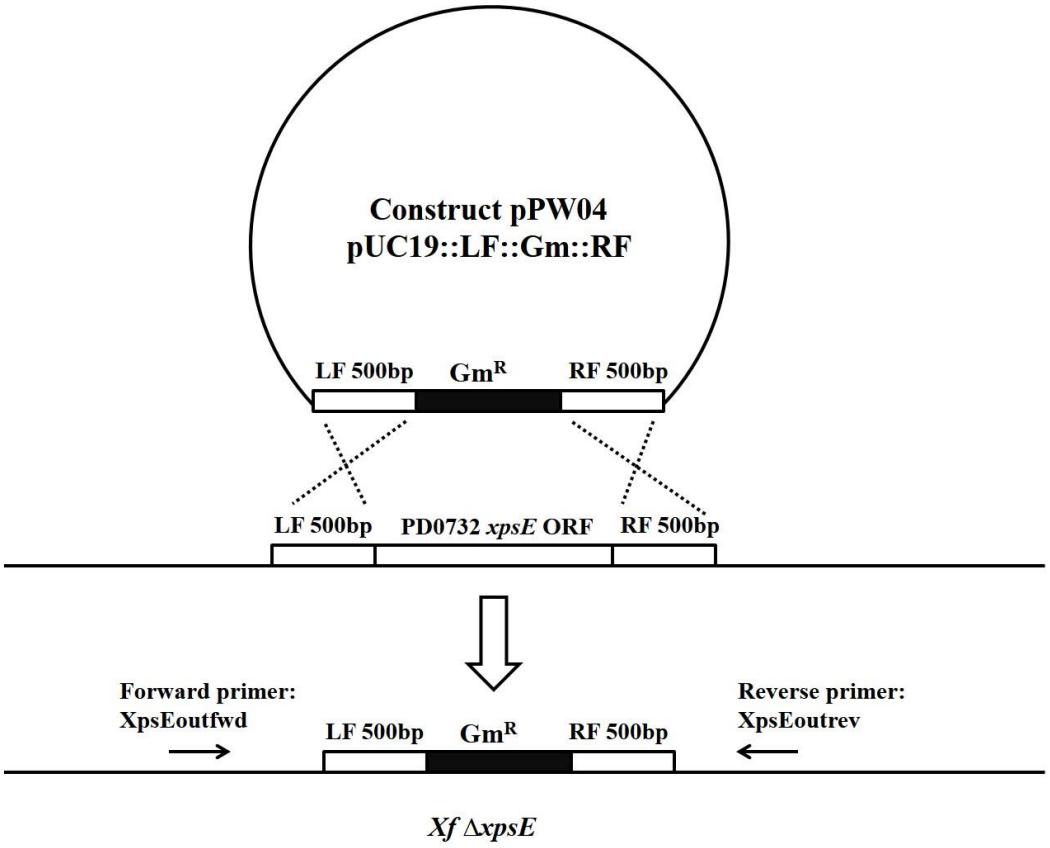


Figure 3.2 Diagram indicating the gene replacement strategy utilized to create Δ*xpsE*. Forward primer XpsEoutfwd and reverse primer XpsEoutrev are designed to anneal to the DNA flanking *xpsE* upstream 500bp (LF 500bp) and downstream 500bp (RF 500bp) separately. Gm^R: gentamicin resistance sequence; LF 500bp: *xpsE* ORF upstream 500bp DNA sequence; RF 500bp: *xpsE* ORF downstream 500bp DNA sequence.

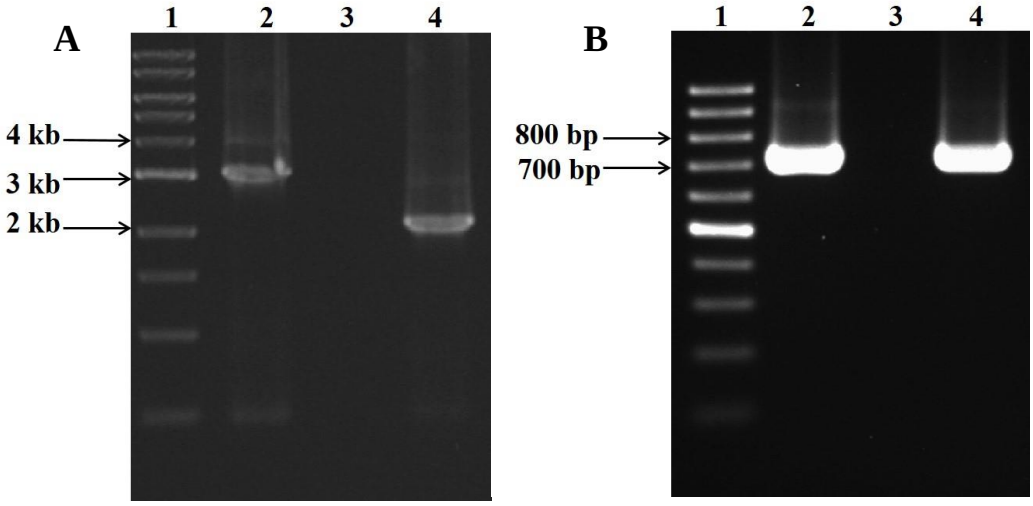


Figure 3.3 Gel pictures showing PCR confirmation results for Δ*xpsE*. A: agrose gel electrophoresis of PCR products obtained using primers XpsEoutfwd and XpsEoutrev; Lane 1, 1 kb DNA ladder; lane 2 and 4, PCR products obtained using genomic DNA template of *Xf* Temecula1 WT and Δ*xpsE*, lane 3, negative control. B: agrose gel electrophoresis of PCR products obtained using primers RST-31 and RST-33; Lane 1, 100 bp DNA ladder; lane 2 and 4, PCR products obtained using genomic DNA template of *Xf* Temecula1 WT and Δ*xpsE*, lane 3, negative control.

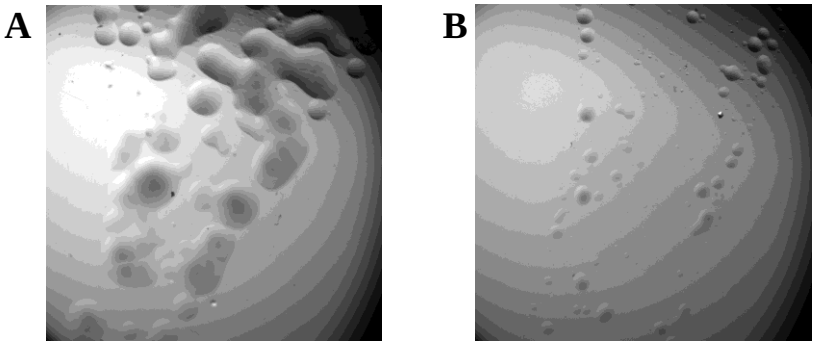


Figure 3.4 Pictures showing colony morphology on the XFM-pectin solid medium. A: WT morphology on the XFM-pectin medium; B: Δ*xpsE* morphology on the XFM-pectin medium. Compared with WT, Δ*xpsE* does not show significant induced EPS production.

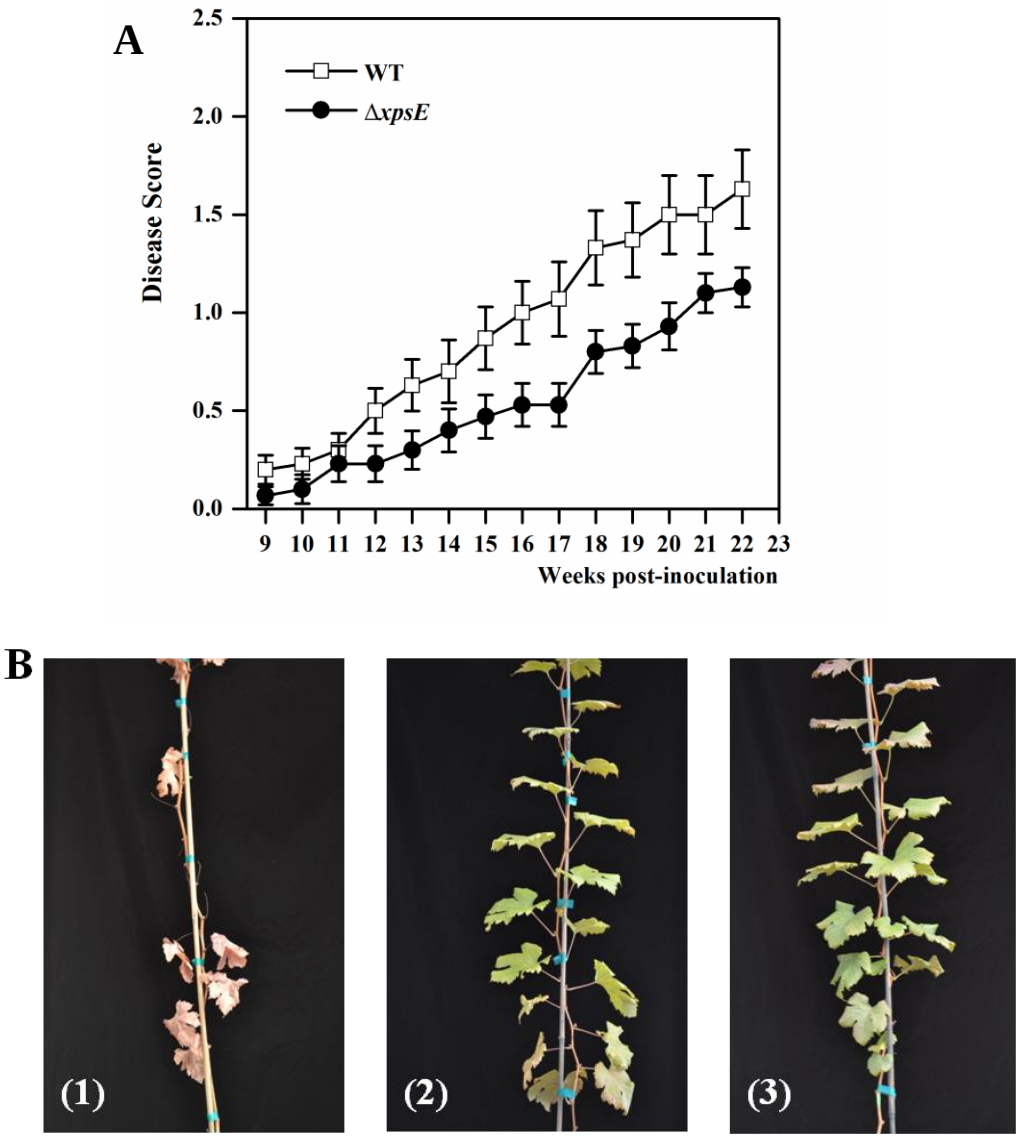


Figure 3.5 Virulence of Δ*xpsE* in *Vitis vinifera* cv. Merlot in year 2012. A. Disease progress curve indicates that Δ*xpsE* is significantly compromised in virulence as compared to WT. The error bars indicate standard error of means. B. Representative pictures of vines with (1) WT inoculation; (2) Δ*xpsE* inoculation; or (3) 1×PBS inoculation.

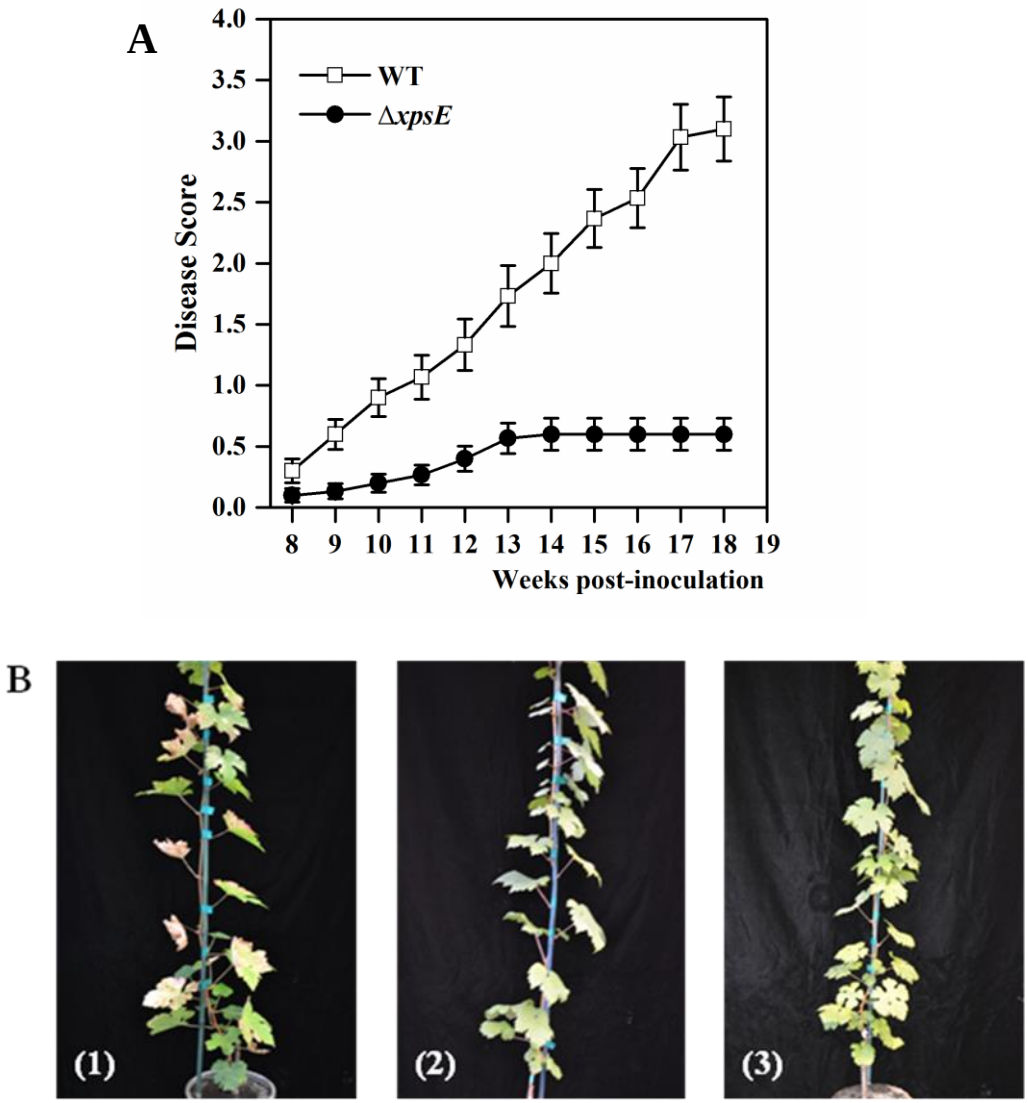


Figure 3.6 Virulence of Δ*xpsE* in *Vitis vinifera* cv. Cabernet Sauvignon in year 2013. A. Disease progress curve indicates that Δ*xpsE* is significantly compromised in virulence as compared to WT. The error bars indicate standard error of means. B. Representative pictures of vines with (1) WT inoculation; (2) Δ*xpsE* inoculation; or (3) 1 ×PBS inoculation.

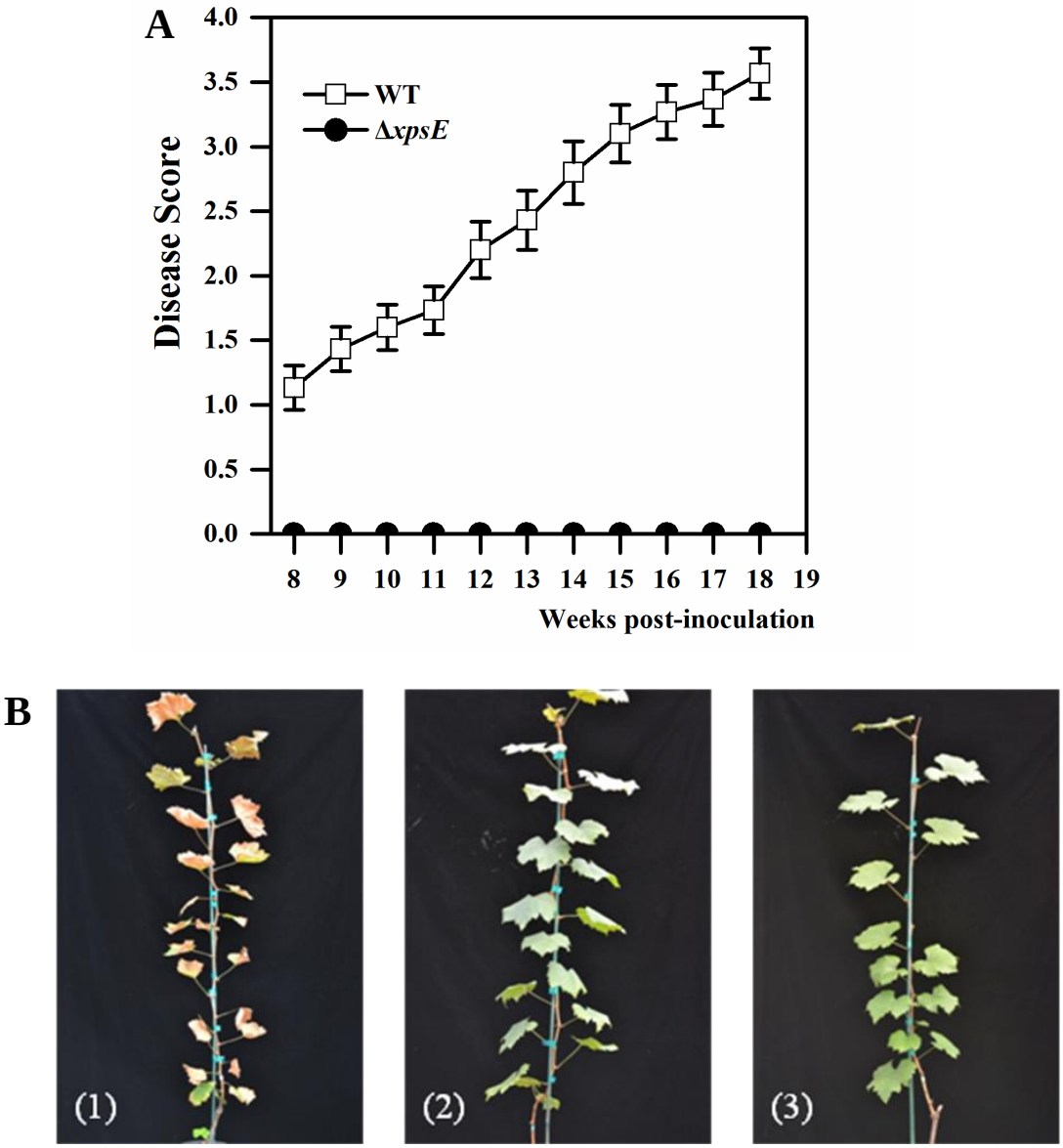


Figure 3.7 Virulence of Δ*xpsE* in *Vitis vinifera* cv. Chardonnay in 2013. A. Disease progress curve indicates that Δ*xpsE* is significantly compromised in virulence as compared to WT. The error bars indicate standard error of means. B. Representative pictures of vines with (1) WT inoculation; (2) Δ*xpsE* inoculation; or (3) 1×PBS inoculation.

Subassembly	Protein component	Function	Reference
Outermembrane complex	GspD	Provides a channel for exporting proteins into extracellular space	Jha et al., 2005; Korotkov et al., 2012
	GspS	Anchor protein D in the outer-membrane	Jha et al., 2005; Korotkov et al., 2012
Pseudopilus	GspG	Make up the pseudopilus	Korotkov et al., 2012
	GspH		
	GspJ		
	GspI		
	GspK		
	GspO	Cleaves and methylates the N-terminus of proteins GspG, GspH, GspI, GspJ and GspK	Campos et al., 2013
Inner-membrane platform	GspM	Compose inner membrane complex	Korotkov et al., 2012
	GspL		
	GspF		
	GpsC		
	GspE	An ATPase, provides energy through ATP hydrolysis	Filloux, 2004; Korotkov et al., 2012
	GspN	Unknown	Korotkov et al., 2012
	GspA	Transfer energy from ATP hydrolysis to the secretion step	Filloux, 2004
	GspB		Filloux, 2004

Table 3.1 List of the T2SS component proteins in gram-negative bacteria.

Table 3.2 Bacterial strains, plasmids and primers used in this study		
	Characteristics	**Reference**
Bacterial Strains		
Xylella fastidiosa	subsp. *fastidiosa* , wild-type isolate from grape	(Guilhabert et al. 2001)
Temecula1		
Temecula Δ*xpsE*	*xpsE* gene deleted, Gm^R*	This study
Escherichia coli TOP10	F^- *mcr* A Δ(*mrr* -*hsd* RMS-*mcr* BC) φ80*lacZ* ΔM15 Δ*lac* X74*rec* A1	Invitrogen
	ara D139 Δ(*ara* -*leu*) 7697 *gal* U *gal* K *rps* L (Str^R) *end* A1 *nup* G λ-	
Plasmids		
pUC19	Ap^R	Invitrogen
pAX1Cm	Cm^R, Ap^R	(Matsumoto et al. 2009)
pPW04	Ap^R, Gm^R	This study
pPW10	Cm^R, Ap^R	This study
pBBR1MCS-5	Gm^R	(Kovach et al. 1995)
Primers		
XpsE LF fwd EcoRI	5'-GCTGT**GAATTC**GAATGGACAGATGCGTCGGC-3'	This study
XpsE LF rev Gm	5'-TTCCACGGTGTGCGTCATTCGGACACCATAAA-3'	This study
XpsE Gm fwd	5'-TTTATGGTGTCCGAATGACGCACACCGTGGAA-3'	This study
XpsE Gm rev	5'-ATTGGCAACCACACCTTTAGGTGGCGGTACTT-3'	This study
XpsE RF fwd Gm	5'-AAGTACCGCCACCTAAAGGTGTGGTTGCCAAT-3'	This study
XpsE RF rev HindIII	5'-TCTAC**AAGCTT**AATCAGCCAGCCGCTGCAG-3'	This study
XpsEoutfwd	5'-CAATGCGCAGGTGTTGTCCAAAGA-3'	This study
XpsEoutrev	5'-ATTAATCACCTTGGCACGCACCAC-3'	This study
RST-31	5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3'	(Minsavage et al. 1994)
RST-33	5'-CACCATTCGTATCCCGGTG-3'	(Minsavage et al. 1994)
*Ap^R, Gm^R,Str^R and Cm^R indicate resistance to ampicillin, gentamicin streptomycin and chloramphenicol		
**The restriction enzyme sequence in the primer was underlined and specified with bold type.		

Table 3.2 Bacterial strains, plasmids and primers used in Chapter 3.

Gene	Locus Tag	Protein	Predicted Signal Peptide	Predicted Leaderless Secretory Protein
pglA	PD1485	polygalacturonase precursor	No	Yes
engXCA	PD1851	endo-1,4-β-endoglucanase	Yes, cleavage site between position 25 and 26	
egl	PD2061	endo-1,4-β-endoglucanase	Yes, cleavage site between position 27 and 28	
engXCA	PD1856	extracellular endoglucanase precursor	No	Yes
guxA	PD0529	cellulose 1,4-beta-cellobiosidase	No	Yes
xylA	PD1829	family 3 glycoside hydrolase	Yes, cleavage site between position 26 and 27	
pspB	PD0313	serine protease	Yes, cleavage site between position 26 and 27	

Table 3.3 *In silico* analysis for putative T2SS secreted proteins. Firstly, predicted proteins were analyzed for the N-terminal signal peptide by program SignalIP (Petersen et al., 2011). If no signal peptide was identified, it was further analyzed by program SecretomeP (Bendtsen et al., 2005) for being leaderless secretory protein. Proteins with SecP scores higher than 0.5 were considered as leaderless secretory proteins by the program SecretomeP.

WT Extracellular Proteins (Locus Tag)	Δ*xpsE* Extracellular Proteins (Locus Tag)
Outer membrane protein MopB (PD1709)	Outer membrane protein MopB (PD1709)
Outer membrane protein OmpW (PD1807)	Outer membrane protein OmpW (PD1807)
Serine protease PspB (PD0313)	DnaK protein (PD1370)
Conserved hypothetical protein (PD1063)	
TonB-dependent receptor (PD1283)	

Table 3.4. Identification of extracellular proteins in the liquid medium by mass spectrometry. WT and Δ*xpsE* extracellular proteins were isolated from modified XFM-pectin liquid medium (without BSA). Proteins present in WT strain, but absent in Δ*xpsE* extracellular space were highlighted with bold type. At least two different peptides (ions score>22) were used to identify the same protein. Individual ions scores > 22 means identity or extensive homology ($p<0.05$).

Distance	Time post-inoculation	WT (CFU/g tissue)	Δ*xpsE* (CFU/g tissue)
POI	14 weeks	$(7.38 \pm 3.92) \times 10^5$	0
One petiole	21 weeks	$(2.85 \pm 1.85) \times 10^7$	0

Table 3.5 A mutation in *xpsE* affected *in planta* survival. No bacteria were recovered from Δ*xpsE*-inoculated plants at the point of inoculation (POI) and one petiole above POI in Merlot grapevines in year 2012. A two-tailed Wilcoxon rank-sum test was used for the comparison of each isolation location. (Average ±SE). SE: standard error. Compared with WT, Δ*xpsE* colonization ability has greatly reduced ($p<0.05$).

Distance	Time post-inoculation	WT (CFU/g tissue)	Δ*xpsE* (CFU/g tissue)
POI	13 weeks	$(2.46 \pm 0.66) \times 10^8$	0

Table 3.6 A mutation in *xpsE* affected *in planta* survival. No bacteria were recovered from Δ*xpsE*-inoculated plants at the point of inoculation (POI) in Chardonnay grapevines in year 2013. A two-tailed Wilcoxon rank-sum test was used for the comparison. (Average ±SE). SE: standard error. Compared with WT, Δ*xpsE* colonization ability has greatly reduced ($p<0.05$).