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Tolerance to oxidative stress is required for maximal xylem colonization by the xylem-limited bacterial phytopathogen, *Xylella fastidiosa*

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SUMMARY

Bacterial plant pathogens often encounter reactive oxygen species (ROS) during host invasion. In foliar bacterial pathogens, multiple regulatory proteins are involved in the sensing of oxidative stress and the activation of the expression of antioxidant genes. However, it is unclear whether xylem-limited bacteria, such as *Xylella fastidiosa*, experience oxidative stress during the colonization of plants. Examination of the *X. fastidiosa* genome uncovered only one homologue of oxidative stress regulatory proteins, OxyR. Here, a knockout mutation in the *X. fastidiosa oxyR* gene was constructed; the resulting strain was significantly more sensitive to hydrogen peroxide (H_2O_2) relative to the wild-type. In addition, during early stages of grapevine infection, the survival rate was 1000-fold lower for the *oxyR* mutant than for the wild-type. This supports the hypothesis that grapevine xylem represents an oxidative environment and that *X. fastidiosa* must overcome this challenge to achieve maximal xylem colonization. Finally, the *oxyR* mutant exhibited reduced surface attachment and cell–cell aggregation and was defective in biofilm maturation, suggesting that ROS could be a potential environmental cue stimulating biofilm development during the early stages of host colonization.

Keywords: grapevines, oxidative stress, Pierce's disease, xylem.

INTRODUCTION

Xylella fastidiosa subspecies *fastidiosa*, 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). Typical symptoms of PD include leaf marginal necrosis, leaf blade drop, leaf scorch, cordon dieback, stunting and vine death (Varela *et al.*, 2001). In addition to grapevine, other subspecies of *X. fastidiosa* infect many other plant hosts, such as citrus, maple, alfalfa, peach, plum, sycamore, elm, almond, coffee and oleander (Hopkins, 1989; Hopkins and Purcell, 2002). Historically, *X. fastidiosa* has been geographically limited to the Americas. However, the recent findings of *X. fastidiosa* in olive groves in Italy and on ornamental plants in France underscore its importance as a re-emerging global plant pathogen (Almeida and Nunney, 2015; Saponari *et al.*, 2013). *Xylella fastidiosa* is delivered directly into the xylem of new host plants by insect vectors during feeding (Chatterjee *et al.*, 2008). In systemically colonized hosts, *X. fastidiosa* multiplies and moves into adjacent xylem vessels (Chatterjee *et al.*, 2008; Newman *et al.*, 2003; Pérez-Donoso *et al.*, 2010). This process requires enzymatic degradation of the primary plant cell wall material that comprises the pit membranes, which separate xylem vessels from one another (Pérez-Donoso *et al.*, 2010; Roper *et al.*, 2007b). In grapevine, *X. fastidiosa* can effectively colonize the xylem tissue to high titres. As part of the colonization process, *X. fastidiosa* forms aggregates and xylem wall-adherent biofilms (Roper *et al.*, 2007a). These biofilms are thought to contribute to bacterial survival in the xylem and to the vascular occlusions that lead to poor hydraulic conductivity in the plant (Chatterjee *et al.*, 2008; Thorne *et al.*, 2006). There is probably both acute and prolonged production of reactive oxygen species (ROS) in the xylem from a variety of sources, including the plant innate immune system and plant developmental processes (Bolwell *et al.*, 2002; Hilaire *et al.*, 2001; Lamb and Dixon, 1997; Ros Barcelo, 1998, 2005). The exposure of the invading pathogen to ROS in the xylem could impinge on bacterial developmental processes important for xylem colonization, such as biofilm formation.

ROS are toxic to bacteria causing damage to DNA, RNA, proteins and lipids (Cabiscol *et al.*, 2000; Imlay, 2015; Storz and Imlay, 1999). In aerobic bacteria, oxidative stress usually results in the induction of scavenging enzymes that lead to the detoxification of specific ROS. For example, catalase detoxifies hydrogen peroxide (H_2O_2), alkyl hydroperoxide reductase reduces various organic hydroperoxides and superoxide dismutase (SOD) catalyses a dismutation reaction, which converts superoxide anion (O_2^-) into oxygen and H_2O_2 (Imlay, 2015). Sequence analyses have suggested that several of these antioxidant enzymes are encoded by *X. fastidiosa*, including alkyl hydroperoxide reductase (*ahpC* and *ahpF*), catalase (*cpeB*), superoxide dismutase (*sodA* and *sodM*)

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and a thiol-dependent peroxidase (*ohr*) (Van Sluys *et al.*, 2003). Indeed, genetic and biochemical studies have confirmed that the *X. fastidiosa cpeB* gene encodes a functional catalase, which contributes to tolerance to exogenous H_2O_2 (Matsumoto *et al.*, 2009). The presence of these antioxidant-encoding genes in the genome suggests that *X. fastidiosa* experiences significant oxidative stress in the plant or insect vector, or in both environments.

Although basal levels of antioxidant enzymes are commonly maintained in the absence of oxidative stress, many bacteria possess regulatory mechanisms that allow the induction of antioxidant enzymes to higher levels on exposure to ROS. These highly inducible oxidative stress defence responses are largely controlled by redox-sensing transcription factors, such as OxyR (Imlay, 2015). OxyR belongs to the LysR family of bacterial regulators encoded by most Gram-negative (both aerobes and anaerobes) and some Gram-positive bacteria (Mongkolsuk and Helmann, 2002; Schell, 1993; Storz and Imlay, 1999; Storz and Tartaglia, 1992; Storz *et al.*, 1990b). The redox-responsive OxyR transcription factor is activated through the formation of a disulfide bond between two cysteines in response to H_2O_2 (Schell, 1993). The *X. fastidiosa* OxyR protein shares 81% identity with the *X. campestris* pv. *campestris* OxyR protein, but only 47% identity with the *Escherichia coli* OxyR protein, and contains the highly conserved N-terminal DNA-binding domain typical of OxyR proteins (Svintradze *et al.*, 2013; Toledo *et al.*, 2011). Moreover, the *X. fastidiosa* OxyR protein sequence shows homology in the region identified in the *E. coli* OxyR protein as the redox switch (cysteine at positions 199 and 208) (Zheng *et al.*, 1998). In the presence of H_2O_2, the redox switch activates OxyR by forming a disulfide bond between two cysteine residues in the protein (Zheng *et al.*, 1998). We speculate that a similar mechanism is used to activate the *X. fastidiosa* OxyR protein. Following activation, OxyR binds to regulatory regions of a variety of antioxidant genes, thereby facilitating their transcription (Storz *et al.*, 1990a; Zheng *et al.*, 1998, 2001). OxyR has also been implicated in bacterial biofilm formation (Burbank and Roper, 2014; Hennequin and Forestier, 2009; Honma *et al.*, 2009; Shanks *et al.*, 2007). Another transcriptional regulator is SoxR and its partner SoxS, which can respond to superoxides and superoxide-generating compounds in enteric bacteria (Imlay, 2008). Both OxyR and SoxR aid in the restoration of oxidant balance within the cell. Activation of the SoxR/S system also confers resistance to nitric oxide, organic solvents and some antibiotics (Chou *et al.*, 1993; Greenberg *et al.*, 1990). Interestingly, although SoxR is present in the genomes of closely related *Xanthomonas* sp., it is conspicuously absent from the *X. fastidiosa* genome (Van Sluys *et al.*, 2003). Indeed, OxyR is the only annotated redox-sensing transcription factor encoded by *X. fastidiosa*, suggesting that it is an important component of the oxidative stress response. In this study, an *X. fastidiosa oxyR* mutant was used as a tool to gauge the redox status of the xylem. We demonstrate that the *X. fastidiosa oxyR* mutant is compromised in the ability to colonize grapevine xylem at 11 weeks post-inoculation. We also show that the initiation of biofilm formation in *X. fastidiosa* is dependent on OxyR. Taken together, these results suggest that entry into biofilm formation is linked to the sensing of oxidative stress via OxyR, which would be especially important for adherence to the xylem wall and for protection against exogenous stress during early establishment of the bacterial population *in planta*.

RESULTS

OxyR protects *X. fastidiosa* against H_2O_2 stress

To confirm the role of OxyR in the response of *X. fastidiosa* to oxidative stress, an insertion mutation in the *oxyR* gene was generated and used to compare the properties of this knockout mutant with those of the wild-type strain. Sensitivity to H_2O_2 was examined using a disc diffusion method (Matsumoto *et al.*, 2009). The diameter of the inhibition zone around the H_2O_2-infused disc was significantly larger for the *X. fastidiosa oxyR* mutant relative to the wild-type ($P < 0.05$), indicating that the *oxyR* mutant is more sensitive to H_2O_2 (Fig. 1A). Exposure to H_2O_2 also had an impact on the growth of the *oxyR* mutant in liquid culture. The survival percentage of the *oxyR* mutant was significantly lower than that of the wild-type ($P < 0.05$) (Fig. 1B). Thus, as expected, the *X. fastidiosa* OxyR protein facilitates survival following exposure to H_2O_2.

OxyR contributes to host colonization, but does not affect overall virulence

To determine the role of OxyR in *X. fastidiosa* pathogenicity, populations of the *oxyR* mutant following infection of grapevines were examined. At 11 weeks post-inoculation, the bacterial titres of the *oxyR* mutant and the wild-type control were quantified by real-time polymerase chain reaction (PCR) from the petiole immediately above the point of inoculation (POI). The average population of the *oxyR* mutant was 3.80×10^5 colony-forming units (CFU)/g tissue, which was significantly lower than the average population of the wild-type, which was 2.48×10^7 CFU/g tissue (Fig. 2). Interestingly, over the course of the disease bioassay, there was no significant difference in virulence observed between the wild-type and *oxyR* mutant (Fig. S1, see Supporting Information). This suggests that, although a mutation in *oxyR* impacts survival in the host, the *oxyR* mutant cells that survive are able to move systemically and eventually achieve bacterial titres above the threshold necessary to incite PD symptoms.

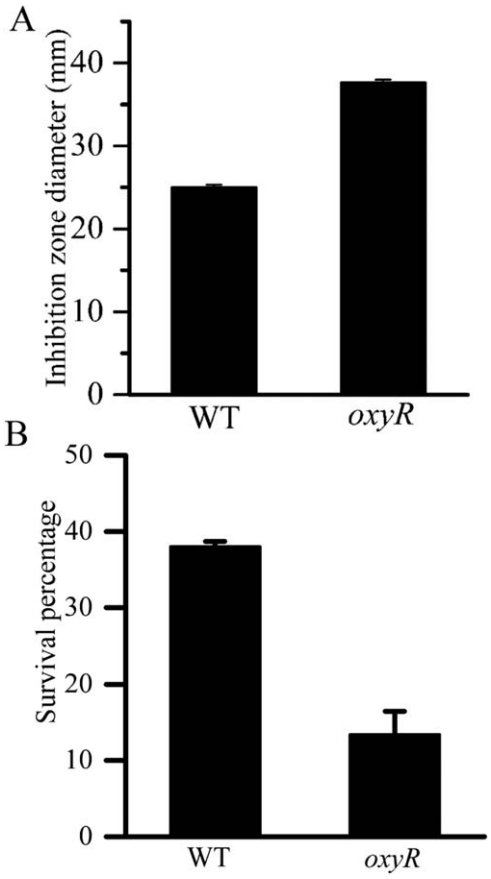

Fig. 1 OxyR protects *Xylella fastidiosa* on exposure to H_2O_2 stress. (A) Sensitivity to H_2O_2 was examined using a disc diffusion method as described by Matsumoto *et al.* (2009) with slight modifications. The graph shows a quantitative comparison of the mean inhibition zone diameters; error bars represent standard errors. Compared with the wild-type (WT), the *oxyR* mutant is significantly more sensitive to H_2O_2 ($P < 0.05$). Three independent biological replications with three technical replications each were performed. The results were analysed by Tukey's test following one-way analysis of variance (ANOVA). (B) The ability of the WT and *oxyR* mutant to survive exposure to H_2O_2 was compared. Cultures of each strain were treated with H_2O_2 (final concentration, 0.5 mM) for 30 min. Cultures without H_2O_2 treatment served as controls. The results indicate that the *oxyR* mutant mean survival percentage on exposure to H_2O_2 stress is significantly lower than that of the WT ($P < 0.05$). Error bars indicate the standard errors of the means. Results were analysed by a two-tailed Student's *t*-test. Two independent biological replications were performed. Three technical replications were included for each biological replication.

A mutation in *oxyR* affects surface attachment, cell–cell aggregation and biofilm formation

To better understand how the presence of OxyR might impact colonization, phenotypes of the *oxyR* mutant in surface attachment and cell–cell aggregation, two properties important for *X. fastidiosa* pathogenicity, were examined. The ability to attach to a solid surface was evaluated using the crystal violet method in which attached cells are 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 relative to the wild-type, indicating that the *oxyR* mutant exhibited reduced attachment to a glass surface (Fig. 3A). Quantitative measurements of attachment confirmed that the *oxyR* mutant was compromised in surface attachment ($P < 0.05$) (Fig. 3A). Cell–cell aggregation for the wild-type and *oxyR* mutant in glass tubes was also evaluated using a previously described protocol (Burdman *et al.*, 2000). Compared with the wild-type, the *oxyR* mutant cells were significantly reduced in the ability to aggregate ($P < 0.05$) (Fig. 3B).

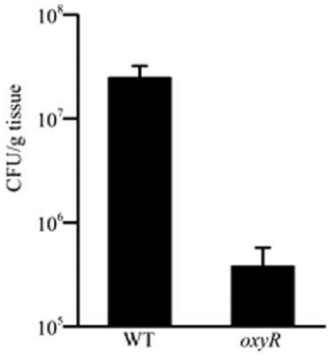

Fig. 2 OxyR promotes colonization in grapevines. At 11 weeks post-inoculation, populations of wild-type (WT) and *oxyR* mutant cells were quantified from leaf petioles near the point of inoculation (POI) in Thompson seedless grapevines. Compared with WT-inoculated grapevines, the *oxyR* mutant-inoculated grapevines harboured a significantly lower titre of bacteria ($P < 0.05$), indicating that the *oxyR* mutant was compromised in xylem colonization. Error bars indicate standard errors. The results were analysed by a two-tailed Wilcoxon rank-sum test. CFU, colony-forming unit.

The *oxyR* mutant was next examined for defects in surface attachment, cell–cell aggregation and biofilm formation. The development of a three-dimensional biofilm was evaluated on a glass slide using confocal laser scanning microscopy (CLSM) over an 8-day time course with images being captured after 2, 4, 6 and 8 days of propagation. After 2 days, the *oxyR* mutant cells were sparsely attached to the glass slide relative to wild-type cells, for which numerous cells were attached and beginning to form aggregates. Over the course of 8 days, the *oxyR* mutant was unable to assemble into a mature three-dimensional biofilm, in contrast with the wild-type (Fig. 4A), leading to a significant difference in biofilm thickness, with the wild-type forming a thicker biofilm than the *oxyR* mutant (Fig. 4B). Taken together, these studies indicate that the *X. fastidiosa oxyR* mutant is defective in key properties important for the initial stages of biofilm formation and biofilm maturation. Thus, coping with oxidative stress in an OxyR-dependent manner plays a key role in the temporal regulation of biofilm formation and potentially biofilm dissolution, both critical processes for the systemic xylem colonization by *X. fastidiosa*.

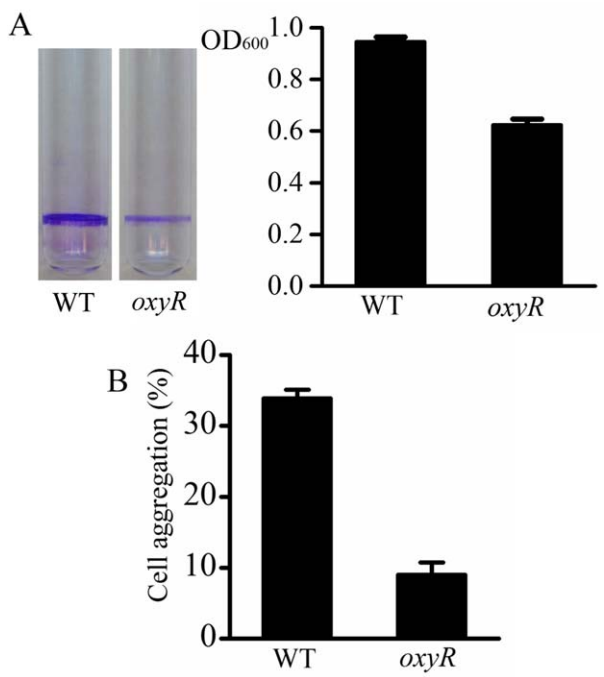

Fig. 3 OxyR mediates surface attachment and cell–cell aggregation. The ability to attach to a glass surface was examined using a crystal violet staining method, as described previously (Espinosa-Urgel *et al.*, 2000). (A) Left: crystal violet-stained wild-type (WT) and *oxyR* mutant cells attached to the glass surface. Right: quantification of crystal violet eluted from stained cells that were attached to the glass surface. The *oxyR* mutant was compromised in attachment to the glass surface when compared with WT ($P < 0.05$). Error bars indicate standard errors. OD_{600}, optical density at 600 nm. (B) Impact of the *oxyR* mutation on cell–cell aggregation. Cell aggregation percentage was determined for the WT and *oxyR* mutant in glass tubes as described previously (Burdman *et al.*, 2000). When compared with WT, the *oxyR* mutant was significantly reduced in its ability to aggregate ($P < 0.05$). Error bars indicate standard errors of the mean.

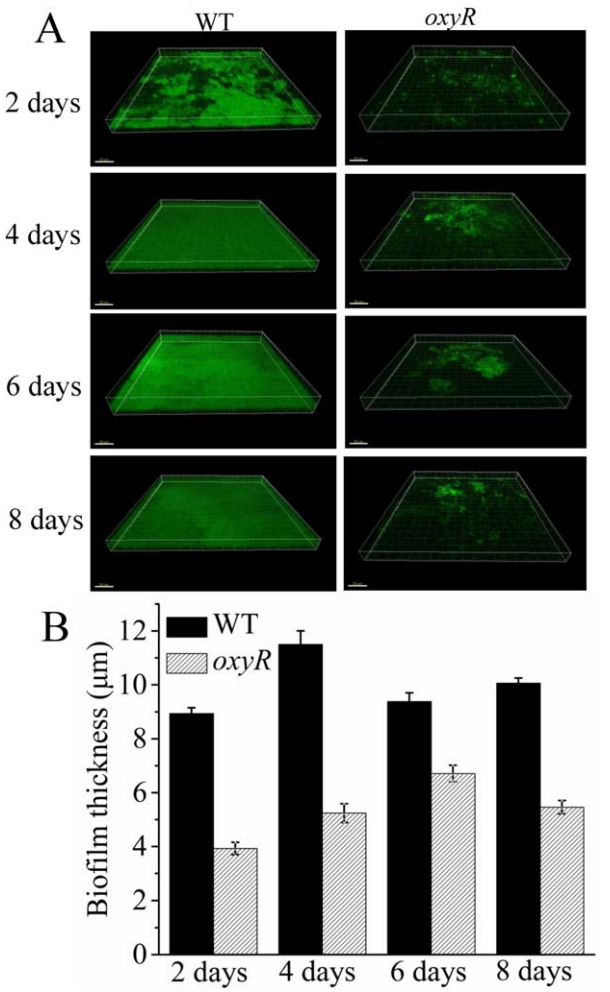

Fig. 4 A mutation in *oxyR* affects biofilm initiation and three-dimensional architecture. (A) Three-dimensional biofilm formation was compared between the wild-type (WT) and *oxyR* mutant by confocal laser scanning microscopy (CLSM), as described previously (Roper *et al.*, 2007a). Images represent the three-dimensional view of either the WT or *oxyR* mutant at 2, 4, 6 and 8 days of propagation on glass slides. Compared with WT, the *oxyR* mutant shows fewer attached cells and was unable to develop into a mature biofilm. Representative photographs are shown here. Scale bars represent 50 μm in all photographs. (B) The thickness of the biofilm captured by CLSM was quantified using Imaris software (Bitplane USA, South Windsor, CT, USA). The *oxyR* mutant biofilm showed significantly reduced thickness ($P < 0.05$) at the different time points (2, 4, 6 and 8 days). Error bars represent standard errors. Results were analysed by a two-tailed Student's *t*-test.

OxyR is co-transcribed with two alkyl hydroperoxide reductase subunits

According to the Prokaryotic Operon DataBase (ProOpDB), *X. fastidiosa oxyR* is predicted to reside as the 3′ proximal gene in a three-gene operon (*ahpC–ahpF–oxyR*) (Taboada *et al.*, 2012). The gene products of *ahpC* and *ahpF* are predicted to form a protein complex with alkyl hydroperoxide reductase activity. In contrast, the Database of Prokaryotic Operons (DOOR) predicts that *X. fastidiosa oxyR* resides as the 3′ proximal gene in a two-gene operon (*ahpF–oxyR*) (Mao *et al.*, 2009). A similar operon structure has been reported for *X. campestris* pv. *phaseoli* (Mongkolsuk *et al.*, 1997). To distinguish between these possibilities, the transcripts within the three-gene cluster (*ahpC–ahpF–oxyR*) were mapped using reverse transcription-PCR (RT-PCR). For this analysis, cDNAs corresponding to the different regions within the three-gene cluster were amplified (Fig. 5). First, individual genes were amplified, indicating that all three genes were transcribed: *ahpC* (lane A, 426 bp), *ahpF* (lane B, 568 bp) and *oxyR* (lane C, 494 bp). Using other combinations of the primers, cDNAs containing *ahpC–ahpF* (lane D, 1687 bp), *ahpF–oxyR* (lane E, 1954 bp) and *ahpC–ahpF–oxyR* (lane F, 3073 bp) were also detected. Finally, the presence of the transcript *ahpC–ahpF–oxyR* (lane F) confirmed the gene order in the operon and indicated that *ahpC*, *ahpF* and *oxyR* genes could be transcribed as one polycistronic mRNA.

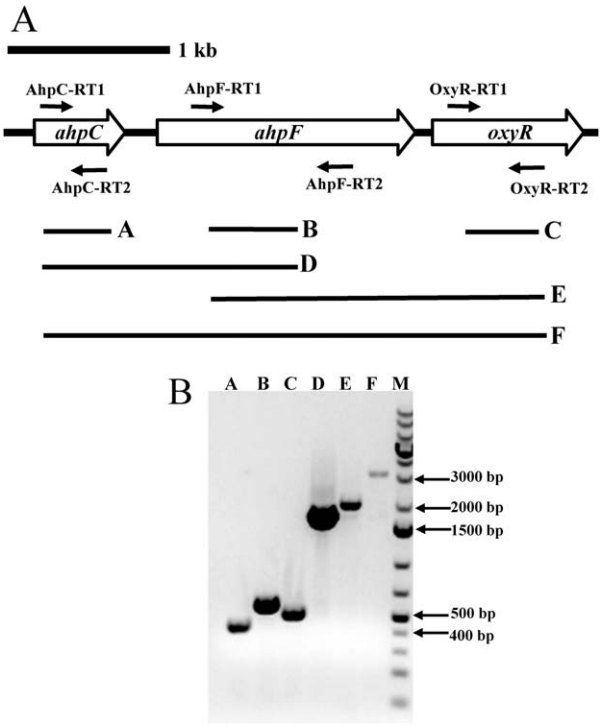

Fig. 5 Transcriptional analysis of *ahpC–ahpF–oxyR* by reverse transcription-polymerase chain reaction (RT-PCR). (A) The open arrows indicate open reading frames for the three genes. *ahpC* (PD0745) and *ahpF* (PD0746) are predicted to encode subunits C and F of alkyl hydroperoxide reductase, whereas *oxyR* (PD0747) is annotated as an oxidative stress transcriptional regulator. The primer (AhpC-RT1, AhpC-RT2, AhpF-RT1, AhpF-RT2, OxyR-RT1 and OxyR-RT2) annealing sites are indicated above and below the three genes. The labelled lines (A, B, C, D, E, F) indicate the locations of RT-PCR products relative to the three genes. (B) A gel showing RT-PCR detection results. Lane M contains a 1-kb DNA plus ladder (Life Technologies, Grand Island, NY, USA). Products in lanes A–F correspond to the labelled products A–F indicated in (A). The presence of product F verifies that genes *ahpC*, *ahpF* and *oxyR* are organized as an operon.

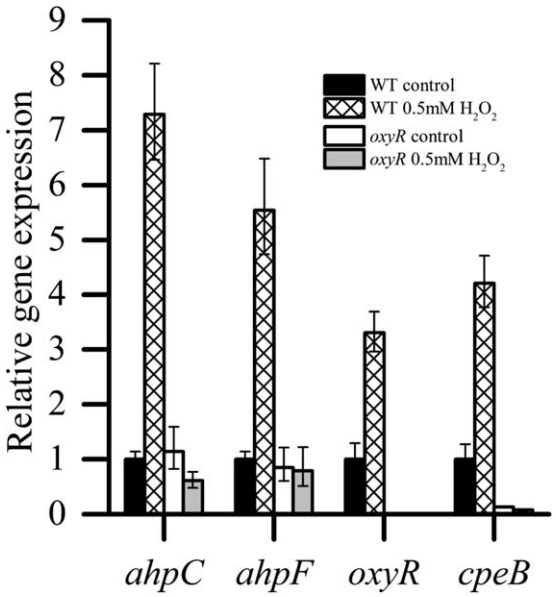

Fig. 6 OxyR is a positive regulator of antioxidant genes. Liquid cultures containing either the wild-type (WT) or *oxyR* mutant were treated for 10 min with H_2O_2 (final concentration, 0.5 mM); untreated cultures were also prepared to serve as controls. The primers AhpC-RT1, AhpC-RT2, AhpF-RT1, AhpF-RT2, OxyR-RT, OxyR-RT2, CpeB-RT1 and CpeB-RT2 were used for quantitative polymerase chain reaction (qPCR) analysis. In the WT strain, the expression of *ahpC*, *ahpF*, *cpeB* and *oxyR* was significantly increased after H_2O_2 treatment when compared with the untreated control ($P < 0.05$). However, induction of these genes was not observed in the *oxyR* mutant ($P > 0.05$). Error bars represent the standard deviation (SD). Results were analysed by a two-tailed Student's *t*-test. The experiment was repeated twice independently with similar results.

OxyR positively regulates enzymes involved in ROS detoxification

The induction of ROS-detoxifying enzymes, such as catalases, alkyl hydroperoxide reductases and superoxide dismutases, is an important part of the bacterial oxidative stress response. Examination of the genome suggested that homologues to some of these enzymes are present in *X. fastidiosa*. Two of these genes (*ahpF* and *ahpC*), which are co-transcribed with *oxyR*, are predicted to encode subunits of an alkyl hydroperoxide reductase. *Xylella fastidiosa* has also been shown to encode a functional catalase (Matsumoto *et al.* 2009). All three of these enzymes are involved in the degradation of H_2O_2 and are known in other bacteria to be positively regulated by OxyR (Flores-Cruz and Allen, 2011; Nakjarung *et al.*, 2003; Zheng *et al.*, 1998, 2001). To determine whether OxyR modulates the expression of these genes in response to H_2O_2 in *X. fastidiosa*, the transcript levels of *cpeB*, *ahpF* and *ahpC* in the *oxyR* mutant and wild-type were compared using quantitative RT-PCR. Following H_2O_2 treatment, the expression levels of *cpeB*, *ahpF* and *ahpC* were all significantly higher in the wild-type strain (Fig. 6). However, this induction was absent in the *oxyR* mutant, confirming that OxyR is required for the regulation of these genes in response to H_2O_2. Thus, OxyR modulates the expression of these key detoxifying enzymes as part of the defence against exogenous oxidative stress.

DISCUSSION

Plants often produce ROS, such as H_2O_2 and superoxide anions (O_2^-), in an effort to protect themselves against pathogens as part of the plant defence response (Bolwell and Wojtaszek, 1997; Lamb and Dixon, 1997). In addition to ROS associated with plant immunity, ROS also arise in plant tissues whenever oxygen enters cells and is oxidized by intracellular redox enzymes (Zheng *et al.*, 1999). Consequently, as xylem sap is the major aqueous pathway of oxygen in the plant, it is a potential source of oxygen radicals (Gansert, 2003). The xylem may also contain developmentally

related ROS that are derived from the lignification process, which occurs during xylem maturation (Ros Barcelo, 1998), and from non-lignifying xylem parenchyma cells, which provide H_2O_2 necessary for the lignification of adjacent differentiating xylem vessels (Ros Barcelo, 2005). Thus, it seems likely that bacteria residing in the xylem will be exposed to ROS generated from multiple sources.

Little is known about the redox status of the xylem sap and even less is known about how it might fluctuate during pathogen invasion of xylem tissue. Studies on the xylem-dwelling bacterium *Ralstonia solanacearum* have indicated that it experiences oxidative stress following its infection of tomato. However, because *R. solanacearum* also occupies other plant tissues, it is difficult to ascertain how much of the oxidative stress experienced by *R. solanacearum* during infection is a result of ROS exposure in other tissues versus exposure to ROS in the xylem (Flores-Cruz and Allen, 2009). In contrast, *X. fastidiosa* is a xylem-limited bacterium residing primarily in the non-living xylem tissue. This non-living tissue cannot mount a defence response on its own. However, adjacent to the non-living tissue, there are living xylem parenchyma cells that are responsible for the storage of water, mineral nutrients and carbohydrates and for response to wounding (Myburg *et al.*, 2013). It is possible that these living xylem parenchyma cells initiate an oxidative burst in response to the presence of *X. fastidiosa* in the grapevine xylem. Indeed, infection of rice xylem by the related bacterium *Xanthomonas oryzae* pv. *oryzae* elicits defence responses from adjacent living parenchyma cells, including the accumulation of a pathogen-induced peroxidase that has been speculated to play a role in the production of toxic ROS intermediates. However, it is not known whether these toxic ROS intermediates accumulate to levels sufficient to be detrimental to *X. oryzae* survival within the xylem (Hilaire *et al.*, 2001).

Our studies using the *oxyR* mutant suggest that ROS are present in sufficient quantity within grapevine xylem to impact the survival of *X. fastidiosa* during early infection. This implies that tolerance to ROS is an important aspect of multiplication within the xylem during the early invasion process. However, *X. fastidiosa* may be more sensitive than other pathogens to the ROS concentration in the xylem. A successful insect transmission event can occur even when a sharpshooter inoculates very few *X. fastidiosa* (less than 100 cells) into a xylem element (Hill and Purcell, 1995a). These bacterial cells need to survive long enough to express the genes necessary for successful colonization. Most sharpshooters prefer to feed primarily on the new growth of grapevine shoots (Redak *et al.*, 2004). As the shoot develops, the new tissue lignifies rapidly and probably contains high concentrations of developmentally related ROS. Thus, to meet the physiological demands of xylem colonization, *X. fastidiosa* probably detoxifies ROS immediately on inoculation. In our infection studies, *X. fastidiosa* was injected into xylem using mechanical needle inoculation, which introduces approximately 4×10^6 bacterial cells into the xylem. Although this cell number is several orders of magnitude higher than the numbers of cells introduced by the insect vector, we still observed an impact of ROS on bacterial survival. At 11 weeks post-inoculation, 87% of the plants inoculated with the wild-type control showed detectable *X. fastidiosa* populations. In contrast, only 33% of the plants inoculated with the *oxyR* mutant showed a detectable *X. fastidiosa* population, and these infected plants harboured 1000-fold fewer bacteria than the wild-type-infected plants. Thus, the vulnerability to ROS, regardless of the source, affects the ability of the *oxyR* mutant to reach wild-type titres within the xylem.

Interestingly, despite the overall colonization defect, there was no significant difference in virulence observed between the *oxyR* mutant and the wild-type parent over the course of the 18-week virulence study. This suggests that, although the growth of the *X. fastidiosa oxyR* mutant has been compromised, the mutant is still able to achieve bacterial numbers above the threshold necessary to cause PD symptoms. The fully virulent phenotype observed with the *X. fastidiosa oxyR* mutant is not unique. A fully virulent phenotype has also been reported with the *oxyR* mutant in *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) (Miguel *et al.*, 2000). However, in *R. solanacearum* (Flores-Cruz and Allen, 2011), *Pantoea stewartii* (Burbank and Roper, 2014) and *X. campestris* pv. *campestris* (Charoenlap *et al.*, 2011), the absence of OxyR has a profound impact on pathogenicity. Thus, the role played by OxyR in pathogenicity is not simple and varies depending on the bacterium, the host plant and the plant tissue entry points. Indeed, in the xylem phase of infection of corn seedlings by *P. stewartii*, OxyR-dependent regulation is critical for the elicitation of the wilting symptoms that are a result of xylem colonization, but is not involved in the elicitation of water-soaked lesions that occur specifically in the leaf apoplast (Burbank and Roper, 2014).

Mutations in *oxyR* can also have a major impact on biofilm formation (Hennequin and Forestier, 2009; Honma *et al.*, 2009; Seib *et al.*, 2007; Shanks *et al.*, 2007). This is a result of the role of OxyR in the regulation of biofilm-associated behaviours, such as fimbrial or exopolysaccharide (EPS) production (Burbank and Roper, 2014; Hennequin and Forestier, 2009; Honma *et al.*, 2009). Because bacterial cells that are members of a biofilm are better able to survive exposure to a variety of stresses, such as oxidative stress (Elkins *et al.*, 1999; Hassett *et al.*, 1999), it is not surprising that many plant-pathogenic bacteria choose to enter the biofilm mode during the early colonization process (Bogino *et al.*, 2013). During the initial stage of infection, when bacterial cell numbers are low, we speculate that OxyR induces the production of scavenging enzymes that detoxify ROS, thereby helping *X. fastidiosa* to survive as a non-biofilm-associated planktonic cell. Following this stage, it would be beneficial for *X. fastidiosa* to form xylem wall-adherent biofilms that would allow more permanent

Table 1 Bacterial strains and plasmids used in this study.

Bacterial strain	Characteristics	Reference
Xylella fastidiosa Temecula1	ssp. *fastidiosa*, wild-type isolate from grape	Guilhabert *et al.* (2001)
X. fastidiosa oxyR mutant (PW31)	Temecula1 containing *oxyR::kan-2*, Km^R*	This study
Escherichia coli DH10β	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74*rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ^-	Invitrogen, Carlsbad, CA, USA
Plasmid		
pUC19	Ap^R	Invitrogen, Carlsbad, CA, USA
pAX1Cm	Ap^R, Cm^R	Matsumoto *et al.* (2009)
pPW01	Ap^R, pUC19-*oxyR*	This study
pPW02	Ap^R, Km^R, pUC19-*ompR::kan-2*	This study

*Ap^R, Km^R, Cm^R and Str^R indicate resistance to ampicillin, kanamycin, chloramphenicol and streptomycin, respectively.

tolerance to chronic exposure to ROS and potential fluctuations in the redox status in the xylem. Our biofilm studies conducted under laboratory conditions indicate that OxyR serves as part of the mechanism that signals *X. fastidiosa* attachment to surfaces and to itself, both early steps in biofilm development. Indeed, the *oxyR* mutant cells were sparsely attached to glass slides and exhibited an uneven distribution of cells over the glass surface; these cells also failed to build upon themselves over time, which resulted in a significantly thinner biofilm relative to wild-type cells, as observed by CLSM. At present, it is unclear whether OxyR regulates biofilm-related phenotypes directly or indirectly in *X. fastidiosa*. However, it should be possible to resolve this issue in future transcriptomics and gene-specific promoter-binding studies.

The identification of the signals controlling *X. fastidiosa* biofilm formation is critical for an understanding of the progression of disease symptoms within the host. Biofilm formation in xylem results in heavily occluded vessels that are compromised in their ability to facilitate the movement of xylem sap throughout the plant. The replenishing flow of xylem sap is a major source of nutrients for both the plant and for xylem-dwelling organisms, including *X. fastidiosa*. Consequently, after *X. fastidiosa* has established as a biofilm community in a given xylem vessel, it must eventually initiate the exploratory phase of systemic colonization to move out of the original vessel before it becomes too occluded, and to escape into a new vessel. The ability of individual *X. fastidiosa* cells to move from a heavily colonized vessel to an open vessel is essential for the bacterium to gain nutrients and thrive. It has been postulated that individual *X. fastidiosa* cells can escape the biofilm into a new vessel, because a subset of the biofilm-associated cells can adjust their adhesiveness to successfully release from the biofilm. This decreased attachment allows a sector of the bacterial population to readily move within the xylem network during the exploratory stage of xylem colonization (Clifford *et al.*, 2013; Guilhabert and Kirkpatrick, 2005; Ionescu *et al.*, 2014; Newman *et al.*, 2004). The *oxyR* mutant does not attach well to surfaces or to itself, and thus appears to be locked in the exploratory phase of infection. Based on our data, it is tempting to speculate that OxyR could act as a regulatory switch that enables *X. fastidiosa* to sense a plant-associated molecular cue (ROS) and prevent bacterial cells from being killed by exogenous stresses encountered in the new xylem vessel. OxyR may also play an important role in adjusting bacterial adhesiveness in response to these stresses and in a host context-dependent manner to facilitate movement from one xylem conduit to another.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and PCR primers

All bacterial strains, plasmids and PCR primers employed in this study are listed in Tables 1 and 2.

Media and growth conditions for bacterial strains

Xylella fastidiosa was grown in liquid or solid PD3 medium (Davis *et al.*, 1981) at 28 °C. Transformants were selected using PD3 supplemented with either 5 μg/mL kanamycin or 5 μg/mL chloramphenicol. *Escherichia coli* strains were cultured at 37 °C in Luria–Bertani (LB) medium (Bertani, 2004). *Escherichia coli* transformants were selected using LB supplemented with either 50 μg/mL kanamycin or 100 μg/mL ampicillin.

Construction of the *oxyR*::*kan* mutant

PCR primers OxyRfwdEcoRI and OxyRrevHindIII were used to amplify the wild-type *oxyR* gene (PD0747) open reading frame (ORF) with its flanking regions from *X. fastidiosa* genomic DNA (GCA_000007245.1) to yield a 1.3-kb amplicon. Following digestion with *Eco*RI and *Hind*III, the amplicon was inserted into the *Eco*RI and *Hind*III sites of pUC19, thereby creating pPW01. Construct pPW01 was mutagenized using the EZ-Tn5 <KAN-2> insertion kit (Epicentre Technologies, Madison, WI, USA). Following mutagenesis, the plasmid was isolated and transformed into *E. coli* DH10β cells. Candidate constructs, which were identified by selection on LB solid medium containing 50 μg/mL kanamycin, were extracted from *E. coli* transformants and sequenced bidirectionally using two transposon-specific primers, KAN-2 FP-1 and KAN-2 RP-1. Based on the sequencing results, one construct was selected for further characterization and named

Table 2 Primers used in this study.

Primer	Sequence (5′ → 3′)	Reference
OxyRfwdEcoRI	GCAGT*GAATTC*CAGATTGTTATTGCGATG*	This study
OxyRrevHindIII	TCGAC*AAGCTT*CAATTTCAAAACGCTCC*	This study
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC	Epicentre Technologies, Madison, WI, USA
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG	Epicentre Technologies, Madison, WI, USA
OxyRoutfwd	GTATTGCAACGCAAGCTACGCAGT	This study
OxyRoutrev	ACGTGCAGAGTCGAGATGGGTAAT	This study
16s rRNA-RT1	AAGGGTGCGTAGGTGGTTATTTA	This study
16s rRNA-RT2	CGCTTTCGTGCCTCAGTGTC	This study
OxyR-RT1	CCGAGGTTGAGGAAATGAAGGAAG	This study
OxyR-RT2	GATACCCACATTAGCAACGACCAT	This study
AhpC-RT1	TGGGCTGTGTTTGTTTTCTATCCT	This study
AhpC-RT2	TTTCCCGCCTTCTTCCCACTTAGC	This study
AhpF-RT1	TGGCTATTGACGGTGCATTGTTCC	This study
AhpF-RT2	ACCCCCTTGTTTCGATACTCATCC	This study
CpeB-RT1	GGCGCGGCTCCAGCAGACAAC	This study
CpeB-RT2	TGATAGGCGCCGGCAGGACTTTT	This study

*The restriction enzyme sequence in the primers is shown in italic type.

pPW02. In construct pPW02, the transposon was inserted between nucleotides 619 and 620 within the *oxyR* ORF and resulted in the *oxyR* mutation, *oxyR::kan*−2.

To generate the *oxyR* mutant, *X. fastidiosa* electrocompetent cells were prepared as described previously (Matsumoto *et al.*, 2009) and electroporated with 200 ng of pPW02 DNA. Transformants were selected on PD3 solid medium containing 5 μg/mL kanamycin. Replacement of the chromosomal wild-type *oxyR* gene with the *oxyR*::*kan*−2 mutation was confirmed by PCR using the primers OxyRoutfwd and OxyRoutrev. The resulting amplicons were sequenced, confirming the gene replacement event. The resulting strain, which is referred to as the *oxyR* mutant, was designated as PW31 (Table 1).

Measurement of H_2O_2 sensitivity

Disc diffusion assay

A disc diffusion method was employed to test H_2O_2 sensitivity as described previously (Matsumoto *et al.*, 2009) with minor modifications. In brief, strains were grown for 7 days on PD3 solid medium, harvested, resuspended in PD3 liquid medium and adjusted to an optical density at 600 nm (OD_{600}) of 0.1. A cell suspension (500 μL) was mixed with 5 mL of PD3 top agar (0.8% agar), gently vortexed and poured onto PD3 solid medium. An autoclaved Whatman disc paper was placed in the centre of the plate. Ten microlitres of 100 mM H_2O_2 were pipetted onto the paper to provide the H_2O_2 stress source. The diameter of the inhibition zone surrounding the disc was measured following 7 days of incubation at 28 °C. Three replications were performed for each strain.

H_2O_2 survival assay

Xylella fastidiosa survival in the presence of H_2O_2 stress was evaluated using a modified version of a previously established protocol (Pericone *et al.*, 2003). In brief, the *X. fastidiosa* strains were harvested from PD3 solid medium after 7 days of incubation at 28 °C and resuspended in 1 × phosphate-buffered saline (PBS). Harvested cells were adjusted to $OD_{600} = 0.25$ in 1 × PBS. Then, 500 μL of cell culture and 500 μL of PBS were mixed in a 50-mL tube (BD Biosciences, San Jose, CA, USA). At time zero, 5 μL of 100 mM H_2O_2 were added to 1 mL of the mixture (final H_2O_2 concentration, 0.5 mM). Cells without H_2O_2 treatment served as controls. Tubes were shaken at 100 rpm at 28 °C for 30 min and then incubated on ice. Cell cultures were serially diluted using PBS. Then, 20 μL of each dilution from the H_2O_2-treated or untreated cells were placed in a stripe pattern onto PD3 solid medium and incubated at 28 °C for 10 days. The number of 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)].

Host colonization and virulence assays

Xylella fastidiosa wild-type and *oxyR* mutant cells were grown on solid PD3 medium for 7 days and harvested in PBS. The concentrations of the resulting bacterial suspensions were adjusted to $OD_{600} = 0.25$ to ensure that the same number of cells were present in each inoculum. *Vitis vinifera* cv. Thompson seedless grapevines were needle inoculated with 20 μL of bacterial suspension using a 20-gauge syringe needle as described previously (Hill and Purcell, 1995b). Grapevines were inoculated twice on opposite sides of the stem with one of the following: *X. fastidiosa* wild-type, the *oxyR* mutant or 1 × PBS. Three biological replications were performed, and 10 technical replications were included for each biological replication. The inoculated plants were rated weekly for disease severity using an established PD severity scale (Guilhabert and Kirkpatrick, 2005; Hopkins, 1985; Roper *et al.*, 2007b). On this scale, plants are assigned a score of 0–5: 0, no PD symptoms (healthy); 1, one or two leaves exhibiting marginal necrosis; 2, two or three leaves showing heavy marginal necrosis; 3, 50% or more of leaves displaying marginal necrosis and a few matchsticks (attached petioles whose leaf blades have abscised); 4, all leaves displaying significant scorch symptoms and numerous matchsticks; 5, dead vine.

To determine the bacterial titre in the plant, wild-type and *oxyR* mutant populations were quantified from petioles collected from the POI

at 11 weeks post-inoculation, as described previously (Roper *et al.*, 2007b). CFUs were enumerated after 10 days of incubation at 28 °C.

Surface attachment assay

A crystal violet staining method was used to compare the surface attachment of the *oxyR* mutant and wild-type, as described previously with minor modifications (Espinosa-Urgel *et al.*, 2000). Briefly, cells were grown for 7 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 7 days of static incubation at 28 °C, 100 μL of a 1% crystal violet solution was added to each tube and the cultures were incubated for 20 min. The supernatant was then removed and the tube was washed three times with double-distilled H_2O. The cells that remained attached to the tube were eluted using 1 mL of 95% ethanol; OD_{600} was measured in a spectrophotometer. Three independent biological replications were performed and one representative culture tube is shown in Fig. 3A. Twelve technical replications were included in each biological replication. The results were analysed by a two-tailed Student's *t*-test.

Cell–cell aggregation assay

Cell–cell aggregation was examined for both the *oxyR* mutant and wild-type using a previously described protocol (Burdman *et al.*, 2000) with minor modifications. Briefly, each strain was grown for 7 days on solid PD3 medium, harvested and resuspended in PD3 liquid medium. The cell concentration was then adjusted to $OD_{600} = 0.1$. The resulting cell suspensions were diluted 1 : 10 in 1 mL of PD3 liquid medium in glass tubes. After 10 days of static incubation at 28 °C, the cultures were agitated slightly and allowed to settle for 20 min. OD_{540} values turbidity of culture suspension and turbidity of homogenized culture (ODs and ODt) were measured as described previously (Burdman *et al.*, 2000). The cell aggregation percentage for each strain was calculated using the following formula: aggregated cell percentage = 100(ODt − ODs)/ODt. Four independent biological replications were performed and one representative replication is shown in Fig. 3B. Three technical replications were included in each biological replication. The results were analysed by a two-tailed Student's *t*-test.

Temporal three-dimensional biofilm architecture

The kinetics of three-dimensional biofilm formation were evaluated using CLSM, as described previously (Roper *et al.*, 2007a). In brief, glass slides were vertically set in 50-mL Falcon tubes containing bacterial liquid cultures. Following 2, 4, 6 or 8 days of incubation, the glass slides were removed and gently heated to fix the biofilm. The fixed biofilm was stained with 20 μM Syto9 (Invitrogen, Carlsbad, CA, USA) for 15 min, rinsed with 1 × PBS and mounted in Slowfade mounting fluid (Invitrogen). 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 488-nm laser. Three technical replications were performed for each strain at each time point (2, 4, 6 or 8 days). Ten images were captured for each replicate along the *z*-axis with a 0.4-μm constant interval. Imaris software (Bitplane USA, South Windsor, CT, USA) was used to create a three-dimensional view of the biofilms and to calculate their thickness.

Analysis of *ahpC–ahpF–oxyR* organization by RT-PCR

Four-day-old *X. fastidiosa* wild-type cells were harvested from PD3 solid medium and resuspended in 4 mL of PD3 liquid medium. One millilitre of bacterial culture was inoculated in 500 mL PD3 medium and incubated at 28 °C with 100 rpm shaking. After 3 days, 10 mL of culture were transferred to a 15-mL tube; transcription was stopped by the addition of rifampicin to a final concentration of 80 μg/mL. The cells were washed with 1 mL of cold Ambion RNA later solution (Life Technologies, Grand Island, NY, USA) and the pellets were stored at −80 °C. Wild-type total mRNA was isolated from the cell pellets using Trizol Reagent (Life Technologies) following the manufacturer's protocol. Genomic DNA was eliminated from the samples using the genomic DNA wipeout buffer provided in the Quantitech reverse transcription kit (Qiagen, Chatsworth, CA, USA). cDNA was synthesized from 1 μg of RNA using random hexamer primers provided in the Quantitech reverse transcription kit (Qiagen), as described by the manufacturer.

To determine whether *ahpC*, *ahpF* and *oxyR* were in the same operon, cDNA from this region of the chromosome was examined using the primer pairs AhpC-RT1/AhpC-RT2, AhpF-RT1/AhpF-RT2, OxyR-RT1/OxyR-RT2, AhpC RT-1/AhpF RT-2, AhpF RT-1/OxyR RT-2 and AhpC RT-1/OxyR RT-2, as indicated in the legend to Fig. 5 and in Table 2. Each 12.5-μL PCR contained 1 μL double-distilled H_2O, 1 μL forward primer (5 μM) and 1 μL reverse primer (5 μM), 5 μL cDNA template (10 ng), 2.5 μL of 5 × LongAmp Taq reaction buffer, 1.5 μL of 2.5 mM deoxynucleoside triphosphate (dNTP) and 0.5 μL of LongAmp DNA polymerase (NEB Biolabs, Ipswich, MA, USA). Amplicons were generated using the following PCR programme: 94 °C for 30 s (one cycle), followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 65 °C for 5 min, and a final extension at 65 °C for 10 min (one cycle).

Gene expression analysis using quantitative PCR

Xylella fastidiosa cells were grown for 7 days in 10 mL of PD3 liquid medium. A 1-mL aliquot of the cultures was inoculated into 100 mL of PD3 liquid medium (1 : 100 dilution), and the resulting culture was incubated at 28 °C with aeration (100 rpm) for 3 days. Then, 10 mL of culture were transferred to a 15-mL tube and H_2O_2 was added to a final concentration of 0.5 mM. After 10 min, rifampicin (final concentration, 80 μg/mL) was used to stop transcription. Cells were then washed with 1 mL of cold Ambion RNA later solution (Life Technologies) and the pellets were stored at −80 °C for 24 h. Total mRNA from the wild-type and *oxyR* mutant was isolated from frozen cell pellets using Trizol Reagent (Life Technologies); cDNA was prepared as described in the RT-PCR analysis section above.

Real-time PCRs were carried out in a DNA Engine Opticon2 machine (MJ Research Incorporation, Waltham, MA, USA) using the following conditions: 98 °C for 3 min (one cycle), followed by 98 °C for 10 s and 60 °C for 20 s (40 cycles). Melting curve analysis was carried out from 60 to 95 °C (in 0.2 °C increments for 1 s). Each 20-μL PCR contained 7.6 μL diethylpyrocarbonate (DEPC)-treated water, 0.2 μL forward primer and 0.2 μL reverse primer (10 pM), 2 μL cDNA template (50 ng) and 10 μL 2 × SSoFast Evagreen Supermix (Bio-Rad, Hercules, CA, USA). The 16S rRNA gene

served as the internal normalization control. Primer pairs AhpC-RT1/AhpC-RT2, AhpF-RT1/AhpF-RT2, OxyR-RT1/OxyR-RT2, CpeB-RT1/CpeB-RT2 and 16s rRNA-RT1/16s rRNA-RT2 were used for expression analysis of genes *ahpC*, *ahpF*, *oxyR*, *cpeB* and 16S rDNA individually. Each reaction was performed in duplicate. The data were analysed by the previously described method (Livak and Schmittgen, 2001).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 A mutation in OxyR does not have a significant impact on *Xylella fastidiosa* virulence in grapevines. *Vitis vinifera* cv. Thompson seedless grapevines were needle inoculated twice on opposite sides of the stem with one of the following: *X. fastidiosa* wild-type (WT), the *oxyR* mutant or 1 × phosphate-buffered saline (PBS). Three biological replications were performed, and 10 technical replications were included for each biological replication. The inoculated plants were rated weekly for disease severity using a scale of 0–5 with 0 = healthy and 5 = dead or dying. No significant difference in virulence was observed between the WT and *oxyR* mutant.