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Characterization of an oxidative stress response regulator, homologous to *Escherichia coli* OxyR, from the phytopathogen *Xylella fastidiosa*

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ABSTRACT

The OxyR oxidative stress transcriptional regulator is a DNA-binding protein that belongs to the LysR-type transcriptional regulators (LTTR) family. It has the ability to sense oxidative species inside the cell and to trigger the cell's response, activating the transcription of genes involved in scavenging oxidative species. In the present study, we have overexpressed, purified and characterized the predicted OxyR homologue (*orf xf*1273) of the phytopathogen *Xylella fastidiosa*. This bacterium is the causal agent of citrus variegated chlorosis (CVC) disease caused by the 9a5c strain, resulting in economic and social losses. The secondary structure of the recombinant protein was analyzed by circular dichroism. Gel filtration showed that XfoxyR is a dimer in solution. Gel shift assays indicated that it does bind to its own predicted promoter under *in vitro* conditions. However, considering our control experiment we cannot state that this interaction occurs *in vivo*. Functional complementation assays indicated that *xfoxyR* is able to restore the oxidative stress response in an *oxyr* knockout *Escherichia coli* strain. These results show that the predicted *orf xf*1273 codes for a transcriptional regulator, homologous to *E. coli* OxyR, involved in the oxidative stress response. This may be important for *X. fastidiosa* to overcome the defense mechanisms of its host during the infection and colonization processes.

Introduction

Oxidative stress plays an important role in the plant defense system against phytopathogens invasion and colonization [1]. Reactive oxygen species (ROS) (H_2O_2, organic peroxides, and superoxide anions) are generated by the metabolism of the plant cell in response to microbial infection. The ROS are capable of oxidizing many cellular components including DNA, lipid membranes and proteins resulting in cell damage [1–3]. These ROS are also generated by normal aerobic metabolism [4]. Therefore, for a phytopathogen, the ability to remove these ROS could be advantageous, allowing it to avoid the harmful effects of its hosts so called oxidative burst defense mechanism [2,5].

In *Escherichia coli*, the role and mechanism of action of the OxyR transcriptional regulator has been well-characterized [6–8]. This transcription regulation factor belongs to the family of LysR-type transcriptional regulators (LTTRs), the largest known family of prokaryotic DNA-binding proteins [9], and has the capacity to sense low amounts of intracellular hydrogen peroxide [10]. Proteins from this family have a highly conserved N-terminal DNA binding domain and a C-terminal regulatory domain that has a cleft for the association of a co-inducer molecule. This association with the co-factor is necessary for the transcription activity of the molecule which is activated by a structural change in the protein. The structural change affects its oligomerization and activity toward the promoter of the regulated gene [11]. Many classes of molecules can act as co-inducers including aminoacids, ions, carbohydrates and lipids. The co-inducers are normally involved in or are intermediate products in the pathway regulated by the LTTR [9]. Exposing the *E. coli* OxyR to oxidative stress leads to the formation of an intra molecular disulfide bond between cysteine residues in positions 199 and 208 and in this case, the covalent modification acts as the co-inducer molecule [8]. Data from the crystal structure of *E. coli* OxyR revealed that this covalent bond causes a large structural change in the regulatory C-terminal domain of the molecule [12]. The change is also implicated in the oligomerization of dimers bound to the promoter sequence leading to the transcriptional activation or repression of the regulated gene [12]. It was also shown that OxyR functions when bound to the DNA molecule as a dimer of dimers [13] The oxidized form of *E. coli* OxyR acts as a positive transcriptional regulator acting on the expression of at least nine hydrogen peroxide-inducible genes including catalase (*katG*), glutathione reductase (*gor*), glutaredoxin (*grxA*), alkyl

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hydroperoxide reductase (*ahpCF*), a regulatory RNA (*oxyS*), ferric uptake regulation (*fur*) and a nonspecific DNA-binding protein (*dps*) [14–17]. There is also a negative feedback loop described for the LTTRs family [9] in which the binding of the dimeric protein to its own promoter in a co-inducer independent way blocks its transcription. This feedback loop may be involved in the regulation of a divergently transcribed gene, which occurs simultaneously with the *oxyR* gene repression. In *E. coli*, the *oxyR* lacks this divergent regulation mechanism but still shows negative auto-regulation [11].

In the present work, the predicted homologue of the OxyR transcriptional regulator in the phytopathogen *Xylella fastidiosa* was biochemically and functionally characterized. *X. fastidiosa* is a Gram-negative, xylem limited phytopathogen, responsible for many diseases in economically relevant crops (citrus variegated chlorosis in citrus, Pierce's disease in grape, "phony peach" in peaches among others) [18–21]. The *orf xf*1273 (936 bp, 311 aminoacids, pI 6.42) predicted to be an OxyR homologue in the *X. fastidiosa* Comparative Genome Database (<www.xylella.lncc.br>), was cloned and the protein it encodes was expressed and purified. We demonstrate that the predicted *X. fastidiosa* OxyR is able to functionally complement an OxyR knockout *E. coli* strain and despite the observed interaction with its own promoter under *in vitro* conditions, we cannot assure that this interaction occurs *in vivo* and that the predicted *xfoxyR* promoter is functional. This report, in addition to describing and providing information about another LTTR protein, contributes to an initial understanding of the bacterial resistance mechanism based on scavenging reactive oxygen species generated by its host defense system.

Methods

Alignment of xfoxyR with E. coli and X. campestris homologues

Sequences were obtained from the NCBI data bank (<www.ncbi.nih.gov>) and aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

Cloning of xfoxyR in expression vectors

The *xfoxyR orf* was amplified with specific primers containing endonuclease restriction sites (forward with the *BamH*I site, 5′-AAGGATCCATGAACCTGCGTGACTT-3′ and reverse with the *Eco*RI site, 5′-GGAATTCCTACCGACCTTTGTACAGCA-3′) for specific insertion of the DNA fragment into the expression vector. The amplified product was purified (GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare, Buckinghamshire, UK) and cloned in pET28 (Novagen, Madison, WI, USA). The insertion of the desired *orf* in the expression vectors was verified by PCR using specific primers and flanking primers (T7 promoter and terminator). The positive clones were sequenced using the same set of primers.

Expression and extraction of recombinant XfOxyR$_{6His\text{-}tag}$ in E. coli

Plasmids containing *xfoxyR* were transformed into BL21(DE3) *E. coli* expression strain. Positive clones were incubated overnight in LB media with 30 μg ml^{-1} of kanamycin at 37 °C, 300 rpm. They were then transferred to 1 L LB media with the same antibiotic concentration. Expression, extraction and purification steps were performed based on the literature for the *Xanthomonas campestris phaseoli* [22] and *E. coli* OxyR [23]. When cultures reached and O.D. of 0.6–0.8 at 600 nm, protein expression was induced by adding 5.6 mM lactose to the media and further incubation at 28 °C, 200 rpm for 4 h. Cells were harvested by centrifugation at 3000*g* for 15 min at 4 °C and used for protein extraction. Initial extraction was performed using buffer containing Tris 20 mM pH 8.0, 500 mM NaCl, 1 mM PMSF (phenylmethanelsulfonyl fluoride, Sigma Chemical, St Louis, MO, USA) 1 mg/ml lysozyme, 0.1 mM EDTA. Cell suspensions were incubated on ice for 30 min on ice following cell lysis by sonication. Soluble protein fractions were recovered by centrifugation at 27,000*g* for 40 min at 4 °C. Purification of XfOxyR$_{6His\text{-}tag}$ was performed by a single step affinity chromatography using Ni–NTA column (Qiagen, Hilden, Germany). The presence of the desired protein in the soluble fraction was analyzed by SDS–PAGE.

Circular dichroism measurements

Circular dichroism (CD) spectra of the purified XfOxyR$_{6His\text{-}tag}$ protein were measured using a Jasco J-810 Spectropolarimeter dichrograph (Japan Spectroscopic, Tokyo, Japan). The far-UV CD spectra were generated at 20° C using XfOxyR$_{6His\text{-}tag}$ protein at 13.6 μM in 10 mM sodium phosphate buffer pH 8.0. The assays were carried out using a quartz cuvette with a 2 mm path length. Ten accumulations within the 260–185 nm range at a rate of 50 nm/min at were recorded. Deconvolution and statistical analysis of the CD spectra were performed using the Dichroweb server [24–26].

Size exclusion chromatography

To assess the oligomeric state of purified XfOxyR$_{6His\text{-}tag}$, size exclusion chromatography was performed using a Superdex 200 HR10/30 prepacked column (GE Healthcare, Uppsala, Sweden). After equilibration of the column with buffer containing 20 mM Tris, 500 mM NaCl, 0.1 mM EDTA and 200 mM imidazole, the sample (250 μL) was loaded at a flow rate of 0.75 mL/min. The calibration curve was prepared using thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) (GE Healthcare) as molecular weight standards.

Electrophoretic mobility shift assay

For the DNA–protein interaction analysis, the 104 bp region upstream of the *xfoxyR* gene start codon, corresponding to its promoter, was cloned into the pGEM-T Easy vector (Novagen, Madison, WI, USA) using specific primers (forward: 5′-TGAGCCAATGCAGTACAGCGGTTTA-3′, reverse: 5′-CATAAAGCCAAACCTCGCGGCAAG-3′). As a control, we amplified and cloned into the pGEM-T Easy vector the final 162 bp fragment from the *xf*1272 *orf*, localized upstream of the *xfoxyR* promoter (forward: 5′-GTGTTCGCTGCTGGTGATGCGA-3′, reverse: 5′-TCACGCTGCCTGTGCCTGAGC-3′). The cloned fragments were amplified, purified (by standard ethanol precipitation and with GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare, Buckinghamshire, UK) and used in shift assays. We used 100 ng of the DNA fragment in each shift reaction, 10 mM $MgCl_2$, 100 mM DTT (only for reduced conditions) and a gradient of purified XfOxyR$_{6His\text{-}tag}$ in a final volume of 40 μL (to reach this final volume XfOxyR$_{6His\text{-}tag}$ extraction buffer was used). Shift reactions with 50, 100 and 150 mM NaCl were also tested (data not shown). Different DNA:protein molar ratios were used (1:1, 1:2, 1:4, 1:8, 1:12, 1:16 and 1:20). Due to the protein's instability XfOxyR$_{6His\text{-}tag}$ was used in shift reactions immediately after purification by affinity chromatography without a further dialysis step. Reactions were incubated at room temperature for 45 min. Before loading onto the gel, 8.7% glycerol was added to the reaction. The DNA–protein interaction was analyzed in 1% agarose gels run in TAE buffer (40 mM Tris–acetate, 1 mM EDTA). Densitometry analysis was performed with Kodak Electrophoresis Documentation and Analysis System (EDAS).

Functional complementation assay

To analyze the ability of the *xfoxyR* gene product to act as an oxidative stress regulator we performed a functional complementation assay using an *oxyR* mutant *E. coli* strain (GS047 – MC4100 ▲*oxyr::kan*), kindly provided by Dr. Gisela Storz. The *xfoxyR orf* was cloned into pGEM-T Easy vector (Novagen) and the construct was transformed into *E. coli* ▲*oxyR* mutant strain and into a wild type strain. The clones obtained were analyzed by PCR using specific primers for *xfoxyR* listed above. The response of transformed cells to oxidative stress was analyzed by the hydrogen peroxide diffusion disc method [27]. Cells were grown overnight and plated (200 μL) in LB medium supplemented with 1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside). A 5 mm diameter Whatman disc with 10 μl hydrogen peroxide (3.5%) was then put on the plates which were incubated overnight at 37 °C. The growth inhibition zones were measured and compared with those obtained for the wild type strain and control clones (harboring the pGEM-T Easy vector only). Experiments were performed in triplicate and the collected data was analyzed by Student's *t*-test.

Results and discussion

Alignment of XfOxyR with homologues

Alignment of the *X. fastidiosa*, *E. coli* and *Xanthomonas campestris pv.campestris* OxyR aminoacid sequences (Fig. 1) showed a high degree of conservation (81% identity between *X. fastidiosa* and *X. campestris* and 47% identity between *X. fastidiosa* and *E. coli*). The N-terminal sequences (generally the first 90 aminoacids) presented more identities in agreement with the fact that among LTTRs the DNA binding domain is highly conserved [11]. Another interesting feature observed is the conservation of the redox switch composed of two cysteines in all sequences (located in positions 199 and 208 in the *E. coli* OxyR sequence). Prediction of secondary structure for XfOxyR using the PSIPRED server [28] also showed, for the N-terminal domain, a helix–turn–helix (HTH) pattern composed of three α-helices followed by two β-sheets which matches a LTTR DNA binding domain (data not shown).

Expression and extraction of recombinant

XfOxyR$_{6His\text{-}tag}$ in E. coli

The *xfoxyR* gene was successfully cloned in the expression vector. The positive clones were sequenced and no alteration in the DNA sequence was found. XfOxyR$_{6His\text{-}tag}$ was successfully expressed and purified according to the described methodology. The purity of the protein was assessed by 12% SDS–PAGE (Fig. 2). The recombinant XfOxyR$_{6His\text{-}tag}$ was obtained at a satisfactory purity level and concentration (∼1 mg/ml).

XfOxyR$_{6His\text{-}tag}$ initial characterization

The secondary structure of the purified protein was assessed by CD and a signal indicating the presence of α-helices and β-sheets was obtained (Fig. 2) showing that the protein has a secondary fold and could be used for further experiments. The oligomeric state of most LysR family proteins is a dimer, however, gel filtration and sedimentation experiments showed that *E. coli* OxyR is a tetramer in solution [13] although a dimeric state was also described [27]. Size exclusion chromatography showed that XfOxyR$_{6His\text{-}tag}$ is a dimer in solution right after elution from affinity chromatography. The predicted molecular weight for the recombinant protein is 38.64 kDa and we obtained an elution peak corresponding to a protein with 60,48 kDa which is close to the expected dimer molecular weight (Fig. 3). The purified sample showed a small fraction of protein aggregates that were almost completely removed when the sample was incubated with 50 mM DTT for 30 min. We also observed that increasing protein concentration leads to its aggregation and precipitation even under reducing conditions.

This behavior may be explained by the nature of the protein itself. Each molecule has four cysteines, two of them are in a conserved position (Cys199 and Cys208 that compose the redox switch in *E. coli* OxyR) forming the so called oxi-redox switch of the protein. Under oxidizing conditions and high protein concentration (*in vitro* conditions) the oxidation of the cysteines is favored and so is the oligomerization of molecules. This oligomerization process may be favored by the C-terminal domain of the molecule, involved in the formation of dimers and tetramers when bound to DNA [11]. This domain, under *in vitro* conditions, may favor non-specific oligomerization leading to aggregation. This also may explain the short stability period of the purified protein *in vitro* that tends to aggregate quickly (around 30 min at room temperature when at a concentration higher than 1 mg/ml) and the fact that addition of a reducing agent (DTT or β-mercaptoetanol) delays this process.

XfOxyR$_{6His\text{-}tag}$ does not interact with its own predicted promoter

We looked for the ability of the purified XfOxyR$_{6His\text{-}tag}$ to interact with its own promoter using an eletrophoretic mobility shift assay. Interaction of XfOxyR$_{6His\text{-}tag}$ with the 104 bp DNA fragment between the *xfoxyR* start codon and the previous *orf* (xf1272) was observed under reducing and non-reducing conditions (Fig. 5). The DNA fragment harbored the TATA box site and the predicted LysR type recognition sequences (a degenerated false palindrome, T-N$_{11}$-A). With similar methodologies used to show the interaction of *Legionella pneumophylla* OxyR ortholog with *ahpC2*

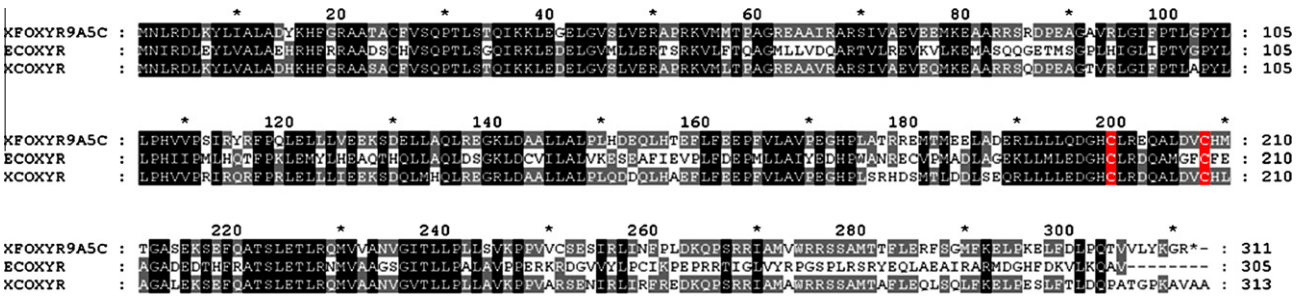


Fig. 1. ClustalW2 alignment of OxyR sequences from *X. fastidiosa* strain 9a5c (XFOXYR9A5C), *Escherichia coli* (ECOXYR), *Xanthomonas campestris* (XCOXYR). Regions conserved in the three sequences (black) and regions conserved only in two sequences (gray) are highlighted. Greater similarity between *X. fastidiosa* and *X. campestris* OxyR sequences can be observed. The oxi-redox switch, composed of two cysteines is indicated in red in all three sequences.

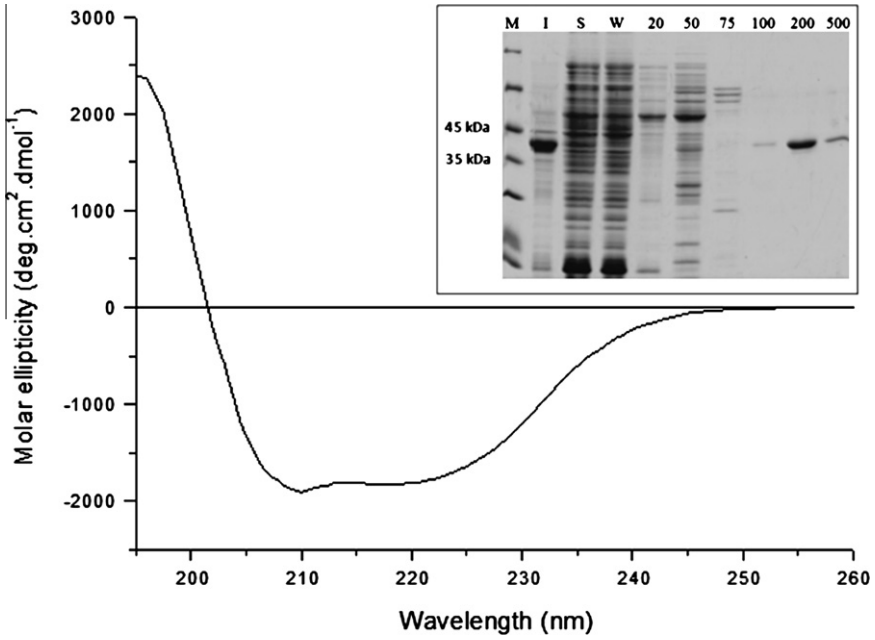


Fig. 2. Circular dichroism curve obtained for purified XfOxyR$_{6His\text{-}tag}$ in 10 mM sodium phosphate buffer pH 8.0. In detail, 12% SDS–PAGE. Purification of XfOxyR$_{6His\text{-}tag}$. M, fermentas unstained protein molecular weight marker; I, insoluble fraction; S, soluble fraction; W, wash fraction. 20–500: elution steps with 20–500 mM imidazole. XfOxyR$_{6His\text{-}tag}$ predicted weight: 38,64 kDa.

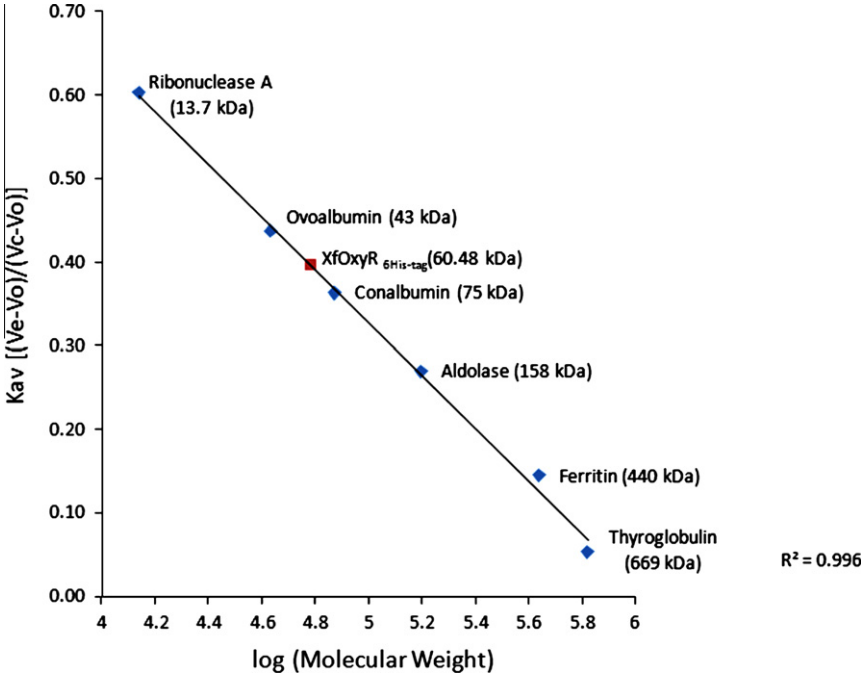


Fig. 3. Size exclusion chromatography calibration curve for XfOxyR$_{6His\text{-}tag}$.The estimated molecular mass for XfOxyR$_{6His\text{-}tag}$ is indicated in the trend line as a red square. Estimated molecular weight for the XfOxyR$_{6His\text{-}tag}$ dimer: 60.48 kDa. Monomer molecular weight: 38,64 KDa. Expected dimer molecular weight: 77,28.

promoter [29], we were able to show that XfOxyR$_{6His\text{-}tag}$ interacts *in vitro* with its own promoter in both, oxidized and reduced form but with different affinities in each case. As a control DNA fragment we used the last 162 bp from the previous *orf* (*xf1272*). Because it is a coding region, we expected no interaction between purified XfOxyR$_{6His\text{-}tag}$ and the control fragment. We performed the same DNA:protein molar ratio gradient (1:1, 1:2, 1:4, 1:8, 1:12, 1:16 and 1:20) with the *xfoxyR* promoter and the control fragment under reducing and oxidizing conditions to analyze its interaction with the purified protein and the affinities involved in each case. Interaction was observed with both fragments (the *xfoxyR* promoter and the control fragment) under reducing and non-reducing conditions. Shifted bands were observed from 1:2 to 1:20 DNA:protein molar ratios. The interaction of XfOxyR with the control fragment showed shifted bands with less intensity and lower shift compared to those seen with the *xfoxyR* promoter at the same

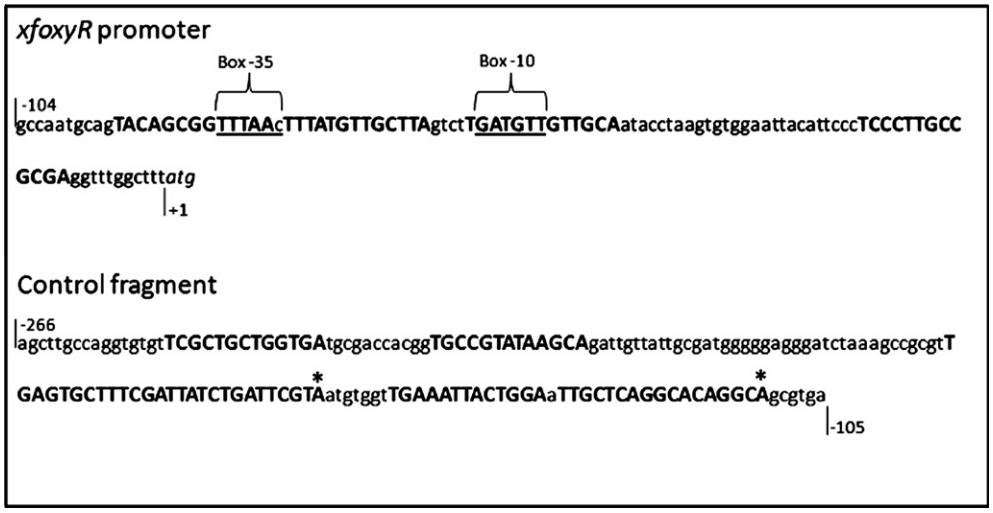
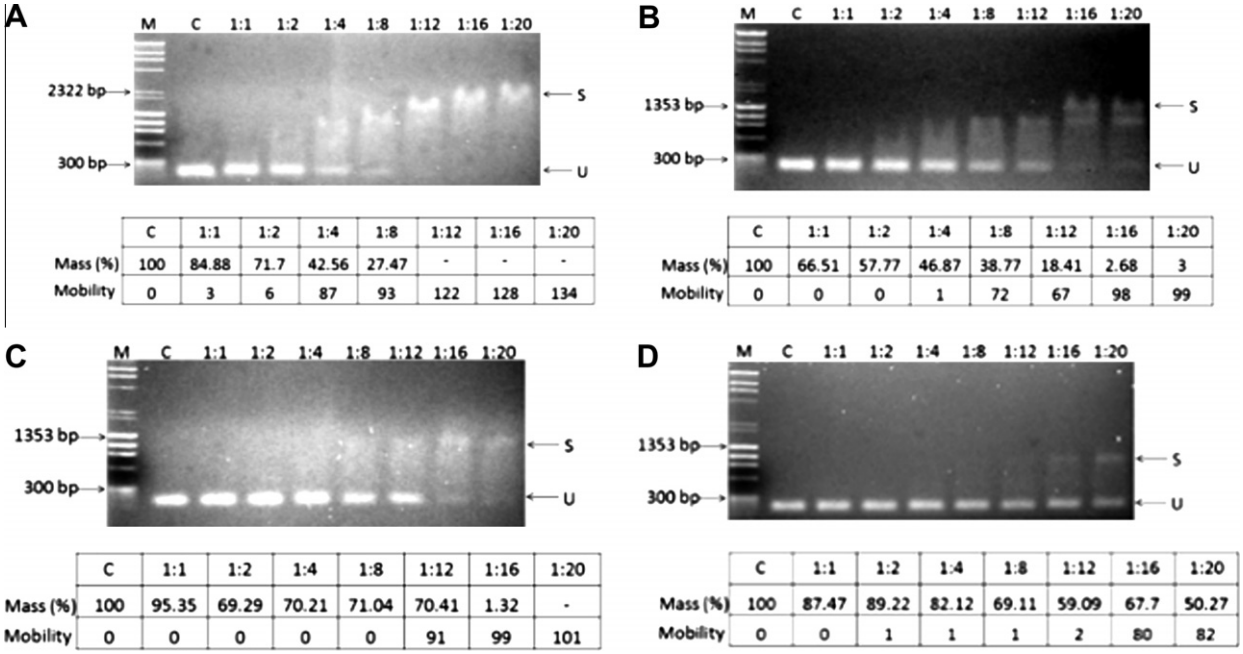


Fig. 4. Diagram showing the DNA fragments used in shift assays. The predicted Box-10 and Box-35 for *xfoxyR* promoter are indicated. +1 indicates the beginning of *xfoxyR* coding region. In both fragments LTTR predicted binding site (T-N_{11}-A) are indicated in uppercase letters. * Indicates that two or more binding sites are overlapped.



	C	1:1	1:2	1:4	1:8	1:12	1:16	1:20
Mass (%)	100	84.88	71.7	42.56	27.47	-	-	-
Mobility	0	3	6	87	93	122	128	134

	C	1:1	1:2	1:4	1:8	1:12	1:16	1:20
Mass (%)	100	66.51	57.77	46.87	38.77	18.41	2.68	3
Mobility	0	0	0	1	72	67	98	99

	C	1:1	1:2	1:4	1:8	1:12	1:16	1:20
Mass (%)	100	95.35	69.29	70.21	71.04	70.41	1.32	-
Mobility	0	0	0	0	0	91	99	101

	C	1:1	1:2	1:4	1:8	1:12	1:16	1:20
Mass (%)	100	87.47	89.22	82.12	69.11	59.09	67.7	50.27
Mobility	0	0	1	1	1	2	80	82

Fig. 5. Interaction of $XfOxyR_{6His\text{-}tag}$ with its own predicted promoter and control fragment (coding region from previous *orf xf1272*). Reducing and non-reducing conditions were tested in a DNA:protein molar ratio gradient. (A) *xfoxyR* promoter with reduced $XfOxyR_{6His\text{-}tag}$. (B) control fragment with reduced $XfOxyR_{6His\text{-}tag}$. (C) *xfoxyR* promoter with non-reduced $XfOxyR_{6His\text{-}tag}$. (D) control fragment with non-reduced $XfOxyR_{6His\text{-}tag}$. (C) control reaction with DNA fragment and no protein. 1:1,1:2,1:4,1:8, 1:12, 1:16 and 1:20:gradient of DNA:protein molar ratio. Shift bands (S) and unbound DNA fragment (U) are indicated. M: Molecular Weight Marker λ/ϕx. Densitometry analysis is shown bellow each respective gel. Mass (%) indicates the decrease in mass of the unbound fragment (in percentage). Mobility indicates the relative shift observed for the shifted bands with highest molecular weight in comparison with the unbound fragment (unbound fragment = 0 mobility).

molar ratio, indicating a lower affinity interaction for the control fragment. As mentioned, this fragment was chosen as a control for being a coding region. However, the binding site described for LysR family proteins is a pseudo-palindrome, T-N_{11}-A, that is often found to form part of an imperfect, dyadic region and such sequences were found in the *xfoxyR* promoter and in the control fragment as well. Indeed, more T-N_{11}-A sequences were found in the control fragment than in the *xfoxyR* promoter (Fig. 4). Initially, for *E. coli* OxyR it was found that it recognizes seemingly dissimilar sequences by using a multidegenerate recognition code [27], but in a more recent work [30] it was described a consensus binding site composed of ATAGnt elements spaced at 10 bp intervals. It was also reported a similar binding site for the of *X. campestris pv. phaseoli* OxyR in the *ahpC-oxyR* operon promoter (22). However, by sequence analysis such sequences could not be found in the *xfoxyR* promoter or in the control fragment. Another consideration to be made is the described operon organization of the *X. campestris pv. phaseoli oxyR orf*, which lies organized in a head-to-tail fashion with the *ahpF* and *ahpC orfs* coding for a 57 kDa flavoprotein and a 22 kDa protein respectively and both are transcriptionally regulated by OxyR. Together, they form a alkyl hydroperoxide reductase (AhpR), an enzyme responsible for the reduction of

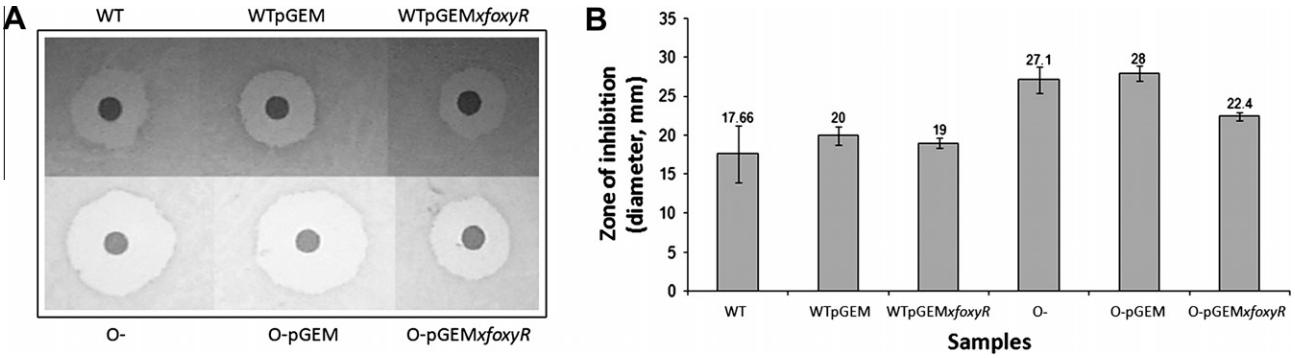


Fig. 6. H_2O_2 disk diffusion assay. (A) Photos showing the zones of growth inhibition by H_2O_2. (B) Histogram showing the results of the H_2O_2 growth inhibition assay. WT: *E. coli* MC4100 strain. WTpGEM: *E. coli* MC4100 strain transformed with pGEM-T Easy vector. WTpGEM *xfoxyR*: *E. coli* MC4100 strain complemented with *xfoxyR* cloned into the pGEM-T vector. O-: GS047 MC4100Δ*oxyR::kan* knockout strain. O-pGEM: GS047 MC4100Δ*oxyR::kan* knockout strain transformed with pGEM-T Easy vector. O-pGEM *xfoxyR*: GS047 MC4100Δ*oxyR::kan* knockout strain complemented with *xfoxyR* cloned into the pGEM-T vector. The recovery of H_2O_2 resistance can be shown by the fact that no statistically significant difference could be noted between wild type strain harboring only the pGEM vector (WT pGEM) and the knockout strain complemented with the *xfoxyR* gene: O-pGEM *xfoxyR* ($P = 0.124$).

organic peroxide to the corresponding alcohols. The *ahpC orf* is monocistronic while the *oxyR* and *ahpF* are transcribed together in the *ahpF-oxyR* operon. This unusual genome organization of the OxyR protein is highly conserved in Xanthomonas spp [31]. Therefore, gel shift assays performed with *X. fastidiosa* OxyR and its predicted promoter show that they interact under the *in vitro* conditions tested. However, we cannot state that this interaction occurs *in vivo* considering the facts that only LTTRs binding motifs were found in the *xfoxyR* promoter sequence and they were also found in the control fragment, which may have caused the observed shifted bands. No OxyR binding motifs similar to those described for *E. coli* or *X. campestris* were found in both fragments. Finally, the *xfoxyR orf* is in a similar genome organization compared to *X. campestris oxyR* with the *orfs Xf*1271 and *Xf*1272 coding for AhpF and AhpC respectively while the *Xf*1273 *orf* corresponds to *xfoxyR*. Considering the phylogenetic proximity of both species (*X. fastidiosa* and *X. campestris* that are enclosed in the *Xanthomonadales* group) [32] and the similarity shown in their OxyR protein sequence (see Fig. 1) we can consider that the operon organization of *oxyR orf* observed for *X. campestris* could also be present in *X. fastidiosa*. In this scenario, xfoxyR would be transcriptionally regulated by the promoter region upstream the *orf Xf*1272 (that codes for AhpF). For this characterization, further experiments involving shift assays, DNA footprinting experiments with *X. fastidiosa ahpC* and *ahpF* promoter regions would lead to a best understanding and characterization of the genome organization of *xfoxyR* as well as its transcriptional regulation and DNA recognition mechanism by the encoded protein.

XfOxyR acts as a transcriptional regulator in vivo activating the oxidative stress response

Once we confirmed that XfOxyR acts as a DNA-binding protein under the *in vitro* conditions tested, we looked for its role as an oxidative stress response regulator *in vivo*. The *xfoxyR orf* cloned into pGEM-T Easy vector (Novagen) was used to transform an *oxyR* defective *E. coli* strain (GS047 – MC4100 ▲*oxyR::kan*). Functional complementation was observed using the H_2O_2 diffusion disc assay (Fig. 6). Comparison of the growth inhibition zones allowed us to conclude that the *oxyR* defective strain that received the *xfoxyR* gene cloned into pGEM-T vector was significantly more resistant to hydrogen peroxide killing than the *oxyR::kan* mutant harboring only the pGEM-T vector ($P = 0.0167$). This functional complementation could also be concluded from the fact that no statistically significant difference was found when comparing the wild type strain harboring only the pGEM-T vector and the mutant strain complemented with the *xfoxyR* ($P = 0.124$). Therefore, OxyR from *X. fastidiosa* does function as an oxidative stress sensor transcriptional regulator, able to functionally complement an OxyR defective *E. coli* strain, probably involved in the transcriptional activation of many genes involved in scavenging oxidative molecules.

Conclusions

Oxidative stress stands as a defense mechanism for plants in response to infection by bacteria. Therefore, for a phytopathogen, it is essential to count on a metabolic pathway capable of scavenging all the reactive oxygen species generated by its host or those originated from its own metabolism as well. We describe here, the characterization of the oxidative stress response transcriptional regulator OxyR from *X. fastidiosa*. This transcriptional regulator has been characterized in many bacteria [6,11,22,23,29] and is shown to be involved in activating the transcription of many genes involved in the oxidative stress response. Based on our results, we show that the *xfoxyR orf* (*xf1273*, according to the *Xylella fastidiosa* Genome DataBase) encodes a 311 aminoacids polypeptide with a secondary structure signal that indicates the presence of α-helices and β-sheets in agreement with *in silico* predictions. Under reducing conditions, the purified $XfOxyR_{6His\text{-}tag}$ protein appears as a dimer in solution. However, at elevated concentrations in solution and under non-reducing conditions, it tends to aggregate quickly, probably due to nonspecific disulfide bond formation in its C-terminal domain, a domain that is involved in the dimerization process described for *E. coli* OxyR. Shift assays showed that purified $XfoxyR_{6His\text{-}tag}$ interacts with its own promoter *in vitro*, but it also interacts with an intragenic DNA fragment used as control. This indicates that there is no clear evidence that $XfOxyR_{6His\text{-}tag}$ interacts with its own promoter that would suggest an auto-regulation mechanism. Considering the genomic organization of the *oxyR orf*, described for *X. campestris* and its phylogenetic proximity to *X. fastidiosa*, it may also be possible that *xfoxyR* is transcribed in an operon with the *ahpF orf*. Finally, complementation studies revealed that the *xfoxyR* gene is able to restore the oxidative stress response to an OxyR defective *E. coli* mutant strain, indicating that the coded protein indeed acts as an oxidative stress transcriptional regulator. For a phytopathogen like *X. fastidiosa*, a molecular mechanism that senses small amounts of oxidative molecules inside the cell may play an important role during the infection and colonization processes, helping to overcome the host defense system. Considering the biological relevance of this transcriptional factor

to *X. fastidiosa* metabolism and pathogenicity, further studies will uncover the mechanisms that underlie the oxidative stress response that allows this bacterium to infect and colonize successfully their hosts.

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