OxyR contributes to virulence of *Acidovorax citrulli* by regulating anti-oxidative stress and expression of flagellin FliC and type IV pili PilA

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In many bacteria, OxyR acts as a transcriptional regulator that facilitates infection via degrading hydrogen peroxide (H2O2) generated by the host defense response. Previous studies showed that OxyR also plays an important role in regulating biofilm formation, cell motility, pili relate-genes expression, and surface polysaccharide production. However, the role of OxyR has not been determined in *Acidovorax citrulli* strain xjl12. In the current study, the qRT-PCR and western blot assays revealed that the expression level of oxyR was significantly induced by H2O2. The oxyR deletion mutant of *A. citrulli* was significantly impaired bacterial tolerance to oxidative stress and reduced catalase (CAT) activity. In addition, oxyR mutant resulted in reduced swimming motility, twitching motility, biofilm formation, virulence, and bacterial growth in planta by significantly affecting flagellin and type IV pili-related gene (*fliC* and *pilA*) expression. The qRT-PCR assays and western blot revealed that OxyR positively regulated the expression of *fliC* and *pilA*. Furthermore, bacterial one-hybrid assay demonstrated that OxyR directly affected pilA and *fliC* promoter. Through bacterial two-hybrid assay, it was found that OxyR can directly interact with PilA and FliC. These results suggest that OxyR plays a major role in the regulating of a variety of virulence traits, and provide a foundation for future research on the global effects of OxyR in *A. citrulli*.

**KEYWORDS**

*Acidovorax citrulli*, OxyR, oxidative stress, virulence traits, virulence

**Introduction**

The Gram-negative bacterium *Acidovorax citrulli* is the causal agent of bacterial fruit blotch (BFB; Schaad et al., 1978, 2008; Willems et al., 1992), a threatening disease of cucurbit crop species worldwide (Schaad et al., 2003; Burdman and Walcott, 2012). According to Hopkins and Thompson (2002), the bacterium is transmitted and spread by infected seeds,
which are the primary inoculum sources for BFB outbreaks. Seed treatments can effectively reduce the spread of disease but often fail to eradicate pathogens in seeds (Dutta et al., 2012). Therefore, understanding the mechanisms of host-pathogen interactions is of great importance for effective BFB management. The interaction between plants and pathogens is that pathogens infect host cells with a variety of virulence traits to acquire nutrients and water for survival and development, while plants prevent the invasion of pathogens through defense responses (Chisholm et al., 2006; Jones and Dangl, 2006; Kunkel and Chen, 2006). *A. citrulli* utilizes multiple virulence traits to infect plant cells, e.g., type II secretion system (T2SS; Johnson, 2010), type III secretion system (T3SS; Ren et al., 2009; Johnson et al., 2011), type VI secretion system (T6SS; Tian et al., 2014), type IV pili (T4P; Bahar et al., 2009), polar flagella (Bahar et al., 2011), and quorum sensing (QS; Wang et al., 2016). Therefore, the hallmark of successful pathogen infection is the ability to effectively inhibit the immune systems of plants (Kunkel and Chen, 2006).

The immune system of plants against pathogens is primarily based on two immune defense mechanisms. Plant cells can detect conserved molecules of the pathogen called pathogen (microbe)-associated molecular patterns (PAMPs/MAMPs) using pattern recognition receptors (PRRs) to inhibit the survival of the pathogen (Zipfel, 2008, 2009). This response is collectively termed PAMP-triggered immunity (PTI). However, some pathogens have evolved the ability to evade or inhibit PTI by secreting a protein called an effector (Block and Alfano, 2011). Thus, plants have evolved a secondary defense called effector-triggered immunity (ETI) to specifically recognize effectors secreted by pathogens (Dodds and Rathjen, 2010). Plant immune responses to pathogens infection include oxidative burst, rapid changes in gene expression, and cell wall reinforcement (Zipfel, 2008; Yu et al., 2016).

The oxidative burst involves the production of reactive oxygen species (ROS), including hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), and hydroxyl radical (HO$^\cdot$). ROS plays an important role in plant defense, such as acting as signaling molecules that form oxidative cross-linkages in plant cell walls to prevent pathogen invasion (Torres, 2010). Therefore, anti-oxidative activity is essential for the successful growth and survival of pathogens under environmental stresses (Herbrard et al., 2009). OxyR is a DNA-binding transcription factor that not only acts as an activator of genes encoding peroxide-detoxifying enzymes, but also against oxidative stress (Jo et al., 2015; Ishiga and Ichinose, 2016). OxyR regulates many genes concerned with defense against hydrogen peroxide (H$_2$O$_2$), e.g., *katA* and *katB* (encoding catalases A and B), *katG* (encoding hydroperoxidase 1), *dps* (encoding DNA-binding protein from starved cells), and *ahpCF* (encoding an alkyl hydroperoxide reductase; Ochsner et al., 2000; Hishinuma et al., 2006; Italiani et al., 2011; Xia et al., 2017). In addition, OxyR also plays a critical role in regulating biofilm formation, pili relate-genes expression, mucosal colonization (Hennequin and Forestier, 2009), and surface polysaccharide production (Shin et al., 2020) in pathogenic bacteria.

At the moment, the role of OxyR in *A. citrulli* is still unclear. In this study, we identified *oxyR* (Aave_0594) from the AAC00-1 genome (GenBank accession number NC_008752) to functionally characterize OxyR in *A. citrulli*. We evaluated the regulatory mechanism and function of OxyR in oxidative stress resistance, as well as its contribution to host virulence. The qRT-PCR assay demonstrated that OxyR regulates the oxidative stress-related gene of catB(Aave_3137) and *ahpC*(Aave_1375). In addition, qRT-PCR, western blot, bacterial one-hybrid, bacterial two-hybrid, and phenotype assay showed that *oxyR* affects the twitching motility, biofilm formation, and swimming motility by positively regulating the expression of pilA (Aave_4679) and *fliC* (Aave_4400) genes and interaction in *A. citrulli*. This study revealed that OxyR is one of the essential virulence factors that supports *A. citrulli* pathogenesis in melon.

**Materials and methods**

**Bacterial strains and growth conditions**

*A. citrulli* wild-type and its derived mutants were routinely cultured in Luria-Bertani (LB) medium at 28°C with shaking at 220 rpm, with or without 1.5% (wt/vol) agar (Sambrook et al., 1989). All *Escherichia coli* strains were cultured in LB medium at 37°C. The optical density of cell suspensions at 600 nm was used to track the growth of bacteria. All strains were stored at −80°C for long-term storage. Following final concentrations of antibiotics were provided: 100 μg/ml rifamycin (Rif), 50 μg/ml kanamycin (Km) and 100 μg/ml gentamicin (Gm). Supplementary Table S1 lists all of the bacterial strains and vectors used in this study.

**Construction and complementation of deletion mutants of Acidovorax citrulli**

Deletion mutations of *oxyR*, *catB*, *ahpC*, *pilA*, and *fliC* were generated using homologous recombination in *A. citrulli*, as described previously (Liu et al., 2019). Briefly, based on the AAC00-1 genome sequence, two flanking regions (upstream and downstream) of target genes were generated by PCR amplified using the primer pairs and cloned into pEX18GM. A Km fragment downstream) of target genes were generated by PCR amplified using homologous recombination in *A. citrulli*. The recombinant vector was transformed into *A. citrulli* xjl12. On LB plates with 10% (wt/vol) sucrose, Rif (100 mg/ml), and Km (50 mg/ml), we successfully picked the transformed colonies, which were further confirmed by PCR amplification using primers F1 and R2 (data not shown). A proven mutant was chosen for additional investigation.

The online promoter prediction website was used to predict the promoter of genes for complementing the deletion mutant of...
A. citrulli. A pair of specific primers, comp-F/R, was created to amplify a region including the gene and its predicted promoter site. The amplicon was subcloned into pMD19-T and this fragment was sequenced to check for base mutations. Following the appropriate restriction enzyme digestion, the fragment was cloned into the expression vector pBRR1MCS-5 (Kovach et al., 1995). Then, the recombinant vector was transferred into gene mutants. Transformants were screened on LB agar plates with Gm (50 μg/ml) and Km (50 μg/ml). Finally, the complementation strain was confirmed by PCR and picked for further research. All primer sequences used in this study are listed in Supplementary Table S2.

Growth curve assay

The bacterial growth assay was carried out according to the instructions (Liu et al., 2019). In brief, A. citrulli strains, containing the wild-type (WT) strain, the oxyR mutant, and the oxyR complementation strain, were all cultured in LB liquid medium overnight at 28°C with shaking at 220 rpm. These strains were then diluted to a final cell density (OD_{600}=0.01) in 25 μl of fresh LB medium. The diluted cells were cultured at 28°C with shaking at 220 rpm. The bacterial populations were investigated by measuring OD_{600} at 2 h intervals for 24 h. The experiments were performed in triplicate and repeated three times.

Catalase activity assay

The catalase activity was analyzed using a protocol previously described (Jittawuttipoka et al., 2009). The bacterial strains were cultured in LB broth overnight at 28°C with shaking at 220 rpm. The bacterial centrifugation was adjusted to an OD_{600}=1.0 with fresh LB broth. The bacterial cells were chilled at 4°C, collected by concentration at 6,000 g for 10 min, and then re-suspended in 50 mmol KH_{2}PO_{4}. The re-suspended cells were crushed by sonication until the suspension became clear. The cell extracts were separated by centrifuging at 12,000 g for 30 min, and the upper layer liquid containing protein was collected into a new tube. Before and after adding H_{2}O_{2} to a final concentration of 10 mm, 100 μl of protein was combined with 1 ml of ddH_{2}O, and the optical density of this combination was measured at 240 nm. By using an extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm, the catalase activity was determined. Under the assay conditions, one unit of catalase activity was defined as the amount of activity required to degrade 1 μmol of H_{2}O_{2} per minute. The experiment was repeated three times with three biological replicates of each treatment.

Detection of H_{2}O_{2} in melon leaves

The H_{2}O_{2} was detected by DAB staining as previously reported (Thordal-Christensen et al., 1997; Yu et al., 2016). Briefly, overnight cultures of A. citrulli strains were collected by centrifugation and adjusted to a concentration of OD_{600}=0.3 with sterile double-distilled water, and cells were infiltrated into melon leaves grown for 1 week. The leaf sections (3–5 mm) at 24 h post-inoculation were cut and placed in water with 0.01% Triton-X-100 and DAB at 1 mg/ml, then the leaves were incubated for 8 h at room temperature. Finally, leaves were boiled with 95% ethanol for 10 min and then rinsed with water, and the presence of H_{2}O_{2} was visualized as reddish brown colored spots by a light microscope. The experiment was repeated three times with three biological replicates of each treatment.

H_{2}O_{2} sensitivity assay

The diameters of the zones of inhibition for A. citrulli WT and derived strains on LB agar plates containing different concentrations of H_{2}O_{2} were measured to investigate the roles of oxyR, catB, and ahpC in A. citrulli sensitivity to H_{2}O_{2}. All tested strains were cultured in LB broth at 28°C with 220 rpm shaking until OD_{600} reached 1.0. 100 μl of LB agar medium and 1 ml of cell suspension were well combined, then poured into Petri dishes. An approximately 0.4 cm-diameter sterilized paper disk was put on the middle of each plate following the solidification of these LB agar plates, and 5 μl of various H_{2}O_{2} concentrations (1, 5, and 10%) were applied to the disk. After a 24 h incubation at 28°C, the diameters of H_{2}O_{2} inhibition zones were measured. Each treatment in this experiment was replicated three times.

Hypersensitive response assays

To test A. citrulli strains’ potential to induce HR, cell suspensions were injected into Nicotiana benthamiana leaves. All tested strains were grown in LB and washed with sterile water, then adjusted to OD_{600}=0.3. Approximately 100 μl of cell suspensions was syringe-infiltrated into the N. benthamiana leaves growing at 28°C, and HR was noticed after 24 to 72 h. Each experiment was repeated three times.

Twitching motility assay

The twitching motility of A. citrulli strains was assessed as described previously (Bahar et al., 2009), with the following adjustments. A. citrulli strains were adjusted to a concentration of approximately 1 × 10^{6} CFU/ml (Dilute 10^{3} fold from OD_{600}=0.3) with sterilized double-distilled H_{2}O. All strains were grown on 1%NA agar plates for 72 h, and twitching motility was observed by Stereo Fluorescence Microscope (Nikon). The characteristic of twitching motility was the formation of a thin and light halo around the colony.

Assays for biofilm formation were carried out in the manner described by Liu et al. (2019). The strains were cultured in LB broth at 28°C and adjusted to OD_{600}=1.0. The OD_{600} was then
determined after 48 h at 28°C by adding 40 μl of the cell suspension to 4 ml of LB broth in a 12-well polystyrene plate. After the cell medium had been decanted, the plate had been dried for 20 min at 80°C. Biofilms were then dyed for 30 min at room temperature with 1% crystal violet. Biofilm production was suggested by a ring of violet precipitate on the plate's interior wall. After dissolving the biofilm with 5 ml of ethanol, the OD₆₀₀ was measured.

Swimming motility was assayed as described previously (Liu et al., 2019). A. citrulli strains were cultured in LB broth at 28°C and adjusted to OD₆₀₀ = 0.3. The middle of 0.3% agar plates received 3 μl of each bacterial cell suspension. After 3 days of incubation at 28°C, the diameters of the swimming halos on the agar plates were measured. Each experiment was run three times with three replicates of each strain tested.

**Electron microscopy**

Transmission electron microscopy (TEM) was used to visualize polar flagella of bacteria grown in culture. Specimens for TEM were prepared as previously described (Bahar et al., 2009).

**RNA isolation and quantitative real-time PCR analysis**

The tested bacterial strains were cultured at 28°C with shaking at 220 rpm. The cells were collected and adjusted to OD₆₀₀ = 1.0, then harvested by centrifugation at 10,000 × g for 1 min. Total RNA was extracted using the bacterial RNA kit (OMEGA) and DNase-treated RNA with reverse transcription using the HiScript III RT SuperMix reagent Kit with gDNA Wiper (Vazyme). The cDNA was diluted to 50 ng/μl and used for quantitative real-time (qRT)-PCR with ChamQ Universal SYBR qPCR Master Mix (Vazyme) in an ABI PRISM 7500 real-time PCR machine (Applied Biosystems). In this study, the 16S ribosomal RNA gene of A. citrulli was used as an internal control. qRT-PCR amplification was conducted according to the following program: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, and a final melting curve analysis step from 60 to 95°C. Three biological replicates for each gene were used in triplicate during the experiments. We calculated the fold change in gene expression using the comparative 2⁻∆∆ct method.

**Western blot assay**

To determine the protein expression of pilA (Aave_4679) and fliC (Aave_4400) in the absence of oxyR, respectively, the plasmid (pBBR1-MCS5) carrying the fragment of pilA and fliC (contain itself native promoter) fusion with a Flag tag was introduced into the A. citrulli wild-type and oxyR mutants. The bacterial strains were grown in LB broth at 28°C with shaking at 220 rpm. The bacterial concentration was adjusted to an OD₆₀₀ = 1.0 with fresh LB broth. The cells were chilled at 4°C, and harvested by concentration at 6,000 g for 10 min, while cell sediment was collected for intracellular secreted protein assay. Cell sediment was re-suspended in 100 μl phosphate-buffered saline (PBS), and then 100 μl Radio Immunoprecipitation Assay (RIPA) lysates was added (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), added at 10 μl per milligram of cells). The cell lysates were heated at 100°C for 10 min. Then, cell lysates were collected by centrifuge with a rate of 12,000 g for 5 min and frozen at −80°C. Cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore, Red Bank, NJ, United States) using the semi-dry blot machine (Bio-RAD, CA, United States). After blocking with 5% milk in Tris-buffered saline containing Tween 0.05% (TBST, pH = 7.5) for 1 h at room temperature, the membrane was probed with a monoclonal antibody specific for the Flag tag (1:5000; Abmart, Shanghai, China), followed by detection with an HRP-conjugated anti-rabbit secondary antibody (No. M21002, Abmart, Shanghai, China). Immunoblots were developed using HyGlo HRP ECL Detection kit (MDBio Inc., Qingdao, China) and visualized using an automatic multi-function image analysis system Tanon-6,600 (Tanon, Shanghai, China). As a loading control, a duplicate protein gel was incubated in staining solution with shaking overnight and then incubated in destaining solution with shaking until the bands could be observed clearly.

**Bacterial one-hybrid assay**

In the present study, we proved the potential interaction between the transcriptional regulator OxyR and the promoter of the flic and pilA using the bacterial one-hybrid reporter system, which consists of two plasmids pTRG and pBXcmT and E. coli XL1-Blue MRF’ kan strain (Wang et al., 2018). In particular, the flic promoter region (344 bp) and pilA promoter region (333 bp) were cloned into pBXcmT, generating the recombinant vector pBXcmT-flic and pBXcmT-pilA (Supplementary Table S1), respectively. Similarly, the coding region of OxyR (966 bp) was cloned into pTRG, creating the final construct pTRG-OxyR. The vectors pBXcmT-P_flic, pBXcmT-P_pilA, and pTRG-OxyR were transformed into XL1-Blue MRF’ strain, respectively. If the direct physical binding occurs between OxyR and the flic or pilA promoter, the transformed E. coli strain containing both pBXcmT-P_flic or pBXcmT-P_pilA and pTRG-OxyR grows well on the selective medium, which is a minimal medium containing 5 mm 3-amino-1,2,4-triazole, streptomycin at 8 μg/ml, tetracycline at 12.5 μg/ml, chloramphenicol at 34 μg/ml, and Km at 30 μg/ml (Wang et al., 2018). Furthermore, the cotransformant containing pBX-R2031/pTRG-R3133 served as a positive control (Xiu et al., 2016; Wang et al., 2018), while the cotransformants containing the empty pTRG and pBXcmT-P_flic or pBXcmT-P_pilA were used as a negative control. The cotransformants containing the pTRG-OxyR and empty pBXcmT were used as another negative control in the present study. All cotransformants...
strains were spotted onto the selective medium plates and placed at 28°C for 3 to 4 days, then photographed.

**Bacterial two-hybrid assay**

The bacterial two-hybrid reporter system was applied to examine the potential interaction between OxyR and FliC/PilA. The bacterial two-hybrid reporter system contains three components: plasmids pTRG and pBT and *E. coli* XLI-Blue MRF’ kan strain. In this study, the coding region of FliC (1,479 bp) and PilA (507 bp) was cloned into pBT, generating the recombinant vector pBT-FliC and pBT-PilA (Supplementary Table S1), respectively. Similarly, the coding region of OxyR (966 bp) was cloned into pTRG, creating the final construct pTRG-OxyR. The vectors pBT-FliC, pBT-PilA, and pTRG-OxyR were transformed into XL1-Blue MRF’ kan strain, respectively. If the direct protein–protein interaction occurs between OxyR and FliC or PilA, the transformed *E. coli* strain containing both pBT-FliC or pBT-PilA and pTRG-OxyR grows well on the selective medium, which is similar to the components of bacterial one-hybrid system. Furthermore, the cotransformant containing pBT-GacS/pTRG-GacS served as a positive control, while the cotransformants containing the empty pTRG and pBT-FliC or pBT-PilA worked as a negative control. The cotransformants containing the pTRG-OxyR and empty pBT worked as another negative control in the present study. All cotransformants strains were spotted onto the selective medium and grown at 28°C for 3 to 4 days, then photographed.

**Virulence and colonization of *Acidovorax citrulli* assay**

In this study, two inoculation methods, including injection of melon seedling cotyledons and seed-to-seedling transmission assay, were applied to examine the virulence of *A. citrulli* strains. For injection of melon seedling cotyledons, overnight cultures of *A. citrulli* strains were collected by centrifugation and adjusted to a concentration of about 1×10⁶ CFU/ml with sterile double-distilled water. Each strain was injected into 10 melons (cv. Huanghou) with 1×10³ CFU/ml, and the homogenate was spread onto LB plates with appropriate antibiotics. After incubating at 28°C for 24 to 96 h, colonies were counted. According to earlier reports (Tian et al., 2014), the surface of the seed was disinfected with 70% ethanol for 5 min before inoculation. The front end of the seed was opened and each bacterial cell suspension (approximately 1×10⁷ CFU/ml) was implanted into 15 melon seeds (Huanghou). Additionally, 15 melon seeds treated with sterilized water served as a negative control. All melon seeds were incubated for 24, 48, 72, and 96 h at 28°C with 100% RH on moist blotter papers. All melon seeds were milled and ground at 28°C for 3 to 4 days, then photographed.

**Results**

**OxyR is present in the *Acidovorax citrulli* AAC001 genome**

A gene encoding OxyR protein was identified in the genome of *A. citrulli* based on comparisons of amino acid sequences from other plant pathogenic bacteria. The *oxyR* open reading frame (ORF) was 966 bp in length and located in the *A. citrulli* AAC001 genome at nucleotide position 651,313 to 652,278 (GenBank accession number NC_008752). The NCBI BLAST was used to perform BLASTP sequence homology analysis. Multiple sequence alignment shows that the amino acid sequence of OxyR from *A. citrulli* has a high identity among the tested bacteria including *E. coli*, *Pseudomonas aeruginosa*, *P. syringae pv. tomato*, and *Xanthomonas oryzae pv. oryzae* with 40, 49, 48, and 43% amino acid sequence identities, respectively (Supplementary Figure S1). Subsequently, we constructed *A. citrulli* oxyR mutant (*AcΔoxyR*) and complementation strain *AcΔoxyR* (pBBR-OxyR) by homologous recombination. We monitored bacterial growth in Luria-Bertani (LB) liquid media. The growth rate of *AcΔoxyR* was similar to that of WT and *AcΔoxyR* (pBBR-OxyR) strains (Supplementary Figure S2).

**Acidovorax citrulli** OxyR is required for catalase activity and response to H₂O₂

To determine the roles of the *oxyR* gene mediating H₂O₂ detoxification, we measured the catalase activities of the *A. citrulli* WT, *AcΔoxyR*, and *AcΔoxyR* (pBBR-OxyR; Figure 1A). The catalase activity of *AcΔoxyR* was significantly reduced as compared with the WT, while the complementation strain *AcΔoxyR* (pBBR-OxyR) restored to the WT level. In addition, we examined the expression level of *oxyR* in WT under H₂O₂ stress. Our results demonstrated that the expression of *oxyR* was significantly increased after 30 min.
of treatment with 1 mm H$_2$O$_2$ (Figure 1B). In addition, the western blot displayed that the expression of OxyR was not detected in both WT and AcΔoxyR without H$_2$O$_2$ treatment (Figure 1C). However, when supplement with exogenous H$_2$O$_2$, OxyR expression of WT was significantly increased than AcΔoxyR (Figure 1C). These results suggested that OxyR plays an important role in catalase production and response to oxidative stress in A. citrulli.

**AcΔoxyR elicit H$_2$O$_2$ production in melon**

We detected the production of H$_2$O$_2$ in melon leaves at 24 h post-inoculation of WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) by using 3, 3’-diaminobenzidine (DAB) staining. The red spots formed by DAB staining in all areas of the melon leaves inoculated with bacteria represented the accumulation of H$_2$O$_2$, while the ddH$_2$O control had no accumulation of H$_2$O$_2$ (Supplementary Figure S3). As a result, these findings suggest that WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) elicit H$_2$O$_2$ production in melon at the early stages of infection.

**Role of Acidovorax citrulli OxyR in H$_2$O$_2$ tolerance**

To investigate whether A. citrulli OxyR plays an important role in H$_2$O$_2$ response, we examined the WT, AcΔoxyR, AcΔahpC, AcΔcatB, and their complementation strains AcΔoxyR (pBBR-OxyR), AcΔahpC (pBBR-AhpC), and AcΔcatB (pBBR-CatB) to H$_2$O$_2$ sensitivity based on the diameter of the inhibition zone (Figure 2A). Both the AcΔoxyR and AcΔcatB mutants showed significantly increased sensitivity, while the AcΔahpC mutant showed significantly decreased sensitivity of H$_2$O$_2$ as compared with the WT (Figure 2B). The H$_2$O$_2$ sensitivity of all complementation strains AcΔoxyR (pBBR-OxyR), AcΔahpC (pBBR-AhpC), and AcΔcatB (pBBR-CatB) was similar with WT (Figure 2B). Furthermore, qRT-PCR assay showed that catB and ahpC expression levels were significantly down-regulated in AcΔoxyR under H$_2$O$_2$ stress environment (Figure 2C). In addition, the expression level of catB was significantly up-regulated in AcΔahpC under H$_2$O$_2$ stress environment.
These results suggested that OxyR played a role in protecting *A. citrulli* cells in H$_2$O$_2$ stress.

**OxyR is involved in *Acidovorax citrulli* HR induction and positively regulates expression of genes related to T3SS**

To determine whether OxyR contributes to the *A. citrulli* type III secretion system (T3SS), we examined the HR induction of WT and Ac$\Delta$oxyR on *Nicotiana benthamiana*. At 24 h post-infiltration (hpi), both the WT and Ac$\Delta$oxyR did not induce HR. HR induction on *N. benthamiana* by Ac$\Delta$oxyR was delayed than that induced by the WT at 48 hpi (Figure 3A). Furthermore, qRT-PCR was used to confirm the regulatory effect on the T3SS-related genes. We identified that T3SS genes including *hrpG*, *hrcC*, *hrcN*, and *hrcQ* were significantly down-regulated in Ac$\Delta$oxyR relative to the WT strain (Figure 3B). These results indicate that OxyR positively regulates the expression of genes related to T3SS.

**Role of OxyR in twitching motility, biofilm production and swimming motility of *Acidovorax citrulli***

Previous studies have confirmed that motility and biofilm play a key role in the virulence of *A. citrulli*. Previous studies have reported that *pilA* is required for biofilm formation for scoliosis, while *fliC* is required for swimming motility in *A. citrulli* M6 strain (Bahar et al., 2009, 2011). We investigated the role of OxyR in *A. citrulli* twitching motility, biofilm production, and swimming motility. Transparent halos around colonies formed by *A. citrulli* strains by twitching motility on NA plates were observed after 72 h at 28°C. In NA, Ac$\Delta$oxyR exhibited a phenotype similar to that of Ac$\Delta$pilA (i.e., no twitching-typical haloes were formed around the colonies; Figure 4A). In addition, the biofilm production of both Ac$\Delta$oxyR and Ac$\Delta$pilA was significantly decreased than the WT and Ac$\Delta$pilA (pBBR-ila; Figure 4B; Supplementary Figure S4). The swimming ability of Ac$\Delta$oxyR and Ac$\Delta$fliC was completely lost (Figure 4C), and Ac$\Delta$oxyR did not produce polar fagella (Figure 4D). However, the complementation strain Ac$\Delta$oxyR (pBBR-OxyR) formed the same...
phenotype as oxyR mutants AcΔoxyR in twitching motility, biofilm production and swimming motility. Furthermore, the qRT-PCR revealed that the expression levels of fliC, pilA, fls, and flgM were down-regulated in the AcΔoxyR as compared with WT (Figure 4E). These results indicated that oxyR gene plays a role in twitching motility, swimming motility, and biofilm production in A. citrulli.

**OxyR positively regulates the expression of FliC and PilA**

To determine the relationship of OxyR to PilA and FliC, we performed the western blot, bacterial one-hybrid, and bacterial two-hybrid assays. The western blot assay indicated that the expression of PilA and FliC was down-regulated in AcΔoxyR (Figure 5A). In addition, a newly-developed bacterial one-hybrid system (Wang et al., 2018) was carried out to test the potential direct interaction between OxyR and the fliC and pilA promoter (P_fliC and P_pilA). We observed that the growth of E. coli strain containing both OxyR and P_fliC was similar to positive control on the selective medium, whereas the negative controls failed to grow (Figure 5B). The same observation occurred on the test E. coli strain containing...
both OxyR and P_pilA (Figure 5C). These results indicated that direct binding of OxyR on P_pilA occurred under the test conditions. To verify whether OxyR interacts with PilA, the bacterial two-hybrid system was performed. Bacterial two-hybrid assays displayed that the growth of E. coli strain containing both OxyR and PilA was similar to positive control on the selective medium (Figure 5D). The same results were observed on selective media for E. coli strains containing OxyR and FliC (Figure 5E). These results indicated that OxyR interacts with PilA and FliC.

Deletion of OxyR in Acidovorax citrulli displayed decreased virulence and bacterial growth on melon

To investigate the effect of oxyR in virulence of A. citrulli on melon, WT, AcΔoxyR, and complementation strain AcΔoxyR (pBBR-OxyR) infiltrated into cotyledons of melon seedlings. In both two virulence assays, AcΔoxyR did not induce BFB symptoms on melon cotyledons (Figures 6A, B). The colonization ability of A. citrulli WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) strains on melon seedlings was determined. At 0, 24, 48, 72, and 96 hpi, bacterial populations in seedling tissues were evaluated. The average cell populations of A. citrulli WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) strains were approximately 2.95 × 10^5, 50, and 80 CFU/g, respectively, by 48 hpi. By 96 hpi, the mean populations of WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) strains were approximately 2.57 × 10^4, 95, and 100 CFU/g, respectively (Figure 6C). In melon cotyledons, the population growth of AcΔoxyR was noticeably decreased than WT strain. Additionally, by monitoring the bacterial populations on artificially inoculated seeds throughout the first 96h of seed germination, we assessed the effect of oxyR in A. citrulli colonization of melon seeds. At 0, 24, 48, 72, and 96h after planting, the bacterial populations on the emerging tissues of melon seedlings were enumerated. The average cell populations of WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) strains were approximately 7.59 × 10^4, 40, and 32 CFU/g, respectively, at 48h after planting (Figure 6D). The average populations of WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) were approximately 5.25 × 10^5, 20, and 25CFU/g, respectively, by 96h after planting (Figure 6D). Compared to the WT strain, AcΔoxyR had a much lower capacity to colonize germinating melon seedlings (p < 0.05). These results demonstrated that OxyR was required for the full virulence of A. citrulli on melon.

Discussion

OxyR plays an important role in oxidative stress, alginate biosynthesis, T3SS-related gene expression, and virulence of many plant-pathogenic bacteria, including Xanthomonas oryzae pv. oryzae (Yu et al., 2016), Pseudomonas syringae pv. tomato (Ishiga and Ichinoe, 2016), and Xanthomonas campestris pv. phaseoli (Loprais et al., 2000). However, to date, there have been no reports regarding the role of OxyR in Acidovorax citrulli. In this study, we functionally characterized the role of oxyR in A. citrulli wild-type strain xjl12 by constructing an oxyR mutant (AcΔoxyR) and a corresponding oxyR-complementation strain AcΔoxyR (pBBR-OxyR). In Bacteroides fragilis (Sund et al., 2007), and Haemophilus parasuis (Wen et al., 2018), ΔoxyR exhibited markedly impaired growth compared to the WT strain. Conversely, the deletion of oxyR did not affect the
Role of oxyR in Acidovorax citrulli virulence and bacterial growth on melon. (A) Melon seedling cotyledons inoculated by injection with A. citrulli wild-type (WT), AcΔoxyR, and AcΔoxyR (pBBR-OxyR; ~1×10^3 CFU/ml) strains, and double-distilled H_2O (ddH_2O) as a negative control (NC). Seedlings were observed for bacterial fruit blotch symptoms at 5 days post-inoculation (dpi). (B) Melon seeds were inoculated by soaking in bacterial cell suspensions (~1×10^6 CFU/ml) of A. citrulli WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR). BFB symptoms were observed 7 days after planting. (C) Bacterial suspensions (~1×10^3 CFU/ml) of each strain were injected into cotyledons of melon seedlings and populations were quantified at 0, 24, 48, 72, and 96 h postinoculation. (D) Melon seed were inoculated with A. citrulli strains (~1×10^3 CFU/ml) planted under conditions conducive for bacterial fruit blotch development and bacterial populations on emerging seedling tissues were quantified at 0, 24, 48, 72, and 96 h after planting. Experiments were repeated three times with similar results.

Reactive oxygen species (ROS) can damage all cellular components, including protein, DNA and membrane lipid (Imlay and Linn, 1988; Fridovich, 1997). Therefore, pathogenic bacteria successfully infect plant tissues in part by depending on their abilities to resist ROS, including H_2O_2 (Cabiscol et al., 2000). OxyR was identified as the primary H_2O_2 sensor responsible for H_2O_2 resistance (Jo et al., 2015). Previously reported that bacterial CAT was involved in the H_2O_2-degradation pathway and increased tolerance to oxidative stress (Loprasert et al., 1996). In this study, the AcΔoxyR strain was significantly reduced the CAT activity as compared with the WT. Furthermore, the transcription and translation levels of oxyR were induced by exogenous H_2O_2. These results indicated that oxyR was activated by H_2O_2 and then play a key role in the H_2O_2 degradation pathway. Moreover, the accumulation of H_2O_2 was observed in melon leaves infected by A. citrulli WT, AcΔoxyR, and AcΔoxyR (pBBR-OxyR) strains, suggesting that the ability of A. citrulli strains to degrade H_2O_2 is a key determinant of host infection by A. citrulli strains.

OxyR is a transcription factor regulates several genes involved in anti-oxidative stress, such as katA and katB (encoding catalases A and B) and alpC and alpF (encoding an alkyl hydroperoxide reductase) (Ochsner et al., 2000; Hishinuma et al., 2006). To investigate the sensitivity to H_2O_2 of oxyR and its regulatory gene, catB and alpC that encode a catalase and an alkyl hydroperoxide reductase, respectively, were deleted. In this study, oxyR and catB mutants were significantly reduced the tolerance to H_2O_2. In contrast, alpC mutant was significantly increased the tolerance to H_2O_2. These results showed that deletion of alpC may be due to increased expression of other oxidative stress-related genes thus triggering compensation mechanism. Using a qRT-PCR assay, we demonstrated that catB and alpC were down-regulated in AcΔoxyR. In addition, catB was significantly up-regulated in AcΔalpC. These results suggest that oxyR may play a key role in the H_2O_2 degradation pathway.
represented that OxyR might be on the top of the antioxidant stress regulatory system by controlling the expression of other oxidative stress-related genes. In the future, we would further analyze the antioxidant stress pathway of oxyR in *A. citrulli*.

Deletion of *oxyR* displayed reduced virulence and the ability to colonize melon seedlings. *AcΔoxyR* may influence other virulence factors, e.g., T3SS, biofilm, and swimming motility. The T3SS is major pathogenicity and virulence factors in *A. citrulli* (Johnson et al., 2009; Bahar and Burdman, 2010). In *Pst* DC3000, the expression of *hrpL* and *corR* were down-regulated in *oxyR* mutant (Ishiga and Ichinose, 2016). In this study, the T3SS-related genes including *hrpG*, *hrcN*, *hrcC*, and *hrcQ* were significantly down-regulated in *AcΔoxyR* relative to the WT strain. This result displayed that OxyR positively regulated the expression of T3SS-related genes that contribute to virulence.

In previous reports, *pilA* was required for twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli* (Bahar et al., 2009). In *A. citrulli*, *fliC* encodes the flagellin subunit and plays an important role in swimming motility (Bahar et al., 2011). The formation of biofilm and swimming motility was significantly inhibited in the *oxyR* mutant (Chung et al., 2016). In this study, *AcΔoxyR* and *AcΔpilA* were deficient in twitching motility and biofilm formation. Furthermore, *AcΔoxyR* was completely lost the ability of swimming motility and it is similar to the deletion of *fliC*. The complementation strain *AcΔoxyR* (pBBR-OxyR) formed the same phenotype as *AcΔoxyR* in twitching motility, biofilm produce, and swimming motility. Similar results existed in the virulence assay, *AcΔoxyR* and complementation strain *AcΔoxyR* (pBBR-OxyR) both reduced virulence and bacterial growth in *plantae*. These results indicated that deletion of *oxyR* in *A. citrulli* may be affected the function of adjacent genes. Therefore, complementation strain *AcΔoxyR* (pBBR-OxyR) cannot restore the function of adjacent genes, thus losing these phenotypes of twitching motility, biofilm production, and swimming motility. The speculation will be further studied in the future. Moreover, complementation strain *AcΔoxyR* (pBBR-OxyR) could restore the tolerance to *H₂O₂*, but not restore the phenotypes of biofilm, swimming, and twitching motility, thus failing to restore the virulence of *A. citrulli*. This result indicates that OxyR affects the virulence in *A. citrulli* by regulating multiple virulence traits.

To clarify whether OxyR directly regulates motility and biofilm and thus affects the virulence of *A. citrulli*, we demonstrated the interaction between *oxyR* and *pilA* and *fliC* by bacterial one-hybrid system and bacterial two-hybrid system. We demonstrated that the PilA and FliC proteins were significantly reduced in *AcΔoxyR*, indicated that OxyR positively affects *pilA* and *fliC*. In addition, bacterial one-hybrid system assay indicated the direct interaction between OxyR and the *pilA* and *fliC* promoter. Interestingly, the interaction between OxyR, PilA, and FliC was proved using bacterial two-hybrid system assay. In this study, we demonstrated that direct binding of OxyR on *pilA* and *fliC* promoter and protein–protein interactions occurred under the test conditions. These results showed that OxyR was directly affected *pilA* and *fliC* expression, thus affecting twitching motility, biofilm, and swimming motility of *A. citrulli*.

In summary, we observed that OxyR is involved in the regulation of many virulence factors in *A. citrulli*, including oxidative stress response, CAT activity, T3SS, swimming motility, twitching motility, and biofilm formation (Figure 7). We also demonstrated that OxyR directly binds *fliC* and *pilA* promoter and interacts with FliC and PilA, thus responding to influence swimming motility, twitching motility, and biofilm in *A. citrulli*.
Therefore, AcΔoxyR displayed reduced virulence and ability to colonize melon seedlings by significantly affecting anti-oxidative stress as well as expression of flagellin and type IV pilus-related gene (fliC and pilA). In the future, we will further investigate the global expression network of the *A. citrulli* OxyR.

**Data availability statement**

The original contributions presented in the study are included in the article/Supplementary material; further inquiries can be directed to the corresponding authors.

**Author contributions**

JW, JL, YZ, MS, and JF designed the experiments. JW, GY, YT, and BH performed the experiments and analyzed the data. JW and JL wrote the manuscript. YT and BH revised the manuscript and provided guidance for the experiments. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.977281/full#supplementary-material

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