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# Necessity of OxyR for the Hydrogen Peroxide Stress Response and Full Virulence in *Ralstonia solanacearum*▽†

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**The plant pathogen *Ralstonia solanacearum*, which causes bacterial wilt disease, is exposed to reactive oxygen species (ROS) during tomato infection and expresses diverse oxidative stress response (OSR) genes during mid-stage disease on tomato. The *R. solanacearum* genome predicts that the bacterium produces multiple and redundant ROS-scavenging enzymes but only one known oxidative stress response regulator, OxyR. An *R. solanacearum oxyR* mutant had no detectable catalase activity, did not grow in the presence of 250 μM hydrogen peroxide, and grew poorly in the oxidative environment of solid rich media. This phenotype was rescued by the addition of exogenous catalase, suggesting that *oxyR* is essential for the hydrogen peroxide stress response. Unexpectedly, the *oxyR* mutant strain grew better than the wild type in the presence of the superoxide generator paraquat. Gene expression studies indicated that *katE*, *katG*, *ahpC1*, *grxC*, and *oxyR* itself were each differentially expressed in the *oxyR* mutant background and in response to hydrogen peroxide, suggesting that *oxyR* is necessary for hydrogen peroxide-inducible gene expression. Additional OSR genes were differentially regulated in response to hydrogen peroxide alone. The virulence of the *oxyR* mutant strain was significantly reduced in both tomato and tobacco host plants, demonstrating that *R. solanacearum* is exposed to inhibitory concentrations of ROS *in planta* and that OxyR-mediated responses to ROS during plant pathogenesis are important for *R. solanacearum* host adaptation and virulence.**

Plants produce reactive oxygen species (ROS) in response to invading pathogens, and an effective oxidative stress response (OSR) contributes to the fitness of phytopathogenic bacteria (18, 39, 40, 51). However, research on the role of regulators of the OSR in the virulence of plant-pathogenic bacteria is limited and contradictory. In *Agrobacterium tumefaciens*, *oxyR* is necessary for tumorigenesis (34), but *Erwinia chrysanthemi* does not need *oxyR* for soft-rot virulence (32). OxyR is a redox-sensing LysR family transcriptional regulator that has been well characterized in several bacteria (23, 43, 52). In the absence of oxidative stress, OxyR is reduced and acts as a repressor of several genes, including *oxyR* itself (47, 52). In the presence of hydrogen peroxide, the conserved cysteines of OxyR (C199 and C208, in *Escherichia coli*) form a disulfide bond that changes its conformation and converts OxyR into a transcriptional activator (52, 53). In *E. coli*, OxyR-regulated genes include catalase (*kat*), alkyl hydroperoxide reductase (*ahp*), glutaredoxin (*grx*), and glutathione reductase (*gor*) (53).

The plant-pathogenic bacterium *Ralstonia solanacearum* causes bacterial wilt disease on many economically important crops, including tomato (19). Multiple quantitative virulence factors contribute to the disease (11); however, little is known about how *R. solanacearum* adapts to its host environment, which is a critical prerequisite for pathogen success. An *in vivo* expression technology (IVET) screen performed on *R. solanacearum* during mid-phase tomato disease revealed that this bacterium encounters a stressful environment while in the host (7). Mutagenesis of stress response genes identified in this screen significantly decreased *R. solanacearum*'s virulence on tomato (8).

Host plants generate ROS in the form of hydrogen peroxide in response to infection by *R. solanacearum* (13, 30), so this pathogen experiences oxidative stress during pathogenesis. The available *R. solanacearum* genomes predict multiple and redundant OSR genes (13), including an *in planta*-induced peroxidase, Bcp (7), and Dps (nonspecific DNA binding protein from starved cells), which is induced by tomato root exudates and is regulated by OxyR (9). However, mutants lacking either *bcp* or *dps* are only slightly reduced in virulence (9, 13). These findings suggest that either the concentrations of ROS *in planta* are sublethal or that the bacterium's remaining OSR genes compensate for the loss of Bcp and Dps.

We created an *R. solanacearum* mutant lacking *oxyR*, the only apparent OSR regulator in this bacterium (12, 13). We hypothesized that OSR genes would be differentially expressed in the *oxyR* mutant background in *R. solanacearum*, thereby contributing to host adaptation and virulence in host adaptation and virulence. We found that *R. solanacearum oxyR* is essential for survival of hydrogen peroxide stress, for hydrogen peroxide-inducible gene expression, and for full bacterial wilt virulence. Further, OxyR regulates at least five OSR-related genes, including itself.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* strains were grown in LB medium (33) at 37°C. *R. solanacearum* strains in this study were derived from a wild-type tomato isolate, the type strain K60 (phylotype II, sequevar 7) (25). *R.*

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*solanacearum* strains were grown in CPG broth (20) or CPG solid medium with 0.05% (wt/vol) tetrazolium chloride (25). Antibiotics were added to cultures at the following concentrations: 25 μg/ml kanamycin, 12.5 μg/ml gentamicin, and 15 μg/ml tetracycline; catalase (MP Biomedicals, Solon, OH) was added at 100 units/plate. Growth curve analyses were performed in minimal Boucher's minimal medium (BMM) (6) with 0.2% (wt/vol) glucose and supplemented with either 250 μM hydrogen peroxide or 5 μM paraquat (Ultra Scientific, North Kingstown, RI). Overnight cultures of *R. solanacearum* strain K60 and *oxyR* mutant K2690 grown in BMM with glucose to an optical density at 600 nm ($OD_{600}$) of 0.1 were exposed to 150 μM hydrogen peroxide for 15 min before RNA extraction. *In planta* growth of *R. solanacearum* strains was assessed by quantifying multiplication of the organism in tobacco leaves (*Nicotiana tabacum* cv. Bottom Special) as previously described (46). The bacterial strains, selected plasmids, and primers used in this study are listed in Table S1 in the supplemental material.

**DNA manipulations.** DNA isolation, PCR, cloning, and Southern hybridization were carried out by standard protocols (4). Competent cells of *E. coli* and *R. solanacearum* were created as described previously (1). Unless otherwise noted, chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), and molecular biology reagents and kits were purchased from Promega (Madison, WI).

**Oligonucleotides and sequence analysis.** DNA and protein sequences were analyzed using Biology Workbench (http://workbench.sdsc.edu/), NCBI BLAST (2), NEB cutter (http://tools.neb.com/NEBcutter2/index.php), and the genomic databases of *R. solanacearum* strains UW551 (http://vision.biotech.ufl.edu/mycap/jsp/project/description.jsp?projectID=1) and GMI1000 (http://bioinfo.genotoul.fr/annotation/iANT/bacteria/ralsto/). Primers were purchased from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed at the University of Wisconsin Biotechnology Center (Madison, WI).

**Construction of K2690.** A 1,048-bp DNA fragment containing the *oxyR* gene was amplified by PCR using primers listed in Table S1 in the supplemental material. The resulting fragment was AT cloned into pSTBlue-1, creating pSToxyR, and sequenced. The *aacCI* gentamicin resistance gene cassette from pUCGM was introduced into a unique SacI site in *oxyR*, creating pSToxyR::*aacCI*. The resulting mutant allele was introduced into *R. solanacearum* through triparental mating using the helper plasmid pRK600 in *E. coli* HB101 and donor plasmid pSJYoT carrying the *oxyR*::*aacC1* EcoRI fragment from pSToxyR::*aacCI*. Transconjugants were plated on media containing gentamicin and catalase, which was necessary to permit growth of the *oxyR* mutant strain. Allelic replacement of the *oxyR* gene in *R. solanacearum* was confirmed by PCR and Southern blot analysis (data not shown). The confirmed *oxyR* mutant was called K2690.

**Complementation of K2690.** The *oxyR* and promoter primers (see Table S1 in the supplemental material) were used to amplify a 1,953-bp fragment containing the *oxyR* gene and its upstream region from *R. solanacearum* biovar 3 phylotype I strain GMI1000. The resulting fragment was AT cloned into pSTBlue-1 and sequenced. Using EcoRI sites, the 1,953-bp fragment was subcloned into the low-copy-number plasmid pUFJ10, which is stably maintained in *R. solanacearum* (15), creating pUFJ10::*oxyR*. For *trans*-complementation, pUFJ10 and pUFJ10::*oxyR* were introduced into K2690 and wild-type *R. solanacearum* by electroporation.

**Virulence assays.** Tomato plants of susceptible cultivar Bonny Best and the wilt-tolerant line Hawaii 7996, and tobacco plants of the susceptible cultivar Petit Havana were inoculated using a naturalistic soil soak assay as previously described (45). Briefly, a suspension of bacteria was poured into the pots of unwounded 15-day-old tomato plants or 30-day-old tobacco plants. Plants were kept in a growth chamber with 12-h day/12-h night cycles at 28°C, and disease symptoms were evaluated daily for 14 days using a 0 to 4 disease index. The assay was performed in triplicate with 16 plants per treatment in each assay for tomato plants and in duplicate with 10 plants per treatment in each assay for the tobacco plants.

**Plating efficiency and catalase activity.** Overnight cultures of *R. solanacearum* strains were collected by centrifugation and resuspended in water, and the $OD_{600}$ was adjusted to 0.1. The bacterial suspensions were serially diluted and plated on CPG plates with and without catalase. Results presented are the average of three independent experiments, each done in duplicate. Catalase activity was assayed visually by adding 1 ml 880 mM hydrogen peroxide to 3 ml of *R. solanacearum* overnight cultures adjusted to an $OD_{600}$ of 1.0 with fresh CPG. The experiment was repeated four times.

**RNA extraction, cDNA synthesis, and quantitative RT-PCR.** After exposure to hydrogen peroxide for 15 min, *R. solanacearum* cells were treated with 1.25 ml of 5% ethanol (EtOH)-phenol solution per 10 ml of culture to preserve the RNA profile (26) and collected by centrifugation. Total nucleic acids were isolated with MasterPure complete DNA and RNA purification kit (Epicentre Biotechnologies, Madison, WI), followed by four Baseline-ZERO DNase treatments (Epicentre Biotechnologies, Madison, WI). RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and RNA quality was assessed with a 2100 Bioanalyzer (Agilent Technologies Deutschland GmbH). cDNA was synthesized from 1 μg RNA with SuperScript III first-strand synthesis system for reverse transcriptase PCR (RT-PCR) (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed in 25-μl volumes with 2 μl of cDNA, a 400 nM concentration of each primer, and 12.5 μl of Power SYBR green master PCR mix (Applied Biosystems, Warrington, United Kingdom) in an ABI 7300 real-time PCR system (Applied Biosystems). Primers were designed to amplify 100- to 200-bp fragments (see Table S1 in the supplemental material). Standard curves with known DNA concentrations were used to determine primer pair efficiency, and primer products were verified with melting-curve analysis according to the ABI real-time PCR system software. All samples were run in triplicate, and RNA with no reverse transcriptase was used as a control for genomic DNA contamination. Relative fold changes in expression of each target gene were calculated with the Pfaffl method (37), using the wild-type strain in the absence of hydrogen peroxide as the calibrator sample and three constitutively expressed normalization reference genes (*glmS*, *gyrB*, and *pcrA*) (48). Results presented are the average of two independent RNA extractions per tested condition. Significant values are those with a $P$ value of $\leq 0.05$ and at least a 1.5-fold difference from that of the wild type in the absence of hydrogen peroxide.

**Statistical analysis.** Statistical analysis was performed with Minitab statistical software (Minitab, Inc.) and GraphPad Prism software (GraphPad Software, Inc.).

## RESULTS

**Cloning of *R. solanacearum oxyR*.** The *R. solanacearum* GMI1000 and UW551 genomes contain a predicted *oxyR* gene with 96% amino acid identity. We used the *R. solanacearum* phylotype II, sequevar 1 strain UW551 genome sequence (15) to design primers to clone *oxyR* from the related phylotype II strain K60. Sequence analysis revealed that K60's OxyR is 99% identical at the amino acid level to that of UW551. Multiple sequence alignments among four other characterized OxyR proteins demonstrate high conservation in the area surrounding the two essential hydrogen peroxide-responsive cysteines (Fig. 1).

**Mutagenesis of *R. solanacearum oxyR*.** To understand the role of *oxyR* in the OSR and virulence of *R. solanacearum*, we disrupted the strain K60 *oxyR* open reading frame (ORF) with a gentamicin resistance cassette. The resulting *oxyR* mutant strain, named K2690, grew as well as its wild-type parent in minimal medium with glucose as a sole carbon source (Fig. 2A) or in susceptible tobacco leaves (cultivar Bottom Special; data not shown), demonstrating that this mutant is not an auxotroph and suffers no general growth defects. However, K2690 had a significantly ($P = 0.003$) lower ability to form colonies on rich medium plates without catalase than the the wild type ($3.17 \times 10^6$ CFU/ml versus $1.02 \times 10^8$ CFU/ml, respectively); this phenotype was rescued by adding exogenous catalase to the plates (data not shown). Consistent with this observation, broth cultures of K2690 had no detectable catalase activity, assessed visually as rapid formation and accumulation of bubbles after the addition of hydrogen peroxide. Both these phenotypes were returned to the wild type following introduction of a wild-type copy of *oxyR* in *trans*.

***R. solanacearum* required *oxyR* for growth in the presence of hydrogen peroxide.** To determine the role of *oxyR* in the *R. solanacearum* hydrogen peroxide stress response, we cultured wild-type strain K60 and *oxyR* mutant K2690 in minimal medium with glucose as a sole carbon source in the absence or presence of 250 μM hydrogen peroxide. The wild-type strain

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E.chrysanthemi ---MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLF
E.coli         ---MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLF
A.tumefaciens  MIALSMKHLRYFDALAKIGHFGRAAEACAISQPALSLQIRELEELIGAPLVERGSRQIRL
R.sol_K60      ---MTLTELKYIVAVARERHFGRAAEACFVSQPTLSVAIKKLEDELAVQIFERGASEVSV
P.syr_DC3000   ---MTLTELRYIVTLAQEQHFGHAAERCHVSQPTLSVGVKKLEDELGVLIFERSKSAVRL
                  :.: .*.*: ::*.  ** :**: * :***:**  :::**: :.. :.**    : .
E.chrysanthemi TQAGLLLVEQARTVLREVKVLKEMASQQGEAMSGPLHIGLIPTVGPYLLPQIIPMLHRAF
E.coli         TQAGMLLVDQARTVLREVKVLKEMASQQGETMSGPLHIGLIPTVGPYLLPHIIPMLHQTF
A.tumefaciens  TALGDEFAERTRAILRSVDELQDLARAGHGPLSGRLRIGVIPTVAPYLLPQVIKTLTRHY
R.sol_K60      TPVGEQIVTQAQRVLEQTMAIREIAKQGMDPLAGPLRLGVIYTIGPYLLPALVKQMIDTV
P.syr_DC3000   TPVGEGIVAQAQKVLEQAQGIRELAQAGKNQLTAPLKVGAIYTVGPYLFPHLIPQLHRVA
               *  *  :. ::: :*...  ::::*      ::. *::* * *:.***:* ::  :
E.chrysanthemi PKLEMYLHEAQTHQLLAQLDSGKLDCAILAMVKESEAFIEVPLFDEPMKLAIYQDHPWAN
E.coli         PKLEMYLHEAQTHQLLAQLDSGKLDCVILALVKESEAFIEVPLFDEPMLLAIYEDHPWAN
A.tumefaciens  PGLEARPREAVTQKLIEDLLEARLDMAIVALPVSEPALEEVPLFSEEFILVRPMEDAGMP
R.sol_K60      PQMPLMLQENFTARLVELLKQGEIDCAIMAEPFPEAGLMTVPLYDEPFVVAVPRGHALAD
P.syr_DC3000   PQMPLYIEENFTHVLRDKLRNGELDAVIIALPFNEADVLTLPLYDEPFSVLMPAGHPWTQ
               * :    .*  *  *   * ...:* .*:*    .  .  :**:.* : :     ..
E.chrysanthemi RERVAMSDLSGEK-LLMLEDGHCLRDQAMGFCFQAGADED------THFRATSLETLRNM
E.coli         RECVPMADLAGEK-LLMLEDGHCLRDQAMGFCFEAGADED------THFRATSLETLRNM
A.tumefaciens  ---VPSADKLGEMRLLLLEEGHCFRNQALSFCSTTNAPPR------VLMEGSSLSTLVQM
R.sol_K60      SQSVDPESLKQQT-MLLLGNGHCFRDHVLNVCPELSRFAQNADGIQKTFEGSSLETIRHM
P.syr_DC3000   KETIDASALNDKS-LLLLGEGHCFRDQVLEACPTLGKGNEGAR--HTTVESSSLETIRHM
                  :       :  :*:* :***:*::.:  *   .            ...:**.*: :*
E.chrysanthemi VAAGSGITLLPSLAVP-QERIRDGVCYLPCYKPEPKRTIALVYRPGSPLRGRYEQLADSV
E.coli         VAAGSGITLLPALAVP-PERKRDGVVYLPCIKPEPRRTIGLVYRPGSPLRSRYEQLAEAI
A.tumefaciens  VGAGIGVTLIPQMAVD-METRLSTVSVFRLAEPRPSRTIGIVWRKSNPLSAQFAHISEIV
R.sol_K60      VASGVGITVLPRTSVPDMHPSTDLLAYVPFQEPVPDRRVVLAWRKSFTRVAAIEAVAKAV
P.syr_DC3000   VASGLGISILPLSAVDSHHYAPGVIEVRPLTPPVPFRTVAIAWRASFPRPKAIEILADSA
               *.:* *::::*  :*   .   . :       * * * : :.:* . .       ::.
E.chrysanthemi REHMQLHMEKLSAQSA--
E.coli         RARMDGHFDKVLKQAV--
A.tumefaciens  RECG---LQKLGLAP---
R.sol_K60      AQCSLPGIRRLPSTPLVH
P.syr_DC3000   RLCSVARPKNVAS-----
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FIG. 1. Sequence of *Ralstonia solanacearum* OxyR. ClustalW multiple sequence alignment of OxyR sequences from *R. solanacearum* strain GMI1000 and related bacteria. Boldface letters indicate the conserved active cysteines in OxyR. An asterisk indicates ful conservation, a colon indicates conservation of strong groups, and a period indicates conservation of weak groups. GenBank accession numbers are as follows: *Erwinia chrysanthemi*, CAB40388; *Escherichia coli* K-12, AAC76943.1; *Agrobacterium tumefaciens*, AAK88806; *Ralstonia solanacearum* K60, JN382247; and *Pseudomonas syringae* DC3000, AA053620.1.

suffered a slight growth delay in the presence of hydrogen peroxide but ultimately reached the same cell density as that in medium without hydrogen peroxide. However, strain K2690 did not grow in the presence of 250 μM hydrogen peroxide (Fig. 2A). The wild-type growth pattern was restored when a copy of *oxyR* was introduced into strain K2690 in *trans* (Fig. 2A). K2690 grew slowly in the presence of 150 μM hydrogen peroxide, reaching a final density well below that of the wild-type strain (data not shown).

***R. solanacearum* strain K2690 grew better than the wild type in the presence of paraquat.** To test the role of *oxyR* in the superoxide stress response of *R. solanacearum*, we cultured K2690 in minimal medium in the absence or presence of a 5 μM concentration of the superoxide-generating chemical paraquat. Growth of wild-type K60 was severely affected in the presence of paraquat. Unexpectedly, in the presence of paraquat, K2690 grew significantly better than the wild-type strain ($P < 0.05$) starting at 28 h postinoculation (Fig. 2B). It did not, however, reach densities comparable to those in the absence of paraquat. The growth of K2690 in the presence of paraquat was reduced to wild-type levels after a wild-type copy of *oxyR* was added in *trans.*

***R. solanacearum* needs *oxyR* for full virulence.** To determine the role of *oxyR* in bacterial wilt virulence, we performed a naturalistic disease assay on susceptible and tolerant tomato and susceptible tobacco plants. The tomato line Hawaii 7996 is horizontally resistant to bacterial wilt, but it readily forms latent infections (16). Under the conditions tested, K60 killed most Hawaii 7996 plants by 14 days postinoculation, but strain K2690 was significantly delayed in disease symptom onset and development, killing approximately half the infected plants by day 14 (Fig. 3A). A similar disease progress curve was observed in the highly susceptible tomato cultivar Bonny Best, where K2690 was also significantly less virulent than the wild type, although the difference between the *R. solanacearum* strains was smaller (data not shown). K2690 was also significantly reduced in virulence on another *R. solanacearum* host, tobacco ($P = 0.05$) (Fig. 3B).

**Oxidative stress response genes were differentially regulated in an *R. solanacearum oxyR* mutant.** To identify genes directly

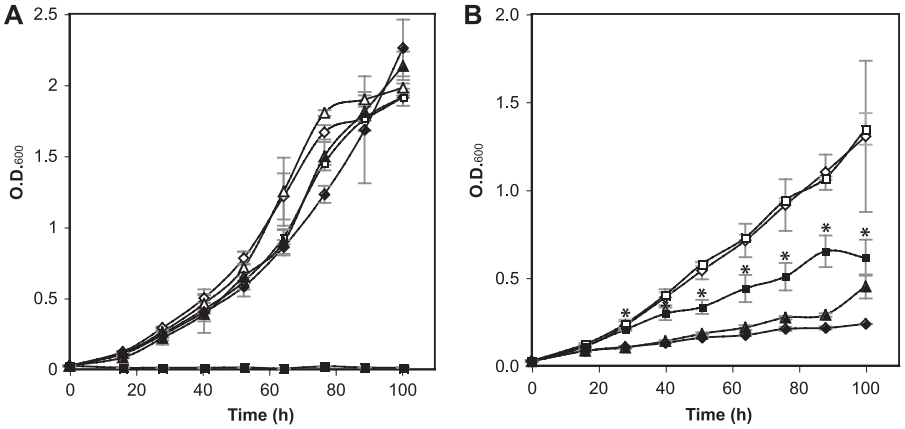


FIG. 2. Effect of oxidative stress on *R. solanacearum* strain growth in minimal medium. Growth of wild-type K60 carrying empty vector pUFJ10 (diamonds), *oxyR* mutant K2690 carrying empty vector pUFJ10 (squares), and K2690 complemented with pUFJ10::*oxyR* (triangles) strains in the absence (empty symbols) and presence (filled symbols) of 250 μM $H_2O_2$ (A) or 5 μM paraquat (B) is shown. Results are the average of two independent experiments, with two replicates for each treatment in each experiment; bars represent standard errors of the means. An asterisk indicates a statistically significant difference according to Student's *t* test ($P < 0.05$).

or indirectly regulated by OxyR and/or hydrogen peroxide in *R. solanacearum*, we performed quantitative RT-PCR (qRT-PCR) to measure expression of genes selected from homologs of known oxidative stress response and OxyR-regulated genes in other bacterial species. RNA was extracted from early-log-phase cells exposed, or not, to 150 μM hydrogen peroxide for 15 min. Relative fold changes in expression of tested genes are presented in Table 1. In the presence of hydrogen peroxide, *R. solanacearum* cells had increased expression of *oxyR*, catalase genes *katG* and *katE*, the alkyl hydroperoxide reductase gene *ahpC1*, and the glutaredoxin gene *grxC*. Induction of *ahpC1* and both catalase genes was dependent on the presence of *oxyR*. However, expression of *grxC* and *oxyR* itself was enhanced in the absence of *oxyR*. In contrast, *bcp*, *sodB*, and *sodC* gene expression was reduced in the presence of hydrogen peroxide, independent of the presence of *oxyR*.

## DISCUSSION

A growing body of work demonstrates the importance of the oxidative stress response in eukaryote-bacterium interactions, including pathogenic ones (18, 36, 38–40, 42, 44, 51). However, there is scant and contradictory information about the role of the OSR regulator OxyR in the virulence of plant-pathogenic bacteria (33, 34). We previously found that the bacterial wilt pathogen *R. solanacearum* is exposed to ROS during host infection and that it possesses multiple and redundant ROS scavenging enzymes, some of which are induced in the host (13). However, the presence of multiple and redundant pre-

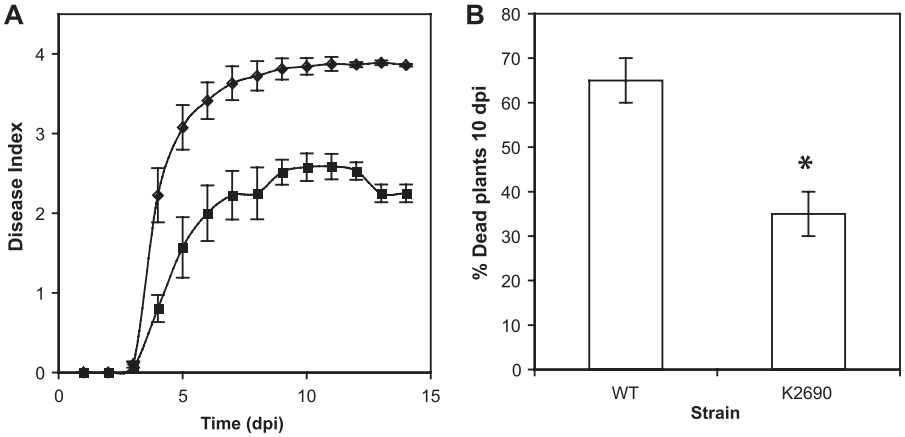


FIG. 3. Effect of *oxyR* mutation on *R. solanacearum* virulence. Disease progression curves of *R. solanacearum* strains on wilt-tolerant tomato line Hawaii 7996 (A) and on wilt-susceptible tobacco cultivar Petit Havana SRI (B) following soil soak inoculation of unwounded plants, with bacterial cell suspensions of strain K60 (diamonds) and *oxyR* mutant K2690 (squares). Disease symptoms were evaluated daily for 14 days using a 0 to 4 disease index. (A) Inoculum was ~$5 \times 10^7$ CFU/gram of soil. Results shown are the average of four independent experiments, with 16 plants per treatment per assay; bars indicate standard errors of the means. (B) Inoculum was ~$3 \times 10^7$ CFU/gram of soil. Results shown are the average of two independent assays, with 10 plants per treatment per assay; bars represent standard errors of the means. An asterisk indicates a statistically significant difference according to Student's *t* test ($P = 0.05$).

TABLE 1. Relative fold change in expression of *R. solanacearum* genes in response to 150 μM hydrogen peroxide and *oxyR*

| Locus[b] | Gene | Fold change in expression[a] | | | Function[c] |
|---|---|---|---|---|---|
| | | K2690 without $H_2O_2$ | With $H_2O_2$ | | |
| | | | K60 | K2690 | |
| RRSL03242 | *oxyR* | **15.49** | **5.79** | **24.55** | Transcriptional regulator |
| RRSL01521 | *katE* | **−64.39** | **1.62** | **−265.45** | Catalase |
| RRSL01521 | *katG* | **−1.94** | **27.92** | **−4.38** | Catalase |
| RRSL01699 | *ahpC1* | 4.94 | **3.26** | **−3.22** | Alkyl hydroperoxide reductase subunit |
| RRSL00135 | *bcp* | 1.54 | **−3.14** | **−4.55** | Peroxidase |
| RRSL03306 | *grxC* | 3.82 | **4.12** | **15.38** | Glutaredoxin |
| RRSL03640 | *sodC* | 1.27 | **−2.19** | **−3.05** | Cu,Zn superoxide dismutase |
| RRSL01451 | *sodB* | 1.23 | **−6.14** | **−9.29** | Fe superoxide dismutase |

[a] Fold change in expression relative to the expression in parent strain K60 grown in the absence of hydrogen peroxide. Boldface numbers are statistically significantly different from gene expression in K60 growing without hydrogen peroxide ($P \leq 0.05$), according to Student's *t* test, and are at least 1.5-fold different.
[b] Gene locus tag in the *R. solanacearum* UW551 genome.
[c] Putative function was assigned by similarity to proteins in the public databases.

dicted OSR genes in the genome made it impossible to assess the importance of the OSR in *R. solanacearum* by deleting individual structural genes. Here we investigated the role of a predicted OSR regulator, *oxyR*, in this pathogen's virulence and ROS susceptibility.

We found that *R. solanacearum* possesses an *oxyR* homolog that was necessary for survival in the presence of hydrogen peroxide. The *oxyR* gene product was also required for wild-type virulence levels on susceptible and tolerant tomato plants and on susceptible tobacco plants, consistent with our previous observation of elevated hydrogen peroxide levels in plants infected with *R. solanacearum* (13). It is known that hydrogen peroxide generated in the plant's defensive oxidative burst triggers host defense expression at the site of infection and in distant tissues, leading to systemic acquired immunity (3). However, neither the concentrations of hydrogen peroxide that induce host defense activation nor the concentrations of ROS experienced by pathogens during infection are known. Our results indicate that during host infection, *R. solanacearum* encounters inhibitory concentrations of ROS and that it needs a functional OSR to overcome them. In addition, the strikingly poor ability of K2690 to form colonies on agar plates, and this mutant's lack of detectable catalase activity, suggests that *oxyR* is an essential part of the hydrogen peroxide stress response in *R. solanacearum* as in other bacteria.

The plating defect of *oxyR* mutants is well documented for several bacteria (29, 32, 35, 50). This defect is believed to result because bacteria beginning to grow on plates launch a global stress response that includes genes in the OxyR regulon (10). In *Pseudomonas aeruginosa*, the plating defect was attributed to autooxidizable components in the medium (17). This likely explains why supplementing the plates with catalase recovers the plating defect of K2690 to wild-type levels. Low-temperature-induced viable but nonculturable (VBNC) *R. solanacearum* cells are partially restored to growth on plates by the addition of catalase to the agar plates (49). Oxidative damage to cell components is hypothesized to induce the VBNC state (5). The recovery of K2690 colonies on plates with added catalase suggests that under these conditions, a proportion of the *oxyR* mutant cells could be in a VBNC state and that oxidative stress may trigger the VBNC state in *R. solanacearum*.

The increased growth rate of strain K2690 in the presence of the superoxide-generating agent paraquat was unexpected. Although paraquat severely reduced growth of wild-type strain K60, in the presence of paraquat, the *oxyR* mutant grew better than the wild type. This phenotype resulted in loss of *oxyR*, since the introduction of a wild-type copy of *oxyR* into K2690 reduced its growth in paraquat to wild-type levels. *R. solanacearum* genomes predict the presence of superoxide dismutases (Sod), which convert superoxide into hydrogen peroxide (14); however, the Sod genes were expressed at similar levels in wild-type and K2690 cells. We speculate that *R. solanacearum* has superoxide stress response genes other than *sod* that are induced in the *oxyR* mutant background.

OxyR, sublethal concentrations of hydrogen peroxide, or both, appear to regulate several putative *R. solanacearum* OSR genes. Expression of *bcp*, *sodB*, and *sodC* genes was reduced in the presence of hydrogen peroxide regardless of the presence of *oxyR*, suggesting that these genes are not under the control of OxyR.

Following hydrogen peroxide exposure, *oxyR* and *grxC* were induced in K2690 and in the wild-type strain, suggesting that OxyR acts as a repressor of these genes. *grxC* encodes a putative glutaredoxin that, accompanied by glutathione, helps maintain the redox status of the cell in *E. coli* (27) and reduces oxidized OxyR, creating a feedback loop regulation of OxyR activity (52, 53). Our results suggest that *R. solanacearum* uses a similar mechanism to regulate OxyR activity and gene expression. The bacterium's two catalase genes, *katE* and *katG*, and *ahpC1* were induced by hydrogen peroxide in an OxyR-dependent manner. These results are consistent with the lack of detectable catalase activity in K2690 and with OxyR-dependent activation of *kat* and *ahpC* genes in other bacteria (22–24, 31, 35, 50, 53). *ahpC1* is predicted to encode one of two predicted alkyl hydroperoxide reductase systems in *R. solanacearum*, which in *E. coli* is the primary scavenger of endogenous and/or low concentrations of hydrogen peroxide (41). We found that *ahpC1* expression trended higher in K2690 in the absence of hydrogen peroxide than in its presence, although the difference was not statistically significant. This could indicate that *R. solanacearum* OxyR is both a repressor and an activator of *ahpC1*. OxyR has been reported to activate and repress transcription of a target gene in other bacterial systems (21, 23, 28). This putative *ahpC1* expression pattern

might explain the unexpectedly strong growth of K2690 in the presence of paraquat. Consistent with this hypothesis, overexpression of *ahpC* in an *Xanthomonas campestris oxyR* mutant resulted in increased superoxide resistance compared to that in an *oxyR* mutant alone (50).

In summary, the diverse phenotypes of an *R. solanacearum oxyR* mutant indicate that this regulator plays a central role in the oxidative stress response of this plant pathogen. In particular, an OxyR-mediated OSR is needed for full bacterial wilt virulence on several different plant hosts. Our *in vitro* gene expression data suggest that much of the OxyR regulon is conserved among several bacterial species. However, global gene expression studies of an *oxyR* mutant during disease development are needed to fully decipher the biological importance of this regulon. Once the full set of OxyR-regulated and oxidative stress-responsive genes are identified, it will be possible to more conclusively determine the specific mechanisms used by *R. solanacearum* to adapt to the oxidative host environment.

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