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Agrobacterium tumefaciens soxR Is Involved in Superoxide Stress Protection and Also Directly Regulates Superoxide-Inducible Expression of Itself and a Target Gene▽

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Inactivation of *Agrobacterium tumefaciens soxR* increases sensitivity to superoxide generators. *soxR* expression is highly induced by superoxide stress and is autoregulated. SoxR also directly regulates the superoxide-inducible expression of atu5152. Taken together, the physiological role of *soxR* and the mechanism by which it regulates expression of target genes make the *A. tumefaciens* SoxR system different from other bacterial systems.

Agrobacterium tumefaciens is a soil and phytopathogenic bacterium which causes crown gall tumors in a variety of plants (28). Reactive oxygen species generated from both internal metabolism and external sources are highly toxic to bacterial cells (9). The bacterial oxidative stress response requires coordination of various cellular activities. SoxR is a member of the MerR family of transcriptional regulators. It has a central role in sensing and regulating gene expression in response to superoxide and nitrosative stresses (4, 21). In *Escherichia coli*, these responses involve a two-stage process (17, 27). Superoxide anions or reactive nitrogen molecules oxidize SoxR, which in turn activates *soxS*, which encodes an AraC-type transcriptional regulator. Oxidized SoxS upregulates transcription of at least 15 genes in the SoxRS regulon (5). Many of the genes (e.g., *sodA* and *nfo*) in the regulon are directly responsible for removal of superoxide anions or repair of superoxide-damaged macromolecules. However, recent reports indicate that in *Pseudomonas*, *soxR* has no clear physiological roles in superoxide stress protection (11, 15, 19, 20) even though it directly regulates expression of superoxide-inducible genes (15). Moreover, SoxR acts directly on target promoters without the involvement of SoxS (15, 20). These findings suggest that SoxR proteins in different bacteria have diverse physiological roles and different mechanisms by which the regulator regulates gene expression. In this report, we demonstrate the physiological function of *A. tumefaciens soxR* in superoxide stress protection. Moreover, SoxR directly regulates its own promoter and promoters of other genes in its regulon.

Physiological analysis of a *soxR mutant*. The role of *soxR* in physiological protection against superoxide stress was evaluated with *A. tumefaciens* wild-type (NTL4) and *soxR* mutant (PW01) strains (8). The oxidant resistance levels during different stages of growth were determined using a growth inhibition zone assay for exponential-phase cells and a plate sensitivity test for stationary-phase cells (22). The results indicate that during the exponential growth phase, the *soxR* mutant was more sensitive to killing effects of superoxide generators, menadione (MD) and paraquat, than the parental strain (NTL4) (Fig. 1A). Similarly, the stationary-phase levels of resistance to menadione also significantly decreased for the mutant (Fig. 1B). The reduced-resistance-to-superoxide-killing phenotype at both phases of bacterial growth for the mutant could be complemented by expression of a functional *soxR* gene from the pSoxR plasmid (Fig. 1A and B, PW01/pSoxR). The *soxR* mutant showed no significant alterations in the levels of resistance to other oxidants, such as H_2O_2, cumene hydroperoxide, and *t*-butyl hydroperoxide (tBOOH), at both phases of bacterial growth (data not shown). Menadione and paraquat are redox cycling agents capable of generating intracellular superoxide anions. The inactivation of *soxR* renders *A. tumefaciens* more susceptible to superoxide stress, suggesting that the gene has roles in the regulation of protective genes involved in alleviating superoxide stress.

The phenotype of reduced resistance to superoxide generator killing for PW01 was partially due to loss of its ability to upregulate the expression of *sod* genes. We have previously shown that the total levels of superoxide dismutase (SOD) in *A. tumefaciens* could be increased in response to the presence of sublethal concentrations of menadione (8). The induction of SOD activities by superoxide anions is abolished in the *soxR* mutant (8).

Regulation of *A. tumefaciens* NTL4 *soxR* expression. In *E. coli*, SoxR is a sensor of superoxide stress and a transcriptional regulator that coordinates the superoxide stress response (4). The only *E. coli* SoxR target gene is *soxS* (4, 21). *A. tumefaciens soxR* is located immediately downstream of *bfrA*, encoding an exogenous ferric siderophore receptor-like protein, and is upstream of *amyA*, encoding an α-amylase (data not shown) (10,

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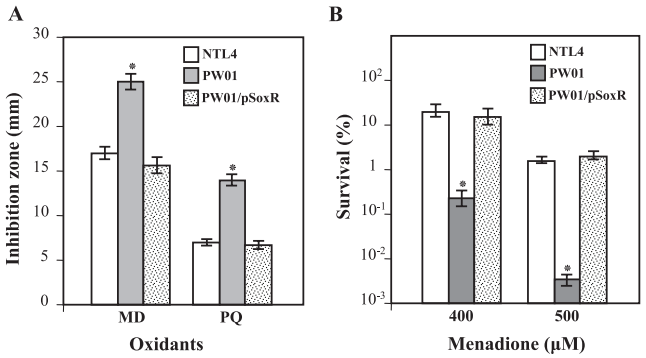

FIG. 1. Determination of the resistance levels toward superoxide generators in *A. tumefaciens* strains. A, levels of resistance of exponential-phase *A. tumefaciens* strains to superoxide generators were determined (22) by mixing exponential-phase cells with molten top agar and overlaying this mixture on LB plates. Sterile 6-mm-diameter paper discs soaked with 5 μl of 1.0 M paraquat or 1.0 M MD were placed on the surface of the cell lawn, and the zones of growth inhibition were measured after 24 h of incubation at 30°C. B, level of resistance of the stationary-phase cells to MD killing was determined using a plate sensitivity assay (22). Serial dilutions of stationary-phase cells were made in 50 mM sodium phosphate buffer (pH 7.0), and 10 μl of each dilution was spotted onto an LB agar plate containing 400 or 500 μM MD. The plates were incubated at 30°C for 24 h before the results were read. Percentage survival is defined as the number of cells grown on plates containing MD divided by the number of cells grown on plates without MD multiplied by 100. The significances of differences among treatments were statistically determined using one-way analysis of variance and post hoc pairwise comparison with the least significant difference test. An asterisk indicates a P value of <0.01 compared with results for the NTL4 strain. NTL4, *A. tumefaciens* parental strain; PW01, *soxR* mutant; PW01/pSoxR, PW01 harboring pSoxR.

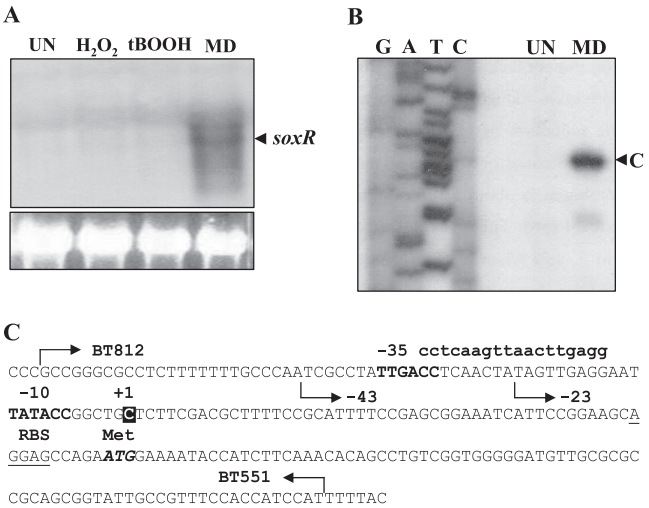

FIG. 2. Menadione induced *soxR* expression and identification of *soxR* promoter and regulatory regions. A, Northern blot of the total RNA (10 μg) samples prepared from exponential-phase cultures of *A. tumefaciens*; uninduced (UN) or induced with 250 μM H_2O_2 (H_2O_2), 200 μM *t*-butyl hydroperoxide (tBOOH), or 200 μM menadione (MD) for 15 min. These samples were then separated, blotted, and hybridized with a ^{32}P-labeled *soxR*-specific probe. The level of 16S rRNA as a loading control is shown underneath the autoradiograph of the Northern blot. B, primer extension of RNA extracted from uninduced (UN) or MD-induced (MD) cultures was previously described (2). The experiment was performed using the ^{32}P-labeled oligonucleotide primer BT551 (5′ ATGGATGGT GGAAACGGC3′). C, T, A, and G are sequence ladders. The arrowhead and +1 indicate the *soxR* transcription start site. C, regulatory regions of *soxR*. The −35 and −10 regions are shown in bold capital letters. RBS and Met represent the ribosome binding site and the translation start site of *soxR*, respectively. A putative SoxR box that shared homology with the *E. coli* SoxR binding site is shown in small letters. +1 indicates the transcription start site.

25). BLASTP (23) searches of the *A. tumefaciens* genome sequence (10, 25) with an *E. coli* SoxS amino acid sequence (1) failed to identify a SoxS homolog. We determined transcriptional organization, expression patterns in response to oxidant treatments, and regulation of *soxR*. The Northern blot results reveal positively hybridized *soxR* mRNA approximately 600 bp in length, indicating that the gene was transcribed as a monocistronic mRNA (Fig. 2A). *soxR* expression patterns in response to oxidant treatments showed that only a superoxide generator, menadione, highly induced its expression (Fig. 2A). Treatments of the bacteria with either H_2O_2 or organic hydroperoxide had no effect on *soxR* expression levels.

Next, the *soxR* transcriptional start site was localized using primer extension with total RNA samples extracted from uninduced and menadione-induced cultures. A 139-bp primer extension product was easily detected in the menadione-induced RNA sample compared to results with the uninduced RNA sample (Fig. 2B). The sizes of the products placed *soxR* transcription initiation at the C nucleotide, 59 nucleotides upstream of the *soxR* translational start codon, ATG (Fig. 2C). The primer extension results also confirmed the results of Northern blot analysis. That is, menadione highly induced *soxR* expression, and this induction was due to increased transcription from the *soxR* promoter. Analysis of the sequence upstream of the transcription initiation site revealed the sequence motifs TTGACC and TATACC, which corresponded to the −35 and −10 promoter regions, respectively (Fig. 2C). The two regions were separated by 19 bases which are atypical for *A. tumefaciens* promoters (2, 16). The intervening sequence between the promoter regions had an inverted repeat sequence motif that shared 14 out of the 18 bases that matched the consensus sequence of the *E. coli* SoxR binding site (17). The atypical spacing of the −10 and −35 regions of the *A. tumefaciens soxR* promoter and the position of the putative SoxR box suggest that *A. tumefaciens* SoxR most likely uses mechanisms similar to those of *E. coli* SoxR to repress and activate expression of a target promoter (14). This suggests that SoxR might bind and regulate its own expression.

In vivo *soxR* promoter analysis was done by cloning a 204-bp *soxR* promoter fragment amplified from *A. tumefaciens* genomic DNA using primers BT812 (5′GCCGGGCGCCTCT TTTTT3′) and BT551 (5′ATGGATGGTGGAAACGGC3′) upstream of a promoterless *lacZ* gene in a low-copy-number plasmid, pUFR027lacZ (16). This clone will be henceforth referred to as pP_{soxR}. The *soxR* promoter activities in response to various oxidant treatments were determined for *A. tumefaciens* NTL4 and the PW01 *soxR* mutant harboring pP_{soxR} (PW01/pP_{soxR}). For NTL4 carrying pP_{soxR} (NTL4/pP_{soxR}), menadione treatment greatly induced (greater than 10-fold) *soxR* promoter activity, while other treatments with H_2O_2 or tBOOH did not significantly alter the promoter activity (Fig. 3A). Furthermore, menadione induction of the *soxR* promoter observed with NTL4/pP_{soxR} was abolished with PW01/pP_{soxR}

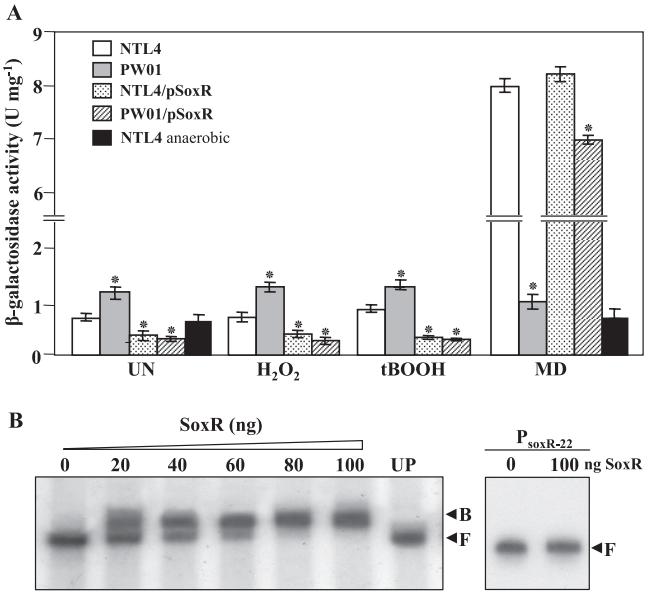

FIG. 3. In vivo promoter analysis and binding of SoxR to the promoter fragment. A, in vivo *soxR* promoter analysis. Exponential-phase cultures of *A. tumefaciens* strains harboring pP_{soxR} (NTL4, PW01, NTL4/pSoxR, or PW01/pSoxR) uninduced (UN) or treated with oxidants (as described in the legend to Fig. 2A) were harvested for crude lysate preparation and analysis of β-galactosidase activity (16). For anaerobic cultures, cells were grown anaerobically for 8 h (7), and MD was then added to the induced culture, which was returned to anaerobic conditions for 30 min before crude lysate preparation and enzyme assay. β-Galactosidase activity is expressed in μmol *p*-nitrophenol generated at 25°C in 1 min per mg protein (18). An asterisk represents a *P* value of <0.01 compared with results for the uninduced condition. B, SoxR binding to the *soxR* promoter. Gel mobility shift reactions were carried out by adding 3 fmol of labeled probe (^{32}P-labeled 207-bp *soxR* promoter fragment) to a 25-μl reaction mixture [20 mM Tris (pH 7.0), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μg ml^{-1} bovine serum albumin, 5 μg ml^{-1} calf thymus DNA, 0.5 mg ml^{-1} poly(dI/dC)]. Purified oxidized SoxR (0 to 100 ng protein) was added, and the reaction mixture was incubated at 25°C for 15 min. Protein-DNA complexes were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 4°C. UP indicates reaction mixtures containing 1 μg unlabeled *soxR* promoter in addition to SoxR (80 ng). $P_{soxR-22}$ is the 45-bp-deleted *soxR* promoter fragment use in the binding reactions. F indicates free probe; B indicates bound probe.

(Fig. 3A). Nonetheless, the constitutive expression phenotype of the PW01/pP_{soxR} strain could be complemented by the *trans* expression of a functional *soxR* gene from the plasmid vector, pSoxR (Fig. 3A). An important question regarding the regulation of gene expression by SoxR is whether the regulator senses and responses to superoxide stress. This was addressed by performing menadione induction of NTL4/pP_{soxR} under aerobic and anaerobic conditions. The results are shown in Fig. 3A; anaerobic growth conditions abolished menadione induction of the *soxR* promoter. Since superoxide anion generation by menadione is abolished under anaerobic conditions, the results supported the role of SoxR as a sensor and a transcription regulator of superoxide stress.

Next, a 5′ deletion of the *soxR* promoter was performed to localize regions required for the menadione induction of *soxR* promoter activity. A deleted *soxR* promoter fragment with the sequence upstream of the −43 region removed was cloned in front of *lacZ*, resulting in $pP_{soxR-43}$. NTL4/$pP_{soxR-43}$ specified menadione-inducible β-galactosidase activity similar to the activity specified by similarly treated NTL4/pP_{soxR} (data not shown). Thus, the SoxR-dependent induction of the *soxR* promoter required no additional sequence upstream of the −35 promoter region. This is consistent with the proposed location of the putative SoxR binding site between the −10 and −35 regions of the promoter (Fig. 2C). The in vivo promoter analysis results are consistent with the results of *soxR* Northern blotting (Fig. 2A) and *soxR* primer extension (Fig. 2B) showing menadione induction of *soxR* promoter activity. Furthermore, the upregulation of the promoter by superoxide anions requires a functional *soxR* gene.

Examination of the basal levels of β-galactosidase activity specified by pP_{soxR} for NTL4 and PW01 revealed that inactivation of *soxR* resulted in increased transcription from the *soxR* promoter, as indicated by higher β-galactosidase activity (1.09 U mg^{-1} protein) for PW01 than for NTL4 (0.67 U mg^{-1} protein). In addition, the high level of *soxR* expression repressed the *soxR* promoter activity, as revealed by the decrease in β-galactosidase activity for NTL4/pP_{soxR}/pSoxR (0.48 U mg^{-1} protein). The data suggest that reduced SoxR represses its own promoter.

Genetically, *soxR* appears to be autoregulated. This, coupled with the presence of a putative SoxR box located in the vicinity of the *soxR* promoter, strongly suggests that SoxR could directly bind to its own promoter and regulate its own expression. An in vitro DNA mobility shift assay was performed to investigate the ability of the purified SoxR protein to bind the *soxR* promoter. *A. tumefaciens* SoxR was overexpressed in *E. coli* BL21 using the pETsoxR vector that contained full-length *soxR* in the pETBlue-2 vector (Novagen). Oxidized SoxR was purified over a P-11 phosphocellulose ion exchange column and was eluted with 0.5 M KCl (12, 26). Twenty to one hundred nanograms of oxidized SoxR was incubated with a radioactively labeled 270-bp fragment containing the *soxR* promoter fragment in a binding buffer (2). The results shown in Fig. 3B demonstrate that SoxR specifically bound to the *soxR* promoter. The specificity of the SoxR binding to the promoter was shown by the ability of unlabeled *soxR* promoter fragment to act as a competitor target DNA and prevent binding of the protein to its target site. In addition, the DNA gel shift experiment was repeated using a deleted *soxR* promoter fragment that had the −35 region, and half of the putative SoxR binding site was removed to position −22 ($P_{soxR-22}$). No binding of SoxR to the deleted promoter fragment was detected (Fig. 3B). These results clearly show that SoxR binds to its own promoter and the binding requires the sequence spanning the −35 and −10 regions, where a putative SoxR box is located.

Regulation of *A. tumefaciens soxR* expression is likely to be an important component of the overall superoxide stress response. In the absence of superoxide stress, reduced SoxR binds to its own promoter and represses transcription. Upon exposure to superoxide anions, SoxR becomes oxidized, and the oxidized form of the regulator binds to the SoxR box and activates transcription from the promoter (21). The observations with *A. tumefaciens* are consistent with SoxR function as a superoxide sensor and a transcriptional regulator.

Characterization of atu5152, a gene in the SoxR regulon. The lack of a SoxS homolog in *A. tumefaciens* raises an important question on how SoxR regulates the target genes. It also

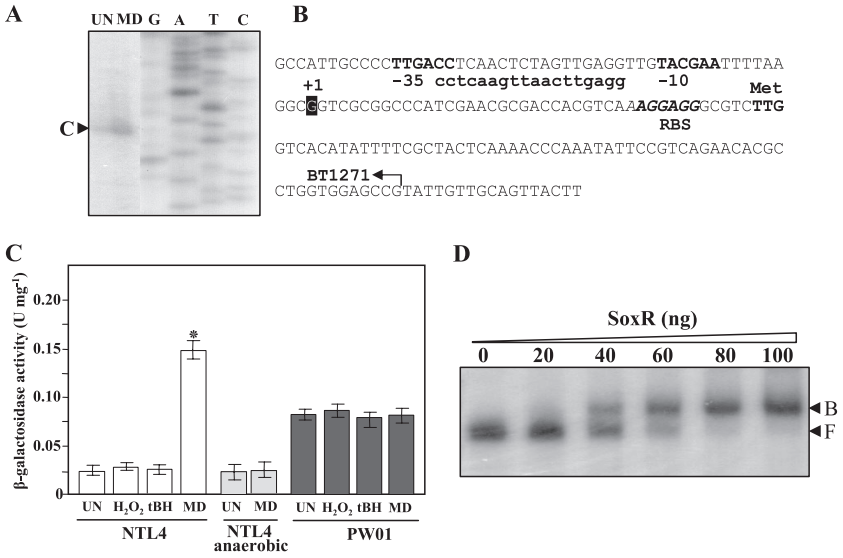

FIG. 4. *soxR* directly regulates atu5152. A, primer extension analysis of atu5152. Primer extension was performed on total RNA samples isolated from uninduced (UN) or menadione-induced (MD) cultures. The BT1271 (5′CGGCTCCACCAGGCGTGTT3′) primer was used. B, regulatory regions of atu5152. The promoter −10 and −35 regions are shown in bold. The transcription start site (+1) and a putative SoxR box between the conserved promoter regions are shown. RBS and Met represent the ribosome binding site and the translation initiation site of atu5152, respectively. C, in vivo atu5152 promoter analysis. Exponential-phase cultures of *A. tumefaciens* NTL4 and PW01 harboring pP_{5152} were treated with oxidants either aerobically or anaerobically as for Fig. 3A. Crude lysate preparations and β-galactosidase activity assays were performed as previously described (16). An asterisk represents a *P* value of <0.01 compared with results for the uninduced condition. D, binding of SoxR to the atu5152 promoter. A gel mobility shift assay was performed using an increased amount of purified oxidized SoxR (ng protein) as described for Fig. 3C except that the 212-bp ^{32}P-labeled atu5152 promoter fragment was used.

implies that *A. tumefaciens* probably has mechanisms for regulating superoxide-inducible gene expression different from those found in enteric bacteria (4) but which could be similar to those of members of *Pseudomonas* spp. (15, 19, 20). The findings presented here showing that SoxR bound to a binding site with a sequence motif similar to that of an *E. coli* SoxR box located between *soxR* promoter regions allowed us to find putative SoxR-regulated genes by searching the *A. tumefaciens* genome sequence (25) with a slightly degenerate SoxR box consensus sequence, CCTCAACTATAGTTGAGG (14). Putative SoxR boxes located in the flanking sequences between the annotated open reading frames were selected for further study. Putative SoxR boxes were identified in front of at least three open reading frames, namely, Atu4762, Atu4895, and Atu5152. atu4762 encodes a manganese-iron SOD. Atu4895 and Atu5152 are uncharacterized 135-amino-acid membrane proteins which share a high score of identity in their amino acid sequences (69%). The BLASTP searches conducted so far indicated that Atu4895 and Atu5152 are found only in *A. tumefaciens*. Analysis of the putative amino acid sequence using the TopPred program (available at http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (3) suggests that these proteins contain three transmembrane motifs (data not shown). The role of SoxR in the regulation of atu5152 was determined. Primer extension was performed using an atu5152-specific primer and total RNA from uninduced and menadione-induced cultures. A 105-nucleotide primer extension product was detected in the menadione-induced RNA sample (Fig. 4A). This placed the transcription start site at a G residue 42 bp from the translational start codon of atu5152 (Fig. 4A and B). Analysis of the atu5152 promoter showed a putative SoxR binding site with the sequence CCTCAACTAGAGTTGAGG, in which 14 out of 18 bp match the *E. coli* SoxR box and which was located between the −10 and −35 regions, which were separated by 19 bp that are atypical for *A. tumefaciens* promoters (Fig. 4B). The location of the SoxR box and the atypical spacing between the −10 and −35 regions fit well with the proposed model for the mechanism by which *E. coli SoxR* regulates the target promoter (13). In vivo atu5152 promoter analysis was done by cloning a 212-bp atu5152 promoter fragment amplified from *A. tumefaciens* genomic DNA using primers BT1125 (5′AAACCTCTCATCTCGCCC3′) and BT1126 (5′CAATACGGCTCCAACCAG3′) upstream of *lacZ i*n pUFR027lacZ (16). This clone was named pP_{5152}. The promoter activity specified by NTL4 harboring pP_{5152} was induced fivefold by menadione treatment, and this induction was abolished in PW01 harboring the plasmid (Fig. 4C). Furthermore, the SoxR-dependent menadione induction of the atu5152 promoter was also abolished when the experiments were performed under anaerobic conditions, indicating that superoxide anions are essential for menadione-induced promoter activity (Fig. 4C). Moreover, the basal β-galactosidase levels for PW01 harboring pP_{5152} were threefold higher than those for the NTL4 parental strain (Fig. 4C). We extended the analysis of SoxR regulation of the atu5152 promoter by performing a DNA mobility shift assay using the 212-bp atu5152 promoter fragment and purified SoxR. The results shown in Fig. 4D show that SoxR specifically bound to the atu5152 promoter fragment.

The results presented here indicate that atu5152 is a SoxR target gene to which SoxR directly binds, regulating its expression in response to superoxide stress. In an uninduced condition, re-

duced SoxR binds to the atu5152 promoter and represses its expression. Exposure to superoxide anions oxidizes SoxR and converts it into a transcriptional activator that activates transcription from the atu5152 promoter. In the initial experiments, the atu4895 and atu5152 mutants did not show significant alterations in superoxide stress resistance levels (data not shown). The physiological roles of these genes remain unclear. Our data illustrate the function of *A. tumefaciens* SoxR as a global transcriptional regulator that senses superoxide stress and directly controls the expression of genes in the regulon.

Currently, there are distinct *E. coli* and *Pseudomonas* paradigms for SoxR-mediated gene regulation. For *E. coli*, oxidation of SoxR leads to activation of the only known SoxR target gene, *soxS* (4, 21). SoxS, a transcription regulator, in turn activates genes involved in superoxide stress protection and repair (17, 21, 27). Thus, an *E. coli soxR* mutant showed increased sensitivity to superoxide stress killing (24). For *Pseudomonas aeruginosa* and *Pseudomonas putida*, reduced and oxidized SoxR directly binds to target promoters, where it can either activate or repress its target genes (15, 19, 20). However, a detailed analysis of SoxR-regulated genes indicates that they are not involved in superoxide stress protection and repair (6, 19, 20). This is reflected in the observation that *Pseudomonas soxR* mutants do not show altered resistance to superoxide generators. Like *Pseudomonas*, *A. tumefaciens* SoxR senses and responds to superoxide stress by directly binding to the promoters of target genes and, depending on the redox state of the protein, either activates or represses gene expression. However, unlike the case with *Pseudomonas*, some of the SoxR target genes in *A. tumefaciens* are involved in superoxide stress protection and repair. Hence, an *A. tumefaciens soxR* mutant is hypersensitive to superoxide stress-generating agents. Taken together, the results reported here show that the *A. tumefaciens* SoxR paradigm differs from either of the existing paradigms and illustrate that different bacteria use different variations of the SoxR system to sense and respond to stresses.

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