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 H2O2, cumene hydroperoxide, and tert-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 generating 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,
Northern blot analysis. That is, menadione highly induced the expression of the soxR gene in A. tumefaciens strains. The primer extension results also confirmed the results of Northern blotting (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. NTLA, A. tumefaciens parental strain; PW01, soxR mutant; PW01/pSoxR, PW01 harboring pSoxR.

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). The experiment was performed with serial dilutions of exponential-phase cells of A. tumefaciens; uninduced (UN) or induced with 250 μM H2O2 (H2O2), 200 μM t-butyl hydroperoxide (tBOOH), or 200 μM menadione (MD) for 15 min. These samples were then separated, blotted, and hybridized with a 32P-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 32P-labeled oligonucleotide primer BT515 (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.

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). 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. NTLA, 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 H2O2 (H2O2), 200 μM t-butyl hydroperoxide (tBOOH), or 200 μM menadione (MD) for 15 min. These samples were then separated, blotted, and hybridized with a 32P-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 32P-labeled oligonucleotide primer BT515 (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.

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′ GCCGGGGCGGCTT TTTT3′) and BT515 (5′ ATGGATGGT GGAAACGGC3′) upstream of a promoterless lacZ gene in a low-copy-number plasmid, pUFR027lacZ (16). This clone will be henceforth referred to as pPsoxR. The soxR promoter activities in response to various oxidant treatments were determined for A. tumefaciens NTL4 and the PW01 soxR mutant harboring pPsoxR (PW01/pPsoxR). For NTL4 carrying pPsoxR (NTL4/pPsoxR), menadione treatment greatly induced (greater than 10-fold) soxR promoter activity, while other treatments with H2O2 or tBOOH did not significantly alter the promoter activity (Fig. 3A). Furthermore, menadione induction of the soxR promoter observed with NTL4/pPsoxR was abolished with PW01/pPsoxR.
by performing menadione induction of NTL4/pPsoxR under aerobic and anaerobic conditions. This was addressed by examination of the basal levels of β-galactosidase activity specified by pPsoxR 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/pPsoxR/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 SoxS 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 demonstrated 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 SoxS binds to its own promoter and represses transcription. Upon exposure to superoxide anions, SoxS 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 SoxS function as a superoxide sensor and a transcriptional regulator.

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

(Fig. 3A). Nonetheless, the constitutive expression phenotype of the PW01/pPsoxR 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 responds to superoxide stress. This was addressed by performing menadione induction of NTL4/pPsoxR under aerobic and anaerobic conditions. The results are shown in Fig. 3A; aerobic 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 transcriptional 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 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 pPsoxR 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/pPsoxR/pSoxR (0.48 U mg\(^{-1}\) protein). The data suggest that reduced SoxR represses its own promoter.

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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 showed 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, CCTCAACTAGAGTTGAGG (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 (UN) and menadione-induced (MD) cultures. The BT1271 (5’CGGCTCCACCAGGCGTGT3’) 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 pP5152 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 32P-labeled atu5152 promoter fragment was used.

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’CGGCTCCACCAGGCGTGT3’) 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 pP5152 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 32P-labeled atu5152 promoter fragment was used.

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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 hyporesponsive 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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