Multiple Superoxide Dismutases in *Agrobacterium tumefaciens*: Functional Analysis, Gene Regulation, and Influence on Tumorigenesis[∇]

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***Agrobacterium tumefaciens* possesses three iron-containing superoxide dismutases (FeSods) encoded by distinct genes with differential expression patterns. SodBI and SodBII are cytoplasmic isozymes, while SodBIII is a periplasmic isozyme. *sodBI* is expressed at a high levels throughout all growth phases. *sodBII* expression is highly induced upon exposure to superoxide anions in a SoxR-dependent manner. *sodBIII* is expressed only during stationary phase. Analysis of the physiological function of *sods* reveals that the inactivation of *sodBI* markedly reduced levels of resistance to a superoxide generator, menadione. A mutant lacking all three Sod enzymes is the most sensitive to menadione treatment, indicating that all *sods* contribute at various levels towards the overall menadione resistance level. Sods also have important roles in *A. tumefaciens* virulence toward a host plant. A *sodBI* but not a *sodBII* or *sodBIII* mutant showed marked reduction in its ability to induce tumors on tobacco leaf discs, while the triple *sod* null mutant is avirulent.**

Agrobacterium tumefaciens is a gram-negative aerobic bacterium commonly found in rhizospheres. The bacterium is the causative agent of crown gall disease on a variety of dicotyledonous plants. During infection, the bacterium transfers part of its Ti plasmid, the T-DNA, into the plant cell, resulting in tumor formation (12).

Superoxide anion ($O_2^{\cdot-}$) is a toxic by-product of aerobic metabolism mainly produced by a partial reduction of oxygen molecules by electrons leaking from the respiratory chain. It can also be generated by auto-oxidation of redox-cycling compounds such as menadione (MD), plumbagin, and the herbicide paraquat and by the host defense response against invading microorganisms. The toxicity of a superoxide anion arises from its ability to oxidize and leach iron from iron-sulfur cluster (4Fe-4S)-containing proteins, thereby inactivating enzymes such as aconitase, 6-phosphogluconate dehydratase, and fumarases (28). In addition, the released iron could participate in the Fenton reaction, leading to increased production of hydroxyl radicals, which can cause damage to many macromolecules including DNA (28). Superoxide anions also can be dismutated to H_2O_2 , which itself is reactive to macromolecules and can participate in the Fenton reaction.

Bacteria have evolved an impressive array of antioxidant defenses to deal with superoxide anion. Superoxide dismutase (Sod; EC 1.15.1.1) is a metalloenzyme catalyzing the dismuta-

tion of superoxide anions to form H_2O_2 , a substrate of catalases, and molecular oxygen. The enzymes can be divided according to their metal cofactors into four classes, namely, the manganese (MnSod), the iron (FeSod), the copper-zinc (Cu/ZnSod), and the nickel (NiSod) (19). Several gram-negative bacteria produce multiple Sod enzymes which differ in terms of metal cofactor, subcellular location, and regulation of gene expression. *Escherichia coli* has three Sods: MnSod (encoded by *sodA*), FeSod (encoded by *sodB*), and Cu/ZnSod (encoded by *sodC*). The first two enzymes are cytoplasmic, while the latter locates to the periplasmic space (19). FeSod is constitutively expressed, while MnSod is produced in the presence of oxygen, upon response to accumulation of intracellular superoxide anion, or upon a change in bacterial growth phase. Consistent with this pattern of regulation, *sodA* is a member of the SoxRS regulon (19, 34). The contribution of Sods to bacterial virulence has been investigated for several animal pathogens. In most cases, inactivation of genes encoding Sods impairs bacterial survival in tested models (32). Although increased production of reactive oxygen species (ROS) is also a common feature in plant defense systems, the roles of Sod as a virulence factor in phytopathogenic bacteria are still unclear, due to the limited number of investigations and variations observed in different plant-microbe interactions. In *Xanthomonas campestris*, the expression of an MnSod is induced during bacterial invasion into the host plant (42). However, the role of this enzyme in pathogenicity is not clear. Deletion of *sodA* in *Erwinia chrysanthemi* reduced the size of necrotic lesions on African violets, while the ability to infect potato was unaffected (40). However, Sod has a key protective role in the symbiotic process of *Sinorhizobium meliloti* (41). In this communication, we report the characterization of three Sod isozymes in *A. tumefaciens*. Mutants lacking one or all three of the Sod

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TABLE 1. Bacterial strains and primers used in this study

Strain or primer	Description or sequence	Source/reference or purpose
<i>A. tumefaciens</i> strains		
NTL4	A pTiC58-cured derivative of strain C58 Δtet_{C58}	35
PW01	NTL4 <i>soxR</i>	16
PS1	NTL4 <i>sodBI::lacZ</i>	This study
PS2	NTL4 <i>sodBIII::lacZ</i>	This study
Primers		
BT534	CAGCACTACAACCGTC	Forward primer for <i>sodBI</i> fragment
BT535	TCCAAGCTTGCCGTCTT	Reverse primer for <i>sodBI</i> fragment
BT639	AAAAGGAGTAAAAACCATG	Forward primer for full-length <i>sodBI</i>
BT638	GCCTTACTTGCGAGCGGCT	Reverse primer for full-length <i>sodBI</i>
BT641	GCGCAGTCCGGCATATTCA	Forward primer for <i>sodBII</i> fragment
BT640	ACCAGCCACGCCAGCCCG	Reverse primer for <i>sodBII</i> fragment
BT644	CGGGATCGTCAAGGAATC	Forward primer for <i>sodBIII</i> fragment
BT645	GGTGCCGAAGTCCGTGTC	Reverse primer for <i>sodBIII</i> fragment
BT709	CATGAGGAGGATATGATGG	Forward primer for full-length <i>sodBIII</i>
BT710	TCGCCTATTGACCTCAAC	Reverse primer for full-length <i>sodBIII</i>
BT711	AGTTGACAAAGGAATACCC	Forward primer for full-length <i>sodBII</i>
BT729	GGCTCAAATCAGAGGCGG	Reverse primer for full-length <i>sodBII</i>
BT793	GTGTGGCAAGGCGCACATA	Upstream primer for <i>sodBII</i> promoter
BT817	AGGGGCGAGCGCATCAT	<i>sodBII</i> primer extension and reverse primer for <i>sodBII</i> gel mobility shift assay
BT1142	CGGGATCCGTCGACGAGCGATAATCC	Forward primer for <i>sodBII</i> promoter and gel mobility shift assay
BT1269	GCGACATGTAAGGTGCAAG	<i>sodBI</i> primer extension

isozymes showed moderate to severe defects in their abilities to induce tumors on tobacco leaves.

(Parts of this work are from the dissertation of P.S. submitted for a Ph.D. degree from Mahidol University.)

MATERIALS AND METHODS

Bacterial growth conditions. *Agrobacterium tumefaciens* NTL4 (a pTiC58-cured derivative of C58 Δtet_{C58}) (35) and the mutant strains were grown aerobically in Luria-Bertani (LB) medium at 30°C with continuous shaking at 150 rpm. To ensure comparable growth states, overnight cultures were inoculated into fresh LB medium to give an optical density at 600 nm (OD_{600}) of 0.1. Exponential-phase (OD_{600} of 0.6 after 4 h of growth) and stationary-phase (OD_{600} of about 5 after 30 h of growth) cells were used as indicated in all experiments. The oxidant-induced experiments were performed by adding either 250 μ M H_2O_2 , 250 μ M *tert*-butyl hydroperoxide, or 200 μ M MD to exponential-phase cultures. These cultures were grown for an additional 15 min for Northern analysis and 30 min for enzymatic assays.

Molecular biology techniques. General molecular genetics techniques, including genomic DNA preparation, plasmid preparation, restriction endonuclease digestion, ligation, transformation in *E. coli*, agarose gel electrophoresis, and Southern and Northern blot analyses were performed using standard protocols (38). Plasmids were purified for DNA sequencing using Qiagen Miniprep kits (Qiagen, Germany). Routinely, *A. tumefaciens* was transformed by electroporation as previously described (35).

Primer extension. Primer extension experiments for *sodBI* were performed using 10 μ g of total RNA, 200 U of Superscript III reverse transcriptase (Invitrogen), and 32 P-labeled oligonucleotide primer BT1269 (Table 1). The sizes of the extension products were determined on sequencing gels (38) by comparison to dideoxy sequencing ladders generated using a PCR sequencing kit with labeled pUC/M13 forward primer and pGEM-3Zf(+) as the template (Applied

Biosystems). Primer extension of *sodBII* was performed using the same protocol described for *sodBI* except the total RNA samples were extracted from uninduced and MD-induced cultures and the 32 P-labeled oligonucleotide primer BT817 was used.

Construction of *A. tumefaciens* *sodBI*, *sodBII*, and *sodBIII* mutants and full-length genes. *A. tumefaciens* *sodBI*, *sodBII*, and *sodBIII* mutants were constructed by insertion inactivation using suicide plasmid pKNOCK (1). DNA fragments (~200 bp) of the target gene were amplified in a PCR using NTL4 genomic DNA as a template. The specific primers for *sodBI* (BT534 and BT535), *sodBII* (BT640 and BT641), and *sodBIII* (BT644 and BT645) were designed based on the nucleotide sequences corresponding to putative open reading frames (ORFs) Atu0876 (accession no. AAL41890), Atu4583 (accession no. AAL45377), and Atu4726 (accession no. AAL45520), respectively (51). The PCR products were ligated into pGEM-T Easy (Promega) prior to the subcloning of the EcoRI fragments into the pKNOCK vector. The recombinant plasmids were transferred into NTL4 by conjugation (1). The putative mutants were screened by PCR using two primers flanking the insertion sites and confirmed by Southern blot analysis. The full-length genes were amplified from NTL4 genomic DNA with primers BT638 and BT639 for *sodBI*, BT711 and BT729 for *sodBII*, and BT709 and BT710 for *sodBIII* by use of *Pfu* polymerase (Promega). The PCR products were cloned into broad-host-range plasmid pBBR1MSC4 (31) and digested with EcoRV, giving pSodBI, pSodBII, and pSodBIII. The cloned *sods* were all sequenced.

***sodBI*, *sodBII*, and *sodBIII* promoter-*lacZ* fusion.** Promoter regions of *sodBI* and *sodBIII* were fused to suicide promoterless plasmid vector pVIK112 (30). The *sodBI* fragment was amplified using BT534 and BT535 primers and cloned into pGEM-T Easy (Promega). The EcoRI-SpeI blunt fragment was subsequently cloned into pVIK112 digested with EcoRI and SmaI. The resultant plasmid was introduced into *A. tumefaciens* NTL4. Homologous recombination between the *sodBI* fragment on the nonreplicating plasmid and on the chromosome generated a *sodBI::lacZ* fusion. The strain containing this fusion was desig-

nated PS1. Strain PS2, containing a *sodBIII::lacZ* fusion, was obtained using the same protocol, but the *sodBIII* fragment was amplified with BT644 and BT645 primers prior to cloning into pVIK112. The *sodBII* promoter-*lacZ* gene fusion was constructed in a low-copy-number promoter-probed vector, pUFR027lacZ (36). The *sodBII* promoter region was amplified using BT640 and BT793 primers and *A. tumefaciens* NTL4 genomic DNA as a template. The 680-bp PCR product was first cloned into pGEM-T Easy (Promega) before the EcoRI fragment was subcloned into pUFR027lacZ digested with the same enzyme to generate pSodBII.

SoxR purification. *A. tumefaciens* SoxR was overexpressed in *E. coli* BL21 by use of pETsoxR (15), which contains 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 as previously described (15). The purified *A. tumefaciens* SoxR showed a UV spectrum that was similar to the spectrum obtained from *E. coli* oxidized SoxR (25).

Gel mobility shift assay. Gel mobility shift reactions were performed by preparing 3 fmol of labeled probe (³²P-labeled 220-bp *sodBII* promoter fragment amplified using BT1142 and BT817 primers) in 25 μ l of reaction buffer [20 mM Tris (pH 7.0), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μ g ml⁻¹ bovine serum albumin, 5 μ g ml⁻¹ calf thymus DNA, 0.5 mg ml⁻¹ poly(dI-dC)]. Various amounts of purified SoxR (15) were 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 $\times$ Tris-borate-EDTA buffer at 4°C and visualized by exposure to X-ray film.

Determination of oxidant resistance. Levels of resistance to oxidants were determined using a plate sensitivity assay as previously described (15). Serial dilutions of bacterial cultures were made in 50 mM sodium phosphate buffer, pH 7.0, for stationary-phase cells and in LB broth for exponential-phase cells, and 10 μ l of each dilution was spotted onto an LB agar plate containing 0.5 mM MD. The plates were incubated at 30°C for 48 h before the results were recorded. The resistance levels were expressed as percent survival, defined as the CFU on plates containing oxidant divided by the CFU on plates without oxidant multiplied by 100.

Resistance towards extracellular superoxide anions was assayed using the xanthine-xanthine oxidase system as described previously (47). Stationary-phase cells were treated with 4.3 mM xanthine, 1 U ml⁻¹ xanthine oxidase, and 1 U *Xanthomonas* KatA monofunctional catalase (11) for 30, 60, and 90 min at 30°C. Bacterial cells surviving the treatment were scored using standard viable cell counts on LB agar plates.

Sod activity gels. To visualize Sod activity, 50 μ g proteins from crude samples were separated by nondenaturing electrophoresis performed using a 10% polyacrylamide gel (pH 8.5) with a 5% stacking gel (pH 6.9) followed by staining with nitroblue tetrazolium-riboflavin photochemical stain as described by Beauchamp and Fridovich (5). In the enzyme inhibition tests, the gel was incubated with 10 mM H₂O₂, sodium azide, or potassium cyanide for 45 min prior to enzyme activity staining. Comparison of Sod activities and the percents inhibition of Sod activities were estimated using densitometry analysis.

Alkaline phosphatase assays. Alkaline phosphatase assays were performed according to the method described by Garen and Levinthal (21), in which the activity was determined by monitoring an increase in OD₄₁₀ resulting from the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol. One unit is defined as the amount of enzyme capable of releasing 1 micromole of *p*-nitrophenol per min at 25°C and pH 8.0.

Virulence assay. Virulence assays were performed as described previously (44) with some modifications. In brief, *Nicotiana tabacum* leaf explants and the wild type or *sod* mutants of *A. tumefaciens* were cocultivated for 48 h on hormone-free Murashige and Skoog (MS) medium containing 60 μ g ml⁻¹ acetosyringone. The infected leaf discs were then transferred to fresh MS medium containing 200 μ g ml⁻¹ Timentin (ticarcillin disodium-clavulanate potassium; GlaxoSmithKline) to inhibit bacterial growth. After 14 days of incubation in the dark, the numbers of tumors on each leaf explant were scored from 0 to 5 by comparison to a standard, with 0 representing tumors induced by NTL4 without a Ti plasmid (pCMA1) and 5 representing tumors induced by NTL4 bearing the Ti plasmid pCMA1. At least 20 leaf explants were used for each strain tested, and each experiment was repeated three times.

RESULTS AND DISCUSSION

***A. tumefaciens* has three FeSods.** A BLASTP (3) search of the annotated genome sequence of *A. tumefaciens* C58 (51) with *E. coli* Sod amino acid sequences available at <http://www.ncbi.nlm.nih.gov/> identified three ORFs, namely, Atu0876

(SodBI), Atu4583 (SodBII), and Atu4726 (SodBIII), with high sequence homology. *sodBI* (formerly *sodI* [49]) is located on the circular chromosome, while *sodBII* and *sodBIII* (formerly *sod2* [49]) are on the linear chromosome. SodBI, SodBII, and SodBIII share 43.5%, 42.5%, and 36.8% identities, respectively, to *E. coli* iron-containing Sod (SodB). Percentage identities among *A. tumefaciens* SodB are 46.7% (SodBI and SodBII), 35.5% (SodBI and SodBIII), and 35.2% (SodBII and SodBIII). The analysis of the primary structure of these putative Sods by use of the BLAST program (3) showed that they belong to the Fe/MnSod family. Multiple alignments of these ORFs with known Fe- and Mn-containing Sods by use of CLUSTAL W (46) revealed that all amino acid residues involved in metal binding and the enzyme active sites are conserved in the three *A. tumefaciens* Sods (Fig. 1). The presence of conserved key amino acid residues described in the work of Santos et al. (39) also suggests that *sodBI*, *sodBII*, and *sodBIII* encode FeSod isozymes.

The inhibition of Sod activity by H₂O₂, cyanide, and azide has been used to differentiate metal cofactors of various Sods (19). Sod activity gels and various inhibition tests were performed to establish the metal cofactor of the three putative *A. tumefaciens* Sods by use of crude extracts prepared from a triple *sod* null mutant (*sodBI sodBII sodBIII* mutant) harboring either pSodBI, pSodBII, or pSodBIII (Fig. 2A). The results demonstrate that all three putative genes encode enzymes with Sod activity, as shown by a positive reaction in Sod activity gels. Furthermore, treating the Sod activity gels with 10 mM H₂O₂ inhibited all *A. tumefaciens* Sod isozymes, while these enzyme activities were resistant to treatment with cyanide and azide (Table 2). We conclude on the basis of inhibition tests and sequence analysis that SodBI, SodBII, and SodBIII are FeSods. In this regard, *A. tumefaciens* differs from most other bacteria which express cytoplasmic Sods, either Fe or Mn or both Fe and Mn Sods. Under superoxide and peroxide stress conditions, bacteria generally chelate free iron and reduce iron uptake to minimize the participation of free iron in various free radical-generating reactions. The physiological advantage to *A. tumefaciens* of having only FeSods is not clear. It could be that the FeSod is functionally better for the bacterium in the soil, rhizosphere, or plant wound site environment.

Subcellular localization of SodBI, SodBII, and SodBIII. The amino acid sequences of SodBI and SodBII lack detectable signal peptide sequences and most likely are cytoplasmic enzymes. By contrast, TatP analysis (6) (available at <http://www.cbs.dtu.dk/services/TatP/>) of the SodBIII sequence showed an extended N terminus and a twin-arginine signal sequence with a potential cleavage site between positions 29 and 30 (AQA-AL), suggesting that the enzyme is localized to the periplasm. We constructed translational gene fusions downstream of the *sodBIII* coding region for a signal sequence with either *phoA* (giving pSodIII-*phoA*) or *lacZ* (giving pSodIII-*lacZ*) in pBBR1MSC4 (31) to confirm the location of the enzyme. The alkaline phosphatase activity specified by NTL4 bearing pSodBIII-*phoA* was 0.35 U mg⁻¹ protein compared to 0.05 U mg⁻¹ protein in NTL4 harboring pPhoA, a clone of *phoA* with a promoter but no signal sequence. No β -galactosidase activity was detected in strains harboring pSodBIII-*lacZ*. These results favor the periplasmic localization for SodBIII. This raises an important question how iron gets inserted into the periplasmic

FIG. 1. Multiple alignments of Sods. Multiple alignments of the deduced amino acid sequences of Sods from *A. tumefaciens* (Atu) (51) with FeSod (SodB) and MnSod (SodA) from *Pseudomonas aeruginosa* (Pae) (24) and from *E. coli* (Eco) (10, 43).

SodBIII. Examination of the SodBIII leader sequence shows twin-arginine (RR) residues in the leader sequence, suggesting that the TAT system could be involved in the transportation of the protein. It is likely that newly synthesized SodBIII is folded with metal insertion in the cytoplasm before being transported to periplasm by the TAT system.

Generally, periplasmic Sods have multiple physiological roles. First, they provide the first line of defense against externally generated superoxide anions. Second, they protect periplasmic enzymes from inadvertently generated superoxide anions from the electron transport chain. In most bacteria, the predominant periplasmic Sods have Cu/Zn metal cofactors (29). Only a few bacteria have been reported to produce periplasmic MnSod (33) or FeSod (18). The BLASTP search of the *A. tumefaciens* genome failed to identify a homolog of Cu/ZnSod. Thus, *A. tumefaciens* has evolved and maintained a FeSod in the periplasm.

The three levels of the three Sods differed during different growth phases as well as in their responses to oxidants. Analysis of total Sod activity in crude extracts taken from an *A. tumefaciens* culture grown in rich medium (LB) at various growth phases by use of Sod activity gels revealed a single Sod activity band (F-Sod) detected during exponential phase, while an additional slower-migrating Sod (S-Sod) activity band was observed during the stationary phase of growth (Fig. 2B). Challenging the exponential cultures with sublethal concentrations of MD, a superoxide generator, caused a substantial increase in the level of F-Sod activity (Fig. 2B). Total Sod activity was

increased from 0.47 ± 0.05 U mg⁻¹ protein in uninduced cells to 1.40 ± 0.10 U mg⁻¹ protein in MD-induced cells. These observations suggest that the expression levels of the three *sods* differ with respect to growth phase and response to oxidants.

To identify the ORFs that encode the Sod activities observed with the activity gels, strains deleted for *sodBI*, *sodBII*, or *sodBIII* were constructed. The results show that the F-Sod activity band was diminished for a *sodBI* mutant (Fig. 2C), while the S-Sod activity band disappeared for a *sodBIII* mutant (Fig. 2B). The identities of these Sods were confirmed by expression of either pSodBI or pSodBIII in the respective mutants; in each case, the complementing plasmids restored the appropriate activity band (data not shown). The *sodBII* mutant did not show significant alterations in F-Sod or S-Sod activity bands. However, the expression of pSodBII in a mutant lacking three *sods* produced an activity band that comigrated with SodBI at the F-Sod activity band position (data not shown).

***sodBI* is highly expressed throughout all growth phases.** As assessed by Northern blot analyses, *sodBI* was transcribed as a monocistronic mRNA and expressed at a high level, as judged by the size and intensity of the Northern blot-hybridized signals (Fig. 3A). Treatments with oxidants did not affect levels of *sodBI* expression. Primer extension was performed to locate the *sodBI* promoter. Two bands of primer extension products were detected in RNA samples prepared from exponential-phase culture. A major band of 110 bp corresponds to a transcription initiation site at a T residue (+1P₁) 49 nucleotides

FIG. 2. Multiple Sods in *A. tumefaciens*. Sod activity gels of *A. tumefaciens* strains. Equal amounts of protein (50 μ g) from crude extracts of NTL4 were loaded and separated on a native polyacrylamide gel. (A) *sodB* null mutant harboring plasmid for high expression of *sodBI* (pSodBI), *sodBII* (pSodBII), and *sodBIII* (pSodBIII). (B) *A. tumefaciens* NTL4 cultivated to different time points (4 h, 8 h, 24 h, and 30 h) and the exponential-phase culture grown under uninduced (UN) and MD-induced conditions. The right panel shows Sod activity from an NTL4 stationary-phase culture and a *sodBIII* mutant. (C) Sod activity from the exponential growth of mutant strains challenged with oxidants. BH, *tert*-butyl hydroperoxide. Arrowheads represent the positions of SodBI, SodBII, and SodBIII activity as indicated.

upstream of the translation initiation codon (Fig. 3B). The *sodBI* promoter had sequence motifs TTGCCC and TATGGT at the -35 and -10 regions, respectively, separated by 17 bp. The *sodBI* promoter resembles typical strong promoters of *A. tumefaciens* genes (13, 15). The second primer extension products corresponded to the 5' end of *sodBI*, located at the G residue ($+1P_2$) 7 nucleotides downstream of $+1P_1$. This gave putative -35 (CAAACA) and -10 (TCCGCT) promoter regions which were atypical of *A. tumefaciens* promoters, suggesting that the observed second primer extension product was a result of premature termination of the reverse transcription in the reaction. The analysis of *sodBI* *in vivo* promoter activity was performed for strain PS1 containing a chromosomal *sodBI* promoter region fused to *lacZ*. The *sodBI* promoter activity was maintained at high levels throughout all growth phases (0.28 U mg^{-1} protein at exponential phase and 0.48 U mg^{-1} protein at stationary phase). Treatment of the cultures with oxidants failed to induce β -galactosidase activity to higher levels, confirming the Northern analysis results (data not shown). *sodBI* is expressed at high levels throughout all growth phases, and the gene does not respond to oxidants.

***sodBII* is a member of the *soxR* regulon.** When an exponential culture of the *sodBI* mutant was treated with MD, increased Sod activity was detected in a Sod activity gel assay (Fig. 2C). The SodBII activity band was barely visible under

normal physiological conditions during all phases of growth (Fig. 2C). In a *sodBI sodBIII* double mutant, treatments of exponentially growing cultures with MD but not other oxidants such as H_2O_2 and organic hydroperoxides strongly enhanced levels of SodBII (Fig. 2C). Northern analysis of *sodBII* expression showed a high level of a hybridized monocistronic mRNA of approximately 0.7 kb in size only in RNA samples from MD-induced cells (Fig. 4A). Treatment of the cultures with other oxidants did not affect the expression of *sodBII*. The expression profile of *sodBII* is similar to those observed for other SoxR-regulated genes in *A. tumefaciens* (15). Hence, Northern analysis was repeated using RNA samples prepared from the uninduced and oxidant-induced *soxR* mutant PW01 (16). The MD-induced expression of *sodBII* was abolished in this mutant (Fig. 4A). This result indicates that the induction of *sodBII* expression with MD is dependent on SoxR. The results also suggest that the activation of *sodBII* expression is responsible for the increase in total Sod activity observed for MD-induced cells.

Next, the location of the *sodBII* promoter was determined using primer extension mapping performed on total RNA extracted from uninduced and MD-induced cultures. A 112-bp major primer extension product, corresponding to the *sodBII* transcript start site ($+1$) located at an A residue 60 bp upstream of the AUG translation start codon, was detected in an MD-induced RNA sample. This product was barely detectable in the RNA sample from uninduced cells (Fig. 4B). This result confirms that the MD-dependent increase in *sodBII* expression is due to increased transcription from the *sodBII* promoter. Examination of the sequence upstream of the $+1$ site revealed the presence of two sequence motifs, TTGACC (-39 to -34) and TAGAAG (-14 to -9), corresponding to the -35 and -10 promoter regions, respectively (Fig. 4B). These promoter motifs were separated by 19 bp, which is typical for SoxR-regulated promoters (26). Furthermore, a search for a SoxR box near the promoter region identified a putative SoxR box with high sequence homology (12 out of 18 bp matched) to a consensus sequence for the *E. coli* SoxR binding site (37) located between the -10 and -35 regions (Fig. 4B).

In *A. tumefaciens*, SoxR binds to its recognition box and directly regulates the expression of genes in its regulon (15). We tested the ability of purified oxidized SoxR protein to bind to the *sodBII* promoter by use of a DNA mobility shift assay. Oxidized SoxR specifically bound to the *sodBII* promoter (Fig. 4C). The binding specificity was shown by the ability of the unlabeled *soxR* promoter fragment but not an excess of heter-

TABLE 2. The H_2O_2 , KCN, and NaN_3 inhibitions of SodBI, SodBII, and SodBIII activities

Sod	Inhibition (%) after treatment with ^a :		
	10 mM H_2O_2	10 mM KCN	10 mM NaN_3
SodBI	92	11	0
SodBII	83	0	0
SodBIII	95	14	0
<i>E. coli</i> SodA	0	14	58
<i>E. coli</i> SodB	100	0	0

^a Sod activities were estimated using Sod activity gel. The percent inhibition was determined using densitometric analysis of Sod activities after treatments by comparison with the activities before treatments.

FIG. 3. Transcriptional analysis of the *sodBI* gene in *A. tumefaciens*. (A) Northern analyses of *sodBI* expression in *A. tumefaciens* NTL4 under uninduced (UN) conditions and induction with H₂O₂, MD, and *tert*-butyl hydroperoxide (BH). 16S rRNA levels were used as loading controls and are shown underneath the autoradiograph of the Northern blot. (B) Primer extension showing the transcriptional start site (+1) of *sodBI*. Arrowhead indicates +1 nucleotide. C, T, A, and G are sequence ladders of pGEM-3Zf (+) from the pUC/M13 forward primer (Applied Biosystems), and the numbers represent the sizes in base pairs of the sequencing products. The arrowheads and +1P₁ and +1P₂ indicate the *sodBI* transcription start sites. The -35 and -10 promoter regions of P₁ and P₂ are underlined and in gray letters, respectively. The translation codon (ATG) and the putative ribosome binding site of *sodBI* are in boldface and italics, respectively.

ologous DNA to compete with SoxR binding to the labeled promoter fragment (Fig. 4C).

sodBII expression was monitored throughout the growth phase by use of the *sodBII::lacZ* fusion in p*SodBII*. The results suggest that *sodBII* is expressed at low levels throughout all phases of growth (data not shown). MD treatment caused an 11-fold increase in β -galactosidase activity (from 0.79 ± 0.03 to 9.08 ± 0.26 U mg⁻¹ protein) in wild-type strain NTL4 harboring p*SodBII*. However, induction by MD was abolished in the *soxR* mutant PW01/p*SodBII* (uninduced, 0.81 ± 0.05 ; MD induced, 0.75 ± 0.05). MD inducibility could be restored to PW01/p*SodBII* by expression of *soxR* from a plasmid (uninduced, 0.87 ± 0.06 ; MD induced, 10.97 ± 0.24). These results are in good agreement with the data obtained from Northern analysis. We speculated that upon exposure to superoxide stress, *A. tumefaciens* SoxR is being oxidized and that the oxidized form of SoxR binds to the SoxR box located between the -10 and -35 regions of the *sodBII* promoter, where it activates the transcription of the gene (14).

Genes in the SoxR regulon can be repressed or activated depending on whether reduced or oxidized SoxR is bound. The data presented here suggest that oxidized SoxR activates the expression of *sodBII*. For *E. coli*, SoxR has been shown to exist in a reduced form in uninduced cells (22). Thus, we extended the investigation and determined the role of reduced SoxR on the *sodBII* promoter by use of in vivo *sodBII* promoter analysis. Measurements of the basal levels of β -galactosidase activities specified by p*SodBII* in NTL4 (0.79 ± 0.03 U mg⁻¹ protein), PW01 (0.81 ± 0.04 U mg⁻¹ protein), and PW01/p*SoxR* (0.87 ± 0.06 U mg⁻¹ protein) showed no significant differ-

ences. These results suggest that reduced SoxR has no direct effects on the *sodBII* promoter. This observation contrasts with previous observations for *A. tumefaciens*, where in vivo-reduced SoxR repressed the expression of *atu5152*, a member of SoxR regulon, and itself (15). Nonetheless, this observation fits well with the SoxR paradigm that reduced SoxR either represses or has no effect on the expression of genes while oxidized SoxR only activates the expression of genes in its regulon.

***sodBIII* is expressed during stationary phase.** In initial experiments, we could not reliably detect *sodBIII* mRNA in Northern blot experiments, suggesting that the gene is expressed at a low level. The expression of *sodBIII* was investigated for *A. tumefaciens* strain PS2 containing the *sodBIII* promoter region fused to *lacZ*. β -Galactosidase activity in PS2 culture was assayed throughout the growth phases. As shown in Fig. 5, *sodBIII* exhibited showed a sixfold increase in β -galactosidase activity, from 0.006 U mg⁻¹ protein at exponential phase to 0.038 U mg⁻¹ protein at stationary phase (30 h). This pattern of *sodBIII* expression is consistent with the observations from activity gels, which showed that SodBIII levels increased during the stationary phase of growth observed in a Sod activity gel assay (Fig. 2B).

A similar pattern of stationary-phase expression for periplasmic Cu/ZnSods has been reported for other bacteria (29). The *sodBIII* expression pattern of *sodBIII* resembles many of the RpoS-regulated genes found in other gram-negative bacteria. However, *A. tumefaciens* lacks an RpoS homologue, and other regulators of stationary-phase gene expression have not been identified.

FIG. 4. Expression analysis of *sodBII* in *A. tumefaciens*. (A) Northern analyses of *sodBII* expression in *A. tumefaciens* NTL4 and a *soxR* mutant (PW01) under uninduced (UN) conditions and induction with H₂O₂, MD, and *tert*-butyl hydroperoxide (BH). 16S rRNA levels were used as loading controls and are shown underneath the autoradiograph of the Northern blot. (B) Primer extension showing the transcriptional start site (+1) of *sodBII*. Arrowhead indicates +1 nucleotide. G, A, T, and C are sequence ladders of pGEM-3Zf(+) from the pUC/M13 forward primer (Applied Biosystems), and the numbers represent the sizes of the sequencing products in base pairs. -10 and -35 regions are boxed. The translation codon (ATG) of *sodBII* is in boldface. The putative SoxR binding site is shaded. (C) Gel mobility shift assay of the *sodBII* promoter fragment with increasing amounts of purified SoxR protein (0 to 100 ng). UP and HD indicate the binding reaction using 80 ng SoxR containing an additional 1 μ g of unlabeled *sodBII* promoter and 1 μ g of heterologous DNA (pUC18 plasmid), respectively. F and B indicate free and bound probes, respectively.

FIG. 5. Transcriptional analysis of the *sodBIII* gene in *A. tumefaciens*. β -Galactosidase activity levels in PS2 containing *sodBIII* promoter *lacZ* fusions were plotted against OD₆₀₀ values at various time points.

Functional analysis of *sods*. In many bacteria, the physiological function of *sod* is to alleviate superoxide stress by lowering the steady-state concentration of superoxide anions. Results presented here show differential expression patterns of the three *sods* as well as differing cellular localizations for these enzymes. The physiological roles of the three *sods* in *A. tumefaciens* are not clear. Hence, insertion inactivation mutants in individual *sod* gene, combinations of individual *sod* mutations, and a triple *sod* null mutant were constructed to evaluate the physiological roles of the three isozymes. The growth rates of the individual *sod* mutants under aerobic conditions in either rich or minimal medium were not significantly different from that for NTL4, the wild type (data not shown). We then tested each mutant for levels of resistance against lethal concentrations of superoxide generators in exponential- and stationary-phase cells. At exponential phase, the inactivation of *sodBI* rendered *A. tumefaciens* 70-fold more sensitive to killing by MD compared to what was seen for NTL4 (Fig. 6A). The phenotype could be complemented by expression of *sodBI*

FIG. 6. Phenotypes of various *A. tumefaciens* *sod* mutant strains. (A) The survivals of *A. tumefaciens* strains on a plate containing 0.5 mM MD were determined for NTL4 and for *sodBI*, *sodBII*, *sodBIII*, *sodBI sodBII*, *sodBI sodBIII*, and *sodBII sodBIII* (null) mutant strains. (B) *A. tumefaciens* NTL4 and *sodBI*, *sodBII*, *sodBIII*, and *sodBI sodBII sodBIII* (null) mutant strains containing the Ti plasmid (pCMA1) were tested for their abilities to induce tumor formation on pieces of tobacco leaves as described in Materials and Methods. Control means leaf explants infected with NTL4 without pCMA1. (C) The *sodBI sodBII sodBIII* mutants (null) harboring pSodBI, pSodBII, pSodBIII, and pSodBIIIΔ29 as indicated were tested for tumorigenesis as described for panel B. Photos were taken after incubation for 14 days.

from pSodBI. The expression patterns and the *sodBI* mutant phenotype suggest that the gene has a housekeeping role in ensuring that the levels of intracellular superoxide anions are kept at low levels throughout the different phases of growth. The inactivation of *sodBI* caused the highest reduction in total Sod activity under uninduced conditions. Hence, the inactivation of the gene results in the most pronounced decrease in the overall resistance to superoxide conferred by the three *sods*.

SodBIII is a periplasmic enzyme that is induced during stationary phase. The inactivation of *sodBIII* alone had no effect on the MD resistance levels during exponential growth. The cellular location of SodBIII suggests that it could have a physiological role in protecting the bacteria from externally generated superoxide anions. To test this hypothesis, a *sodBIII* mutant was treated with xanthine-xanthine oxidase in a killing assay (47) in the presence of bacterial monofunctional catalase, and no difference in the survival rates for the mutant and the parental strain was observed (data not shown). These data suggest that *sodBIII* plays a minor role in protecting the bacteria from superoxide stress during exponential phase. More-

over, the remaining Sod activities could compensate for the lack of SodBIII activity. The inactivation of *sodBII* resulted in a small decrease in MD resistance levels during exponential phase (Fig. 6A), implying that the inducible *sodBII* plays a minor protective role against MD toxicity in uninduced growth conditions.

The presence of multiple Sod activities prevents a clear analysis of the role of each *sod* in protecting the bacteria from superoxide stress. Hence, combinations of double and triple *sod* null mutants were constructed. All double mutants had an additional decrease in MD resistance levels compared to what was seen for the single mutants. The *sodBII sodBIII* mutant was the least affected (3-fold) compared to the *sodBI sodBIII* (20-fold) and the *sodBI sodBII* (100-fold) mutants. Furthermore, the MD resistance level was lower in a *sod* null mutant than in any of the single and double *sod* mutants (Fig. 6A). These findings confirmed that all three *sods* contribute to various degrees to overall resistance to MD during exponential phase. The contribution of each of the *sods* to MD resistance appears to correlate with their expression patterns and levels.

The inactivation of highly expressed *sodBI* had the most effect, and that of weakly expressed *sodBIII* the least effect, on levels of resistance to MD. Additional experiments were done to determine the resistance levels of the mutant strains under acidic pH (5.5) and low-phosphate concentration growth conditions, which mimic the conditions that bacteria encounter during infection of plant wound sites. MD plate sensitivity tests were performed using AB minimal medium, pH 5.5 (9). The results show that all *sodB* mutants had MD resistance profiles similar to those observed using a rich medium (data not shown). This suggests that *sodBs* probably have an important role in the protection of *A. tumefaciens* from superoxide anion during the infection of plant tissues.

In bacteria, stationary-phase cells are generally more resistant to stresses, including superoxide stress, than are exponential-phase cells (7, 17, 48). The mechanisms responsible for this phenotype often are not directly correlated to levels of stress detoxification and protective enzymes, but other factors, such as nonspecific DNA binding proteins and membrane modifications, could be involved (2, 23). Here, unlike what is seen for other bacteria, the resistance of *A. tumefaciens* to superoxide stresses slightly decreased (fivefold) during the stationary phase of growth, despite a sharp increase in the levels of SodBIII and the maintenance of basal levels of SodBI and SodBII (Fig. 5 and 6A). The initial analysis of individual *sod* mutants did not show stationary-phase-specific alterations in MD resistance levels. The decreases in the MD resistance levels during stationary phase in single *sod* mutants were proportional to the decreases in resistance levels observed at the exponential phase. Similarly, the *sodBII sodBIII* double mutant did not show altered stationary-phase resistance to MD compared to what was seen for the other two single mutants. This suggests that in the double mutant the remaining SodBI can compensate for the absence of the two missing Sod activities during stationary phase. Also, *sodBII* appears to play a minor role in the basal levels of stationary phase to MD. Nonetheless, in the absence of *sodBI* and the stationary-phase-specific expression of *sodBIII*, a *sodBI sodBIII* double mutant shows an additional fivefold decrease in resistance to MD. These observations suggest that *sodBI* alone could compensate for the lack of *sodBII* and *sodBIII*. We conclude that the highly expressed *sodBI* is responsible for a large percentage of the total MD stationary-phase resistance. Moreover, *sodBIII* also plays a significant role in the process, while the expression of *sodBII* alone is not sufficient for maintaining stationary-phase resistance levels. The *sod* null mutants show an additional 10-fold decrease in stationary-phase MD resistance levels compared to double *sod* mutants, indicating that *sodBI*, *sodBII*, and *sodBIII* all contribute to various degrees to the overall resistance level.

We assessed the ability of an individual *sod* gene in an expression vector to complement the MD-hypersensitive phenotype of the *sod* null mutant. High levels of expression of *sodBI* and *sodBII* fully complemented the MD-hypersensitive phenotype of the triple *sod* null mutant, while *sodBIII* could only partially protect the mutant from MD toxicity (Fig. 6A). We extended the investigation by removing the signal sequence of pSodBIII, giving pSodBIII Δ 29 (S. Mongkolsuk, P. Saenkhom, and P. Vattanaviboon, unpublished data), which produces SodBIII in the cytoplasm. As shown in Fig. 6A, the expression of cytoplasmic SodBIII from pSodBIII Δ 29 restored

TABLE 3. Virulence assay of *A. tumefaciens* strains on tobacco leaf explants

Strain	Virulence score ^a
NTL4	0 $\pm$ 0
NTL4/pCMA1	4.5 $\pm$ 0.3
<i>sodBI</i> /pCMA1	0.8 $\pm$ 0.2
<i>sodBII</i> /pCMA1	4.3 $\pm$ 0.3
<i>sodBIII</i> /pCMA1	3.8 $\pm$ 0.3
<i>sodB</i> null/pCMA1	0 $\pm$ 0
<i>sodB</i> null/pCMA1/pSodBI	4.5 $\pm$ 0.4
<i>sodB</i> null/pCMA1/pSodBIII	0 $\pm$ 0
<i>sodB</i> null/pCMA1/pSodBIII Δ 29	3.6 $\pm$ 0.4
<i>sodB</i> null/pCMA1/pSodBII	4.1 $\pm$ 0.3

^a The scale of 0 to 5 takes into account both the numbers of tumors and their sizes.

the MD resistance of the triple *sod* null mutant back to the wild-type level. Redox-cycling agents such as MD generate superoxide anions predominantly in the cytoplasm but also in the periplasm (53). In the cytoplasm, superoxide anion toxicity primarily arises from oxidation of iron sulfur clusters in enzymes (such as aconitase [20]) and the transcription factor FNR (45). This is consistent with our observations that cytoplasmic Sods fully protected the bacteria from MD toxicity. Nonetheless, the oxidation of periplasmic proteins provides a minor contribution to MD resistance levels, as shown by the ability of *sodBIII* to provide low-level MD resistance to the triple *sod* null mutant.

The role of *sod* genes in tumorigenesis. *sods* are virulence factors in some pathogenic bacteria (32, 40). Increased production and accumulation of ROS by host plants in response to bacterial invasion is an important part of the active plant defense response. Moreover, plants produce compounds which could cause an increased production of intracellular ROS (50). However, the role of ROS in the plant defense response against *A. tumefaciens* invasion is not clear. Recently, a catalase-peroxidase mutant has been shown to be attenuated in its ability to cause crown gall (52). This observation indicates that ROS play important roles in the *A. tumefaciens*/plant interaction and tumorigenesis. Thus, we tested various *sod* mutants containing the pTiC58 plasmid pCMA1 (27) for their abilities to induce tumors on tobacco leaf discs (44). The *sodBI* mutant was markedly reduced (80%) in its ability to induce tumors on the plant leaf discs (Fig. 6B and Table 3). Inactivation of either *sodBII* or *sodBIII* did not affect the ability to induce tumor formation (Fig. 6B and Table 3). However, the triple *sod* null mutant was avirulent, producing no tumors on leaf discs. Expression of *sodBI*, *sodBII*, and the cytoplasmic form of *sodBIII* (pSodBIII Δ 29) restored the tumorigenicity to the triple *sod* null mutant (Fig. 6B and C). Unexpectedly, the periplasmic *sodBIII* (pSodBIII) failed to complement the phenotype of the mutant. This observation suggests that cytoplasmic Sods have important roles in the virulence of *A. tumefaciens* (Fig. 6C and Table 3). The abilities of various *sod* mutants to form tumors and of different *sod* genes to complement the avirulent phenotype show a pattern similar to the MD resistance phenotype. A reduced induction of *vir* genes has been shown to cause the attenuated tumor-forming phenotype of *A. tumefaciens* mutant strains (44). Thus, we tested whether a triple *sod* null mutant

FIG. 7. Expression of *virB* and *virE* in *A. tumefaciens* strains. *A. tumefaciens* NTL4, the *sodBI sodBII sodBIII* mutant (null), and the null mutant harboring pSodBI plasmid (null/pSodBI) containing a *virB::lacZ* fusion (A) or a *virE::lacZ* fusion (B) were incubated in induction broth (44) with (gray bars) or without (white bars) acetosyringone for 24 h. β -Galactosidase activities were measured.

was defective in the induction of *vir* genes. pSM243cd and pSM358cd plasmids, which contain *virB* and *virE* promoter-*lacZ* fusions, respectively (44), were introduced into the *sod* null mutant harboring pCMA1 and its wild-type parent, NTL4/pCMA1, and the ability of acetosyringone to induce *vir* promoters was determined. As shown in Fig. 7A and B, the uninduced promoter activities of *virB* and *virE* were similar in both NTL4 and the triple *sod* null mutant (*sodBI sodBII sodBIII* triple mutant). Treatment with 100 μ M acetosyringone induced the *virB* promoter by 23-fold in NTL4, while induction was 14-fold in the triple *sod* null mutant (Fig. 7A).

A similar twofold decrease in acetosyringone induction of the *virE* promoter was detected for the mutant compared to what was seen for the wild-type parent (Fig. 7B). pSodBI could partially complement the decrease in acetosyringone-mediated induction of the *virB* and *virE* reporter fusions (Fig. 7A and B). The reduction in acetosyringone induction of *vir* promoters is at least in part responsible for the attenuated virulence of the Sod-deficient mutant (44).

Clearly, cytoplasmic Sod activity is needed for the full virulence of *A. tumefaciens* (Fig. 6C and Table 3). The role of Sod in tumorigenesis is consistent with a previous observation that virulent strains of *A. tumefaciens* have Sod activity higher than that of avirulent strains (4). A strain that lacks Sod likely accumulates intracellular superoxide anions. Many enzymes containing iron-sulfur clusters are extremely sensitive to superoxide anion inactivation due to oxidation and leaching of iron from the cluster. The inactivation of key members of the tricarboxylic acid cycle enzymes such as aconitase and fumarase in the triple *sod* null mutant probably contributes to the avirulent phenotype. A recent study has shown that the inactivation

of citrate synthases in *A. tumefaciens* attenuates tumorigenesis (44). The lack of bacterial virulence could arise from defects in the mutants' abilities to synthesize branched-chain, sulfur-containing, and aromatic amino acids as a consequence of losing *sod* function (8). Alternatively, Sod may be an important factor for *A. tumefaciens* survival from the oxidative burst generated during the infection of plant wound sites. Studies with *Xanthomonas campestris* reveal the induction of *sod* expression during bacterial invasion of its host (42), suggesting the possible role of Sod in the infection.

High levels of superoxide anions are highly toxic to bacteria. *A. tumefaciens* has evolved and maintained three *sods*, each with different expression patterns and two cellular locations. The three superoxide dismutation systems consist of, first, the *sodBI* system, which is a constitutively highly expressed gene that functions as a housekeeping gene. *sodBI* is the major protector of the bacteria against superoxide stress. The second system involves the superoxide-inducible expression of *sodBII*. The major function of this enzyme is most likely during conditions under which intracellular superoxide anions briefly accumulate, thus leading to the oxidation of SoxR and the subsequent activation of *sodBII* to remove the excess superoxide anions. In the third system, *sodBIII* is expressed only during stationary phase, and the enzyme appears to localize to the periplasm. Its major role is probably to protect proteins in the electron transport chains and other proteins in the periplasmic space from damage by superoxide anions during the stationary phase of growth. These systems are complementary to each other and not redundant. Overall, they ensure that superoxide anions are kept at physiologically safe levels throughout exponential growth and stationary phase and during interaction with the host plant.

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