**RESEARCH LETTER**

**Agrobacterium tumefaciens** iron superoxide dismutases have protective roles against singlet oxygen toxicity generated from illuminated Rose Bengal

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**Abstract**

Singlet oxygen is a highly reactive form of molecular oxygen that is harmful to biological systems. Here, the role of three iron-containing superoxide dismutase (sodB) genes is clearly shown in protecting Agrobacterium tumefaciens against singlet oxygen toxicity. A sodBI mutant was more sensitive to singlet oxygen than both wild-type bacteria and a double sodBII–sodBIII mutant strain. Moreover, a sodBI–sodBII double mutant had higher sensitivity to singlet oxygen than a single sodBI mutant, although the double mutant was comparable to a sodB null mutant. High-level expression of sodBI and sodBII fully complemented the singlet oxygen hypersensitivity phenotype of the sodB null mutant, while high-level expression of sodBIII encoding a periplasmic SOD only partially restored the phenotype. Taken together, our data suggest that SodBI and SodBII have novel protective roles against singlet oxygen toxicity through unknown mechanisms.

**Introduction**

**Agrobacterium tumefaciens**, a Gram-negative alphaproteobacterium, is a soil-borne phytopathogen that causes crown gall disease in a variety of dicotyledonous plants worldwide (Hernalsteens et al., 1984).

Singlet oxygen (¹O₂) is an electronically excited state of molecular oxygen and one of the most harmful active oxygen intermediates, capable of rapidly oxidizing biological macromolecules and promoting deleterious damage that leads to cell death. In aerobic cells, singlet oxygen is generated during normal aerobic metabolism (Halliwell & Gutteridge, 1999) as well as from the decomposition of superoxide anions and peroxidized glutathione (Farr & Kogoma, 1991). Singlet oxygen can also be formed from a photodynamic reaction when photosensitizers such as Rose Bengal (RB), methylene blue (MB), eosin or acridine orange are excited by absorbing photon energy and then transfer that energy to molecular oxygen. Indeed, a number of biological molecules, including chlorophylls, flavins, porphyrins, pyridoxals and cytochromes, can function as endogenous photosensitizers (Halliwell & Gutteridge, 1999).

The bacterial response to singlet oxygen has been studied intensively in *Rhodobacter* spp., a photosynthetic bacterium that encounters singlet oxygen stress derived from byproducts of photosynthesis (Anthony et al., 2005; Glaeser et al., 2007). Carotenoids are isoprenoid pigments that protect photosynthetic bacteria from singlet oxygen toxicity by direct quenching of the radical (Glaeser & Klug, 2005). Much less is known, however, regarding singlet oxygen defense systems in other proteobacteria. OxyR is an H₂O₂ sensor and transcriptional regulator that has been shown to play a role in *Escherichia coli* defense against singlet oxygen derived from the photosensitizing system, probably through the protection of antioxidants enzymes [e.g. superoxide dismutase (SOD) and catalase] from damages induced by singlet oxygen (Kim et al., 2002). In addition, elevated singlet oxygen can activate SoxR, thereby triggering the soxRS regulon (Agnez-Lima et al., 2001), the principal genes accounting for the detoxification and prevention of superoxide stress.

In this study, we evaluated the role of SOD in protecting *A. tumefaciens* from singlet oxygen generated from illuminated RB. *Agrobacterium tumefaciens* produces three iron-
containing SOD isozymes with differences in their cellular localization and expression profiles (Saenkham et al., 2007). SodBI and SodBII are cytoplasmic isozymes, while SodBIII is a periplasmic isozyme. sodBI is expressed at relatively high levels throughout all growth phases, while the expression of sodBII, a member of the soxR regulon, is highly induced in response to superoxide. sodBIII is expressed at detectable levels only during the stationary phase. Our results show that SODs have an important role in protecting A. tumefaciens from singlet oxygen toxicity generated from a photosensitizing dye.

Materials and methods

Bacterial growth conditions

Agrobacterium tumefaciens NTL4 (pTiC58-cured derivative of C58, ΔtetC58 Luo et al., 2001) and the mutant strains were grown aerobically in Luria–Bertani (LB) medium at 30 °C with continuous shaking at 150 r.p.m. To ensure comparable growth states, overnight cultures were inoculated into fresh LB medium to an OD600 nm of 0.1. Cells in the exponential phase (OD600 nm of 0.6, after 4 h of growth) and stationary phase (OD600 nm about 5, after 30 h of growth) were used in all experiments, as indicated.

Molecular biology

General molecular genetics techniques, including plasmid preparation, transformation into E. coli and agarose gel electrophoresis, were performed using standard protocols (Sambrook et al., 1989). Agrobacterium tumefaciens was transformed by electroporation as described previously (Luo et al., 2001).

Construction of pSodBIIIΔ29

A mutated sodBIII gene with a deletion of the first 29 codons corresponding to the putative leader sequence was amplified by PCR from pSodBIII plasmid with primers BT1355 (5′-GGAGGATCTGATGCTTTGACACAGCCGA-3′) containing the A. tumefaciens consensus ribosome-binding site and initiation codon ATG and BT710 (5′-TCGCCTATGGACCTCAAC-3′). The PCR product was cloned into pGem-T-Easy (Promega) before the SalI–SaclII fragment was subcloned into a broad-host-range expression plasmid pBBR1MCS4 (Kovach et al., 1995), which was digested with the same enzymes, yielding pSodBIIIΔ29. This construct was verified by DNA sequencing.

Determination of singlet oxygen resistance

The resistance levels to singlet oxygen were determined using a plate sensitivity assay as described previously (Eiamphungporn et al., 2006), but with some modifications. 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. A volume of 10 μL of each dilution was spotted onto an LB agar plate containing 2.5, 5.0 or 7.5 μM RB. The plates were then incubated at 30 °C for 48 h under light generated from two 10 W fluorescent light bulbs (Philips, Indonesia), and the results were recorded. Control experiments were conducted in parallel, but the plates containing dye were incubated in the dark. Resistance levels were expressed as percentage survival, defined as the CFU on plates containing dye divided by the CFU on plates without dye, multiplied by 100.

Enzymatic assays

Crude bacterial lysates were prepared, and protein assays were performed as described previously (Nakjarung et al., 2003). The total protein concentration in the cleared lysates was determined using a dye-binding method (BioRad) before the enzyme assays. β-galactosidase was assayed as described previously and expressed as micromoles of p-nitrophenol generated at 25 °C in 1 min mg⁻¹ of protein (Ochsner et al., 2001). SOD activity was monitored based on a xanthine–xanthine oxidase-coupled reduction of cytochrome c (McCord & Fridovich, 1969). One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50%.

Results and discussion

SodBI and SodBII have protective roles against singlet oxygen toxicity

The singlet oxygen resistance levels in A. tumefaciens mutants lacking functional sodBI, sodBII and sodBIII and a parental strain, NTL4, were tested. Exponential-phase cultures of A. tumefaciens were serially diluted and plated onto LB agar plates containing RB at concentrations ranging from 2.5 to 7.5 μM and incubated under light. The sodBI mutant showed a fivefold decrease in resistance against photoactivated RB (at 7.5 μM) compared with the isogenic NTL4 strain (Fig. 1a). sodBII and sodBIII resistance levels were not significantly altered. We showed previously that SodBI is the principal cytoplasmic SOD, produced at a high level throughout all phases of growth (Saenkham et al., 2007). Inactivation of sodBI drastically reduced the level of total SOD activity in exponential-phase cultures from 0.47 ± 0.05 U mg⁻¹ protein (for NTL4) to 0.13 ± 0.02 U mg⁻¹ protein (Fig. 2a). Total SOD activity was not significantly reduced in either sodBII or sodBIII mutants (Fig. 2a). Lack of either SodBI or SodBIII did not affect the resistance level of A. tumefaciens to the singlet oxygen-generating system. These results imply that a reduction in the total SOD level is likely responsible for the singlet oxygen-sensitive phenotype observed in the
null mutant was highly sensitive to illuminated RB. At a
lower illuminated RB concentration of 2.5 μM, the sodB null
mutant was three orders of magnitude more sensitive than
the NTL4 parental strain, and bacterial growth was com-
pletely inhibited by treatment with 7.5 μM illuminated RB (Fig.
1b). Among the sodB double mutant strains, the sodBI–sod-
BIII mutant was the most sensitive to illuminated RB, with a
sensitivity level similar to that observed in the sodB null
mutant (Fig. 1b). The sodBI–sodBIII double mutant, how-
ever, had a similar resistance to singlet oxygen as the sodBI
single mutant (Fig. 1a and b). These results suggest that,
during the exponential phase of growth, sodBI and sodBIII
exert crucial functions in protecting A. tumefaciens
against illuminated RB, while the expression of sodBIII alone has no
protective role. Control experiments were performed in
parallel, where either plates containing RB were incubated
in the dark or plates without the dye were incubated under
illumination. Neither RB (7.5 μM) nor illumination alone
had deleterious effects on the growth of all tested strains
relative to plates without RB (data not shown).

With regard to the total SOD activity in the mutant
strains, we noticed that the resistance level against singlet
oxygen did not show a direct correlation to the total SOD
activity. SOD activity in the sodBI mutant was drastically
reduced from 0.47 ± 0.05 U mg⁻¹ (in NTL4) to 0.13 ±
0.02 U mg⁻¹ protein, but the mutant showed only slightly
higher sensitivity to the singlet oxygen-generating system.
sodBI–sodBII and sodBI–sodBIII double mutants produced
SOD levels (0.03 ± 0.01 and 0.04 ± 0.01 U mg⁻¹ protein,
respectively) comparable to the sodBI mutant, but they were
significantly more sensitive to singlet oxygen generated from
illuminated RB. These results suggest that there are differ-
ences in the efficiency of different SOD isozymes to detoxify
singlet oxygen. Altogether, at equivalent SOD activities,
SodBIII was more efficient than SodB in protecting cells
from singlet oxygen radicals.

Next, we tested the ability of plasmid-borne expression of
sodBI, sodBII and sodBIII to complement the single oxygen
hypersensitivity phenotype of the sodB null mutant. High-
level expression of sodBI and sodBIII from pSodBII and
pSodBIII plasmids, respectively, fully complemented, while
sodBIII could only partially restore, the hypersensitive
phenotype of the sodB null mutant relative to the parental
NTL4 strain (Fig. 1c). The sodB null mutant harboring
pSodBI had 0.22 ± 0.04 U mg⁻¹ protein total SOD activity,
which was half of the wild-type level of activity (Fig. 2b).
This SodBIII level was not sufficient to protect the mutant
from photoactivated RB toxicity, probably due to the
inefficiency of SodBIII. Alternatively, the major difference
between SodBIII and the other SODs is its subcellular
localization in the periplasm. If the periplasmic localization
of SodBIII is responsible for its inability to fully complement
the singlet oxygen hypersensitive phenotype of a sodB
null mutant, then one would expect that a cytoplasmic version of

Fig. 1. Determination of the resistance levels of Agrobacterium tumefaciens
strains to illuminated RB. (a), (b) and (c) Exponential growth-
phase cultures of A. tumefaciens NTL4 and various sodB mutant strains
were tested for their ability to survive and grow on LB agar containing
the indicated concentrations of RB and incubated under light. Percentage
survival is defined as the CFU on plates containing RB divided by the
CFU on plates without RB, multiplied by 100. ◆, NTL4; ▢, sodB; ▢,
sodBI; ●, sodBII; □, sodBI–sodBII; △, sodBI–sodBIII; ◆, sodBI–sodBIII;
○, null sodB; ▽, null sodB/pSodBI; ▼, null sodB/pSodBII; ▼, null sodB/pSodBI;
◆, null sodB/pSodBIII; x, null sodB/pSodBII A29.

sodBI mutant strain. Next, the resistance levels to illumi-
nated RB were determined in sodBI–sodBII, sodBI–sodBIII
and sodBI–sodBIII double-mutants and a sodB null mutant
(sodBI–sodBIII–sodBIII triple mutant). As expected, the sodB
null mutant was highly sensitive to illuminated RB. At a

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SodBIII should confer greater protection. The pSodBIIIΔ29 plasmid, which encoded a SodBIII protein with the leader peptide truncated in order to prevent translocation of the protein into the periplasm, was used to test this hypothesis. The complementation analysis described above was repeated using the sodB null mutant harboring pSodBIIIΔ29. The results are illustrated in Fig. 2b. The sodB null mutant bearing either pSodBIII or pSodBIIIΔ29 produced comparable levels of total SOD activity, but only the cytoplasmic form of SodBIII was able to fully complement the singlet oxygen hypersensitive phenotype of the sodB null mutant up to the wild-type level. In addition, we observed that the sodB null mutant strain carrying pSodB1 produced a twofold increase in the total SOD activity (1.0 ± 0.05 U mg\(^{-1}\) protein) compared with the wild-type levels, but showed a similar level of singlet oxygen resistance to the A. tumefaciens NTL4 strain (Figs 1c and 2b). This suggests that total SOD activity beyond a certain level affords no additional protection against singlet oxygen toxicity in A. tumefaciens.

The results suggest that cytoplasmic SODs have primary roles in protecting the cell from singlet oxygen toxicity. In addition, the toxicity of illuminated RB was primarily due to singlet oxygen that arose in the cytoplasm and, to a lesser extent, in the periplasm. RB is an anionic dye, and its ability to enter bacterial cells is controversial. Studies in E. coli indicated that the main toxic effect induced by illumination was generated from extracellular RB, while intracellular RB was the principal source of toxicity killing Staphylococcus aureus (Demidova & Hamblin, 2005). This might be due to differences in the envelope structure of Gram-negative and Gram-positive bacteria. The finding that cytoplasmic SODs in A. tumefaciens provide protection from illuminated RB implies that RB must be transported or diffuse along a concentration gradient into the cells. It is unlikely that extracellular singlet oxygen could diffuse through two membranes into the cytoplasm due to its extreme reactivity and short-lived nature (Halliwell & Gutteridge, 1999). Nonetheless, singlet oxygen generated in close proximity to the cell could damage lipids and proteins in the outer and inner membranes (Dahl et al., 1987).

Illumination of photosensitizers in the presence of molecular oxygen generally activates the formation of an excited triplet-state intermediate capable of undergoing reactions belonging to either type I (radical mediated) or type II (singlet oxygen mediated) (Foote, 1991). RB predominantly mediates a type II photodynamic reaction producing singlet oxygen. Photoactivation of RB with laser irradiation (532 nm) generates 80% singlet oxygen and 20% superoxide anion (Lee & Rodgers, 1987). Thus, the role of SOD in protecting against toxicity induced by irradiation of RB could arise from its ability to directly negate the toxicity of singlet oxygen and its ability to dismutate superoxide anion accumulated from the photodynamic reaction.

**Resistance to singlet oxygen of stationary-phase cells**

The resistance levels of stationary-phase A. tumefaciens to singlet oxygen generated from illuminated RB were determined. The results showed that A. tumefaciens NTL4
stationary-phase cells were more than 1000-fold less resistant to illuminated RB than cells in the exponential growth phase (Fig. 3). The total SOD activity in the NTL4 cells was indeed increased as cells entered into the stationary phase (Saenkham et al., 2007). This makes it unlikely that the reduction in singlet oxygen resistance is due to decreased SOD activity. The reduced resistance is probably due to reduced expression of exponential-phase proteins, alterations in the levels of cellular metabolites and membrane modifications in stationary-phase cells that enhance their susceptibility to reactivity of singlet oxygen.

$sodB$ genes still play crucial roles in protection against singlet oxygen toxicity during the stationary phase of growth. Similar profiles of resistance to illuminated RB were obtained in all $sodB$ mutants tested (data not shown). Analogous to exponential-phase cells, cytoplasmic $sodB$I and $sodB$II contributed to the detoxification of photosensitized RB during the stationary phase of growth. $sodB$III appeared to have little protective role, however. Moreover, we noticed that the resistance to illuminated RB of wild-type NTL4 cells during the stationary phase decreased by more than 1000-fold compared with the exponential phase. During the stationary phase, illuminated RB resistance levels in the $sodB$ null mutant ($sodB$I, $sodB$II and $sodB$III triple mutant) were only $<10$-fold lower than the NTL4 level, but the exponential-phase $sodB$ null mutant was more than 500-fold less resistant to illuminated RB than NTL4. This suggests that $sodB$s have only minor roles in protecting stationary-phase cells from singlet oxygen toxicity.

**Evaluation of the ability of singlet oxygen to induce $sodB$ expression**

Phenotypic analysis suggests that SOD is necessary for *A. tumefaciens* to survive under singlet oxygen stress; thus, adaptive expression of $sod$ genes in response to elevated levels of singlet oxygen would benefit the bacteria. We examined the effects of singlet oxygen exposure on expression levels of the three $sodB$ genes. Essentially, exponential cultures of *A. tumefaciens* strains containing $sodB$I, $sodB$II or $sodB$III promoter lacZ fusions were treated with various concentrations of illuminated RB, ranging from 0.1 μM (no inhibition of bacterial growth) to 10 μM (bacterial growth inhibition), and the levels of promoter activity were determined. Control experiments were performed by incubating the induced cultures in the dark. The $sodB$I and $sodB$III promoters were tested in *A. tumefaciens* PS1 and PS2 bearing $sodB$I::lacZ and $sodB$III::lacZ, containing $sodB$I and

![Fig. 3.](image-url) Determination of the resistance level of *Agrobacterium tumefaciens* strains during the stationary growth phase. Percentage survival of *A. tumefaciens* NTL4, $sodB$I–$sodB$II double mutant and $sodB$ null mutant (Null) during exponential (open bar) and stationary (shaded bar) growth phases on LB plates containing 5.0 μM RB and incubated under light.

![Fig. 4.](image-url) Transcriptional analysis of the $sodB$I, $sodB$II and $sodB$III genes in *Agrobacterium tumefaciens*. β-galactosidase activity in cultures of (a) PS1 containing $sodB$I promoter lacZ fusion, (b) *A. tumefaciens* NTL4 harboring pPsodBII containing $sodB$II::lacZ and (c) PS2 containing $sodB$III promoter lacZ fusions in the presence of various concentrations of RB and incubated under illumination. MD indicates induction with 200 μM menadione.
sodBII promoters fused to lacZ, respectively, on the chromosome (Saenkhkham et al., 2007). The sodBII promoter was evaluated in A. tumefaciens NTL4 harboring a pPSodBII plasmid containing a sodBII-lacZ fusion. As illustrated in Fig. 4a–c, none of the sodB promoters could be induced by treating A. tumefaciens cultures with illuminated RB at any concentration tested. These results are consistent with SOD activity assays obtained from A. tumefaciens cultures similarly treated with illuminated RB, where no increase in total SOD activity was observed (data not shown). In addition, we showed previously that sodBII expression was inducible by exposure to superoxide anions in a SoxR-dependent manner (Saenkhkham et al., 2007). Hence, the observation that illuminated RB could not enhance sodBII promoter activity suggests that illuminated RB produced minimal or no superoxide anions that were not sufficient to induce sodBII expression. This supports the hypothesis that protection of A. tumefaciens from illuminated RB toxicity by SOD is not due to superoxide anions being produced as byproducts from the treatment. In E. coli, SoxRS has a central role in sensing and regulating gene expression in response to superoxide, nitrosative stresses and singlet oxygen (Demple, 1997, 2002; Agnez-Lima et al., 2001). The inability of singlet oxygen generated from illuminated RB to activate SoxR indicates that SoxR behaves differently in A. tumefaciens than in E. coli in response to singlet oxygen. Lack of SOD induction by singlet oxygen suggests that the basal levels of SOD provide sufficient protection against singlet oxygen toxicity. This idea is consistent with our observation that the high levels of SOD produced from sodB overexpression did not increase singlet oxygen resistance.

Conclusion

Here we report the pivotal role of cytoplasmic SodBI and SodBII in protecting A. tumefaciens from singlet oxygen toxicity generated intracellularly by illuminated RB in both the exponential and the stationary phases of growth. Cells in the stationary phase were more vulnerable to damage caused by singlet oxygen than cells in the exponential growth phase. Although SOD exerts a protective effect against both superoxide anions and singlet oxygen, the response of A. tumefaciens to singlet oxygen was different from the response to superoxide anion. Challenging cells with superoxide anion induced SoxR-regulated sodBII expression, while singlet oxygen failed to stimulate the expression of any sodB genes.

We have shown previously that the SOD null mutant of A. tumefaciens is avirulent (Saenkhkham et al., 2007). It has been recognized that generation of singlet oxygen through photoactivation of phytoalexins containing phenalenone chromophores is one of the strategies that plants use to combat the invading pathogens (Flors & Nonell, 2006). Thus, the avirulent phenotype of the SOD null mutant could be due to the inability to cope with superoxide anions and singlet oxygen generated from plant active defense response to microbial invasion.

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Reference


