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Major Fe-Superoxide Dismutase (FeSOD) Activity in *Pseudomonas putida* is Essential for Survival Under Conditions of Oxidative Stress During Microbial Challenge and Nutrient Limitation

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Abstract An isolate of *Pseudomonas putida* has been found to aggressively colonize root tips and induce plant resistance to *Fusarium* wilt. However, *P. putida* mutants lacking Fe-superoxide dismutase (SOD) or both FeSOD and MnSOD activities are less competitive in root tip colonization. In the current study, the growth of an FeSOD mutant was found to be more sensitive than that of the wild-type or a MnSOD mutant to oxidative stress imposed by paraquat treatment and culturing with the soil fungus *Talaromyces flavus*, which generates reactive oxygen species. Also, the loss of culturability with an aging stationary-phase culture was greater for a double SOD mutant than an FeSOD mutant, while no reduction in culturability was observed with the wild-type and a MnSOD mutant under the same protracted stationary-phase conditions. Accordingly, it was concluded that FeSOD activity is the major form of SOD in *P. putida* and plays an essential role in survival under stress conditions when increased oxidative stress is encountered.

Key words: Superoxide dismutase, reactive oxygen species, *Talaromyces flavus*, *Pseudomonas putida*

Although certain rhizosphere microbes stimulate plant growth and suppress soilborne plant pathogens by producing various compounds [11, 15], their performance as crop-protecting agents under field conditions is variable. In soil, biocontrol bacteria are exposed to stressful conditions, such as nutrient limitations and competition with other microorganisms. However, to provide plant protection, such bacteria must maintain sufficient populations to express their beneficial traits at levels that have an impact on pathogen performance. As such, protection from oxidative stress may be one of the key processes required for bacterial adaptation to the rhizosphere and survival under the nutrient imbalances inherent in the soil matrix. Bloomfield *et al.* [3] and Dodd *et al.* [7] suggested that increased oxidative pressure occurs within bacterial cells upon transition from a nutrient-poor to a nutrient-rich environment, for example, when organisms in a nutrient-limited soil environment become exposed to the nutrients in root exudates. Conversely, transition to nutrient limitation can also result in oxidative stress due to the formation of singlet oxygen [4], for example, when a microbe is dislodged from a root or the root no longer produces adequate exudates. In addition, oxidative stress can be experienced upon contact with a plant root or interaction with other soil microbes. Roots generate superoxide anions during normal growth, and reactive oxygen species (ROS) are rapidly produced with the challenge of plant tissues [1, 5, 20]. The fungus *Talaromyces flavus* is known to produce reactive oxygen species as part of its survival package [12], and Al-Bekairi *et al.* [2] demonstrated that the glucose oxidase secreted by its mycelium produces hydrogen peroxide and may also be a source of superoxide anions. Thus, the sensitivity of certain pathogens to ROS is thought to be one of the mechanisms that accounts for the biocontrol activity of this fungus [12].

The *Pseudomonas putida* isolate Corvallis colonizes plant roots, induces resistance to *Fusarium* wilt, and increases the expression of plant defense genes [14, 19, 20]. The superoxide dismutase (SOD) activity in the bacterium is important for root colonization; as such, a *sodB* mutant lacking FeSOD activity was found to colonize roots less well than the wild-type during mixed inoculation studies [14], while a mutant lacking both Fe- and MnSODs, a *sodAsodB* mutant, poorly colonized at the root tip even without microbial competition [14]. Accordingly, to further explore the role of Mn- and FeSOD activities in influencing

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the properties related to the survival of this strain in the rhizosphere, the current study examined the effects of mutations in the *sod* genes to ROS generated by *T. flavus* and during prolonged culture conditions.

Previously constructed SOD-deficient mutants of *P. putida* were used in this study [14], thus mutants lacking MnSOD (*sodA*::*Km*), FeSOD (*sodB*::*Km*), and both Fe- and MnSODs (*sodA*::*Gt sodB*::*Km*) were obtained by the insertion of gentamycin (*Gt*) and kanamycin (*Km*) resistant genes into the appropriate open reading frames [14]. To confirm the mutations in the functional SOD genes, the SOD activity was determined in extracts from bacterial cells grown in Kings medium B (KB) broth, while the SOD isozyme composition was determined by staining activities on 7% native polyacrylamide gels using the methods described previously [14]. Previous work by the current authors revealed that hydrogen peroxide-sensitive FeSOD migrates more rapidly than hydrogen peroxide-insensitive MnSOD on native gels [14]. The *sodA* and *sodB* mutants did not produce their respective gene products, plus no detectable activity was observed in extracts of the *sodAsodB* double mutant or in a spectrophotometric analysis (Fig. 1). While FeSOD activity was produced throughout all growth phases, MnSOD was only observed in the late log-phase (Fig. 1 and [14]). However, an enhanced production of MnSOD was observed in the early stationary-phase of the *P. putida* mutant lacking the stationary-phase alternative sigma factor, RpoS, which is known to have a reduced ability to handle oxidative stress [16].

To test the role of SOD in protecting *P. putida* against oxidative stress, the wild-type and SOD-deficient mutants were exposed to ROS, using paraquat to generate an

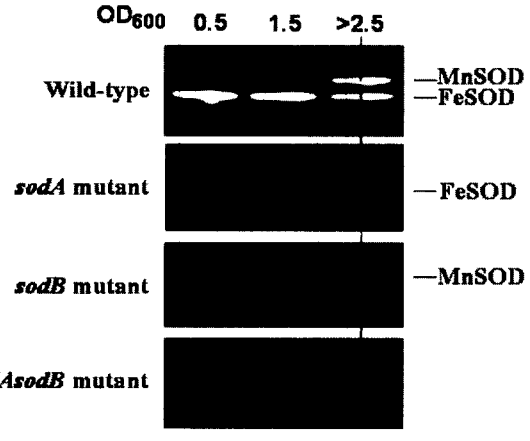

Fig. 1. SOD isozyme composition in cell extracts of wild-type *P. putida* and its SOD mutants.
The bacterial cells, grown in a Kings medium B (KB) broth to a defined growth phase, were collected by centrifugation and extracts prepared by sonication. The proteins (50 µg/lane) were separated by nondenaturing gel electrophoresis and the SOD isozymes detected by activity staining [14]. The data shown are from one of at least three separate studies with the same results.

Table 1. Effect of SOD mutations in *P. putida* on sensitivity to paraquat and hydrogen peroxide.

Strain	Inhibition zone (mm)[a]	
	Paraquat	Hydrogen peroxide
Wild-type	14	30
sodA mutant	15	30
sodB mutant	34	31
sodAsodB mutant	40	32

[a]The bacterial cells were grown in a KB broth at 28°C until the optical density at 600 nm was 0.5. Aliquots (100 µl) of each cell culture were then spread on LB agar plates and sterile 3 mm Whatman paper disks soaked in either 100 mM paraquat or 30% hydrogen peroxide. The plates were incubated for 48 h at 28°C and the diameters of the resulting inhibition zones measured. The values are the means of three separate experiments.

internal source of superoxide anions or hydrogen peroxide in disk assays. The *sodA* mutant only displayed a slightly increased paraquat sensitivity, yet the *sodB* mutant and especially the double mutant were highly sensitive (Table 1). As such, these results confirmed previous findings by the current authors, where 10 µM paraquat in a liquid medium was lethal for the *sodB* mutant, whereas 100 µM paraquat had no effect on the *sodA* mutant [14]. The sensitivity to hydrogen peroxide was similar for all the mutants and the wild-type, although the *sodB* and *sodAsodB* mutants were slightly more inhibited (Table 1). In *E. coli*, MnSOD has been found to be more involved than FeSOD in protecting cells against paraquat and hydrogen peroxide [6], while in *P. aeruginosa*, FeSOD is more important [9]. Interestingly, the current findings also indicated that in *P. putida*, FeSOD alleviated the oxidative stress produced by paraquat more than MnSOD, possibly due to the presensce of a higher FeSOD activity than MnSOD activity. Nonetheless, it was still clear that SOD activity was required for protection against superoxide anion accumulation within the cells exposed to paraquat.

To test the antagonism of bacterial growth by the ROS-producing fungus *T. flavus*, "1-by-1 cm" cubes of fungal mycelium were excised from the growing edge of a potato dextrose agar (PDA) (Difco, Detroit, MI, U.S.A.) culture and transferred to the center of PDA plates. The plates were then incubated for five days to permit fungal growth and the expression of glucose oxidase. Thereafter, the wild-type and SOD-deficient *P. putida* strains were streaked from the edge of the fungal growth to the edge of the plate, and the growth of the bacterial streaks measured two days later to deduce the extent of inhibition. The wild-type and *sodA* and *sodB* mutants grew as full streaks on the PDA plates without the fungus. In contrast, the *sodB* mutant exhibited no growth on the *T. flavus*-plates, while the wild-type and *sodA* mutant only grew partially with growth inhibited within 20–25 mm from the edge of the fungal colony (Fig. 2). The double *sodAsodB* mutant was not tested as it failed to grow on the PDA medium (data not

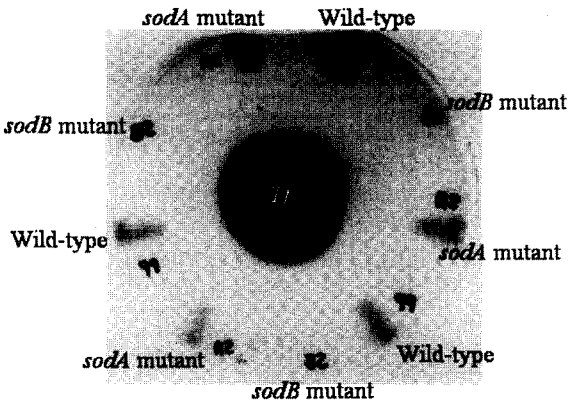

Fig. 2. Growth inhibition of wild-type and SOD mutants of *P. putida* by biocontrol fungus *T. flavus*.
The bacterial strains and fungus were inoculated onto PDA plates and the bacterial growth measured. The data shown are for one of three studies with the same growth inhibitory effects.

shown), possibly because it could not utilize glucose as the sole carbon source [14]. Consequently, the current results suggest that SOD activity provided an essential function that protected the *P. putida* cells against ROS produced by the rhizosphere colonizer *T. flavus*.

The production of enzymes that protect against ROS is one of the features detected in cells as they undergo nutrient deprivation in the stationary-phase. In *P. putida*, an increasing complexity of catalase isozymes has been detected, in part under the control of the stationary-phase sigma factor RpoS [16], and, as demonstrated in Fig. 1, MnSOD is expressed. Therefore, the current study also investigated the long-term culturability of the wild-type and SOD mutants of *P. putida* after growth in two rich media. As such, an iron-sufficient Luria broth (LB) and iron-restricted Kings medium B (KB) medium were used to determine whether the iron level was a complicating factor, as *sodA* expression is known to be conditioned by repression with Fur-Fe^{2+} under iron-replete conditions [13]. The wild-type and *sodB* mutant grew from inoculum levels of between 2–5×10^5 cfu/ml at similar rates in the LB medium and reached a maximum cell density (10^{10} cfu/ml) 48 h after inoculation, then declined slowly to about 10^8 cfu/ml ten days after inoculation (Fig. 3A). Meanwhile, the *sodB* and *sodAsodB* mutants grew slower, reaching the same maximum cell density (10^{10} cfu/ml) 62 h after inoculation (Fig. 3A). Thereafter, the culturability of the *sodAsodB* mutant in LB medium dramatically decreased and no cells were retrieved after seven days (Fig. 3A), whereas the *sodB* mutant exhibited a slower rate of decline with a count of 10^6 cfu/ml seven days post-inoculation and 10^3 cfu/ml ten days post-inoculation. This same pattern of culturability in the stationary-phase was replicated by the cells grown in the KB broth (Fig. 3B). Thus, SOD activity was found to be essential in maintaining long-term culturability as the

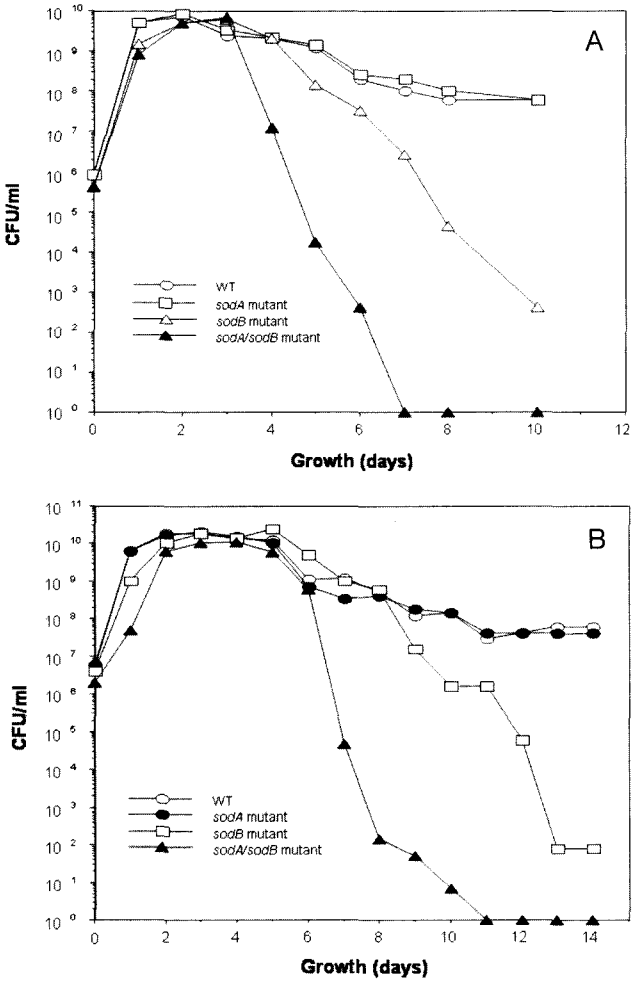

Fig. 3. Effect of mutations in genes encoding SOD on long-term survival of *P. putida* in LB (A) or KB (B) media.
The inocula were made from previously established liquid cultures. The cells were grown from a 12% glycerol-freezer stock in a LB medium for 24 h (OD 600 nm>2.0), then used as the inoculum at a 1:100 (v/v) dilution in a fresh LB medium. After growth to OD 600 nm=0.4, the cells were harvested by centrifugation at 10,000 ×*g* for 10 min, the pellet washed with sterile water, and the cells suspended in LB or KB. The cells were then incubated at 26°C with shaking at 225 rpm. At defined times, samples were removed to determine colony-forming-units based on performing serial dilutions on LB agar plates containing appropriate antibiotics (WT=wild-type). The data are from one of three studies where the same changes in culturability were observed.

cultures aged in both rich media. Furthermore, since the same results were observed in the KB and LB media, iron-regulated processes were not involved.

Accordingly, the results of the current study seem to confirm that FeSOD has a more important function than MnSOD as regards the stress situations encountered in the rhizosphere. However, whether the scenario is conditioned just by the relative proportion of these isozymes in the cells (for instance in the stationary-phase KB grown cells, FeSOD constitutes about 80% of the total activity [14]), or whether the differential properties of the isozymes influence the situation, requires further investigation.

Several other studies have also found that bacterial SOD activities are important during the interaction of a bacterium with a plant host. For example, SOD is rapidly induced when *Xanthomonas campestris* invades *Arabidopsis* leaves [10], the MnSOD expressed by *Erwinia chrysanthemi* is essential for pathogenicity when the plant host is an African violet, yet not when the host is a potato plant [17], and MnSOD is essential for effective nodulation by *Sinorhizobium meliloti* [18]. Furthermore, the conclusion from each of these studies was that the enzyme was needed to combat the oxidative stress encountered when the bacterium came into contact with the plant. The stress imposed during the utilization of plant root exudates has been linked with the enhanced expression of FeSOD in Frankia when in contact with the roots of the symbiotic host *Alnus glutinosa* [8]. Thus, although SODs have been categorized as housekeeping genes, their function may be crucial for cell survival under certain stress conditions imposed by their habitat. Meanwhile, for *P. putida*, it was apparent that FeSOD rather than MnSOD played a key survival role under stress, perhaps due to its stronger expression in the cells.

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