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Pseudomonas aeruginosa sodA and *sodB* Mutants Defective in Manganese- and Iron-Cofactored Superoxide Dismutase Activity Demonstrate the Importance of the Iron-Cofactored Form in Aerobic Metabolism

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The consumption of molecular oxygen by *Pseudomonas aeruginosa* can lead to the production of reduced oxygen species, including superoxide, hydrogen peroxide, and the hydroxyl radical. As a first line of defense against potentially toxic levels of endogenous superoxide, *P. aeruginosa* possesses an iron- and manganese-cofactored superoxide dismutase (SOD) to limit the damage evoked by this radical. In this study, we have generated mutants which possess an interrupted *sodA* (encoding manganese SOD) or *sodB* (encoding iron SOD) gene and a *sodA sodB* double mutant. Mutagenesis of *sodA* did not significantly alter the aerobic growth rate in rich medium (Luria broth) or in glucose minimal medium in comparison with that of wild-type bacteria. In addition, total SOD activity in the *sodA* mutant was decreased only 15% relative to that of wild-type bacteria. In contrast, *sodB* mutants grew much more slowly than the *sodA* mutant or wild-type bacteria in both media, and *sodB* mutants possessed only 13% of the SOD activity of wild-type bacteria. There was also a progressive decrease in catalase activity in each of the mutants, with the *sodA sodB* double mutant possessing only 40% of the activity of wild-type bacteria. The *sodA sodB* double mutant grew very slowly in rich medium and required ~48 h to attain saturated growth in minimal medium. There was no difference in growth of either strain under anaerobic conditions. Accordingly, the *sodB* but not the *sodA* mutant demonstrated marked sensitivity to paraquat, a superoxide-generating agent. *P. aeruginosa* synthesizes a blue, superoxide-generating antibiotic similar to paraquat in redox properties which is called pyocyanin, the synthesis of which is accompanied by increased iron SOD and catalase activities (D. J. Hassett, L. Charniga, K. A. Bean, D. E. Ohman, and M. S. Cohen, Infect. Immun. 60:328–336, 1992). Pyocyanin production was completely abolished in the *sodB* and *sodA sodB* mutants and was decreased ~57% in *sodA* mutants relative to that of the wild-type organism. Furthermore, the addition of sublethal concentrations of paraquat to wild-type bacteria caused a concentration-dependent decrease in pyocyanin production, suggesting that part of the pyocyanin biosynthetic cascade is inhibited by superoxide. These results suggest that iron SOD is more important than manganese SOD for aerobic growth, resistance to paraquat, and optimal pyocyanin biosynthesis in *P. aeruginosa*.

During aerobic respiration, the diversion of electron flow from the electron transport chain can lead to the production of superoxide (O_2^-). O_2^- was once postulated to be a relatively innocuous molecule (13), considering its weak oxidant and reductant properties, but Fridovich and coworkers have shown that its reactivity in biological systems is very significant. Several critical enzymes important in branched-chain amino acid biosynthesis, oxidative defense, and the Krebs cycle are inactivated by O_2^-. These include dihydroxy-acid dehydratase (29), 6-phosphogluconate dehydratase (15), catalase-peroxidase (28), aconitase (16), and fumarases A and B (30). The production of O_2^- within aerobic cells is countered by superoxide dismutase (SOD) (EC 1.15.1.1), which catalyzes the disproportionation of O_2^- to H_2O_2 and O_2 (32). Catalases and peroxidases form a second enzymatic line of defense to dispose of peroxides, which can cause mutations and membrane damage (12).

Escherichia coli, a facultative anaerobe, possesses two cytoplasmic SODs, one of which is cofactored by iron (Fe-SOD) and the other of which is cofactored by manganese (Mn-SOD) (5), and a periplasmic SOD cofactored by copper and zinc (Cu,Zn-SOD) (4). Mutation of the *sodA* (encoding Mn-SOD) or *sodB* (encoding Fe-SOD) gene does not affect the aerobic growth of *E. coli* in rich or minimal medium (7). However, mutations in *sodA* and *sodB* cause slow aerobic growth in rich medium, an auxotrophy for branched-chain amino acids, and an enhanced mutation rate (11).

Pseudomonas aeruginosa is an obligate respirer, capable of utilizing oxygen or nitrate, nitrite, and arginine as terminal electron acceptors (17). Like *E. coli*, *P. aeruginosa* possesses an Fe-SOD (22, 24) and an Mn-SOD (22, 24). The genes encoding these proteins have been cloned and characterized (24). Unlike that of *E. coli*, the *P. aeruginosa* Mn-SOD is expressed only when the organisms are starved for iron by the addition of iron-chelating agents (23) or when they are overproducing alginate, a viscous exopolysaccharide produced in the airways

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Source or reference
Strains		
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	H. Boyer
DH5α	F^+ *F80 ΔlacZ*(*lacZYA-argF*)*U169 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA*	31
SM10	*thi pro hsdR recA*; mobilizer strain	39
P. aeruginosa		
PAO1	Wild type	B. Holloway
PADH2 (*sodA*)	*sodA*::Gm^r mutant of PAO1	This study
PADH3 (*sodB*)	*sodB*::Cb^r mutant of PAO1	This study
PADH4 (*sodA sodB*)	*sodA*::Gm^r *sodB*::Cb^r mutant of PAO1	This study
Plasmids		
pBluescriptKS−	Extended polylinker pUC derivative; Ap^r	Stratagene
pNOT19	pUC19 plus 10-bp *Nde*I-*Not*I adapter in *Nde*I site; Ap^r	37
pNOT322	pBR322 plus 10-bp *Nde*I-*Not*I adapter in *Nde*I site; Ap^r Tc^r	37
pMOB3	*sacB oriT* Km^r Cm^r	37
pUCGM	pUC19 plus ~850-bp Gm^r cassette	38
pDJH7	pKS^- (Bluescript) with 3.4-kb *Pst*I fragment of *P. aeruginosa* containing *sodB*	24
pDJH10	pKS^- (Bluescript) with 1.7-kb *Bam*HI-*Pst*I fragment of *P. aeruginosa* containing *sodA*	This study
pPS252	pNOT19 plus 1.7-kb *Bam*HI-*Pst*I fragment from pDJH7 containing *sodA*	This study
pPS254	pPS252 plus 0.83-kb *Sma*I Gm^r cartridge in *Hin*cII site of *sodA*	This study
pPS255	*Not*I-cut pPS254 ligated to *Not*I site of pMOB3	This study
pPS263	3.4-kb *Pst*I *sodB* fragment from pDJH10 in pNOT322	This study
pPS271	pPS263 plus 1,011-bp blunt-ended *Rca*I fragment from pUC19 inserted into *Sma*I sites of *sodB*	This study
pPS272	*Not*I-cut pPS271 ligated to *Not*I site of pMOB3	This study

[a] Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance; Cm^r, chloramphenicol resistance.

of cystic fibrosis patients (22, 24). However, the regulation of these genes and the relative impact of their gene products upon basic fundamental processes of *P. aeruginosa* under aerobic conditions are unknown.

In this study, we chose a mutant analysis approach to better understand the roles of Fe-SOD and Mn-SOD in the aerobic metabolism of *P. aeruginosa*. We constructed chromosomal *sodA*, *sodB*, and *sodA sodB* mutants of *P. aeruginosa* PAO1 and demonstrate that Fe-SOD is more important than Mn-SOD for (i) aerobic growth, (ii) resistance to the redox-cycling antibiotic paraquat, and (iii) optimal production of its own redox-cycling antibiotic, pyocyanin.

MATERIALS AND METHODS

Bacterial strains and plasmids. Relevant properties of all *E. coli* and *P. aeruginosa* strains and plasmids used in this study are shown in Table 1.

Growth media and conditions. All bacteria were grown from single colony isolates or overnight cultures in either Luria (L) broth, a glucose minimal medium (VBMM [42]), or a low-phosphate succinate medium (LPSM) used for the production of pyocyanin (9). Liquid cultures were grown at 37°C with shaking at 300 rpm or on a roller wheel at 70 rpm unless otherwise indicated, and media were solidified with 1.5% Bacto-agar. Culture volumes were 1/5 or 1/10 of the total Erlenmeyer flask volume to ensure proper aeration. Antibiotics were used for *E. coli* at the following concentrations (in micrograms per milliter): ampicillin, 100; tetracycline, 15; kanamycin, 50; gentamicin, 15; and chloramphenicol, 30. For *P. aeruginosa*, the antibiotic concentrations were 500 μg/ml for carbenicillin and 300 μg/ml for gentamicin.

Mutagenesis of *sodA* and *sodB*. The strategy for mutagenesis of the *P. aeruginosa sodA* and *sodB* genes was facilitated by using gene replacement vectors harboring the *sacB* gene as a counterselectable marker as described by Schweizer (37). Detailed descriptions of the *sodA* and *sodB* mutagenesis schemes are given in Results. Plasmids containing *sodA* or *sodB* that was insertionally inactivated by an antibiotic resistance gene were constructed. Biparental mating with *E. coli* SM10 was used to transfer the plasmids to *P. aeruginosa*, followed by selection for a plasmid antibiotic resistance marker on VBMM without glucose to obtain cointegration with the chromosome at the site of *sod* homology. Subsequent selection on L agar containing 5% sucrose and the appropriate antibiotic was performed, and sucrose- and antibiotic-resistant colonies were selected for further study.

Manipulation of recombinant DNA. DNA transformations were performed with *E. coli* DH5α (Gibco-BRL Corp., Gaithersburg, Md.) and SM10 (39) as plasmid recipients. Recombinants were screened on agar medium containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Restriction endonuclease, alkaline phosphatase, and T4 DNA ligase were used as specified by the vendor (Gibco-BRL). Plasmid DNA was isolated by the alkaline lysis method described by Sambrook et al. (36). Chromosomal DNA was isolated from *P. aeruginosa* as previously described for *Rhizobium meliloti* (33). Southern analysis was performed as previously described (40). Restriction fragments were recovered from agarose gels by using low-melting-temperature agarose (SeaPlaque; FMC Corp., Rockland, Maine) (43) or a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.).

Sensitivity to aerobic growth. The sensitivity of *P. aeruginosa* PAO1 wild-type and *sod* mutant strains to aerobic growth was measured by monitoring cell growth as measured by an increase in optical density at 600 nm (OD_{600}) or Klett units (540-nm-wavelength filter) in L broth and glucose-VBMM. For growth experiments with broth, prewarmed (37°C) medium was inoculated 1:50 (vol/vol) with an overnight culture and incubated at 37°C with shaking at 300 rpm until the optical density reached 0.6. Cultures were then diluted 1:100 in fresh, prewarmed L broth and incubated with shaking at 37°C. For studies of growth in glucose-VBMM, mid-log-phase organisms grown in L broth (60 Klett units) were washed three times with VBMM salts to remove traces of L broth and resuspended in an equal volume of complete glucose-VBMM. Cultures were then diluted 1:100 in fresh, prewarmed glucose-VBMM and incubated with shaking as described above.

Sensitivity to paraquat and H_2O_2. (i) Disk assay. Bacteria were grown under aerobic conditions to mid-log phase in L broth, and 100-μl aliquots were spread on L-agar plates to uniformity. Sterile Whatman filter paper disks (7-mm diameter) were impregnated with 10 μl of 100 mM paraquat or 30% H_2O_2 and placed in triplicate on each plate. Sensitivity to these compounds was determined as zones of clearing surrounding each disk and scored after incubation at 37°C for 24 to 48 h.

(ii) Broth assay. *P. aeruginosa* PAO1 was grown aerobically in LPSM, which promotes pyocyanin biosynthesis (9, 22). When the organisms reached a cell density of 10^8/ml, increasing sublethal concentrations of paraquat were added, and the cultures were incubated an additional 14 h prior to the quantification of pyocyanin (see below).

Cell extract preparation and biochemical assays. Cell extracts of mid-logarithmic-phase organisms or overnight-grown bacteria were prepared from cultures harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. For SOD spectrophotometric assays, the pellet was washed once in cold 50 mM potassium phosphate–0.1 mM EDTA, pH 7.8 (WB), resuspended in cold WB and sonicated in an ice water bath for 20 s with a Branson 450 sonifier (Branson, Danbury,

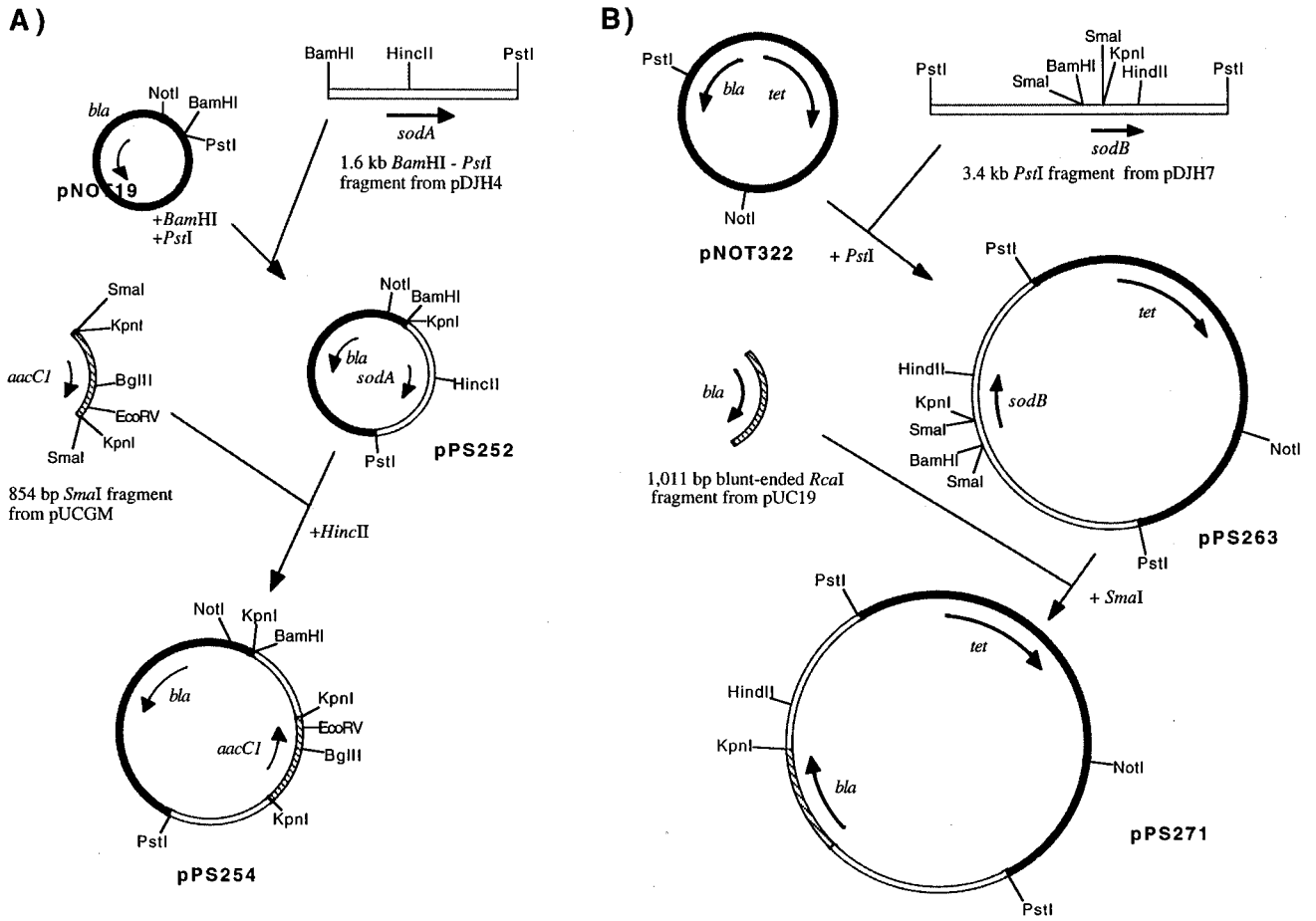

FIG. 1. Schematic summary of plasmid constructions involved in the mutagenesis of the *P. aeruginosa* PAO1 *sodA* (A) and *sodB* (B) genes.

Conn.) at output setting 20. The sonicate was then clarified by centrifugation at 13,000 × *g* for 10 min at 4°C. SOD was assayed by the method of McCord and Fridovich (32), in which 1 U of SOD activity caused a 50% inhibition of the rate of cytochrome *c* reduction by a xanthine oxidase-xanthine-catalyzed O_2^--generating system. Cell extract preparation for native gel electrophoresis was performed as described above except that 50 mM Tris-HCl (pH 7.4) was used as the diluent instead of WB. Gels were then stained for SOD activity as described by Clare et al. (8). Protein concentrations in cell extracts were estimated by the method of Bradford (6), using bovine serum albumin as a standard. Catalase assays were performed by the method of Beers and Sizer (3). Pyocyanin was purified from LPSM culture supernatants of *P. aeruginosa* PAO1 by extraction with chloroform. The chloroform phase was removed and extracted with 0.2 N HCl, which converted pyocyanin to the acidic (red) form. The original culture supernatant was adjusted to pH 7.0 with 2 M Tris-HCl (pH 8.0), and the chloroform-acid extraction procedure was repeated five additional times. The chloroform was removed by evaporation under a stream of air, and the pyocyanin was dissolved in water, filter sterilized, and stored in darkness at 4°C. The amount of pyocyanin in culture supernatants was determined by using an extinction coefficient (E_{690}) of 164 (9).

RESULTS

Mutagenesis of *sodA* and *sodB* of *P. aeruginosa*. A mutant analysis approach was used to evaluate the role of SOD in protecting *P. aeruginosa* against the deleterious effects of elevated O_2^- levels. The goal of this work was to construct *sodA*, *sodB*, and *sodA sodB* mutants and characterize the effects of their mutations on various aspects of aerobic metabolism. To mutagenize the *sodA* gene of *P. aeruginosa*, the scheme in Fig. 1A was followed. An ~1.7-kb *Bam*HI-*Pst*I *sodA*-containing fragment of pDJH10 was cloned into the replacement vector pNOT19 to form pPS252. The gene was then mutagenized by inserting a gentamicin resistance (Gm^r) cassette derived from pUCGM (38) into the unique *Hin*cII site of pPS252. This plasmid, pPS254, was linearized with *Not*I and ligated to the *oriT*- and *sacB*-containing fragment of pMOB3, forming pPS255. This plasmid was then mobilized into *P. aeruginosa* PAO1 and subjected to selection on VBMM (minus glucose) containing gentamicin. The *sodB* mutagenesis scheme is depicted in Fig. 1B. Briefly, the 3.4-kb *Pst*I *sodB*-containing fragment of pDJH7 was cloned into pNOT322, forming pPS263. An ~1.0-kb *Rca*I fragment containing the *bla* (ampicillin resistance) gene of pUC19 was blunt-end ligated into the *Sma*I site within *sodB* to create pPS271. This plasmid was linearized with *Not*I, ligated to the *oriT*- and *sacB*-containing fragment of pMOB3, forming pPS272, and conjugated into *P. aeruginosa* PAO1 by biparental mating. Bacteria that were carbenicillin resistant (Cb^r) and sucrose resistant were used for further study.

To create a *sodA sodB* mutant, the gentamicin-resistant *sodA* mutant was mated with *E. coli* SM10 containing pPS272. Colonies arising on VBMM agar plates containing carbenicillin were streak purified on L agar–5% sucrose–2% potassium nitrate. Obtaining the double mutant required incubation under anaerobic conditions for 4 to 5 days. Colonies that were sucrose, carbenicillin, and gentamicin resistant were selected for further study.

To confirm mutagenesis at the DNA level, genomic Southern analysis was performed with chromosomal DNAs prepared

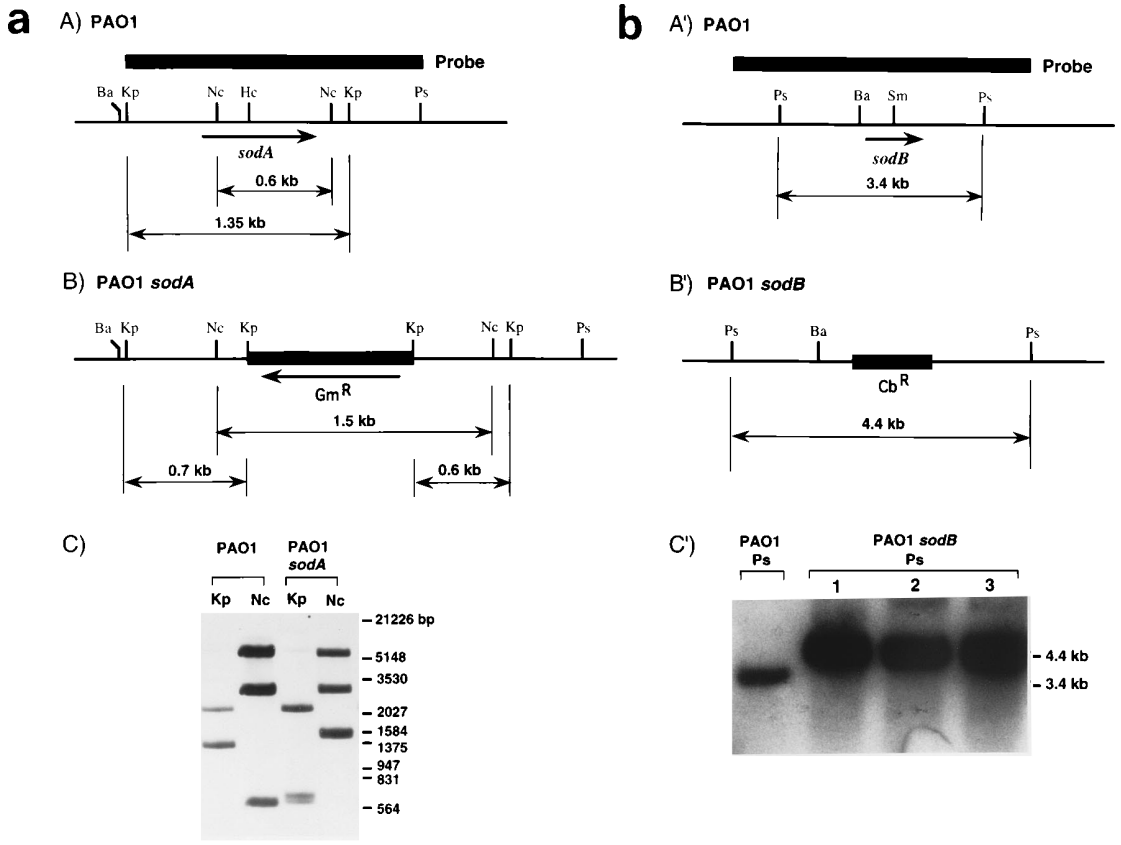

FIG. 2. Genomic Southern hybridization illustrating the replacement of *P. aeruginosa* PAO1 *sodA* with a Gm^r-marked insert (a) and of *sodB* with a Cb^r-marked insert (b). The physical maps of wild-type (panels A and A′) and mutant (panels B and B′) *sodA* and *sodB* regions are depicted. Genomic DNAs were digested to completion (panels C and C′) with *Kpn*I (Kp) or *Nco*I (Nc) for *sodA*; for *sodB*, the wild-type chromosome was cut to completion with *Pst*I (Ps) (lane 1), and three different mutants were also cut with *Pst*I (lanes 2 to 4).

from the various mutants. As shown in Fig. 2A, the *sodA* mutants demonstrated a different pattern after *Kpn*I and *Nco*I restriction analysis. Similarly, the *sodB* gene fragment harboring the ~1-kb Cb^r cassette increased in size by the expected ~1 kb in the three *sodB* mutants used in these experiments (Fig. 2B). After analysis by Southern blot hybridization, the mutants were given the following strain designations: PADH2 (*sodA*), PADH3 (*sodB*), and PADH4 (*sodA sodB*).

After confirmation of *sod* mutagenesis at the DNA level, we then examined the SOD electrophoretic profile of cell extracts derived from these strains. We have previously shown that Fe-SOD migrates more rapidly than Mn-SOD on a native polyacrylamide gel stained for SOD activity (22, 24). As shown in Fig. 3, wild-type *P. aeruginosa* PAO1 produces both Fe-SOD and Mn-SOD after overnight growth in the presence of 100 μM 2,2′-dipyridyl (Fig. 3, lane 1). The *sodA* (Fig. 3, lane 2) and *sodB* (Fig. 3, lane 3) mutants did not produce their respective gene products. The *sodA sodB* double mutant demonstrated no detectable SOD activity (Fig. 3, lane 4).

Mutagenesis of the *P. aeruginosa sod* genes affects SOD and catalase activities. To determine the effect of mutations in the *P. aeruginosa sod* genes on total SOD activity, we prepared cell extracts from aerobically grown wild-type and mutant strains. As shown in Fig. 4A, the wild-type strain PAO1 possessed 40 U of SOD activity per mg. The SOD activity of the *sodA* mutant (34 U/mg) was decreased only 15% from that of the parent strain. In contrast, the *sodB* mutant possessed only 6.8 U/mg and the *sodA sodB* mutant possessed no detectable ac-

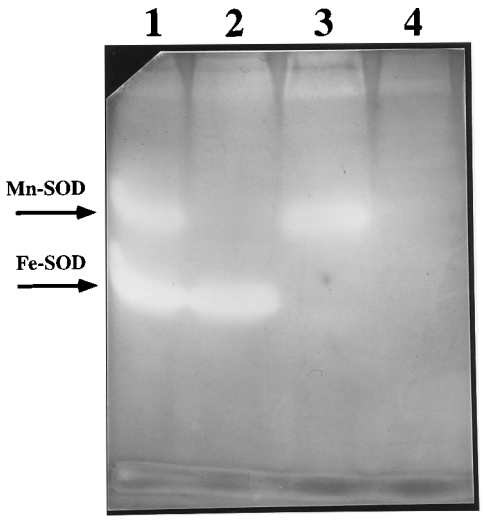

FIG. 3. Native polyacrylamide gel electrophoresis of cell extracts from *P. aeruginosa* stained for SOD activity. Bacteria (*P. aeruginosa* PAO1 wild type and *sodA*, *sodB*, and *sodA sodB* mutants) were grown as described in Materials and Methods for 17 h at 37°C in L broth. Suspensions (10 ml) were centrifuged for 10 min at 10,000 × *g* and washed twice in 20 ml of ice-cold 50 mM Tris-HCl (pH 7.4), and the pellet was resuspended in 1.0 ml of the same buffer. The cells were then disrupted by sonication for 20 s with a Fisher Sonic Dismembrator with a microprobe at setting 20. Cell debris was pelleted by centrifugation at 13,000 × *g* for 20 min at 4°C. Samples (20 μl, approx. 20 μg) were applied to 10% nondenaturing gels and stained for SOD activity by the method of Clare et al. (8). Lane 1, wild-type PAO1 grown in the presence of 100 μM 2,2′-dipyridyl; lane 2, *sodA* mutant; lane 3, *sodB* mutant; lane 4, *sodA sodB* double mutant.

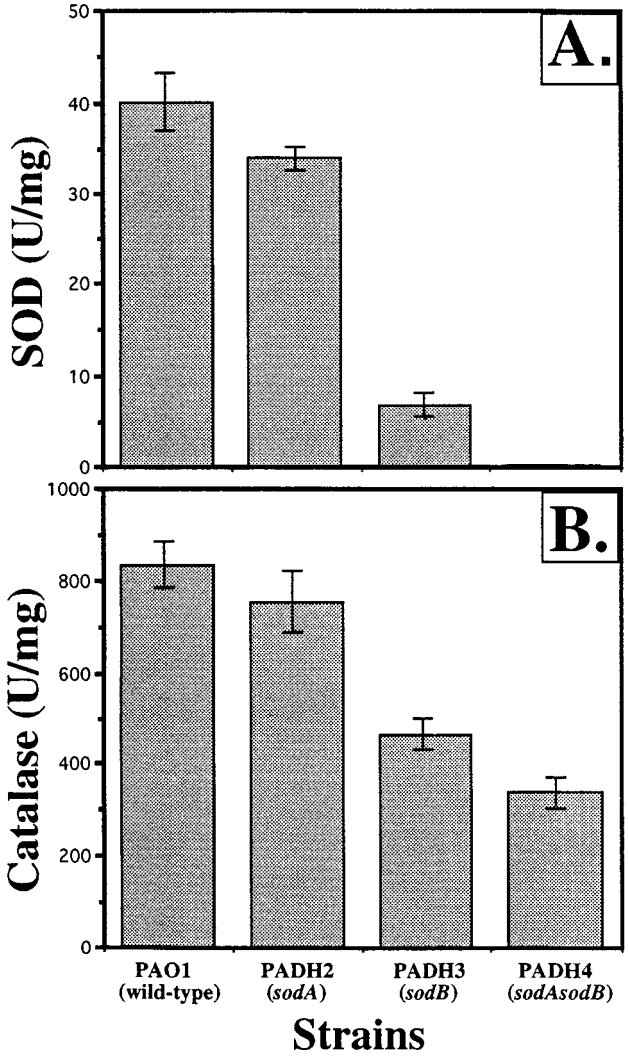

FIG. 4. SOD (A) and catalase (B) activities of *P. aeruginosa* PAO1 wild type and *sod* mutants. Bacteria were grown for 17 h in L broth at 37°C, centrifuged, and resuspended in ice-cold WB (SOD assays) or 50 mM potassium phosphate (pH 7.0) (catalase assays). They were then sonicated as described in Materials and Methods and dialyzed against the above buffers at 4°C for 17 h. SOD activity was assayed as described by McCord and Fridovich (32). Catalase activity was assayed by the method of Beers and Sizer (3) with 17.6 mM hydrogen peroxide. Data are the means and standard errors of three separate experiments.

tivity. Interestingly, like SOD activity, catalase activity decreased in the order PAO1 > PADH2 (*sodA*) > PADH3 (*sodB*) > PADH4 (*sodA sodB*) (Fig. 4B).

A mutation in *sodB* but not *sodA* markedly affects aerobic growth. In *E. coli*, a mutation in either the *sodA* or the *sodB* gene does not alter the aerobic growth of this organism compared with that of wild-type bacteria (7). The *sodC* gene of *E. coli*, encoding a periplasmic Cu,Zn-SOD, has recently been cloned (25a), but a triple *sodA sodB sodC* mutant has not been generated. However, a mutant defective in both *sodA* and *sodB* grows very slowly in rich medium, is auxotrophic for all amino acids (to different degrees [7, 26]), and is susceptible to a high frequency of mutagenic events (11). As shown in Fig. 5A, a mutation in the *sodA* gene has little effect on the aerobic growth profile compared with that of wild-type bacteria. In contrast, a *sodB* mutant grows much more slowly than the *sodA* and wild-type strains. The *sodA sodB* double mutant demonstrated an even lower rate of aerobic growth than the *sodB* mutant. The patterns of growth in glucose minimal medium paralleled those in L broth except that (i) all strains grew less efficiently than in L broth and (ii) the *sodA sodB* double mutant was incapable of growth in this medium (Fig. 5B) unless cultured for 48 h (data not shown). The *sodA sodB* mutant, which grew after 48 h in minimal medium, grew nearly as well as wild-type bacteria, suggesting the presence of external auxotrophy suppressors. As might be predicted, growth of each strain under anaerobic conditions in either medium was unaffected (data not shown).

Effect of mutations in *P. aeruginosa sod* genes on sensitivity to paraquat and H_2O_2. *E. coli* SOD-deficient mutants demon-

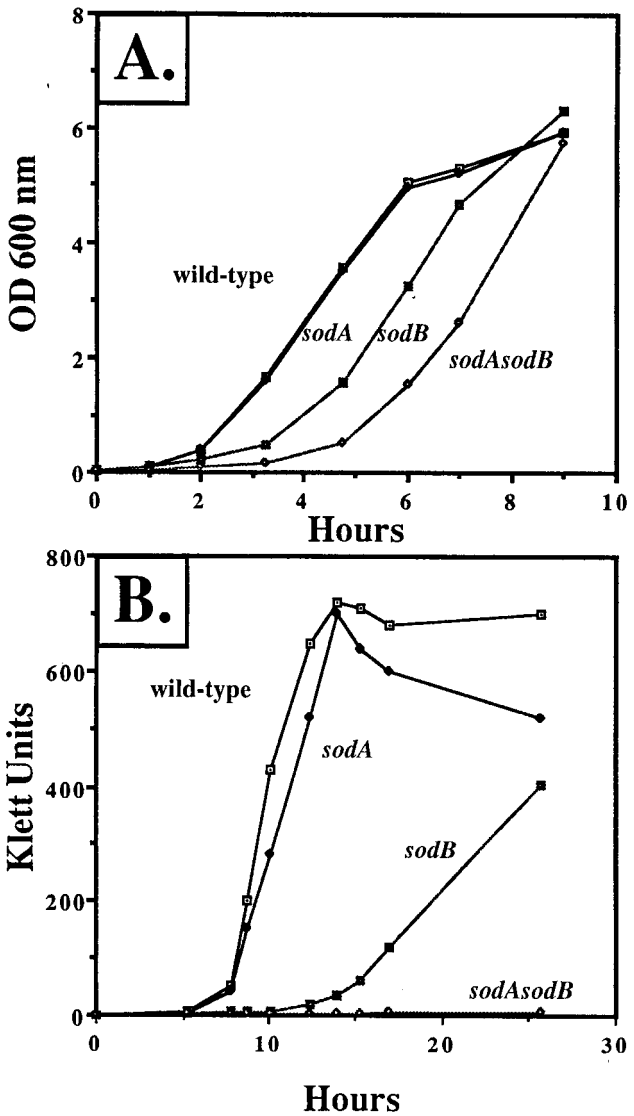

FIG. 5. Effect of mutations in *sod* genes upon aerobic growth of *P. aeruginosa* wild-type and *sod* mutant strains. Bacteria were grown aerobically overnight in L broth (A) or glucose-VBMM (B) at 37°C. Fresh prewarmed medium (culture-to-flask volume ratios, 1:10 for L broth and 1:5 for glucose minimal medium) was inoculated with 1/50 of the final culture volume and allowed to reach an OD_{600} of 0.6 (L broth) and 600 Klett units (glucose-VBMM). At this point, fresh, prewarmed media were inoculated again with 1/100 of the initial culture volume. The bacteria were then grown aerobically at 37°C with shaking at 300 rpm. At intervals, samples were removed and growth was monitored. Samples yielding optical density readings of >1.0 or Klett unit readings of >100 were diluted prior to analysis.

TABLE 2. Effect of *sod* mutations in *P. aeruginosa* on sensitivity to paraquat and H_2O_2 and production of pyocyanin

Strain	Relevant genotype	Inhibition zone[a]		Pyocyanin production[b]
		Paraquat	H_2O_2	
PAO1	*sodA*$^+$ *sodB*$^+$	13.5	30	2.77 ± 0.11
PADH2	*sodA*	15	30	1.20 ± 0.06
PADH3	*sodB*	32	32	<0.1
PADH4	*sodA sodB*	42	34	<0.1

[a] Bacteria were grown until mid-log phase (OD_{600} = 0.6) in L broth at 37°C. Aliquots (100 μl) were immediately plated onto prewarmed L-agar plates. Sterile Whatman no. 1 filter paper disks were impregnated with 10 μl of either 100 mM paraquat (pH 7.0) or 30% H_2O_2 and allowed to incubate for 24 to 48 h at 37°C. Values given are the diameters of zone inhibition (in millimeters) and are the means of three separate experiments.

[b] Bacteria were grown for 17 h in LPSM as described in Materials and Methods. Pyocyanin in culture supernatants was determined spectrophotometrically at 690 nm by using an E_{690} of 164. The results are the means and standard errors of three separate experiments.

strate enhanced sensitivity to the redox-cycling drug paraquat (18–20) in comparison with wild-type bacteria (11). As is the case for *E. coli*, paraquat also causes exacerbated production of intracellular O_2^- in *P. aeruginosa* (22). To test the role of SOD activity in the protection of these mutants against oxidative stress, we exposed the *P. aeruginosa* wild type and *sod* mutants to paraquat and H_2O_2 in a disk assay. As shown in Table 2, a mutation in *sodA* only slightly increased paraquat sensitivity. However, the *sodB* mutant and, to a greater extent, the *sodA sodB* double mutant demonstrated a marked increase in sensitivity to this compound. Regarding sensitivity to H_2O_2, the *sodB* and *sodA sodB* mutants demonstrated only a slightly greater sensitivity than *sodA* and wild-type bacteria. Similarly, when assayed in broth cultures, there was also little difference in killing, with the *sod* mutants demonstrating only an ~1-log-unit sensitivity difference at high concentrations of H_2O_2 (10 and 20 mM) (data not shown).

Effect of *sod* mutations on pyocyanin production. *P. aeruginosa* produces a blue, redox-active antibiotic, pyocyanin, which is toxic to aerobic bacteria but not to *P. aeruginosa* (21, 22). Hassett et al. (22) have shown that the production of pyocyanin is accompanied by an increase in Fe-SOD activity. Thus, we hypothesized that an absence of Fe-SOD would either be lethal to the organism under pyocyanogenic conditions or cause a decreased production of pyocyanin. To test this hypothesis, we grew each organism in LPSM (9), which has previously been used for production of elevated levels of pyocyanin in *P. aeruginosa* culture filtrates (9, 22). As shown in Table 2 and Fig. 6, pyocyanin production by the *sodA* mutant was reduced (43% of that of wild-type bacteria). In contrast, the *sodB* and *sodA sodB* mutant strains produced no detectable pyocyanin. However, these mutants were not sensitive to exogenous pyocyanin in disk sensitivity assays (data not shown), presumably because of the limited ability of pyocyanin to enter *P. aeruginosa* once it is secreted (22). These data suggested that elevated intracellular levels of O_2^- inhibit pyocyanin production. To prove this hypothesis, we added increasing sublethal concentrations of paraquat to aerobic wild-type bacteria grown in LPSM. As shown in Fig. 7, there was a dose-dependent inhibition of pyocyanin biosynthesis by paraquat, suggesting that part of the pyocyanin biosynthetic cascade is inhibited by O_2^-.

DISCUSSION

Elevated levels of O_2^- can be toxic to aerobic cells. This is why virtually all aerobic bacteria possess SOD or compounds

FIG. 6. Effect of mutations in *sod* genes on pyocyanin production in *P. aeruginosa*. Bacteria were grown aerobically at 37°C in LPSM. The photograph shows samples for which pyocyanin values are given in Table 2.

with SOD-like activity, such as elevated levels of glutathione (in *Neisseria gonorrhoeae* [1]) or Mn^{2+} (in *Lactobacillus plantarum* [2]). In this study, we characterized *sodA*, *sodB*, and *sodA sodB* mutants of *P. aeruginosa*. We have previously demonstrated that *P. aeruginosa* possesses both an Fe-SOD (as was previously suggested [41]) and an Mn-SOD (22, 24). Fe-SOD activity is present under all growth conditions, while Mn-SOD activity is elevated only when the organism is starved for iron (22) and/or when it is producing alginate (24), a viscous exopolysaccharide produced within the airways of patients with cystic fibrosis. Since we and others have also cloned and characterized the *sodA* and *sodB* genes of *P. aeruginosa* (24), the goal of this study was to evaluate the contributions of Fe-SOD and Mn-SOD to aerobic growth, sensitivity to the O_2^--generating agent paraquat, and production of the redox-cycling antibiotic of *P. aeruginosa*, pyocyanin.

The rapid growth of the *P. aeruginosa sodA* mutant relative to the *sodB* mutant differed from that of *sodA* and *sodB* mutants of *E. coli* which grew as the wild-type strain in either rich or minimal medium (7). This was the first line of evidence suggesting that *P. aeruginosa* Fe-SOD is more important than Mn-SOD for aerobic growth. This is likely due to the fact that in the *sodA* mutant, only 15% of total SOD activity is lost. In

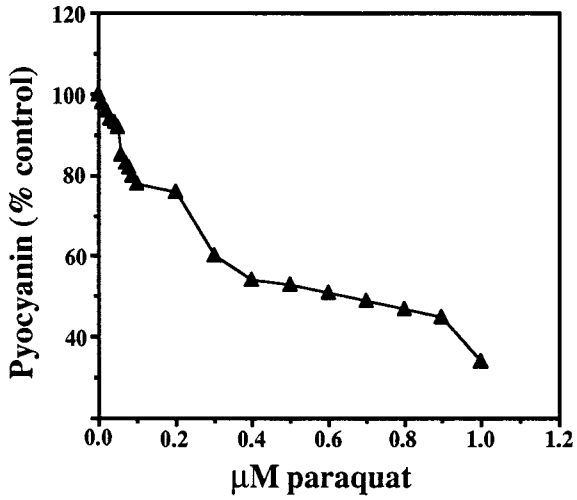

FIG. 7. Effect of paraquat on pyocyanin production by *P. aeruginosa* PAO1. Cells were grown in LPSM at 37°C until mid-log phase (OD_{600} = 0.3), and increasing sublethal concentrations of paraquat were added. The bacteria were incubated at 37°C for an additional 14 h. Pyocyanin was extracted from culture supernatants as described in Materials and Methods.

contrast, inactivation of *sodB* eliminates ~87% of total SOD activity, which undoubtedly places considerable O_2^--mediated stress on these organisms.

The inability of the *sodA sodB* double mutant to grow aerobically (but not anaerobically) in glucose-VBMM for the first 24 h is likely a result of an O_2^--mediated amino acid auxotrophy. *E. coli sodA sodB* mutants are auxotrophic for all amino acids, to various degrees (7, 26). The growth of the *P. aeruginosa sodA sodB* mutant to saturation after 48 h may have resulted from a suppressor mutation (11) which relieved the amino acid auxotrophy. Such a phenomenon has been demonstrated for *ssa-1* mutants of SOD-deficient *E. coli* which are reported to protect the cell envelope in its sensitivity to turgor pressure and suppress amino acid auxotrophies (26).

Interestingly, like SOD activity, catalase activity was also decreased in the *sod* mutants in the decreasing order wild type > *sodA* > *sodB* > *sodA sodB* (Fig. 4B). Under aerobic conditions, elevated levels of intracellular O_2^- would be scavenged by SOD, a product of which is H_2O_2. Consistent production of H_2O_2 by the SOD-catalyzed dismutation of O_2^- would serve as an inducer for catalase activity and would be dependent on the amount of O_2^- and, in turn, SOD in the cell. H_2O_2 would then stimulate catalase activity. In contrast, in SOD-deficient bacteria, the spontaneous dismutation rate of O_2^- at pH 7.8 is only 10^5 M^{-1} s^{-1} (14), ~10^4-fold lower than the SOD-catalyzed rate of 1 to 2×10^9 M^{-1} s^{-1} (27). Another explanation for decreased catalase activity in *sod* mutants is that catalase itself, like many other cellular enzymes (15, 16, 29, 30), is sensitive to O_2^- (28).

The sensitivities of the *sodB* and *sodA sodB* mutants to paraquat, relative to that of the *sodA* mutant and the wild-type strain, again illustrate the importance of Fe-SOD relative to Mn-SOD for aerobic *P. aeruginosa*. *Legionella pneumophila* (35), *Porphyromonas gingivalis* (34), and the cyanobacterium *Synechococcus* sp. strain PCC 7942 (25) also demonstrate a pronounced effect on viability or normal cellular processes when their *sodB* loci are interrupted. Because of the obligately aerobic nature of *P. aeruginosa*, elevated levels of iron would be required, not only as a cofactor for respiratory chain components, but for Fe-SOD and catalase as well. Like that of Fe-SOD activity, the level of catalase activity is very high in *P. aeruginosa* (22). Thus, the likely reason for high levels of Fe-SOD (and catalase) in *P. aeruginosa* relative to Mn-SOD is that iron is in the ferric state (insoluble) under aerobic conditions. To gain adequate amounts for normal cellular processes to occur under aerobic conditions, it would behoove the organism to maintain high concentrations of intracellular iron, perhaps storing it in proteins such as Fe-SOD, catalase, and the Fur protein (41a). In contrast, Mn-SOD activity, unlike that of *E. coli*, is controlled by Fur, as it is increased only in response to iron deprivation (21a). Thus, when iron becomes limiting, the Mn-SOD serves only as a backup because Fe-SOD activity decreases (23).

P. aeruginosa synthesizes pyocyanin, a blue redox-active antibiotic which poisons aerobic cells, much like paraquat (9, 21, 22). It has been shown previously that the level of Fe-SOD activity is elevated in pyocyanogenic *P. aeruginosa* (22). Thus, increased oxidative stress mediated by aerobic pyocyanin redox cycling would require the protective effect of Fe-SOD for optimal production. The inhibition of pyocyanin biosynthesis by the *sodB* mutant (relative to that of the *sodA* mutant and the wild type) supports the growth and paraquat sensitivity experiments. Similarly, exposure of wild-type bacteria to a sublethal dose of paraquat inhibited pyocyanin production. The progressive decrease in pyocyanin production in the order wild type > *sodA* > *sodB* = *sodA sodB* likely reflects increased intracellular O_2^- production with a concomitant O_2^--mediated inactivation of enzymes involved in the pyocyanin (a phenazine) biosynthetic pathway (10). Thus, when *sodB* is mutagenized, *P. aeruginosa* possesses a built-in "circuit breaker" to prevent pyocyanin biosynthesis and potentially lethal oxidative stress. This hypothesis is currently under study.

Taken together, the results of this study suggest that the *P. aeruginosa sodB* gene product, Fe-SOD, is more important than the *sodA*-encoded Mn-SOD for optimal aerobic growth, resistance to paraquat, and production of the redox-cycling antibiotic pyocyanin.

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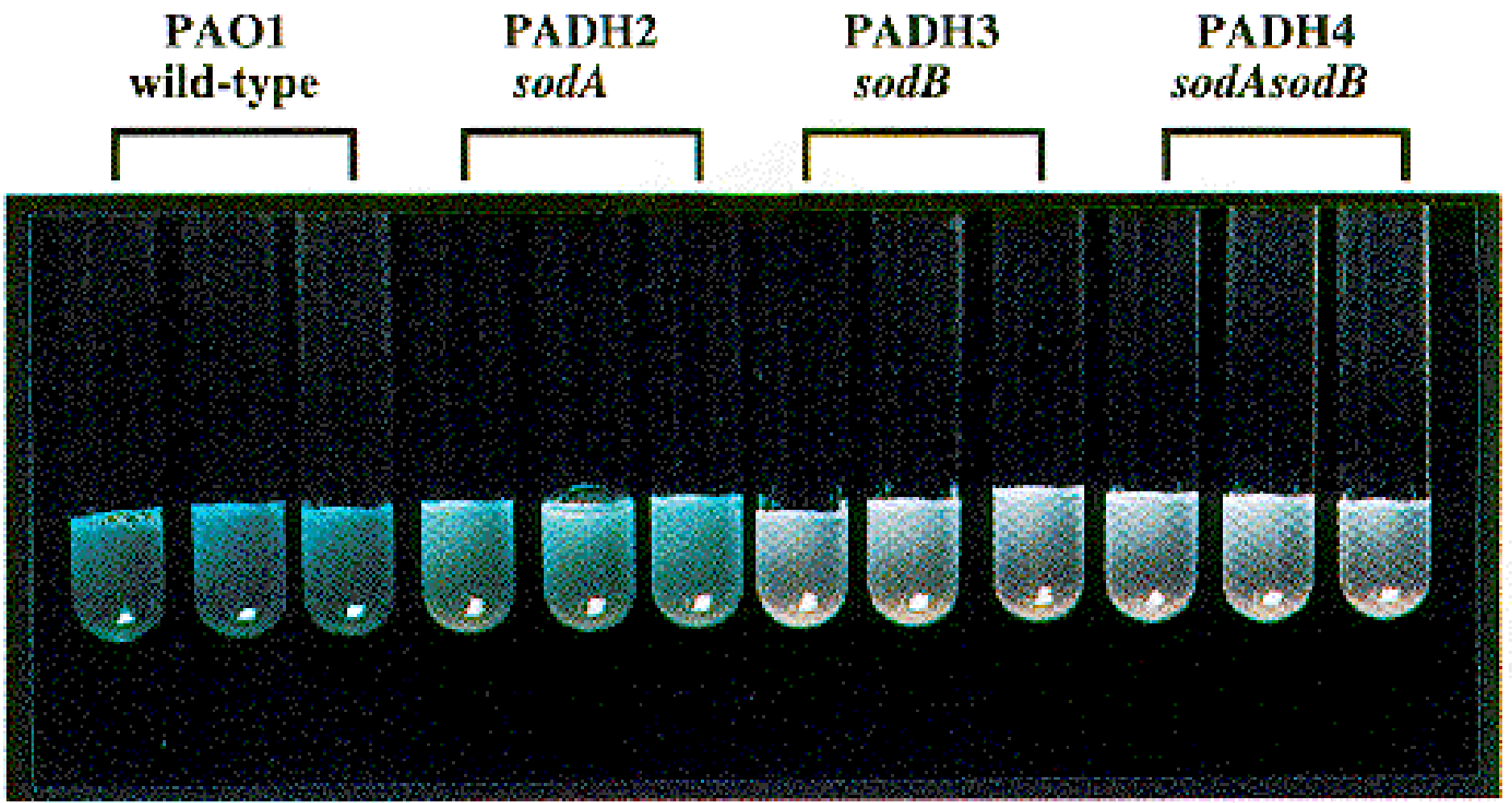
REFERENCES

1. **Archibald, F. S., and M.-N. Duong.** 1986. Superoxide dismutase and oxygen toxicity defenses in the genus *Neisseria*. Infect. Immun. **51:**631–641.
2. **Archibald, F. S., and I. Fridovich.** 1981. Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. J. Bacteriol. **145:**442–451.
3. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195:**133–140.
4. **Belov, L. T., and I. Fridovich.** 1994. *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. J. Biol. Chem. **269:**25310–25314.
5. **Beyer, W., J. Imlay, and I. Fridovich.** 1991. Superoxide dismutases. Prog. Nucleic Acid Res. **40:**221–253.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Carlioz, A., and D. Touati.** 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J. **5:**623–630.
8. **Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 1984. Effects of molecular oxygen on the detection of superoxide radical with nitroblue tetrazolium and an activity stain for catalase. Anal. Biochem. **140:** 532–537.
9. **Cox, C. D.** 1986. Role of pyocyanin in the acquisition of iron from transferrin. Infect. Immun. **52:**263–270.
10. **Essar, D. W., L. Eberly, A. Hadero, and I. P. Crawford.** 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J. Bacteriol. **172:**884–900.
11. **Farr, S. B., R. D'Ari, and D. Touati.** 1986. Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. Proc. Natl. Acad. Sci. USA **83:**8268–8272.
12. **Farr, S. B., D. Touati, and T. Kogoma.** 1988. Effects of oxygen stress on membrane functions in *Escherichia coli*: role of HPI catalase. J. Bacteriol. **170:**1837–1842.
13. **Fee, J. A.** 1980. Is superoxide toxic? Dev. Biochem. **11:**41–48.
14. **Fridovich, I.** 1985. Superoxide dismutases: regularities and irregularities. Harvey Lect. **79:**51–75.
15. **Gardner, P. R., and I. Fridovich.** 1991. Superoxide sensitivity of the *Escherichia coli* 6-phosphogluconate dehydratase. J. Biol. Chem. **266:**1478–1483.
16. **Gardner, P. R., and I. Fridovich.** 1991. Superoxide sensitivity of the *Escherichia coli* aconitase. J. Biol. Chem. **266:**19328–19333.
17. **Haas, D., B. W. Holloway, A. Schamböck, and T. Leisinger.** 1977. The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **154:**7–22.
18. **Hassan, H. M., and I. Fridovich.** 1977. Regulation of the synthesis of superoxide dismutase in *Escherichia coli*: induction by methyl viologen. J. Biol. Chem. **252:**7667–7672.
19. **Hassan, H. M., and I. Fridovich.** 1977. Enzymatic defenses against the toxicity of oxygen and of streptonigrin in *Escherichia coli*. J. Bacteriol. **129:** 1574–1583.
20. **Hassan, H. M., and I. Fridovich.** 1979. Paraquat and *Escherichia coli*: mechanism of production of extracellular superoxide radical. J. Biol. Chem. **254:** 10846–10852.
21. **Hassan, H. M., and I. Fridovich.** 1980. Mechanism of the antibiotic action of pyocyanine. J. Bacteriol. **141:**156–163.
21a. **Hassett, D. J.** Unpublished data.
22. **Hassett, D. J., L. Charniga, K. A. Bean, D. E. Ohman, and M. S. Cohen.** 1992. Antioxidant defense mechanisms in *Pseudomonas aeruginosa*: resis-

tance to the redox-active antibiotic pyocyanin and demonstration of a manganese-cofactored superoxide dismutase. Infect. Immun. **60:**328–336.
23. **Hassett, D. J., P. A. Sokol, H. P. Schweizer, and M. L. Vasil.** Regulation of superoxide dismutase and catalase by the ferric uptake regulatory protein (Fur) in *Pseudomonas aeruginosa*. Submitted for publication.
24. **Hassett, D. J., W. A. Woodruff, D. J. Wozniak, M. L. Vasil, M. S. Cohen, and D. E. Ohman.** 1993. Cloning of the *sodA* and *sodB* genes encoding manganese and iron superoxide dismutase in *Pseudomonas aeruginosa*: demonstration of increased manganese superoxide dismutase activity in alginate-producing bacteria. J. Bacteriol. **175:**7658–7665.
25. **Herbert, S. K., G. Samson, D. C. Fork, and D. E. Laudenbach.** 1992. Characterization of damage to photosystems I and II in a cyanobacterium lacking detectable iron superoxide dismutase activity. Proc. Natl. Acad. Sci. USA **89:** 8716–8720.
25a. **Imlay, J.** Personal communication.
26. **Imlay, J. A., and I. Fridovich.** 1992. Suppression of oxidative envelope damage by pseudoreversion of a superoxide dismutase-deficient mutant of *Escherichia coli*. J. Bacteriol. **174:**953–961.
27. **Klug, D., J. Rabani, and I. Fridovich.** 1972. A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. J. Biol. Chem. **247:**4839–4842.
28. **Kono, Y., and I. Fridovich.** 1982. Superoxide radical inhibits catalase. J. Biol. Chem. **257:**5751–5754.
29. **Kuo, C.-F., T. Mashino, and I. Fridovich.** 1987. α,β-Dihydroxyisovalerate dehydratase: a superoxide sensitive enzyme. J. Biol. Chem. **262:**4724–4727.
30. **Liochev, S. I., and I. Fridovich.** 1992. Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *soxRS* regulon. Proc. Natl. Acad. Sci. USA **89:**5892–5896.
31. **Liss, L.** 1987. New M13 host: DH5αF′ competent cells. Bethesda Res. Lab Focus **9:**13.
32. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase: an enzymic function for erythrocuprein. J. Biol. Chem. **244:**6049–6055.
33. **Meade, H. M., S. R. Long, G. Ruvkun, S. E. Brown, and F. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
34. **Nakayama, K.** 1994. Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis*. J. Bacteriol. **176:** 1939–1943.
35. **Sadosky, A. B., J. W. Wilson, H. M. Steinman, and H. A. Shuman.** 1994. The iron superoxide dismutase of *Legionella pneumophila* is essential for viability. J. Bacteriol. **176:**3790–3799.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:** 1195–1204.
38. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
39. **Simon, R., M. O'Connell, M. Labes, and A. Puhler.** 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods Enzymol. **118:**640–659.
40. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
41. **Steinman, H. M.** 1985. Bacteriocuprein superoxide dismutases in pseudomonads. J. Bacteriol. **162:**1255–1260.
41a. **Vasil, M.** Unpublished observation.
42. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase in *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
43. **Wieslander, L.** 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. **98:**305–309.


PAO1
wild-type
PADH2
sodA
PADH3
sodB
PADH4
sodAsodB