Cloning and Characterization of the *Pseudomonas aeruginosa* sodA and sodB Genes Encoding Manganese- and Iron-Cofactored Superoxide Dismutase: Demonstration of Increased Manganese Superoxide Dismutase Activity in Alginate-Producing Bacteria

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**Pseudomonas aeruginosa** is a strict aerobe which is likely exposed to oxygen reduction products including superoxide and hydrogen peroxide during the metabolism of molecular oxygen. To counterbalance the potentially hazardous effects of elevated endogenous levels of superoxide, most aerobic organisms possess one or more superoxide dismutases or compounds capable of scavenging superoxide. We have previously shown that *P. aeruginosa* possesses both an iron- and a manganese-cofactored superoxide dismutase (D. J. Hassett, L. Charniga, K. A. Bean, D. E. Ohman, and M. S. Cohen, Infect. Immun. 60:328-336, 1992). In this study, the genes encoding manganese (sodA)- and iron (sodB)- cofactored superoxide dismutase were cloned by using a cosmids library of *P. aeruginosa* FRD which complemented an *Escherichia coli* (JH132) strain devoid of superoxide dismutase activity. The sodA and sodB genes of *P. aeruginosa*, when cloned into a high-copy-number vector (pKS⁺), partially restored the aerobic growth rate defect, characteristic of the Sod− strain, to that of the wild type (AB1157) when grown in Luria broth. The nucleotide sequences of sodA and sodB have open reading frames of 612 and 579 bp that encode dimeric proteins of 22.9 and 21.2 kDa, respectively. These data were also supported by the results of in vitro expression studies. The deduced amino acid sequence of the *P. aeruginosa* manganese and iron superoxide dismutase revealed 50 and 67% similarity with manganese and iron superoxide dismutases from *E. coli*, respectively. There was also remarkable similarity with iron and manganese superoxide dismutases from other phyla. The mRNA start site of sodB was mapped to 174 bp upstream of the ATG codon. A likely promoter with similarity to the −10 and −35 consensus sequence of *E. coli* was observed upstream of the ATG start codon of sodB. Regions sequenced 519 bp upstream of the sodA gene revealed no such promoter, suggesting an alternative mode of control for sodA. By transverse field electrophoresis, sodA and sodB were mapped to the 71- to 75-min region on the *P. aeruginosa* PA01 chromosome. Strikingly, mucoid alginate-producing bacteria generated greater levels of manganese superoxide dismutase than nonmucoid revertants, suggesting that mucoid *P. aeruginosa* is responding to oxidative stress and/or changes in the redox status of the cell.
iron chelators (28), denitrification in the presence of paraquat (32), heat shock (31), and changes in the redox potential of the cell (17). On the genetic level, sodA transcription is negatively regulated by the fur, arc, and fnr gene products (18). In contrast, sodB transcription is positively regulated two- to threefold by the fur gene product and is negatively regulated by iron starvation (30). E. coli mutants possessing no SOD activity demonstrate an O₂-dependent auxotrophy for branched-chain and aromatic amino acids (5). Thus, SOD-deficient E. coli cannot grow in minimal media but does grow slowly in rich media under aerobic conditions (5). To begin studies on the role of SOD in P. aeruginosa, we report here the cloning of the sodA and sodB genes and characterization of their gene products. In addition, we demonstrate that mucoid, alginate-producing bacteria generate increased levels of Mn-SOD, suggesting a link between oxidative stress, associated with changes in the redox status of the cell, and alginate production by P. aeruginosa.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All P. aeruginosa strains employed in this study were derived from FRD1, a sputum isolate from a CF patient (12). Relevant properties of all bacterial strains and plasmids used in this study are shown in Table 1.

Growth conditions. All bacteria were grown from single-colony isolates or overnight cultures in either Luria (L) broth (10.0 g of tryptone, 5.0 g of yeast extract, and 5.0 g of NaCl per liter [pH 7.2]), yeast-tryptone-glucose (YTG) medium (10 g of tryptone, 5 g of yeast extract, and 2 g of glucose per liter), a glucose minimal medium (43) containing required amino acids (1 mM), or a high-phosphate succinate medium (19), as required. Liquid cultures were grown at 37°C with shaking at 300 rpm or on a roller wheel unless otherwise indicated, and media were solidified with 1.5% Bacto Agar. Culture volumes were 1/10 of the total Erlenmeyer flask volume to ensure proper aeration. Antibiotics were used for E. coli at the following concentrations (per milliliter): ampicillin, 100 μg; tetracycline, 15 μg; kanamycin, 50 μg; chloramphenicol, 30 μg.

Manipulation of recombinant plasmids. A genomic DNA library from P. aeruginosa FRD1 which contained partially restricted BamHI fragments (20 to 40 kb in size) cloned into the cosmid vector pEMR2 (9) was stored in E. coli HB101 (6). A lysate of λcI857 was prepared on these cells to efficiently package the cosmid clones into phage particles. The library of plasmids was transferred to the sodA sodB mutant of E. coli J1132 by transduction and selection for ampicillin resistance on minimal agar and L agar. Complementation of the Sod− defect was assessed as described below.

Sensitivity to aerobic growth. Relative sensitivity of E. coli sodA sodB cells to aerobic conditions and the effect of the presence of P. aeruginosa sodA and sodB in high copy number were measured by monitoring cell growth at an optical density at 600 nm (OD₆₀₀) in L broth with high aeration. Prewarmed (37°C) L broth was inoculated 1:50 (vol/vol) with an overnight culture and incubated at 37°C with rotary shaking at 300 rpm until the OD reached 0.6. Cultures were then diluted 1:100 in fresh, prewarmed L broth, and the mixtures were incubated with shaking at 37°C. The changing OD₆₀₀ of these cultures was monitored throughout the growth cycle, and samples with an OD₆₀₀ greater than 1.0 were diluted for accurate measurements of turbidity.

DNA sequence analysis and manipulation. DNA sequences were determined by the chain termination technique on double-stranded DNA by using the TaqTrack sequencing system (Promega Corp., Madison, Wis.) and Sequenase (U.S. Biochemical, Cleveland, Ohio). Oligonucleotides used as sequencing primers were synthesized on an Applied Biosystems 380B DNA synthesizer. Templates containing the P. aeruginosa sodA gene on a 1.9-kb XhoI-PstI fragment in pDjH9 and the sodB gene on both a 1.6-kb BamHI-PstI fragment in pDjH4 and a 3.4-kb PstI fragment in pDjH7 (Table 1) were examined. Plasmid DNA was obtained by using a commercially available system (Qiagen, Chatsworth, Calif.) or by centrifugation in cesium chloride-ethidium bromide density gradients. DNA and
inferred protein sequences were analyzed by using the computer program Align (DNASTAR, Madison, Wis.) and a Protein Identification Resource protein data base. In vitro transcription and translation were performed by using a prokaryotic DNA-directed translation system as described by the manufacturer (Promega). For primer extension analysis of sodB, total cellular RNA was isolated from logarithmic-phase cells (OD600 = 0.7) of P. aeruginosa FRD1 and FRD2 grown in L broth by methods previously described (2, 8). A synthetic oligonucleotide (5′-CCTAGGTCAGGACTG-3′) specific for sodB was end labeled with [γ-32P]ATP by polynucleotide kinase as described elsewhere (2) and incubated with 40 μg of RNA for 5 min at 85°C and 3 h at 42°C to permit annealing. This was followed by extension with 200 U of reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) in the presence of deoxynucleoside triphosphates (0.2 mM) for 30 min at 42°C. The extended DNA product was isolated and then analyzed by electrophoresis on 8% denaturing polyacrylamide gels as described previously (45). Dideoxynucleotide sequencing reactions, primed from the same end-labeled oligonucleotide, were electrophoresed in adjacent lanes to identify the start of transcription.

**Cell extract preparation and biochemical assays.** Cell extract of mid-logarithmic-phase 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 10 s with a Branson 450 sonifier (Branson, Danbury, Conn.) at an output setting of 20. The sonicate was then centrifuged at 13,000 × g for 10 min at 4°C. SOD was assayed according to the method of McCord and Fridovich (26), with 1 U of SOD activity causing a 50% inhibition of the rate of cytochrome c reduction by a system generating xanthine oxidase- xanthine-catalyzed O2-generating system. Cell extracts were prepared for native gel electrophoresis as above except that the top running buffer instead of WB was used as a diluent. Gels were then stained for SOD activity according to the method of Clare et al. (7). Protein concentrations in cell extracts were estimated by the method of Bradford (4) with bovine serum albumin as standard.

**Mapping of sodA and sodB.** P. aeruginosa sodA and sodB genes were mapped by the method of transverse alternating-field electrophoresis as previously described (35).

**Nucleotide sequence accession number.** The nucleotide and amino acid sequence data reported in this article have been submitted to the GenBank nucleotide sequence data base and assigned accession number, L25672A and L25675B.

**RESULTS**

**Cloning of sodA and sodB of P. aeruginosa.** To initiate studies on the role of P. aeruginosa SODs in response to oxidative stress, we cloned both sod genes by complementation in a Sod− E. coli strain. A cosmid library of P. aeruginosa FRD1 was transferred to the sodA sodB mutant of E. coli, J1132. This Sod− strain is unable to grow on aerobic minimal medium (5). This is because the enzymes necessary for branched-chain amino acid biosynthesis (i.e., dihydroxy-acid dehydratase [23]) and metabolism (i.e., aconitase [11]) are O2− sensitive in the Sod− mutant and there is no sod gene product to protect these critical enzymes. Colonies of J1132 containing complementing plasmids which restored a Sod+ phenotype were selected by their growth on minimal medium agar plates (29). Such colonies also grew more rapidly on rich media (e.g., L agar), forming large colonies in contrast to the pinpoint colonies of the Sod− strain after overnight growth at 37°C. This suggested a more adaptive response to aerobic growth by clones expressing SOD from P. aeruginosa. Approximately 100 colonies were obtained by this selection and screening process. Plasmids were isolated from 50 of them, and restriction endonuclease patterns were observed.

We have previously shown that the Fe-SOD and Mn-SOD of P. aeruginosa and those of E. coli migrate differently when cell extracts are electrophoresed on 10% native polyacrylamide gels and stained for SOD activity (19). As shown in Fig. 1, P. aeruginosa FRD2 generated both Fe-SOD and Mn-SOD when grown in a high-phosphate succinate medium, a medium which we previously found to enhance Mn-SOD production (Fig. 1, lane 3). Cell extracts from the 100 Sod+ clones were shown to contain predominantly (98%) P. aeruginosa SodB (Fig. 1, lane 4). One clone containing sodB was called pDJH1 (Table 1). In addition, two clones, one of which was called pDJH6, contained DNA encoding both sodA and sodB (Table 2). These SODs were distinct from the bands of Fe-, hybrid, and Mn-SODs in the wild-type E. coli strain, AB1157 (Fig. 1, lane 1), that were absent in the sodA sodB mutant, J1132 (Fig. 1, lane 2). Both pDJH1 and pDJH6 could also be expressed in E. coli HB101, which harbored its own Fe-, hybrid, and Mn-SODs as well as pDJH1 (Fig. 1, lane 3) and pDJH5 (Fig. 1, lane 7). A KpnI deletion derivative of pDJH4, called pDJH5, demonstrated no Fe-SOD activity on native gels stained for SOD activity (data not shown).

**Restriction analysis and DNA sequence of P. aeruginosa sodA and sodB.** Plasmid pDJH9 contains a 1.9-kb XhoI-PstI fragment of pDJH6 which was subcloned into pKS− and contained a ~1.7-kb BamHI-PstI fragment of P. aeruginosa DNA expressing SodA (data not shown). Restriction analysis was also performed on the P. aeruginosa DNA in pDJH9 (Table 1) to determine sites within and flanking the sodA gene. DNA sequence analysis was then performed on both strands of pDJH9 (Fig. 2A and B). The sodA coding region of 612 bp predicts an amino acid sequence of 204 residues and a subunit

**FIG. 1.** Native polyacrylamide gel electrophoresis of cell extracts from E. coli pDJH1 stained for SOD activity. Bacteria [E. coli AB1157 or J1132 (sodA sodB)] were grown as described in Materials and Methods for 17 h at 37°C in L broth plus 100 μg of ampicillin. P. aeruginosa was grown in high-phosphate succinate medium as previously described (19). Suspensions (1.5 ml each) were centrifuged for 2 min at 13,000 × g and washed once in 1.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), and the pellet was resuspended in 100 μl of the same buffer. The cells were then disrupted by sonication for 10 s with a Branson sonifier (model 450, equipped with microtip) in a cooling bath at 4°C. Samples (20 μl each; approximately 20 μg) were applied to 10% nondenaturing gels and stained for SOD activity according to the method of Clare et al. (7). Lane 1, E. coli AB1157; lane 2, E. coli J1132 (ΔsodA sodB); lane 3, P. aeruginosa FRD2; lane 4, J1132(pDJH1); lane 5, J1132(pDJH6); lane 6, AB1157(pDJH1); lane 7, AB1157(pDJH6).
TABLE 2. SOD activities of *P. aeruginosa* and *E. coli* containing sodB clones

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid genotype</th>
<th>SOD activity (U/mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRD1</td>
<td>sodA* sodB*</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>FRD2</td>
<td>sodA* sodB*</td>
<td>65 ± 5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB1157 sodA*</td>
<td>sodB*</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>J1132 sodA</td>
<td>sodB*</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>J1132(pDJH4)</td>
<td>sodB*</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>J1132(pDJH5)</td>
<td>sodB*</td>
<td>ND</td>
</tr>
<tr>
<td>J1132(pDJH7)</td>
<td>sodB*</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>J1132(pDJH6)</td>
<td>sodA* sodB*</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>J1132(pDJH9)</td>
<td>sodA*</td>
<td>45 ± 5</td>
</tr>
</tbody>
</table>

a Bacteria were grown for 17 h in L broth at 37°C, centrifuged, and resuspended in ice-cold 50 mM potassium phosphate–0.1 mM EDTA (pH 7.8). They were then sonicated as described in Materials and Methods and dialyzed against 50 mM potassium phosphate–0.1 mM EDTA (pH 7.8) at 4°C for 17 h.

molecular size of 22.9 kDa, which was supported by the results of in vivo transcription-translation studies (data not shown). The sodA open reading frame (ORF) initiation site is an ATG at position 519 and terminates with a stop codon (TAG) at position 1131. Codon usage of both the sodA and the sodB ORFs was highly reflective of other GC-rich genomes and *P. aeruginosa* genes (45). A putative ribosome-binding (Shine-Dalgarno) sequence, GGAGA, was revealed 11 bp upstream of the ATG codon.

In addition, a 1.6-kb BamHI-PstI fragment of pDJH4 harboring sodB (Fig. 3A) was also sequenced on both strands, as were portions of the 3.4-kb PstI sodB clone (pDJH7), which possessed the putative promoter region (Fig. 3B). The sodB coding region of 579 bp predicts an amino acid sequence of 192 residues and a subunit molecular size of 21.2 kDa. Analysis of 35S-labeled protein derived from pDJH4 after in vitro transcription-translation experiments revealed a protein migrating at 21.2 kDa, in agreement with the predicted molecular weight of the monomeric SodB protein (data not shown). The translation initiation codon of sodB was an ATG at position 224, and the ORF terminated with a stop codon (TGA) at position 802. A putative ribosome-binding sequence identical to that of the sodA gene, GGAGA, was detected 7 bp upstream of the sodB ATG codon. Immediately downstream (15 bp) of the sodB stop codon (TGA) was a region of dyad symmetry which resembles a ρ-independent terminator. This region comprised an 11-mer with an internal run of five A’s.

Identification of a sodB promoter. To determine the transcriptional start site of sodB, additional DNA was sequenced upstream of the ATG translational start codon from pDJH7, which contained a 3.4-kb PstI fragment harboring sodB in pKS— (Fig. 3A). The start of sodB transcription was determined by extension of a primer hybridizing to the 5’ region of the sodB mRNA (Fig. 4). The major transcript started with a G residue at position 51 in Fig. 3B and 4. The potential −10 (TATGAT) and −35 (GTGATA) regions of an RNA polymerase-binding site are also indicated upstream of the transcription start site in Fig. 3B and 4. Interestingly, the amounts of sodB transcript in *P. aeruginosa* FRD1 (mucoid) and FRD2 (nonmucoid) bacteria were identical. As a convenient internal standard, primer extension of the αIP gene was also employed. As expected, there were high levels of transcript in mucoid bacteria and no detectable transcript in nonmucoid bacteria (data not shown).

Mapping of sodA and sodB on the *P. aeruginosa* chromosome. To determine the location of the *P. aeruginosa* sodA and sodB genes on the *P. aeruginosa* PAO1 chromosome, we performed transverse pulsed-field gel electrophoresis (35) on SpeI- and DpnI-digested genomic DNA. Interestingly, both the sodA and the sodB probes hybridized to a 360-kb SpeI fragment and to a 290-kb DpnI fragment of PAO1 genomic DNA, indicating that both genes are located between 71 and 75 min of the *P. aeruginosa* PAO1 chromosome (data not shown). *P. aeruginosa* sodA and sodB gene products show high similarity to other SodA and SodB proteins. To compare the SodA and SodB proteins of *P. aeruginosa* with like proteins from *E. coli*, the predicted protein sequences were aligned as shown in Fig. 5. The SodA and SodB from *P. aeruginosa* demonstrated approximately 50 and 67% similarities to SodA and SodB of *E. coli*, respectively. The deduced amino acid sequence of the *P. aeruginosa* SodB also showed 84% similarity with the SodB protein of *Pseudomonas ovalis* (3). The inferred amino acid sequences of the *P. aeruginosa* SodA and SodB proteins were also subjected to a homology comparison of SodA and SodB proteins spanning different phyla and found to
**P. aeruginosa sodA** was prokaryotic as described above, As or assayedMinimal medium indicated examine the H, By pDJH7. The ribosome-binding or Shine-Dalgarno (S.D.) sequence and promoter bases in the -10 and -35 regions are underlined. Initiation of transcription is indicated by an angled arrow, *start codon, TGA. Palindromic regions are indicated by horizontal arrows.

demonstrate high similarity regardless of whether the source was prokaryotic or eukaryotic (3; data not shown).

**Growth of sodA- and sodB-containing strains of E. coli J1132.** As described above, the E. coli sodA sodB mutant grew on minimal medium agar plates and formed larger colonies on L agar when it carried the P. aeruginosa sodA or sodB gene. To further examine the ability of isolated sodA and sodB genes of P. aeruginosa to complement the Sod- growth phenotype in E. coli J1132, the effects of the P. aeruginosa sodA and sodB expressed in high copy number (pKS-) on the growth of E. coli J1132 in L broth were assessed (Fig. 6). Growth studies have previously been employed to assess various degrees of SOD complementation in Sod- bacteria (5). The cloning of sodA used was from pDJH9, and that of sodB was from pDJH7. By 6 h postinoculation, J1132(pDJH7) had reached a turbidity that was approximately sixfold higher than that of the Sod- strain and was slightly higher than that of the sodA-containing clone, pDJH9. However, the presence of the P. aeruginosa sodA or sodB gene in the sodA sodB mutant did not completely restore growth to the rate of the wild-type strain AB1157, despite the mutant’s greater total SOD activity (Table 2; see below).

**SOD activity in mucoid and nonmucoid P. aeruginosa strains and Sod- E. coli containing various P. aeruginosa sod clones.** To better understand the effects of the growth rate complementation experiments described above, SOD activity was assayed spectrophotometrically by the xanthine-xanthine oxidase-cytochrome c reduction assay (26) in the cell extracts of E. coli J1132 containing such clones in addition to mucoid and nonmucoid strains of P. aeruginosa. It has been previously shown that alginate produced by mucoid P. aeruginosa can act as a scavenger of $O_2^-$ generated by stimulated human macrophages (36). This suggests that mucoid and nonmucoid isogenic strains may behave differently in response to oxidative stress. To test this hypothesis, we measured SOD activity of mucoid, alginate-producing P. aeruginosa FRD1 as well as that of its nonmucoid counterpart, FRD2. Interestingly, both FRD1 and FRD2 possessed nearly fourfold the SOD activity of the wild-type E. coli strain, AB1157 (Table 2). However, P. aeruginosa FRD1 possessed significantly greater activity than FRD2 (85 versus 65 U/mg). Total SOD activity in wild-type AB1157 was similar to that of J1132(pDJH1) but was less than that of J1132 (pDJH6). When sodB and sodA were subcloned onto high-copy-number vectors such as pDJH7 (sodB in pKS-) or pDJH9 (sodA in pKS-), clones used in the growth rate complementation experiment above, SOD activities were approximately doubled (Table 2). For clone pDJH7, which contained sodB under the expression of its own promoter,
SOD activity was increased compared with that for the promoterless clone (pDJH4). Activity expressed by pDJH4 was presumably driven by vector (pKS-) promoter. Interestingly, when we examined SOD activity in alginate-producing (mucoid) and nonmucoid P. aeruginosa on native polyacrylamide gels stained for SOD activity (Fig. 7), we demonstrated that mucoid bacteria generate increased levels of Mn-SOD relative to nonmucoid bacteria in LB broth or YTG medium.

FIG. 5. Amino acid similarity between the SODs of P. aeruginosa and E. coli. EF, E. coli Fe-SOD; EM, E. coli Mn-SOD; PF, P. aeruginosa Fe-SOD; PM, P. aeruginosa Mn-SOD. Highly conserved amino acids are boxed. M, metal-binding region. Reprinted in a modified form from Progress in Nucleic Acid Research (3) with permission of the publisher.

DISCUSSION

To begin characterizing the role of SOD in oxidative stress in P. aeruginosa, we cloned, sequenced, and expressed the sodA and sodB genes encoding Mn-SOD and Fe-SOD. As with SOD sequences from various phyla (3), there was significant amino acid similarity, suggestive of a highly conserved evolutionary link. Interestingly, the P. aeruginosa SodA showed less similarity to the E. coli SodA than did the P. aeruginosa SodB to the E. coli SodB (Fig. 5). Transcription of the sodB gene of P. aeruginosa appears to be under the control of a promoter identified in this study. A primer extension analysis showed that transcription of sodB initiated 174 bp upstream of the ATG translational start codon, and a promoter possessed a 5/6 match at the consensus -10 region and a 4/6 match at the -35 region. Thus, we would predict that the sodB promoter is of the σ^70 variety. In contrast to upstream regions of sodB, we were unable to find any region of DNA 519 bp (to the BamHI site) upstream of the sodA ATG codon which resembled a consen-

FIG. 6. Effects of the P. aeruginosa SodA and SodB on growth of E. coli strains. Bacteria [E. coli AB1157 and J132 Δ(sodA/sodB)] containing either pKS-, pDH7 (sodB plus promoter region), or pDH9 (sodA) were grown overnight in L broth plus 0.1 mg of ampicillin at 37°C. Fresh prewarmed medium (1 volume of culture per 10-volume flask) was inoculated with 1/50 the final culture volume and allowed to reach an OD_{600} of 0.6. At this point, fresh prewarmed media were inoculated again with 1/50 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 spectrophotometrically at 600 nm.

FIG. 7. Native polyacrylamide gel electrophoresis of cell extracts from mucoid versus nonmucoid P. aeruginosa. Bacteria were described in Materials and Methods to an OD_{600} of 1.0 at 37°C in LB broth or YTG medium. Suspensions were clarified and sonicated as for Fig. 1. Samples (40 μg each) were applied to 10% nondenaturing gels and stained for SOD activity according to the method of Clare et al. (7). Lanes 1 and 2, mucoid P. aeruginosa grown in L broth and YTG medium, respectively; lanes 3 and 4, nonmucoid P. aeruginosa grown in LB broth and YTG medium, respectively.
sus – 35/10 promoter region, and identification of its promoter region is currently under investigation.

Because part of the defective phenotype associated with a double mutation in sodA and sodB in E. coli is a slow growth rate in rich medium relative to wild-type bacteria, we also analyzed the efficacy of growth rate complementation after introducing high-copy-number plasmids containing the sodA or sodB gene of P. aeruginosa into this mutant. Clearly, the expressed activity of the P. aeruginosa sodA or sodB gene permitted the oxygen-sensitive, SOD-deficient E. coli mutant, J1132, to grow well under highly aerobic conditions in L broth. Still, why wild-type E. coli (containing 16 U/mg) grew more rapidly than the sodA sodB mutant containing pDJH7 or pDJH9 (which possessed 55 and 45 U/mg, respectively) remains a puzzle. It has been suggested that SOD hyperproducers demonstrate greater sensitivity to aerobic growth than wild-type bacteria (34), due to the concomitant production of greater levels of H₂O₂, a product of SOD. This issue was recently resolved by Liochev and Fridovich (24), who demonstrated that an abnormal increase in Mn-SOD activity can tax the bacterium metabolically by limiting the biosynthesis of other enzymes which are normally stimulated by elevated intracellular levels of O₂⁻ (e.g., glucose-6-phosphate dehydrogenase, endonuclease IV, etc. [13]). In support of the latter hypothesis, the Sod⁺ strain, AB1157, grew slightly more slowly with the 3.4-kb PstI sodB fragment (pDJH7) (data not shown). Alternatively, it is possible that the Fe-SOD and Mn-SOD have different locations or roles, and thus, either alone is insufficient to completely restore the wild-type mutant to the wild-type growth rate. Furthermore, these results could be attributed to cross-species differences.

At present, we know little of how the P. aeruginosa sodA and sodB genes are regulated in response to oxidative stress. Interestingly, both oxygenation and decreased levels of available iron, conditions which increased the biosynthesis of the Mn-SOD in E. coli, also stimulate alginate biosynthesis by P. aeruginosa (22, 41). Alginate is an unbranched (1–4)-linked exopolysaccharide composed of β-D-mannurionate and its C-5 epimer α-L-guluronate. The biosynthesis of alginate by P. aeruginosa occurs almost exclusively in the pulmonary airways of patients afflicted with CF, an event which considerably hastens morbidity and mortality of the disease. In this study, we demonstrate that Mn-SOD activity is increased in alginate-producing P. aeruginosa relative to isogenic nonmucoid bacteria. Typically, the Fe-SOD is the predominant SOD synthesized by P. aeruginosa under routine laboratory conditions (19, 39). However, when iron becomes limiting in the presence of high levels of manganese (300 µM) (19), the Mn-SOD is also produced. Martins et al. (25) and Appanna (1) have recently demonstrated that increased manganese levels (500 µM) also stimulate alginate biosynthesis in P. aeruginosa and exopolysaccharide production in Rhizobium meliloti, respectively. The increase in Mn-SOD activity by mucoid P. aeruginosa suggests that mucoid bacteria are under iron limitation and/or oxidative stress. Being highly negatively charged, alginate can possibly act as an iron-sequestering agent once secreted from the cell, a hypothesis which has been supported by physicochemical studies (21, 37). Therefore, if alginate-producing P. aeruginosa is suffering from iron deprivation, which leads to an increase in Mn-SOD activity, then the addition of purified, Chelex-100-treated P. aeruginosa alginate to nonmucoid bacteria should also lead to an increase in Mn-SOD activity. When we tested this hypothesis, there was no increase in Mn-SOD activity (data not shown). Alternatively, if iron limitation leads to decreased Fe-SOD activity, as was demonstrated with E. coli (30), it is logical to have a backup enzyme such as the Mn-SOD to sequester potentially hazardous levels of O₂⁻ within the cell. This hypothesis is currently being tested in our laboratory. Mn-SOD activity in E. coli is increased during anaerobic growth when there is also an increase in the potential (in millivolts) of the cell (17). If Mn-SOD activity in P. aeruginosa is responsive to changes in cellular redox potential [i.e., increases in NAD(P)⁺/NAD(P)H ratios], then an increase in Mn-SOD activity by mucoid bacteria is likely a response to changes in the redox status relative to nonmucoid bacteria. Thus, the redox status of the bacterium may play an important role in the alginate regulatory cascade and possibly in the control of other P. aeruginosa virulence factors. Relatedly, compounds known to stimulate alginate production by P. aeruginosa may also affect the intracellular redox potential.

We have also demonstrated that both the sodA and the sodB genes of P. aeruginosa map to the 71- to 75-min region of the 75-min P. aeruginosa PAO1 chromosome. In contrast to the locations of sodA and sodB in P. aeruginosa, the locations of sodA and sodB in E. coli are quite distant from each other, being localized to the 88.45- and 36.35-min regions of the 100-min chromosome, respectively (27). Using the cloned sodA and sodB genes and current gene replacement techniques, we are now in the process of constructing sodA and sodB mutants of P. aeruginosa to examine the phenotype of this obligate respirer without these important enzymes which help relieve oxidative stress. The generation of these mutants would allow us to examine the possibility that elevated levels of O₂⁻, which can alter the redox status (or potential [in millivolts]) of the bacterium (17), may indirectly control the expression of various virulence factors including alginate and other antioxidants in P. aeruginosa.

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