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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 cosmid library of *P. aeruginosa* FRD which complemented an *Escherichia coli* (JI132) 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* PAO1 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.**

Pseudomonas aeruginosa is an opportunistic pathogen and a common cause of pulmonary infection in patients afflicted with cystic fibrosis (CF). In the bioenergetically complex arena of the CF lung, several factors suggest that *P. aeruginosa* may be subject to elevated oxidative stress including (i) the enhanced oxygen consumption by airway epithelial cells from CF patients (40), (ii) oxygen reduction products generated by stimulated human phagocytic cells (20), which are increased markedly in the CF airways (38), (iii) the endogenous aerobic metabolism of *P. aeruginosa* in this highly aerobic environment, and (iv) the oxygen reduction products generated through the autoxidation of pyocyanin, a redox-active phenazine antibiotic synthesized under aerobic conditions by nonmucoid *P. aeruginosa* in the CF lung (16, 19). Thus, enzyme systems in *P. aeruginosa* which protect the organisms from such forms of oxidative stress may also contribute to pathogenesis.

P. aeruginosa is an obligate aerobe or, more accurately, an obligate respirer, and is thus subject to endogenous oxidative stress. During the normal aerobic metabolism of dioxygen (O_2), some O_2 can be reduced to form the potentially hazardous superoxide radical (O_2^-) (10, 20). To circumvent this stress, *P. aeruginosa* possesses an iron (Fe) (19, 39)- and a manganese (Mn) (19)-cofactored superoxide dismutase (SOD), metalloenzymes which catalyze the disproportionation of O_2^- to H_2O_2 and O_2. The organism also possesses catalase which detoxifies the H_2O_2, forming O_2 and water.

Studies on the role of SOD in the response of *Escherichia coli* to oxidative stress have included the cloning of genes encoding both Fe-SOD (*sodB*) (33) and Mn-SOD (*sodA*) (42). The regulation of the *E. coli sodA* gene is extremely complex and is affected by oxygen (14), O_2^--generating agents (15),

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli		
AB1157	F⁻ *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx*	B. Bachman
JI132	Same as AB1157 plus (*sodA*::Mu dPR13)25 (*sodB*-Kan)1-2 *proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	J. Imlay
HB101		H. Boyer
P. aeruginosa		
FRD1	Prototrophic, Fe and Mn SOD⁺, mucoid CF isolate	12
FRD2	Nonmucoid revertant of FRD1	12
Plasmids		
pEMR2	pBR322 *cos oriT* Ap^r Km^r Tn*5*	9
pUC18	Amp^r	This laboratory
pBluescriptKS−	Extended polylinker pUC derivative; Ap^r	Stratagene
pDJH1	pEMR2 with a >40-kb *Bam*HI fragment of *P. aeruginosa* DNA containing *sodB*	This study
pDJH2	pEMR2 with a 3.7-kb *Bam*HI fragment of *P. aeruginosa* DNA containing *sodB*	This study
pDJH3	pUC18 with a 3.7-kb *Bam*HI fragment of *P. aeruginosa* DNA containing *sodB*	This study
pDJH4	pKS- with a 1.6-kb *Bam*HI-*Pst*I fragment of *P. aeruginosa* DNA containing *sodB*	This study
pDJH5	pUC18 with a 1.3-kb *Kpn*I-*Pst*I fragment of *P. aeruginosa* containing truncated *sodB*	This study
pDJH6	pEMR2 with a >40-kb *Bam*HI fragment containing *sodB* and *sodA* of *P. aeruginosa* FRD	This study
pDJH7	pKS- with a 3.4-kb *Pst*I fragment of *P. aeruginosa* DNA containing *sodB*	This study
pDJH8	pEMR2 with a ~12-kb *Bam*HI fragment containing *sodA*	This study
pDJH9	pKS- (Bluescript) with a 1.9-kb *Xho*I-*Pst*I fragment of *P. aeruginosa* containing *sodA*	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

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 O_2-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 *Bam*HI fragments (20 to 40 kb in size) cloned into the cosmid vector pEMR2 (9) was stored in *E. coli* HB101 (6). A lysate of λ*c*I857 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* JI132 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 (OD_{600}) 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 OD_{600} of these cultures was monitored throughout the growth cycle, and samples with an OD_{600} 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 *Xho*I-*Pst*I fragment in pDJH9 and the *sodB* gene on both a 1.6-kb *Bam*HI-*Pst*I fragment in pDJH4 and a 3.4-kb *Pst*I 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 (OD_{600} = 0.7) of *P. aeruginosa* FRD1 and FRD2 grown in L broth by methods previously described (2, 8). A synthetic oligonucleotide (5′-CCTCAGGTCAGGACTG-3′) specific for *sodB* was end labeled with [γ-^{32}P]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 extracts 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 clarified by centrifugation 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 O_2-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*, JI132. 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 O_2^- sensitive in the Sod$^-$ mutant and there is no *sod* gene product to protect these critical enzymes. Colonies of JI132 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.

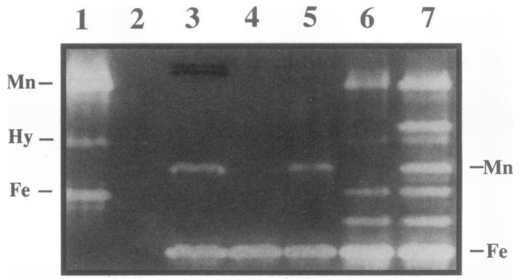

FIG. 1. Native polyacrylamide gel electrophoresis of cell extracts from *E. coli* stained for SOD activity. Bacteria [*E. coli* AB1157 or JI132 Δ(*sodA sodB*)] were grown as described in Materials and Methods for 17 h at 37°C in L broth plus 100 μg of ampicillin. *P. aeruginosa* was grown in high-phosphate 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 at setting 2. Cell debris was pelleted by centrifugation at 13,000 × *g* for 20 min 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* JI132 Δ(*sodA sodB*); lane 3, *P. aeruginosa* FRD2; lane 4, JI132(pDJH1); lane 5, JI132(pDJH6); lane 6, AB1157(pDJH1); lane 7, AB1157(pDJH6).

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-SOD activities in the wild-type *E. coli* strain, AB1157 (Fig. 1, lane 1), that were absent in the *sodA sodB* mutant, JI132 (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 (Fig. 1, lanes 6 and 7). A *Kpn*I 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 *Xho*I-*Pst*I fragment of pDJH6 which was subcloned into pKS− and contained a ~1.7-kb *Bam*HI-*Pst*I 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

TABLE 2. SOD activities of *P. aeruginosa* and *E. coli* containing *sodB* clones[a]

Strain	Plasmid genotype	SOD activity (U/mg)[b]
P. aeruginosa		
FRD1	*sodA*$^+$ *sodB*$^+$	85 ± 7
FRD2	*sodA*$^+$ *sodB*$^+$	65 ± 5
E. coli		
AB1157 *sodA*$^+$ *sodB*$^+$	*sodA*$^+$ *sodB*$^+$	16 ± 4
JI132 *sodA sodB*	*sodA*$^-$ *sodB*$^-$	ND
JI132(pDJH1)	*sodB*$^+$	22 ± 4
JI132(pDJH4)	*sodB*$^+$	40 ± 3
JI132(pDJH5)	*sodB*$^-$	ND
JI132(pDJH7)	*sodB*$^+$	55 ± 7
JI132(pDJH6)	*sodA*$^+$ *sodB*$^+$	30 ± 8
JI132(pDJH9)	*sodA*$^+$	45 ± 5

[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.

[b] Determined by the method of McCord and Fridovich (26). The data are means and standard errors of three different experiments. ND, not detectable.

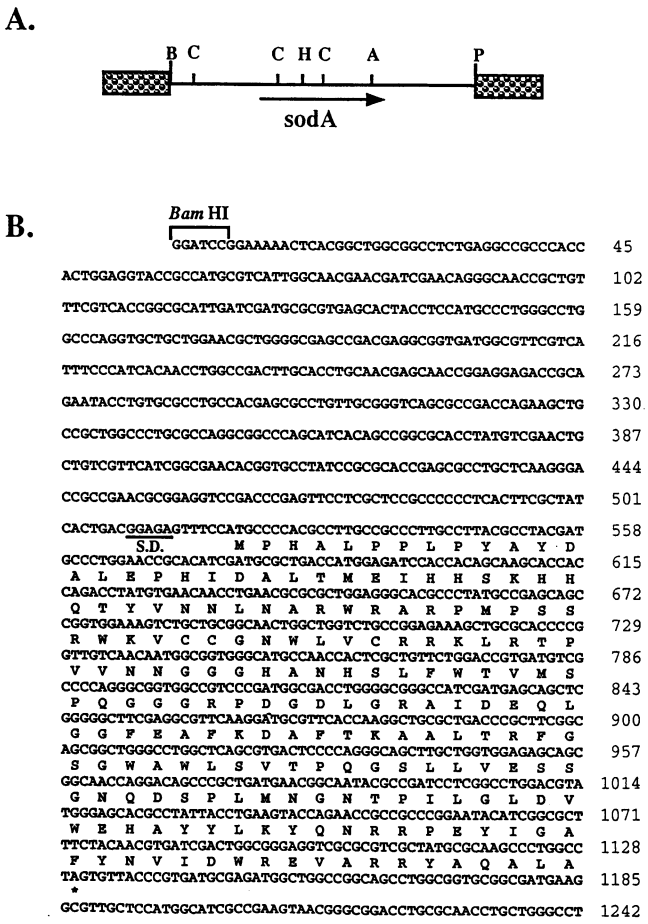

FIG. 2. Partial restriction map (A) and nucleotide sequence (B) of the *sodA* gene of *P. aeruginosa* FRD with upstream sequences and the deduced polypeptide sequence. (A) Restriction map of portions of pDJH9. Abbreviations: B, *Bam*HI; C, *Cla*I; A, *Hae*I; H, *Hin*cII; P, *Pst*I. The ribosome-binding or Shine-Dalgarno (S.D.) sequence of *sodA* is underlined in panel B. *, stop codon.

molecular size of 22.9 kDa, which was supported by the results of in vitro 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 *Bam*HI-*Pst*I fragment of pDJH4 harboring *sodB* (Fig. 3A) was also sequenced on both strands, as were portions of the 3.4-kb *Pst*I *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 ^{35}S-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 *Pst*I 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 *algD* 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 *Spe*I- and *Dpn*I-digested genomic DNA. Interestingly, both the *sodA* and the *sodB* probes hybridized to a 360-kb *Spe*I fragment and to a 290-kb *Dpn*I 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

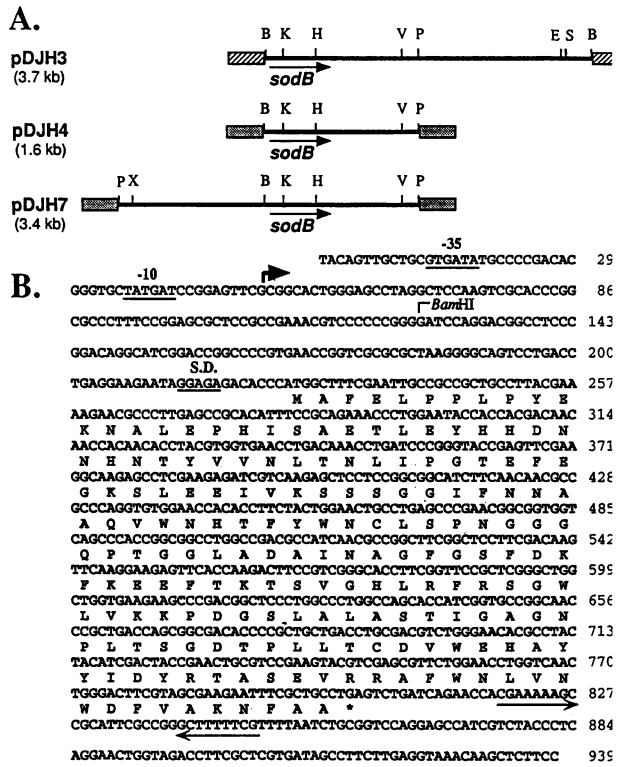

FIG. 3. Partial restriction map (A) and nucleotide sequence (B) of the *sodB* gene of *P. aeruginosa* FRD with upstream sequences and the deduced polypeptide sequence. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*cII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; V, *Pvu*I; X, *Xho*I. The ribosome-binding or Shine-Dalgarno (S.D.) sequence and promoter bases in the −10 and −35 regions are underlined. Initiation of transcription is indicated by an angled arrow. *, stop codon, TGA. Palindromic regions are indicated by horizontal arrows.

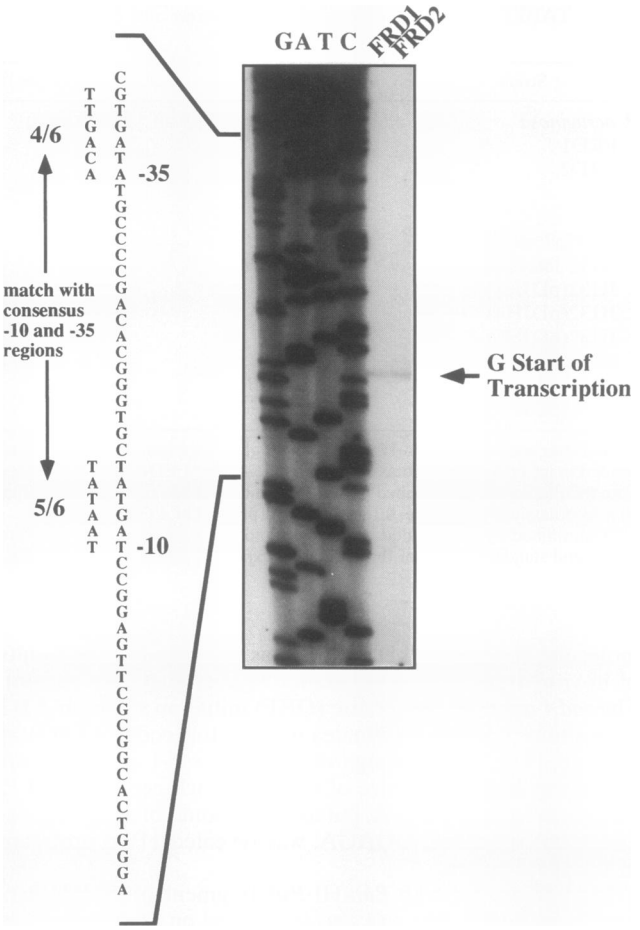

FIG. 4. Sequence and primer extension analysis of the *sodB* promoter region. RNAs from *P. aeruginosa* FRD1 (mucoid) and FRD2 (nonmucoid) were primer extended with reverse transcriptase and a 5′-end-labeled primer. The same primer was used to generate the sequencing ladder with pDJH7 DNA as a template. The size of the primer extension product is indicated (vertical arrows). Also given are the −10 and −35 promoter regions with the frequencies of matching with consensus *E. coli* promoter sequences.

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* JI132. 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* JI132, the effects of the *P. aeruginosa sodA* and *sodB* expressed in high copy number (pKS−) on the growth of *E. coli* JI132 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 clone of *sodA* used was from pDJH9, and that of *sodB* was from pDJH7. By 6 h postinoculation, JI132(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 results 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* JI132 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 JI132(pDJH1) but was less than that of JI132 (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,

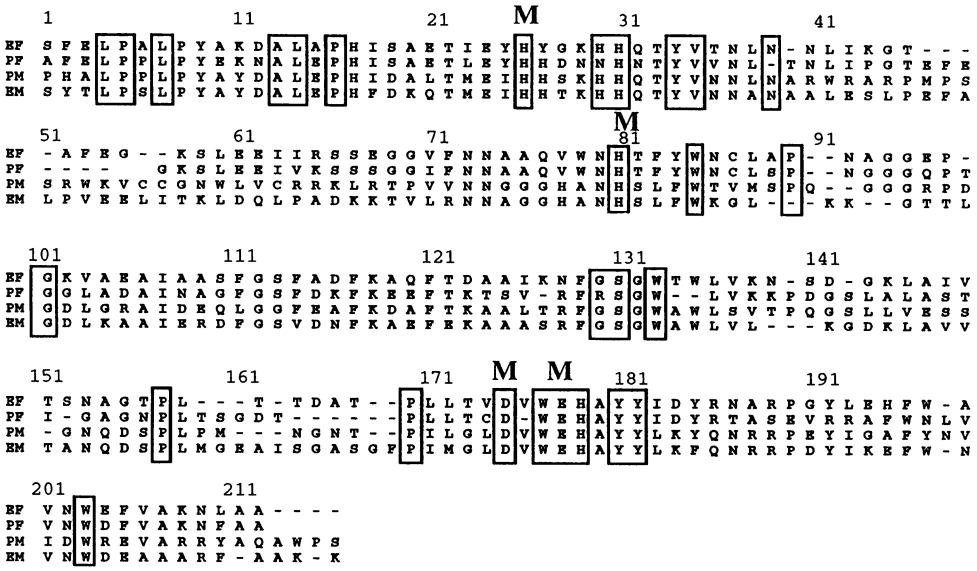

FIG. 5. Amino acid similarity between the SODs of *P. aeruginosa* and *E. coli*. EF, *E. coli* Fe-SOD; EM, *E. coli* Mn-SOD; PF, *P. aeruginosa* Fe-SOD; PM, *P. aeruginosa* Mn-SOD. Highly conserved amino acids are boxed. M, metal-binding region. Reprinted in a modified form from *Progress in Nucleic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

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 L broth or YTG medium.

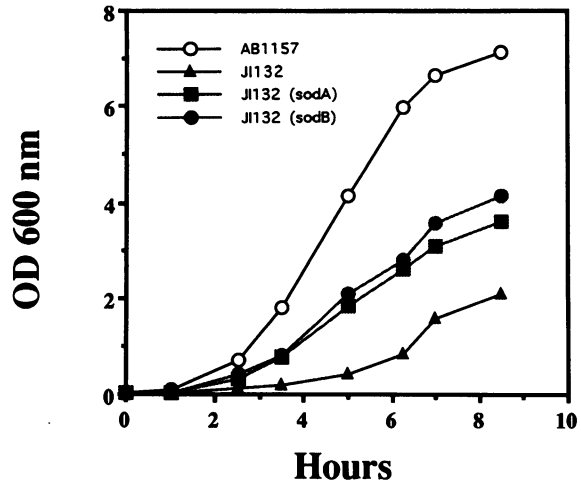

FIG. 6. Effects of the *P. aeruginosa* SodA and SodB on growth of *E. coli* strains. Bacteria [*E. coli* AB1157 and JI132 Δ(*sodA sodB*)] containing either pKS−, pDJH7 (*sodB* plus promoter region), or pDJH9 (*sodA*) were grown overnight in L broth plus 0.1 mg of ampicillin at 37°C. Fresh prewarmed medium (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.

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 *Bam*HI site) upstream of the *sodA* ATG codon which resembled a consen-

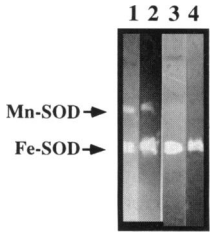

FIG. 7. Native polyacrylamide gel electrophoresis of cell extracts from mucoid versus nonmucoid *P. aeruginosa*. Bacteria were grown as described in Materials and Methods to an OD_{600} of 1.0 at 37°C in L broth or YTG medium. Suspensions were clarified and sonicated as for Fig. 1. Samples (40 μg each) 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 L 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, JI132, 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 H_2O_2, 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 O_2^- (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 double 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-mannuronate 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 O_2^- 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 O_2^-, 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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