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# Characterization of Four Superoxide Dismutase Genes from a Filamentous Cyanobacterium

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**By using an oligonucleotide probe constructed from a conserved region of amino acids located in the carboxyl-terminal end of superoxide dismutase (SOD) proteins, four SOD genes were cloned from the cyanobacterium *Plectonema boryanum* UTEX 485. One of these genes, designated *sodB*, encoded an FeSOD enzyme, while the remaining three genes, designated *sodA1*, *sodA2*, and *sodA3*, encoded MnSOD enzymes. To investigate the expression of these four genes, total cellular RNA was isolated from *P. boryanum* UTEX 485 cells grown under various conditions and RNA gel blot analysis was carried out. Results indicated that *sodB* and *sodA*1 were constitutively expressed, although *sodB* expression was partially repressed in cells grown under conditions of iron stress. *sodA2* transcripts, which were not detectable in control cells, accumulated to high levels in cells treated with methyl viologen or in cells grown under conditions of iron or nitrogen stress. However, under microaerobic conditions, iron and nitrogen stress failed to induce *sodA2*, indicating that multiple factors affect the regulation of *sodA2*. While discrete transcripts were not detected for *sodA3*, hybridization was observed under a number of conditions, including those which increased the accumulation of *sodA2* transcripts. Additionally, there were high levels of the *sodA*3 transcript detected in a *P. boryanum* UTEX 485 mutant strain resistant to methyl viologen treatment.**

The photoproduction of a variety of active species of oxygen is inevitable in photosynthetic organisms. The effects of activated oxygen radicals are dependent on the species involved and the subcellular location of its production. Activation of $O_2$ in the oxygen-evolving photosynthetic system can occur primarily through two pathways. The most direct route results in the production of singlet dioxygen ($^1O_2$) through the interaction of $O_2$ with excited triplet-state chlorophyll in the light-harvesting complex of photosystems I and II (PSI and PSII). Protection against the formation of singlet oxygen usually involves the quenching of the excited singlet state of chlorophyll by carotenoids before it forms triplet chlorophyll (11). The second $O_2$ activation pathway results in the production of the superoxide anion radical ($O_2^-$) by the interaction of $O_2$, via a Mehler-type reaction, with electron acceptors of PSI. $O_2^-$ is not particularly reactive with cellular components, but it has been implicated in lipid peroxidation, enzyme inactivation, and the depolymerization of polysaccharides (17). However, $O_2^-$ is able to react with hydrogen peroxide ($H_2O_2$) in a Haber-Weiss-type reaction to form the highly reactive hydroxyl radical ($OH\cdot$). Once produced, the hydroxyl radical can react indiscriminately to cause lipid peroxidation, the denaturation of proteins, and damage to DNA. Therefore, it is imperative to the survival of photosynthetic organisms to scavenge both $H_2O_2$ and $O_2^-$ immediately to prevent photooxidative damage (7).

Superoxide dismutases (SODs) are metalloenzymes of 17 to 85 kDa that are ubiquitous in aerobic organisms and function by catalyzing the dismutation of $O_2^-$ to $H_2O_2$ and $O_2$. This disproportionation reaction is very efficient and is limited only by the rate of diffusion of $O_2^-$ into the active site of the enzyme and the availability of hydrogen ions (13). There are three types of SOD enzymes, which can be distinguished by the prosthetic metals present at the active site: iron (FeSOD), manganese (MnSOD), and copper/zinc (Cu/ZnSOD). The FeSOD enzyme has been detected in algae, cyanobacteria, and other prokaryotes as well as the stroma of chloroplasts (23, 33). MnSOD has been detected in some but not all cyanobacteria and is widely distributed among prokaryotic and eukaryotic organisms. In cyanobacteria, FeSOD is localized in the cytosol, whereas MnSOD is found primarily in the membrane fraction (23). Sequence homologies suggest that MnSOD and FeSOD evolved from a common ancestral form in early prokaryotes, whereas the Cu/ZnSOD appears to have evolved independently in the green plant line (9).

The importance of SOD in the prevention of photooxidative damage in cyanobacteria has been demonstrated by several studies using mutant strains with altered levels of SOD activity. Steinitz et al. (30) isolated a *Plectonema boryanum* mutant which was resistant to photooxidation. The genetic basis for this mutant appeared to be marginally lower photosensitizer levels and increases in the level of MnSOD activity. Recently, a mutant strain of *Synechococcus* sp. strain PCC 7942 which lacks detectable FeSOD activity has been constructed. In this mutant, photosynthetic electron transport was more sensitive to active $O_2$ species photoinduced in the presence of methyl viologen (MV) and to elevated $O_2$ concentrations (14). Although both photosystems were susceptible to photooxidative stress, the primary site sensitized in the FeSOD mutant was located near PSI, in the return part of PSI cyclic electron flow between the iron-sulfur centers $F_A$ and $F_B$ and cytochrome *f*. The FeSOD mutant was much more sensitive to the presence of MV in the growth medium and exhibited an altered acclimation to high growth irradiance. Notably, the PSI concentration relative to PSII concentration was, at high growth irradiance, 3.5 times higher in the FeSOD mutant than in the wild-type strain (28).

Cyanobacterial SODs have been most extensively studied in the filamentous cyanobacterium *P. boryanum* (6, 23, 30). Ad-

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ditionally, a system for the manipulation and inactivation of genes in *P. boryanum* has recently been developed and used to analyze the function of several genes involved in the biosynthesis of chlorophyll (12). In this communication, we report the isolation and characterization of four *P. boryanum* UTEX 485 SOD genes and that three of these proteins appear to contain $Mn^{2+}$ at their active sites. RNA gel blots have been used to characterize the size and accumulation of the transcripts encoded by these genes.

## MATERIALS AND METHODS

**Strains and culture conditions.** Axenic cultures of *P. boryanum* UTEX 485 were grown in liquid and on solid (1% Difco Bacto Agar) BG-11 medium (4) buffered to pH 8.0 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Liquid cultures were grown at 29°C in 50-ml culture tubes bubbled with filtered air.

**Chromosomal DNA isolation from *P. boryanum* UTEX 485.** Exponentially growing cells (0.5 g [wet weight]) were pelleted by centrifugation and resuspended in a saturated solution of sodium iodide (NaI) for 30 min at 37°C in order to remove the external mucilaginous sheath. The cells were pelleted by centrifugation, washed with distilled $H_2O$, and recentrifuged to remove the NaI. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 5 mM $Na_2EDTA$), and lysozyme was added to a final concentration of 5 mg/ml. The lysis solution was incubated at 37°C for 45 min, and then *N*-lauryl sarcosine was added to a final concentration of 1% (wt/vol). The lysate was extracted twice with Tris-buffered (pH 8.0) phenol, twice with phenol-chloroform (1:1), and twice with chloroform. The DNA was precipitated with ethanol and pelleted. The pellet was washed with 70% ethanol, dried, and resuspended for 2 days at 4°C in 2 ml of distilled $H_2O$. The DNA was further purified by CsCl density gradient centrifugation (27).

**DNA gel blot analysis.** *P. boryanum* UTEX 485 chromosomal DNA was digested with the appropriate restriction enzymes as instructed by the supplier. Digested DNA samples were electrophoresed in 0.7% agarose gels in a Tris-sodium acetate-EDTA buffer system. Transfer of DNA to supported nitrocellulose filters (Schleicher & Schuell, Inc.) was carried out by the methods of Sambrook et al. (27). DNA gel blot hybridization was carried out as described previously (27) in glass hybridization tubes in a Bachofer hybridization incubator/rotator oven.

**DNA probe labeling.** Oligonucleotide probes were 5′ end labeled with [$\gamma$-$^{32}$P]dATP (specific activity, ≥3,000 Ci/mmol), using the enzyme T4 bacteriophage polynucleotide kinase (27). Double-stranded DNA fragments, isolated from low-melting-point preparative agarose gels, were labeled with [$\alpha$-$^{32}$P]dCTP (specific activity, ≥3,000 Ci/mmol), using the T7 Quickprime random primer labeling kit as instructed by the manufacturer (Pharmacia). Unincorporated radiolabeled nucleotides were separated from the labeled probes by Sephadex column chromatography (27).

**DNA sequence determination.** DNA sequencing using both M13 and plasmid templates was carried out by the dideoxynucleotide chain termination method originally developed by Sanger et al. (29). A modified T7 DNA polymerase (Sequenase version 2.0; United States Biochemical) was used in the sequencing reactions with oligonucleotide primers that were constructed by using sequence data derived from previous clones in order to facilitate progressive sequencing of the DNA template. Ambiguities in G-C-rich regions were resolved by substituting dITP or 7-deaza-dGTP for dGTP in the sequencing reactions. The Genetics Computer Group sequence analysis software package (University of Wisconsin) was used to analyze the DNA sequence. Possible open reading frames (ORFs) were compared with sequences in the National Center for Biotechnology Information database, using Blast alignment programs (5).

**RNA analysis.** Total RNA from *P. boryanum* UTEX 485 was isolated by the method described by Laudenbach et al. (18). RNA (5 μg per lane) was resolved by electrophoresis in 1.2% agarose gels under denaturing conditions, transferred to reinforced nitrocellulose filters, and hybridized to radiolabeled DNA probes (31). A 480-bp DNA fragment, derived from a PCR product located between amino acids 55 and 226 (as numbered in Fig. 4) of the SodA1 protein sequence, was used as a *sodA1* gene-specific probe in RNA gel blot analysis. A 475-bp DNA fragment, derived from a PCR product located between amino acids 55 and 226 (as numbered in Fig. 4) of the SodA2 protein sequence, was used as the *sodA2* gene-specific probe. A 560-bp DNA fragment, extending from the *Eco*RI site located near the 5' end of *sodA3* to the *Hin*dIII site located 12 bases downstream of the *sodA3* termination codon (see Fig. 2C), was used as a *sodA3* hybridization probe. A 490-bp *Xba*I-*Hin*cII restriction fragment, consisting of a sequence totally internal to *sodB* (see Fig. 2D), was used as the *sodB* gene-specific probe.

**NTG mutagenesis.** *P. boryanum* UTEX 485 cells were mutagenized by using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) as described by Miller (20). Cells from a 250-ml culture (0.5 g [wet weight]) were pelleted, washed three times in 0.1 M citrate buffer (pH 5.5), and resuspended in 2 ml of the same buffer; 100 μl of a 1-mg/ml stock of NTG in acetate buffer (pH 5.0) was added to the cell suspension, and the mixture was incubated for 30 min at 29°C in the light. The cells were then pelleted and washed twice in 5 ml of fresh BG-11 growth medium. The cells were added to 50 ml of BG-11 in a flask and allowed to recover overnight with shaking. Cells were plated on BG-11 plates containing 500 nM MV. Plates were incubated under light at 29°C for 10 days in order to detect any MV-resistant colonies.

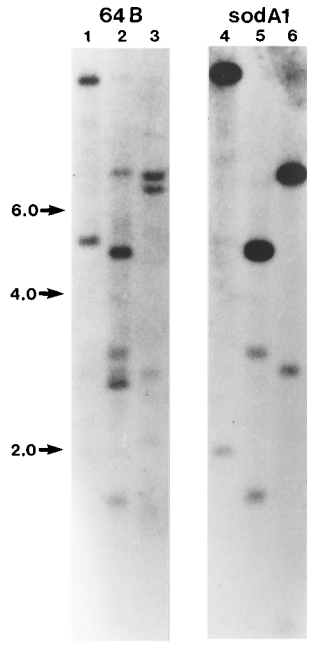


FIG. 1. DNA gel blot hybridization of *P. boryanum* UTEX 485 genomic DNA, using oligonucleotide 64B and a *Hin*dIII-*Sac*I fragment of the *P. boryanum* UTEX 485 *sodA1* gene as radiolabeled DNA probes. Genomic DNA was digested with the restriction enzymes *Bgl*II (lanes 1 and 4), *Eco*RI (lanes 2 and 5), and *Hin*dIII (lanes 3 and 6), electrophoresed on a 0.7% agarose gel, and transferred to a nitrocellulose membrane. Hybridizations were carried out at 37°C for the oligonucleotide 64B and 52°C for *sodA1*. Size markers are in kilobase pairs.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the four SOD genes have been submitted to GenBank under accession numbers U17609 (*sodA1*), U17610 (*sodA2*), U17611 (*sodA3*), and U17612 (*sodB*).

## RESULTS

**Cloning strategy.** On the basis of codon usage data from existing cyanobacterial genes, a 23-base synthetic oligonucleotide containing the sequence 5′ GATGTCTGGGAACACGC TTACTA 3′ (designated 64B) was synthesized by reverse translating a region of conserved amino acids (DVWEHAYY) found in the carboxy-terminal end of known SOD proteins. Oligonucleotide 64B was used as the radiolabeled probe in DNA blot hybridization studies to detect *P. boryanum* UTEX 485 genomic DNA digested with various restriction enzymes. As can be seen in Fig. 1, several strong signals were obtained in each lane. The *sodA* and *sodB* genes from *Escherichia coli* (26, 32) were also used as hybridization probes at low stringency to detect *P. boryanum* UTEX 485 genomic DNA. The results obtained indicated that the 6.5- and 6.75-kb *Hin*dIII bands, which hybridized strongly to oligonucleotide 64B, were also faintly detected by the *E. coli sodB* and *sodA* gene probes, respectively (results not shown). On the basis of these results, a *Hin*dIII 6.0- to 7.0-kb size-fractionated genomic library was constructed by using the cloning vector pUC118. Plasmid DNA was isolated from 200 individual colonies and screened by DNA gel blot hybridization using oligonucleotide 64B as a radiolabeled gene probe. Two clones, containing *Hin*dIII inserts of 6.5 and 6.75 kb, were isolated by this cloning strategy.

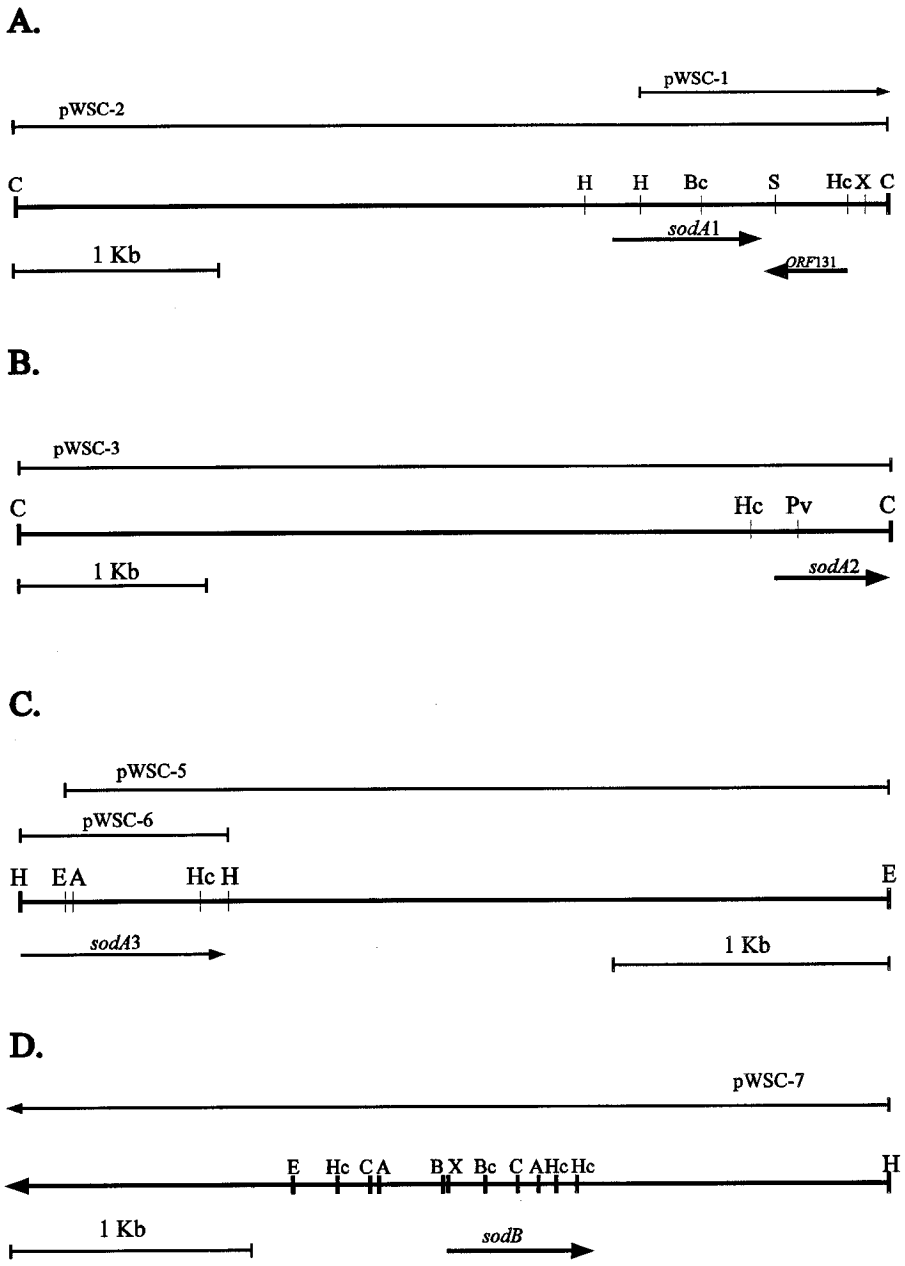


FIG. 2. (A) Restriction map of the 6.75-kb *Hin*dIII clone (pWSC-1) and the 4.5-kb *Cla*I clone (pWSC-2) containing the *sodA1* gene from *P. boryanum* UTEX 485. Only the portion of clone pWSC-1 containing the *sodA1* gene is shown. The length and direction of the *sodA1* coding region are indicated. The length and orientation of the 131-amino-acid ORF downstream of *sodA1* are also shown. (B) Restriction map of the 4.3-kb *Cla*I clone (pWSC-3) containing the *sodA2* gene from *P. boryanum* UTEX 485. The length and direction of the *sodA2* coding region are indicated. (C) Restriction map of the 3.0-kb *Eco*RI clone (pWSC-5) and the 0.75-kb *Hin*dIII clone (pWSC-6) containing the *sodA3* gene from *P. boryanum* UTEX 485. The length and direction of the *sodA3* coding region are indicated. (D) Restriction map of the 6.5-kb *Hin*dIII clone (pWSC-7) containing the *sodB* gene from *P. boryanum* UTEX 485. Only the portion of the clone containing the *sodB* gene is shown. The length and direction of the *sodB* coding region are indicated. The restriction enzyme sites for all panels are abbreviated as follows: H, *Hin*dIII; E, *Eco*RI; Hc, *Hin*cII; C, *Cla*I; A, *Acc*I; B, *Bgl*II; X, *Xba*I; Bc, *Bcl*I; S, *Sac*I; Pv, *Pvu*I.

**Isolation of a *sodA1* clone.** A restriction map of the clone containing the 6.75-kb *Hin*dIII insert, designated pWSC-1, was constructed (Fig. 2A), and oligonucleotide 64B was used as a probe to localize the putative gene to a 0.65-kb *Hin*dIII-*Sac*I fragment. DNA sequence analysis revealed that the *Hin*dIII-*Sac*I fragment contained the partial sequence of an MnSOD gene, designated *sodA1*. An additional clone designated pWSC-2, containing a 4.5-kb *Cla*I fragment, was isolated and used to obtain the complete sequence of *sodA1*.

**Isolation of *sodA2* and *sodA3* clones.** As shown in Fig. 1, several hybridization signals were obtained in each lane when a *P. boryanum* UTEX 485 *sodA1* gene-specific probe was used in low-stringency hybridization experiments. These weaker signals were thought to represent either one or two additional MnSOD genes, since some of these signals were also present in the hybridization results obtained with oligonucleotide 64B as a hybridization probe (Fig. 1). Hybridization studies indicated that one of the weaker signals obtained with the

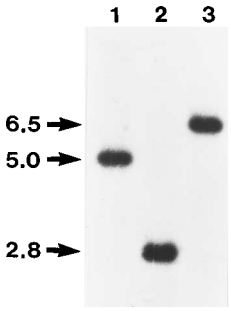


FIG. 3. DNA blot hybridization to *P. boryanum* UTEX 485 genomic DNA, using the *P. boryanum* UTEX 485 *sodB* gene as a $^{32}$P-radiolabeled probe. Hybridization was carried out under low-stringency conditions at 52°C. Genomic DNA was digested with the restriction enzymes *Bgl*II (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3) and electrophoresed on a 0.7% agarose gel before transfer to a nitrocellulose membrane. Size markers are in kilobase parts.

*sodA1* gene probe corresponded to a 4.3-kb *Cla*I fragment (data not shown). A size-fractionated *Cla*I library was screened with *sodA1* and oligonucleotide 64B as radiolabeled gene probes, and a recombinant clone designated pWSC-3 was isolated (Fig. 2B). Nucleotide sequence analysis revealed that an MnSOD gene, designated *sodA2*, was encoded in the pWSC-3 insert.

Since both the *sodA1* and *sodA2* gene probes hybridized weakly to a 3-kb *Eco*RI fragment (results not shown), a size-fractionated *Eco*RI library was constructed in an attempt to clone a third MnSOD gene. Recombinant plasmids were screened by using *sodA1* as a probe under low-stringency conditions. A positive clone, designated pWSC-5, was selected and used for restriction enzyme and sequence analysis (Fig. 2C). DNA sequence analysis revealed that this clone contained the partial sequence of an MnSOD gene (the 5′ end of the gene was missing). Comparison with the two previously identified MnSOD genes indicated that the sequence of this gene was similar, but not identical, to the sequences of *sodA1* and *sodA2*. It was therefore designated *sodA3*.

Since pWSC-5 did not contain the entire *sodA3* gene, further cloning was necessary. A 0.75-kb *Hin*dIII clone, designated pWSC-6, was isolated from a size-fractionated library by using *sodA3* as a gene probe, and sequence analysis confirmed that this fragment contained more sequence toward the beginning of the gene encoding SodA3. However, analysis of the SodA3 reading frame indicated that a methionine residue which could act as the initiation codon was still missing from the sequence. Repeated attempts to clone the intact *sodA3* gene on a larger fragment in *E. coli* have not been successful.

**Isolation of a *sodB* clone.** The second positive *Hin*dIII clone that was originally isolated by using oligonucleotide 64B as a hybridization probe contained a 6.5-kb *Hin*dIII fragment. A restriction map of this clone, designated pWSC-7, is shown in Fig. 2D. DNA gel blot hybridization studies indicated that oligonucleotide 64B hybridized to a 2.1-kb *Hin*dIII-*Acc*I fragment, which was subcloned into M13 mp18 and mp19 and used for sequence analysis. The nucleotide sequence obtained confirmed that the *sodB* gene from *P. boryanum* UTEX 485 was encoded on the pWSC-7 insert. When a radiolabeled *sodB* probe was hybridized at low stringency to *P. boryanum* UTEX 485 genomic DNA, only one band was seen in each of the lanes, indicating that there was only one FeSOD gene (Fig. 3).

**SodA amino acid sequence analysis.** The SodA1 mature protein has a predicted molecular mass of 23,550 Da and a calculated pI of 10.0. The SodA2 protein has a predicted molecular mass of 23,455 Da and a calculated pI of 5.5. The SodA3 mature protein has a predicted molecular mass of 23,828 Da and a calculated pI of 6.9. The deduced amino acid sequences for the three SodA proteins from *P. boryanum* UTEX 485 were aligned with other MnSOD amino acid sequences, and the results are shown in Fig. 4. Since the MnSOD and FeSOD proteins have amino acid similarity, it can be difficult to identify the metal cofactor present in the SOD protein. However, four residues which are positioned in or close to the active site of the SOD enzyme can be used to distinguish MnSOD and FeSOD sequences (8, 24, 25, 34). As can be seen in Fig. 4, the four residues marked with diamonds are consistent with the SOD protein containing an $Mn^{2+}$ cofactor. A comparison among the three Sod proteins from *P. boryanum* UTEX 485 indicates that SodA3 is more similar to SodA2 than to SodA1 (Fig. 5).

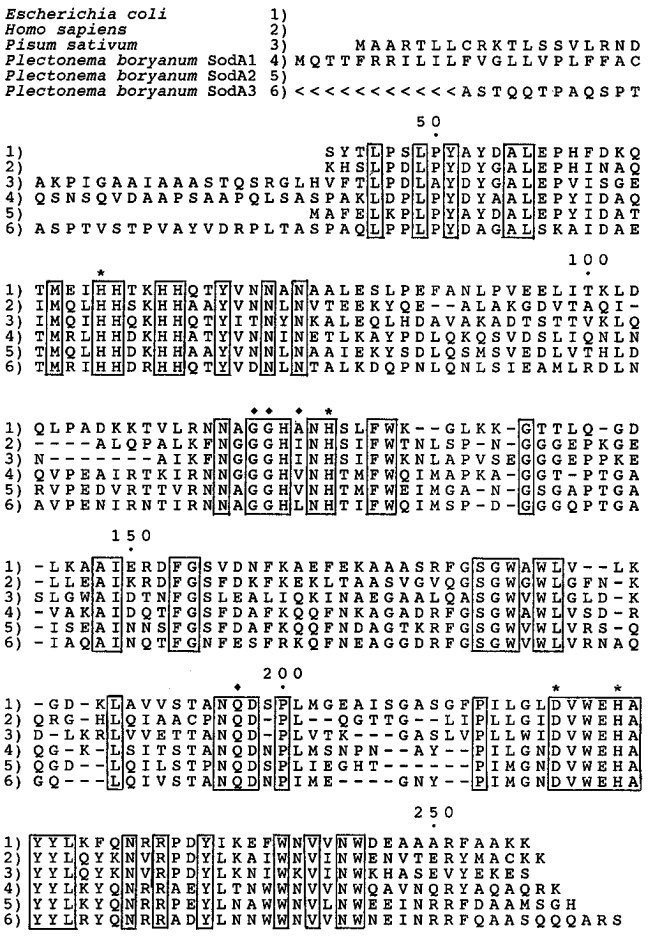


FIG. 4. MnSod amino acid sequence comparison between *P. boryanum* UTEX 485 SodA1, SodA2, and SodA3 and MnSod from *E. coli*, *Homo sapiens*, and *Pisum sativum*. Residues conserved in all six sequences are boxed. The positioning of the $NH_2$ terminus of human MnSOD is according to Barra et al. (8). Asterisks indicate amino acid residues involved in the metal ligand binding. Residues marked with diamonds indicate primary candidates for distinguishing the identity of the metal ligand (Fe or Mn) bound by the SOD enzyme (8, 24, 34). Arrowheads represent regions of sequence which have not been determined. Gaps (represented by dashes) have been introduced into the sequence to maintain optimal alignment.

**SodB amino acid sequence analysis.** The SodB protein contains 199 amino acids with a predicted molecular mass of 21,934 Da and a calculated pI of 5.29. A comparison of the deduced amino acid sequence of *P. boryanum* UTEX 485 SodB

| | *P. boryanum* SodA1 | *P. boryanum* SodA2 | *P. boryanum* SodA3 | *P. boryanum* SodB | *Synechococcus* SodB | *E. coli* SodA | *E. coli* SodB | *H. sapiens* SodA | *P. sativum* SodA |
|---|---|---|---|---|---|---|---|---|---|
| *P. boryanum* SodA1 | 100 | 75.1 (62.9) | 78.0 (64.4) | 64.5 (43.7) | 63.0 (45.5) | 69.2 (53.2) | 63.0 (45.8) | 63.8 (49.5) | 57.8 (42.2) |
| *P. boryanum* SodA2 | | 100 | 80.6 (61.2) | 68.0 (45.2) | 66.0 (45.5) | 69.8 (53.3) | 69.3 (49.0) | 64.3 (49.5) | 57.4 (37.1) |
| *P. boryanum* SodA3 | | | 100 | 67.2 (38.9) | 64.0 (43.5) | 68.8 (48.7) | 68.2 (43.8) | 62.1 (45.6) | 56.8 (39.2) |
| *P. boryanum* SodB | | | | 100 | 73.4 (60.3) | 64.1 (47.2) | 77.0 (59.2) | 62.3 (44.0) | 56.4 (33.8) |
| *Synechococcus* SodB | | | | | 100 | 62.8 (47.5) | 72.8 (55.5) | 55.4 (43.1) | 68.5 (55.0) |
| *E. coli* SodA | | | | | | 100 | 62.4 (46.6) | 64.8 (47.2) | 62.9 (44.8) |
| *E. coli* SodB | | | | | | | 100 | 60.0 (45.4) | 56.5 (36.1) |
| *H. sapiens* SodA | | | | | | | | 100 | 71.7 (55.6) |
| *P. sativum* SodA | | | | | | | | | 100 |

FIG. 5. Comparisons between various SOD amino acid sequences from prokaryotic and eukaryotic organisms. Values represent percent similarity between the amino acid sequences. Values in brackets represent percent identity. *H. sapiens* and *P. sativum* SodA represent mitochondrial MnSOD sequences from humans and garden peas, respectively.

with the sequences from both eukaryotic and prokaryotic FeSODs is shown in Fig. 6. Again, the identity of the *P. boryanum* UTEX 485 *sodB* gene can be confirmed as that of an FeSOD gene by comparing the four residues previously established (8, 24, 34) as the best candidates for distinguishing FeSOD and MnSOD enzymes. Additionally, the highest similarity of *P. boryanum* UTEX 485 SodB is to *E. coli* and *Synechococcus* sp. strain PCC 7942 FeSOD proteins (19, 26), while similarity to MnSOD sequences is at least 10% lower (Fig. 5).

**Characterization of an ORF downstream of *sodA1*.** Analysis of the DNA sequence obtained downstream of *sodA1* revealed a 393-bp ORF encoded on the DNA strand complementary to *sodA1* (Fig. 2A). A comparison of ORF131 of *P. boryanum* UTEX 485 revealed 54% similarity (37.5% identity) between the entire length of ORF131 and the first 130 residues of *E. coli* OmpR (Fig. 7). OmpR is the regulatory component of a two-component regulatory system which contains a sensor component, which monitors environmental change, and a response regulator, which mediates changes in cellular functions in response to the sensor. Identities between the two sequences were found at residues shown to be conserved in regulator components (3). In addition, ORF131 contains the two invariant aspartic acid residues which may be important for activation by phosphorylation (3).

**SodA1 and SodA3 leader sequences.** As shown in Fig. 4, the amino acid sequences of both SodA1 and SodA3 are longer at the $NH_2$-terminal end than previously characterized SOD sequences. Similarity with other SODs begins at approximately residue 42 of SodA1 and to a corresponding region in SodA3. Since previous studies (6, 23) have localized MnSOD activity to the *P. boryanum* membrane fraction, these additional N-terminal residues may encode a signal sequence. The 41-residue, putative signal sequence of SodA1 exhibits similarity to the beginning of a PSI reaction center subunit, PSI-N, from barley (*Hordeum vulgare* L.) (16). The N terminus of PSI-N represents both a transit sequence which directs the subunit into the chloroplast and a signal sequence which directs the protein through the thylakoid membrane, where it can become associated with PSI. Similarity was also revealed between the SodA1 sequence and the signal sequence of the protein PSI-M from *Euglena* sp. Therefore, this region may act as a signal sequence, guiding the protein through or anchoring it to a membrane. The putative signal sequence of SodA1 is hydrophobic (results not shown), and this is characteristic of other prokaryotic signal sequences. (35).

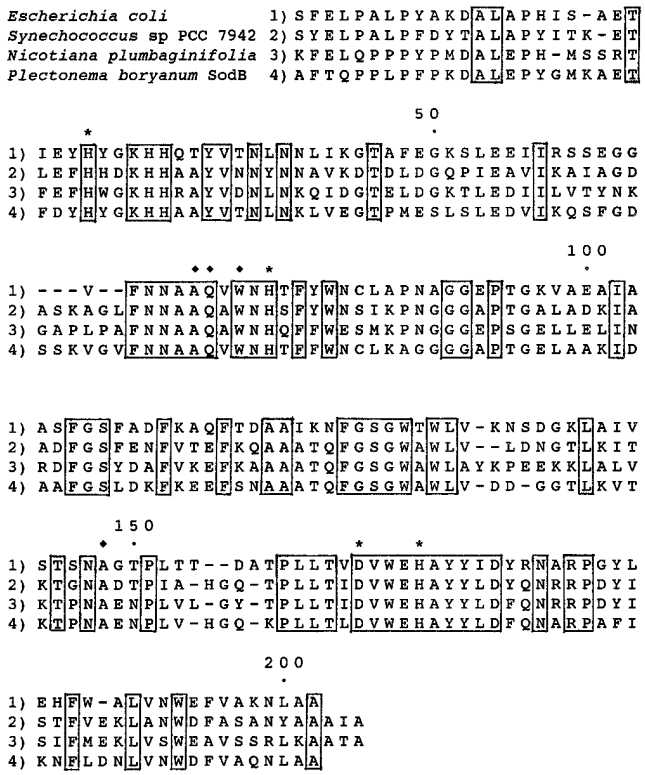


FIG. 6. FeSOD amino acid sequence comparison between *P. boryanum* UTEX 485 SodB and FeSOD from *E. coli*, *Synechococcus* sp. strain PCC 7942, and *Nicotiana plumbaginifolia.* Residues conserved in all four sequences are boxed. Asterisks indicate amino acid residues involved in the metal ligand binding. These residues are conserved between FeSOD and MnSOD (see Fig. 4). Residues marked with diamonds indicate primary candidates for distinguishing the identity of the metal ligand (Fe or Mn) bound by the SOD enzyme (8, 24, 34). Gaps (represented by dashes) have been introduced into the sequence to maintain optimal alignment.

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PhoB    ....MLPKIA VVEDEEALSV LLRYNLEAEG FEVDTILRGD EAEIRLQERL
                 *   **^*^       ***^ ** **    *       ^   **    *^  *
ORF131  MQTLAQMDIL VVDDDPDTRD LLRFMLEDEG AIVTVAPNAK EALSLLEREL  50
              ** ****#*    *^  **    * ^^*    *    ^**  ^    ** **
OmpR    ..MQENYKIL VVDDDMRLRA LLERYLTEQG FQVRSVANAE QMDRLLTRES

PhoB    PDLLILDWML PGVSGIELCR RLRQRPETER LPIIMLTARG EESERVRGLA
        * **^ *   ^ *^^ *^**   *^*^ *  *  ** * *** ^   * ^*   ^*
ORF131  PKLLVSDVAM PEMDGFELIG RVRELPKGET LPAIALTAYA REEDRQAALR  100
          *^* *^ ^  *^ **^ ^    *^*     ^^  ^* * ^** ^   * **   ^*
OmpR    FHLMVLDLML PGEDGLSICR RLR..SQSNP MPIIMVTAKG EEVDRIVGLE

PhoB    TGADDYVVKP FSTPEL...M ARVKAMLRRA KPEVL...
         * ^**^ **      **    ^    *          *^
ORF131  SGFNDYLTKP VDPLEL...I RLVQQYCLAF PPDA*  132
         * ^**^ **   ^* **    * * *           *^*
OmpR    IGADDYIPKP FNPRELLARI RAVLRRQANE LPGAP...
```

FIG. 7. Amino acid sequence comparison of *P. boryanum* UTEX 485 ORF131 with OmpR of *E. coli* and PhoB of *Rhizobium meliloti.* Asterisks indicate identical amino acid residues, while carets indicate conserved amino acid residues. The two invariant aspartic residues important for activation by phosphorylation (3) are in boldface.

**Isolation of an MV-resistant mutant.** Exposure of cyanobacteria to MV preferentially enhances the production of $O_2^-$ radicals. Previous work (28) had shown that a cyanobacterial strain lacking FeSOD was hypersensitive to MV. Thus, one possible mechanism of MV resistance could be altered levels of SOD expression. To test this hypothesis, *P. boryanum* UTEX 485 cells were mutagenized by using NTG and spread on petri plates containing 500 nM MV. Previous platings using wild-type cells had determined that the lethal threshold of MV was 100 nM; therefore, any cells able to remain viable on these plates would be resistant to MV in some way. Four viable colonies were obtained and maintained on MV plates up to a concentration of 1,000 nM.

**Characterization of SOD gene transcripts.** RNA gel blot hybridization experiments were undertaken to determine if all four SOD genes were transcribed constitutively, or if they were differentially regulated in response to various environmental conditions. RNA was isolated from cells grown under 15 different conditions (described below) and the RNA gel blot hybridization results for 9 of these conditions are presented in Fig. 8. Unless otherwise stated, the cells were grown with an irradiance of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 29°C and were bubbled with filtered, humidified air (lane 1). Cells were also grown bubbled with air augmented with 5% $CO_2$ since the increased concentration of $CO_2$ may enhance the Calvin cycle, thereby increasing the abundance of $NADP^+$ available as an electron acceptor at PSI (lane 2). Fluctuations in $CO_2$ levels have previously been shown to affect SOD activity in cyanobacteria (1). The addition of MV to cells was used to induce the production of $O_2^-$ and acted as a control for increased oxidative stress within the cell (lane 3). Cyanobacteria often alter their gene expression in response to environmental stresses such as nutrient deprivation, and several nutrient stresses were therefore tested. Iron stress, which has been shown to alter *E. coli* SOD gene expression, was carried out by growing cells in media with an available iron concentration of $10^{-21}$ M in the absence (lane 4) and presence (lane 5) of external $O_2$. RNA was also isolated from cells which were grown in the absence of nitrate (lane 7), sulfate (results not shown), and carbonate (results not shown). Since ferric ammonium citrate was replaced with $FeCl_3$ (final concentration, $10^{-17}$ M available iron) as the iron source in nitrate-starved cells, cells grown with nitrate and $FeCl_3$ (lane 6) were also analyzed as a control for the nitrate-starved cells. The ability of increased levels of exogenous $H_2O_2$ to affect SOD transcript levels was also monitored (lane 8). An MV-resistant mutant strain of *P. boryanum* UTEX 485 was also tested for changes in SOD mRNA levels (lane 9). To lower internal $O_2$ levels, cells were bubbled with 95% $N_2$–5% $CO_2$ (results not shown). This results in a microaerobic environment within the cell, since the only oxygen present in the cell is that produced in the water-splitting reaction of photosynthe-

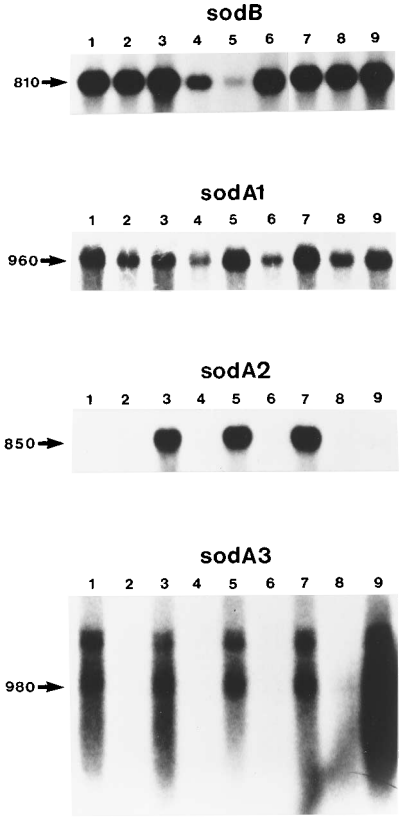


FIG. 8. RNA blot hybridizations of total cellular *P. boryanum* UTEX 485 RNA, using gene-specific radiolabeled probes for *sodB*, *sodA1*, *sodA2*, and *sodA3*. Sizes of transcripts, indicated by arrows, are in bases. Growth conditions were as follows: cultures bubbled with air (lane 1); cultures bubbled with 5% $CO_2$–95% air (lane 2); cultures grown in BG-11 with a final concentration of 100 nM MV for 4 h (lane 3); cultures grown in $10^{-21}$ M available iron medium bubbled with 5% $CO_2$–95% $N_2$ (lane 4); cultures grown in $10^{-21}$ M available iron medium bubbled with air (lane 5); cultures grown in $10^{-17}$ M available iron (lane 6); cultures grown in nitrate-deficient medium with $10^{-17}$ M available iron (lane 7); cultures grown in medium containing 25 $\mu M$ $H_2O_2$ (lane 8); cultures of a *P. boryanum* UTEX 485 mutant resistant to 1,000 $\mu M$ MV (lane 9).

sis. To test the role of SOD as a protective measure during nitrogen fixation, cells were grown under microaerobic conditions in medium lacking nitrate (results not shown). Under these conditions, the cells initially bleached slightly but recovered when the nitrogen-fixing enzymes were induced and began to fix atmospheric nitrogen. It has been previously shown that high-light conditions often induce photooxidative conditions in cyanobacteria (2), and high-light conditions (350 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were therefore used in an attempt to induce one or more of the SOD genes (results not shown). In contrast, low-light conditions (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were also tested, since they may result in reduced photoxidative stress and therefore a decrease in SOD levels in the cell (results not shown).

The results presented in Fig. 8 indicate that *sodA1* and *sodB* were constitutively transcribed. The only exception was cells grown under iron stress conditions (lanes 4 and 5), in which the levels of *sodB* transcripts appeared to be significantly reduced. Slight decreases in transcript levels were also observed in the *sodA1* mRNA levels from cells grown under iron stress in the absence of external $O_2$ (lane 4) and in the presence of $10^{-17}$ M available iron (lane 6).

As shown in Fig. 8, significant levels of *sodA2* mRNA were detected only in cells which were treated with MV (lane 3) or grown under conditions of iron (lane 5) or nitrate (lane 7) stress. Although *sodA2* appears to be induced by iron stress, transcripts were not detected when cells were grown in iron-deficient medium under microaerobic conditions (lane 4). Very low levels of *sodA2* mRNA were detected in air-grown cells (lane 1), but not $CO_2$-grown cells (lane 2), when autoradiograms were overexposed.

The results presented for *sodA3* are inconclusive. No distinct transcripts were seen in the lanes which hybridized to the probe, although a weak signal was visible at approximately 980 bases (Fig. 8). The bands larger than 980 bases are probably due to rRNA shading. Despite the lack of distinct bands, it appeared that *sodA3* transcripts accumulate under specific growth conditions, including those which affect *sodA2* transcription. Interestingly, the *sodA3* transcript is most abundant in the MV-resistant mutant (lane 9).

Conditions for which the data are not shown in Fig. 8 (sulfate and carbonate stress, high and low light, microaerobic and nitrogen fixation conditions) all exhibited transcript levels similar to those of control cells (lane 1) for all four SOD genes.

## DISCUSSION

It has been previously shown that *P. boryanum* contains both FeSOD and MnSOD enzyme activities (6). To further study the role that these enzymes play in protecting the cell from oxidative damage, studies involving SOD-deficient mutants need to be undertaken. *P. boryanum* represents a favorable system for these studies because of the ability to manipulate and inactivate genes in this organism (12). To construct mutant strains, however, clones of each of the genes of interest must first be isolated and characterized. Using an oligonucleotide probe to a conserved region of SOD proteins, we have cloned four SOD genes from *P. boryanum* UTEX 485; amino acid analysis has been used to tentatively identify one gene that encodes an FeSOD and three genes encoding MnSODs. The isolation of three MnSOD genes from *P. boryanum* UTEX 485 is unique, since no other examples of multiple isozymes of SOD have been reported in prokaryotes. The only other report has been the three Cu/ZnSOD isozymes encoded by three nonallelic genes in maize (36).

Both *sodB* and *sodA1* exhibited constitutive expression under most conditions tested, indicating that both are required to scavenge superoxide radicals produced by normal cell metabolism. However, under iron-limiting conditions (Fig. 8, lanes 4 and 5), *sodB* transcripts appear to be markedly reduced. Since iron is the metal cofactor required for FeSOD activity, the reduction in transcript levels likely represents a down regulation of *sodB* expression in response to the lack of available iron within the cell.

*sodA2* transcripts were barely detected in cells grown in complete BG-11 medium bubbled with air, indicating that high levels of SodA2 are not required to scavenge superoxide radicals produced by normal cell metabolism. However, when cells were exposed to MV, *sodA2* was induced to high levels (Fig. 8, lane 3). This finding indicates that SodA2 is regulated in response to increased oxidative stress within the cell. It was also induced to high levels under conditions of iron limitation (lane 5) and nitrate limitation (lane 7). The induction of *sodA2* mRNA in iron stress cells is not completely unexpected. Since the levels of *sodB* mRNA are reduced under iron deprivation, it is possible that *sodA2* is induced to compensate for the loss in FeSOD activity. Additionally, ferredoxin, an iron-sulfur protein which receives electrons from PSI, cannot be synthesized at normal levels under iron-limiting conditions (15). Although it has been shown that cyanobacteria can produce flavodoxin to replace ferredoxin under these conditions, this replacement may result in the increased transfer of electrons to oxygen. This would cause an increase in the production of $O_2^-$ and cause the cell to increase the levels of *sodA2* mRNA. Although iron limitation induces *sodA2* transcripts to high levels, microaerobic conditions nullify this result (Fig. 8, lane 4). This finding indicates that multiple factors are involved in regulating *sodA2* transcription. This is similar to the regulation of *sodA* in enteric bacteria. In *E. coli*, the *sodA* gene is regulated by a complex series of at least six global regulators (10), including ArcA and Fur, either of which is able to bind to the promoter region of *sodA* and repress transcription. Under iron-limiting conditions, Fur repression is removed and *sodA* is transcribed. Similarly, ArcA repression is removed under aerobiosis. The results from our RNA gel blot hybridization studies indicate that a similar system may function in *P. boryanum* UTEX 485. Fur binds to a DNA moiety termed the iron box which overlaps the −35 consensus sequence upstream of *sodA* in *E. coli* (22). No obvious iron box was observed upstream of *P. boryanum sodA2*, although the iron box may not be required since Fur regulation of *sodB* in *E. coli* has been shown to occur in the absence of an iron box (22).

The induction of *sodA2* under nitrate-limiting conditions cannot be easily explained. Under normal conditions, reducing power produced at PSI is used not only to fix $CO_2$ but also to reduce nitrate or nitrite. However, in the absence of these combined nitrogen sources, the excess reducing power could be used to reduce $O_2$, resulting in the production of $O_2^-$ and hence inducing the production of *sodA2*. Alternatively, cells which are nitrogen limited could also be under increased oxidative stress since the cells are energetically restricted by the lack of nitrogen metabolism. It should be noted that cells grown under 95% $N_2$–5% $CO_2$ in the absence of nitrate are able to fix dinitrogen and do not show an increase in the levels of *sodA2* mRNA. This finding indicates that either the presence of a microaerobic environment or the ability to fix dinitrogen is sufficient to prevent the accumulation of *sodA2* mRNA in nitrate-starved cells.

RNA blot hybridization experiments using *sodA3* as a gene-specific probe did not produce discrete RNA bands. Instead, a smearing pattern was observed in the hybridization experiments. However, since hybridization was observed for RNA

isolated under certain conditions and not for others, it was assumed that this hybridization was specific for *sodA3*. The lack of a discrete banding pattern could indicate that the half-life of the *sodA3* transcript is very short, and degradation products are therefore observed. The results indicated that *sodA3* is induced under the same conditions as *sodA2*, suggesting similar regulatory mechanisms. However, the *sodA3* transcript was also detected in control cultures bubbled with air (Fig. 8, lane 1). A puzzling result was that *sodA3* transcripts were not detected in cells grown with $FeCl_3$ (lane 6) or in the presence of $H_2O_2$ (lane 8). The only difference in the media used to grow the cells in lane 6 was the substitution of $10^{-17}$ M ferric chloride for the ferric ammonium citrate present in the media used to grow the cells in lane 1. This was done to confirm that the results observed for the nitrogen-deficient cells was due to the lack of nitrogen and not the concentration or type of iron used. Cells used to isolate RNA in lane 8 were grown under the same conditions as the control cells (lane 1) except that $H_2O_2$ was added to the medium. Thus, there is no apparent explanation for the discrepancy in the appearance of *sodA3* transcripts in lane 1 and not in lane 6 or 8.

*sodA3* transcripts appeared to be induced to their highest levels in the MV-resistant mutant strain (Fig. 8, lane 9). Although our evidence does not rule out additional mechanisms for MV resistance, the increase in the accumulation of *sodA3* mRNA likely plays a large role in MV resistance in *P. boryanum* UTEX 485. The isolation of an MV-resistant mutant containing increased MnSOD activity provides evidence that the genetic manipulation of antioxidant defense systems is a viable strategy to increase tolerance to oxidative stress conditions in photosynthetic organisms.

Downstream of *sodA1*, on the complementary strand, is a 131-amino-acid ORF which exhibits similarity to OmpR and other related proteins (21) (Fig. 7). OmpR and PhoB are members of a family of regulatory proteins that are involved in bacterial two-component regulatory systems. In general, these regulatory components consist of two domains. The N domain is thought to receive the signal from the sensor component and has been shown to be conserved for the majority of regulators. Most regulators that function as transcriptional activators can be grouped into subclasses on the basis of homology within their C domains (3). The C domains of these regulators are thought to be responsible for the target response, in the case of OmpR, for example, transcriptional activation by DNA binding (21). However, the sequence of ORF131 is not long enough to contain a C domain homologous to that of OmpR or related proteins. The role of ORF131, if any, in regulating SOD gene expression remains to be elucidated.

The discovery of four SOD genes in *P. boryanum* UTEX 485 represents a unique opportunity to study the regulation of the cellular response to oxidative stress conditions in cyanobacteria. Gene manipulation strategies will allow the construction and investigation of SOD gene-specific mutants, providing information on the regulation and sites of action of each SOD protein.

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