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Cloning and Expression of the Superoxide Dismutase Gene from the Obligate Anaerobic Bacterium *Desulfovibrio vulgaris* (Miyazaki F)

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We identified the SOD gene in the obligate anaerobic bacterium *Desulfovibrio vulgaris* (Miyazaki F) and constructed a high-level expression system in *Escherichia coli*. A 2.6-kbp DNA fragment isolated from *D. vulgaris* (Miyazaki F) by double digestion with *Eco*RI and *Sma*I contained the SOD gene and part of another open reading frame. The amino acid sequence deduced from the SOD gene, which was composed of 238 amino acid residues, showed high homogeneity with iron-containing SOD (Fe-SOD) and predicted that the amino terminus of this protein would carry an export signal peptide. We produced the precursor form of SOD (PSOD) and the mature form of SOD (MSOD), which lacked the putative signal peptide. In *E. coli*, PSOD was present in insoluble inclusion bodies, and its putative signal peptide was not cleaved. In contrast, MSOD contained one iron per mononer and formed a dimer, which exhibited an SOD activity of 850 U/mg. Furthermore, *D. vulgaris* soluble extract showed a band of SOD activity on native polyacrylamide gel that migrated to the same point as MSOD. The intracellular localization of SOD and its role in *D. vulgaris* are also discussed.

Key words: obligate anaerobe, oxidative stress, recombinant, sulfate-reducing bacteria, superoxide dismutase.

Abbreviations: ICP-AE, inductively coupled plasma-atomic emission; ORF, open reading frame; SOD, superoxide dismutase; SRB, sulfate-reducing bacteria.

Sulfate-reducing bacteria (SRB), which were formerly classified as obligate anaerobes, were long believed to be incapable of surviving under aerobic conditions. These organisms were thought to lack a protective system against oxygen toxicity, which usually includes enzymes such as SOD and catalase, but evidence that they possess both enzymes has been reported (*1–3*). Some SRB were observed to live at the oxic-anoxic interface rather than under perfectly anoxic conditions (*4*). Recent studies have indicated that some strains of *Desulfovibrio* and other sulfate reducers possess a degree aerotolerance (*5–7*). It has been also reported that *Desulfovibrio* species possess an oxygen-reducing system coupled with ATP production (*8*), and their respiration rates are higher than those of aerobic bacteria (*9*). However, although these species are capable of energy-coupled respiration with oxygen, their aerobic growth is absent or poor. When *Desulfovibrio* species were exposed to oxygen, they did not simply migrate to the anoxic regions but accumulated in air saturation concentrations of up to 20% (*10*). Thus, they exhibit both positive and negative responses to oxygen. Oxygen reduction in SRB may play a role in removing oxygen from the environment rather than for energy conservation.

SRB produce substantial amounts of toxic sulfides through reduction of sulfates, which result in sulfide pollution. Contamination of gas and oil, and corrosion of metal and concrete are major problems for the oil and gas industries as well as in the process of sewage treatment. Controlling the activity and growth of SRB is necessary, and the simplest method for this is exposure to air. Therefore, the study of defense mechanisms against oxygen toxicity in SRB is considered important.

An alternative system for reducing oxygen toxicity involving superoxide reductase (SOR) was recently proposed (*11*). SOR, which is only found in anaerobic or microaerophilic organisms, was shown to catalyze the reduction of superoxide to hydrogen peroxide without producing molecular oxygen. Desulfoferrodoxin (Dfx) from SRB is highly homologous with SOR and exhibited superoxide reductase activity (*12*). Defense mechanisms against oxygen toxicity involving SOD and catalase are well known in aerobes. However, molecular characterization of SOD from obligate anaerobic bacteria has not been conducted, and the distinction between the physiological roles of SOD and SOR remains unclear. It was recently reported that the SOD gene from *Desulfovibrio vulgaris* (Hildenborough) had been cloned and sequenced. The deduced amino acid sequence suggested that this protein carries an export signal peptide, and thus SOD was localized in the periplasm. However, active SOD from *D. vulgaris* could not be expressed in *E. coli* (*13*).

To understand the defense mechanisms against oxygen toxicity in anaerobes, we previously characterized a cata-

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Fig. 1. **Nucleotide sequence of the approximately 2.6-kbp *Eco*RI–*Sma*I DNA fragment from *D. vulgaris* (Miyazaki F).** Amino acid sequences deduced from two possible ORFs are shown below the respective nucleotide sequences. Putative sequences serving as promoter regions (–35 region and –10 region) and the ribosome binding site are underlined. The putative transcriptional terminator is shown with arrows. The putative initiation codon that we initially suspected is boxed (see "DISCUSSION").

lase from *D. vulgaris* (Miyazaki F), which functioned as a protective enzyme against hydrogen peroxide (*3*). We now report the molecular cloning of the SOD gene from *D. vulgaris* (Miyazaki F) as well as overproduction and characterization of SOD expressed in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Materials—*E. coli* JM109 (*rec*A1, *sup*E44, *end*A1, *hsd*R17, *gyr*A96, *rel*A1, *thi*, Δ(*lac-proAB*), F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) was used for cloning and expression of the SOD gene. *D. vulgaris* (Miyazaki F) was grown (*14*) and used for genomic DNA preparation. Plasmids pUC18 and the pMK2 (*15*), which are expression vectors carrying the *tac* promoter, were used. Restriction enzymes and modification enzymes were purchased from New England Biolabs, Nippon Gene and Toyobo. [γ-^{32}P]ATP (185 TBq/mmol) was obtained from ICN. Cytochrome *c*, xanthine sodium salt, xanthine oxidase and nitroblue tetrazolium (NBT) were obtained from Sigma. All other reagents were of the highest purity available.

Cloning and Sequencing of SOD Gene—Genomic DNA was prepared from *D. vulgaris* (Miyazaki F) by the method of Saito and Miura (*16*). DNA probe 5′-GACGTATGGGAGCACGCCTACTATCTCGACTAC-3′ (33 mer) was synthesized corresponding to the amino acid sequence D-V-W-E-H-A-Y-Y-L-D-Y, which is the conserved amino acid sequence of Fe-SOD and Mn-SOD from other bacteria (see Fig. 2). Genomic DNA fragments, digested with *Eco*RI and *Sma*I, were separated by agarose gel electrophoresis. Southern blot hybridization with ^{32}P-labeled probes was carried out, and the band hybridizing with the ca. 2.6-kbp *Eco*RI–*Sma*I fragment was detected using a BAS1000 image analyzer (FUJIX). The approximately 2.6-kbp *Eco*RI–*Sma*I fragment extracted from the agarose gel was ligated into the same site of pUC18, and *E. coli* was transformed with the ligation mixture. The plasmid pTN, containing the 2.6-kbp *Eco*RI–*Sma*I fragment, was screened by colony hybridization. The nucleotide sequence of the 2.6-kbp *Eco*RI–*Sma*I fragment was determined by sequencing the restriction fragments subcloned into pUC18 as well as the deletion mutants obtained with exonuclease III and mung bean nuclease. Nucleotide sequencing was performed with a Big-Dye terminator cycle sequencing kit on an ABI 310 sequencer (Perkin Elmer Applied Biosystems).

Construction of Expression Vectors—We constructed a high-level expression system in *E. coli* by connecting a strong promoter (*tac* promoter) to the upstream region of the SOD gene. Plasmid pTN was initially digested with *Eco*RI, blunt-ended with Klenow fragment and then digested with *Dra*I. We ligated an approximately 1.0-kbp fragment containing the SOD gene into pUC18 previ-

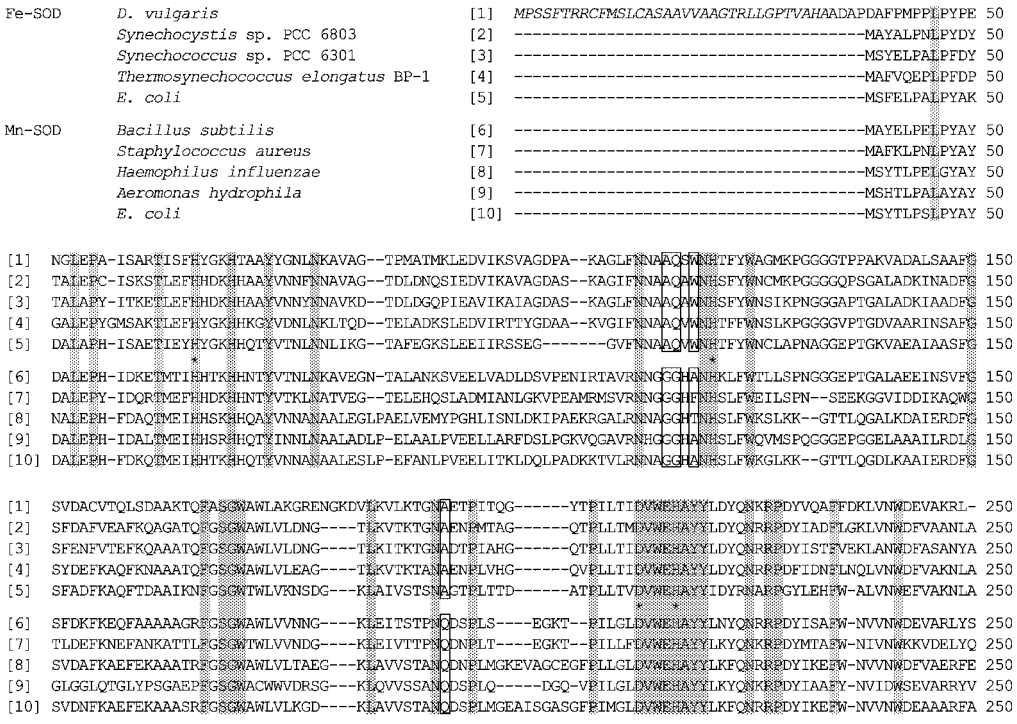

Fig. 2. **Alignment of amino acid sequences deduced from the *D. vulgaris* SOD gene and other SOD genes.** Amino acid sequences of SODs from *Synechocystis* sp. PCC 6803 (*22*), *Synechococcus* sp. PCC 6301 (*23*), *Thermosynechococcus elongatus* BP-1 (*24*), *E. coli* (accession no. J03511 and X03951), *Bacillus subtilis* (accession no. D86856), *Staphylococcus aureus* (accession no. AF273269), *Haemophilus influenzae* (accession no. X73832), and *Aeromonas hydrophila* (accession no. AF317226) are shown. Sequence alignment was carried out using the CLUSTAL W (version 1.8) program. Amino acid residues conserved in all sequences are shadowed. Asterisks show amino acids involved in metal binding. Amino acid residues that characterize Fe-SOD or Mn-SOD are boxed (see text). The italic letters in the amino acid sequence deduced from *D. vulgaris* SOD gene indicate the putative signal peptide.

ously cut with *Sma*I. This plasmid (pTN1000) was used as a template in the PCR below. Nucleotide sequences of the PCR primers were as follows: Super8 was 5′-GGAATTCATATGCCGTCCAGCTTCACC-3′ (27 mer); Super9 was 5′-TGAATTCCATGGCCGACGCCCCCGACGCC-3′ (29 mer); and Super10 was 5′-ACTAAGCTTCTACAACCGCTTGGCCAC-3′ (27 mer). PCR was carried out for 30 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min. The PSOD gene was amplified by PCR using pTN1000 as a template and the primers Super8 and Super10. PCR products were digested with *Eco*RI and *Hin*dIII and ligated into the same site of pUC18. The cloned fragment was sequenced, digested with *Eco*RI and *Hin*dIII, and then ligated into the same site of pMK2 to give the expression vector pMKPSOD. The MSOD gene was prepared according to a similar procedure using the primers Super9 and Super10. The resulting expression vector was designated pMKMSOD.

Expression and Purification of Recombinant SODs—*E. coli* was transformed with pMKPSOD, and transformants were grown in 1.7 ml of Luria-Bertani (LB) medium containing 50 μg/ml ampicillin for 9 h at 37°C. Six flasks containing 167 ml of the same medium were inoculated with 1.7 ml of the culture and incubated overnight with agitation at 37°C. Cells were harvested by centrifugation at 6,000 rpm for 10 min. The cell pellet was suspended in 10 mM Tris-HCl buffer (pH 8.0). The cell suspension was sonicated with a Model 201M sonicator (KUBOTA) at 9,000 Hz, 200 W for 15 min, then centrifuged at 10,000 rpm for 1 h. After removing the membranes and cell debris, the supernatant was ultracentrifuged at 45,000 rpm for 2 h. The supernatant was then dialyzed against 10 mM Tris-HCl buffer (pH 8.0). Expression of MSOD gene was also carried out using this procedure.

For MSOD purification, dialysate was loaded onto a DEAE-cellulose (DE52) column (2.1 × 10.0 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was washed with 100 ml of 10 mM Tris-HCl buffer (pH 8.0), and MSOD was eluted with a linear gradient of NaCl from 0 mM to 100 mM in a total volume of 300 ml of 10 mM Tris-HCl buffer (pH 8.0). MSOD, which was weakly adsorbed by this column, was dialyzed against 10 mM potassium acetate buffer (pH 5.5). Dialysate was loaded onto a CM-cellulose (CM52) column (2.1 × 10.0 cm) equilibrated with 10 mM potassium acetate buffer (pH 5.5). The column was washed with 100 ml of 10 mM potassium acetate buffer (pH 5.5), and MSOD was eluted with a linear gradient of NaCl from 0 mM to 200 mM in a total volume of 300 ml of 10 mM potassium acetate buffer (pH 5.5). The fraction containing MSOD was concentrated with a Centriprep YM-10 (Millipore). MSOD was loaded onto a gel filtration column (Superdex 75 HR10/

30) equilibrated with 200 mM NaCl/10 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 ml/min. Eluted MSOD was dialyzed against 10 mM Tris-HCl buffer (pH 8.5) and concentrated again. Anion-exchange chromatography (TSK gel DEAE-5PW; Tosoh) was then carried out using a Shimadzu HPLC system. The column was washed with 10 mM Tris-HCl buffer (pH 8.5) for 10 min at a flow rate of 0.5 ml/min. The column did not adsorb the purified MSOD, and the fraction was collected and concentrated.

SOD Activity Measurement—SOD specific activity was determined using the standard xanthine oxidase/cytochrome *c* assay at pH 7.8 as described by McCord and Fridovich (*17*). The increase in absorbance at 550 nm was recorded at 25°C with a Beckman DU-640 spectrophotometer. SOD activity was also visualized in 7% nondenaturing polyacrylamide gel by nitroblue tetrazolium activity staining, as described by Beauchamp and Fridovich (*18*). To perform the inhibition study, 5 mM H_2O_2 was added during incubation in 2.45 mM NBT (*19*).

Analytical Methods—Protein concentration was determined by the method of Bradford (*20*). SDS–PAGE was carried out by the method of Laemmli (*21*). Molecular weight of MSOD in the native state was determined by gel filtration chromatography. Molecular weight markers were bovine serum albumin (66,000), carbonic anhydrase (29,000) and horse heart cytochrome *c* (12,400). UV-visible absorption spectrum was recorded using a Hitachi U-3000 spectrophotometer. Metal contents were determined by inductively coupled plasma-atomic emission analysis using a Shimadzu ICPS-1000III.

RESULTS

Nucleotide Sequence of SOD Gene—In order to identify the SOD gene, a ca. 2.6-kbp *Eco*RI–*Sma*I fragment of *D. vulgaris* (Miyazaki F) was cloned and sequenced. The results are shown in Fig. 1. In this fragment, we found an ORF encoding a protein composed of 238 amino acid residues. A potential ribosome binding site (AGGAGA) from nucleotides 265 to 270 in *Eco*RI-*Sma*I fragment was present upstream of the putative initiation codon (ATG), while possible promoter regions from nucleotides 72 to 77 (TTGCAC) and 94 to 99 (TATGGT) in the *Eco*RI–*Sma*I fragment resembled the *E. coli* –35 and –10 regions. Nucleotides 1013 to 1051 comprised the putative transcriptional terminator, forming a stem-and-loop structure. The result of a BLAST homology search indicated that the product of this ORF was highly homologous with Fe-SOD from other bacteria. The deduced amino acid sequence displayed 62, 58, and 55% homology with the Fe-SOD from *Synechocystis* sp. PCC 6803 (*22*), *Synechococcus* sp. PCC 6301 (*23*) and *Thermosynechococcus elongatus* BP-1 (*24*), respectively. We therefore confirmed that this ORF was an SOD gene. In addition, a partial ORF was found at the 3′-terminal region of this fragment, and the deduced amino acid sequence displayed 31% homology with the carboxyl terminus of phosphoenolpyruvate synthase from *Staphylothermus marinus* (*25*), which is involved in gluconeogenesis.

Amino Acid Sequence Comparison—The deduced amino acid sequences of the *D. vulgaris* SOD gene and other SOD genes are compared in Fig. 2. Because the amino acid sequences of Fe-SOD and manganese-containing

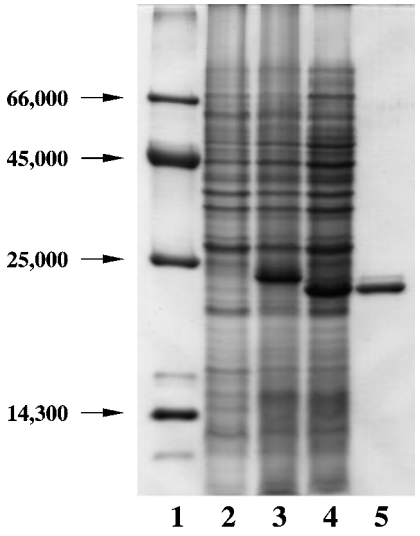

Fig. 3. **Analysis of recombinant SODs by SDS–PAGE.** Gel concentration was 15%. Lane 1, molecular weight marker: bovine serum albumin (66,000), ovalbumin (45,000), α-chymotrypsinogen A (25,000), lysozyme (14,300); lane 2, pMK2/JM109 (negative control); lane 3, pMKPSOD/JM109; lane 4, pMKMSOD/JM109; and lane 5, purified MSOD.

SOD (Mn-SOD) are homologous and the four metal-binding sites (positions 66, 122, 214, and 218 in Fig. 2) are identical, it is difficult to determine which metal is bound based only on the amino acid sequence. The crystal structure of Fe-SOD from *E. coli* indicates that the four metal-binding sites are conserved at the active site (*26*). Comparison of the crystal structure of Fe-SOD and Mn-SOD from *E. coli* shows high correspondence in the active site (*27*). However, several residues (positions 117, 118, 120, and 193 in Fig. 2) that differ between Fe-SOD and Mn-SOD have been reported to be useful in distinguishing them (*28*). The amino acid sequence encoded by the *D. vulgaris* SOD gene contained residues typical of Fe-SOD, suggesting that the product may be classified as an Fe-SOD. Furthermore, we observed a possible signal peptide at the amino terminus of this protein. Analysis of the amino acid sequence using SignalP (http://www.cbs.dtu.dk/services/SignalP/) and PSORT (http://psort.nibb.ac.jp/) programs predicted that this protein would carry an export signal peptide of 34 amino acid residues at the amino terminus, and implied that this product would be localized in the periplasm.

Expression and Molecular Weight of Recombinant SODs—We constructed *E. coli* expression systems of the PSOD gene and the MSOD gene, which lacked the 34 amino terminus residues. Both PSOD and MSOD were detected in transformed *E. coli* crude cell extract by SDS–PAGE (Fig. 3, lanes 3 and 4). However, PSOD was in an insoluble form in this study. The PSOD gene was also cloned into expression vector pUT7, carrying a T7 promoter (*29*), but in this case, PSOD was not detected by SDS–PAGE. The molecular weight of expressed PSOD was estimated to be about 22,700 by SDS–PAGE, which was close to the value (25,500) calculated from the amino acid sequence. These results indicated that PSOD was not digested at the signal peptide processing site in *E.*

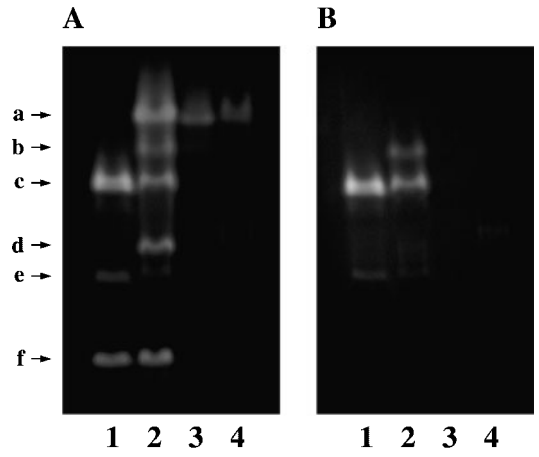

Fig. 4. **(A) Activity staining analysis of SODs. (B) As A but treated with 5 mM H_2O_2; lane 1, pMK2/JM109; lane 2, pMKM-SOD/JM109; lane 3, purified MSOD; and lane 4, *D. vulgaris* soluble extract.** Arrows indicate positions of (a) MSOD, (b) MSOD–*E. coli* Mn-SOD heterodimer, (c) *E. coli* Mn-SOD, (d) MSOD–*E. coli* Fe-SOD heterodimer, (e) *E. coli* MSOD–*E. coli* Fe-SOD heterodimer and (f) *E. coli* Fe-SOD, respectively.

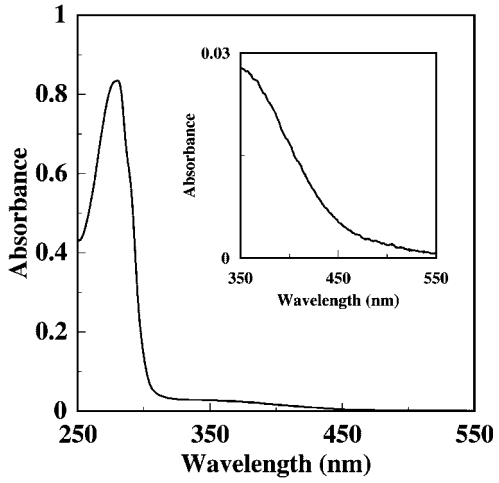

Fig. 5. **UV-visible absorption spectrum of purified MSOD.** The absorption spectrum was recorded in 10 mM Tris-HCl (pH 8.5) at room temperature. The insert shows magnification of absorbance between 350 and 550 nm.

coli. In contrast, MSOD was present in the soluble fraction of cell lysates and showed SOD activity. After the purification using HPLC, MSOD showed a specific activity of 850 U/mg. The molecular weight of MSOD was estimated to be about 21,300 by SDS–PAGE (Fig. 3, lane 5), which was close to the value (22,200) calculated from the amino acid sequence. We also estimated the molecular weight in the native state to be about 43,500 using a Superdex 75 gel filtration column. The value was almost twice that calculated from the amino acid sequence, indicating that the native form of MSOD is a dimer.

Detection of SOD Activity on Native Polyacrylamide Gel—The soluble extract of *E. coli* transformed with pMKMSOD showed an additional three bands of SOD activity on native polyacrylamide gel (Fig. 4A, lane 2, arrows a, b and d), where only three bands were detected in the soluble extract of *E. coli* transformed with pMK2 (Fig. 4A, lane 1, arrows c, e and f). To identify these bands, we investigated the sensitivity of SOD toward hydrogen peroxide, which inhibits Fe-SOD but does not inhibit Mn-SOD (*30*) (Fig. 4B). Of the bands tested, three bands were sensitive to hydrogen peroxide (Fig. 4, arrows a, d and f) but the other bands were insensitive or slightly sensitive (Fig. 4, arrows b, c, and e). *E. coli* Fe-SOD and Mn-SOD were known to form a heterodimer, and then hybrid SOD is resistant to hydrogen peroxide (*30*) (Fig. 4, arrow e). Based on these results, arrow a is the MSOD homodimer, arrow b is the MSOD–*E. coli* MnSOD heterodimer and arrow d is the MSOD–*E. coli* FeSOD heterodimer, respectively. On the other hand, the *D. vulgaris* soluble extract showed only one band of SOD activity, which was sensitive to hydrogen peroxide (Fig. 4, panels A and B, lane 4). After the gel filtration chromatography using Superdex 75, MSOD showed a single band on SDS–PAGE. However, SOD activity staining on native PAGE demonstrated that MSOD was separated into three bands. Therefore, further purification was performed by anion-exchange chromatography using an HPLC system. MSOD then showed a single band of SOD activity on native PAGE, thus confirming the purity of this fraction (Fig. 4A, lane 3). The purified MSOD and *D. vulgaris* soluble extract also showed bands of SOD activity that migrated to the same point (Fig. 4A, arrow a, lanes 3 and 4, respectively).

Spectroscopic Property and Metal Analysis of MSOD—The UV-visible absorption spectrum of MSOD is shown in Fig. 5. The absorption maximum was observed at 280 nm and the absorption coefficient at 280 nm was 1.84 ml mg^{-1} cm^{-1}. In the visible region, absorption was reduced gradually through 500 nm. This spectrum was similar to that of *E. coli* Fe-SOD (*31*) and did not show a specific peak corresponding to *E. coli* Mn-SOD at 473 nm (*32*). To quantify bound metals, ICP-AE analysis was performed, and the results are summarized in Table 1. The results indicated that MSOD contained 0.96 iron atoms per monomer, and no manganese was detected. The molar ratio of bound iron to MSOD was almost 1:1.

Table 1. **Metal analysis of MSOD by ICP-AE.**

	Analyzed concentration (μM)	Ratio
Peptide portion	0.50	1
Iron	0.48	0.96
Manganese	not detected	0
Zinc	not detected	0

DISCUSSION

The gene encoding SOD was cloned and expressed in *E. coli*. The amino acid sequence deduced from the SOD gene was highly homologous with Fe-SOD and the ORF carried a putative signal peptide in the amino terminus. The signal sequence contained a twin-arginine consensus motif, and the twin-arginine translocation (Tat) system is known to export the folded protein, which contains a cofactor, across the cytoplasmic membrane (*33*). A Tat

targeting signal was also found in the periplasmic hydrogenase from *Desulfovibrio desulfuricans* (*34*). The identity of the amino acid sequences between PSOD and *D. vulgaris* (Hildenborough) SOD (accession no. AF034841) was 85%. To construct an expression system for the SOD gene, we first referred to the nucleotide sequence data for the SOD gene from *D. vulgaris* (Hildenborough). Because the initiation codon of the SOD gene was noted in these data, we attempted to express the SOD gene in *E. coli* according to this information, but this resulted in failure (see Fig. 1). We then reconstructed the expression system and succeeded in expressing the SOD gene in *E. coli* using the newly identified initiation codon, which was located upstream of the ATG codon that was initially believed to be the initiation codon. However, PSOD was produced as an inclusion body in *E. coli* and its signal peptide was not digested. This may be because the Tat signal peptide is not recognized by different Tat systems (*35*). Therefore, we believe that the signal peptide in PSOD was not recognized by the *E. coli* Tat system.

In contrast, MSOD expressed in *E. coli* was in a soluble form and showed SOD activity. Purified MSOD, which possessed properties typical of an Fe-SOD, exhibited lower activity (850 U/mg) than *E. coli* Fe-SOD (2,470 U/mg) (*31*). The SOD activity of MSOD may be sufficient for protection against superoxide toxicity, because SRB generally grow in anaerobic conditions. The metal content detected by ICP-AE indicated that purified MSOD contained only a single iron. The metal binding selectivity may be high, in contrast to that of recombinant rubredoxin from *D. vulgaris*. Though native rubredoxin contains a non-heme iron, recombinant protein may contain an iron or a zinc (*36*).

The soluble cell extract of *D. vulgaris* had SOD activity and exhibited the same mobility as MSOD on native PAGE. Only one band of SOD activity was detected in *D. vulgaris*, while it is reported that two separate bands of SOD activity are detectable in *Desulfovibrio gigas* (*2*). Although it is not clear if there is a different SOD in *D. vulgaris*, SOD activity was detected at the same position as MSOD on native PAGE. This suggests that the SOD gene is expressed in *D. vulgaris* and that this SOD always acts as a protective enzyme against superoxide toxicity after the putative signal peptide is cleaved. Furthermore, it was shown that SOD activity in the periplasmic fraction of *D. vulgaris* was five times higher than that in the cytoplasmic fraction (*13*). These results indicated that the location of SOD in *D. vulgaris* is the periplasm and that we were able to produce the mature form of SOD in *E. coli*.

It was reported that the oxygen reduction activity of *Desulfovibrio* species is localized in the periplasmic fraction and that they have a strategy for maintaining anaerobic conditions in their habitats (*37*). When *Desulfovibrio* species are exposed to air, oxygen is removed through reduction in the periplasm, and reactive oxygen species may simultaneously be generated in the periplasm rather than in the cytoplasm. Therefore, SOD must play a main role as protective enzyme against superoxide toxicity in the periplasm. As SOD generates hydrogen peroxide, there must be catalases and peroxidases in the periplasm. Perhaps, NADH- and NADPH-peroxidases, which are contained in the periplasmic fraction, may act instead of catalases and peroxidases (*38*). *D. vulgaris* has both SOD and Dfx (also called 2Fe-SOR), which are commonly thought to scavenge superoxide anions, but their structural homology is uncertain. The defense system in the cytoplasm, including SOR, may be necessary in aerobic conditions. However, in microaerophilic conditions, the defense system against oxygen toxicity present in the periplasm is more important than that in the cytoplasm.

We produced recombinant SOD from a sulfate-reducing bacterium for the first time, and demonstrated that the recombinant SOD possessed similar properties to SODs from other bacteria. Because SOR has only been found in anaerobic or microaerophilic organisms, aerobic organisms may have selected SOD as a protective enzyme against superoxide toxicity during the evolutionary process. Although defense mechanisms against oxygen toxicity exist both in the periplasm and in the cytoplasm, it is still unclear why anaerobes generally cannot grow under aerobic conditions.

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