Cloning of Fe-superoxide dismutase gene from the diazotroph *Azotobacter vinelandii* and the stimulation of its expression under anaerobic conditions in *Escherichia coli*

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To reveal the expression control of iron-containing superoxide dismutase (Fe-SOD) gene (*sodB*), a 10-kbp DNA fragment containing *sodB* was cloned from the nitrogen-fixing bacterium *Azotobacter vinelandii* IFO12018 and sequenced. The *sodB* gene showed an open reading frame of 582 bp, encoding a protein having a molecular mass of 21,377 Da with 193 amino acid residues. The deduced amino acid sequence of the Fe-SOD showed high similarity with those of other bacterial Fe-SODs, especially with that of *Pseudomonas putida*. In the 10-kbp fragment, four annotated ORFs in addition to *sodB* were found. The gene arrangement of *A. vinelandii* resembled that of *P. putida* downstream of *sodB*, but no synteny was observed between *A. vinelandii* and *E. coli.*

***E. coli* harboring the plasmid pSK-HB4, inserted with a 4-kbp *Hin*dIII-*Bam*HI fragment containing *sodB*, expressed *A. vinelandii* Fe-SOD using its own promoter under anaerobic conditions. Oxygenic culture repressed the expression of *A. vinelandii* Fe-SOD in *E. coli*. Occurrence of two stem-loops and the A/U-rich motif in the 5'-UTR and coding region of *sodB* mRNA, required for control by the Fur (ferric uptake regulation) global regulatory system, suggests that *sodB* is under control of the Fur system in *A. vinelandii*. Anaerobic expression of *A. vinelandii sodB* in *E. coli* is discussed in terms of the Fur control.**

Key words: *Azotobacter vinelandii*, reactive oxygen species, *sodB*, superoxide dismutase, Fur.

INTRODUCTION

Superoxide dismutases (SODs) are metalloenzymes that remove superoxides ($O_2^-\cdot$) from cells by catalyzing their dismutation to hydrogen peroxide and oxygen (Fridovich 1995). These enzymes are indispensable to all aerobes and even some anaerobes to protect cells from the deleterious effects of reactive oxygen species (ROS), because the production of ROS within cells is inevitable in normal biological processes such as respiration. SODs are classified into four types according to the metals at active sites: Cu and Zn, Mn, Fe and Ni-containing SODs (Kanematsu and Asada 1994, Youn et al. 1996). Whereas Mn-SODs and Fe-SODs are homologous proteins, the amino acid sequences of CuZn-SODs are different from those of Mn- and Fe-SODs. Four types of SODs show different subcellular localization and expression control, indicating their respective specific physiological functions.

The regulation of SOD expression has been extensively studied in *Escherichia coli* containing Mn-SOD (Keele et al. 1970) and Fe-SOD (Yost and Fridovich 1973) in the cytosol encoded by *sodA* and *sodB*, respectively, and CuZn-SOD (Benov and Fridovich 1994) in the periplasm encoded by *sodC*, as a model system of bacterial response against environmental stresses including oxidative stress. The synthesis of Mn-SOD in *E. coli* is induced by superoxide. Hyperbolic oxygen and redox-cycling agents, such as methyl viologen, that elevate superoxide production also induce Mn-SODs (Fridovich 1995). *E. coli sodA* is controlled by the global transcriptional regulatory proteins such as Fur (ferric uptake regulation), ArcAB (aerobic respiratory control), Fnr (fumarate nitrate reduction) and SoxRS (superoxide response) (Iuchi and Weiner 1996). It has been shown that the *sodA* promoter contains potential binding sites for Fur, ArcA, Fnr and SoxS (Compan and Touati 1993). To the contrary, Fe-SOD was believed to be constitutive, but Niederhoffer et al. (1990) suggested that *E. coli sodB* was also controlled by Fur. While Fur is a transcriptional repressor that down-regulates the expression of target genes, *E. coli sodB* is positively regulated by Fur. Recent studies have shown the involvement of the regulatory small RNA RyhB (Massé and Gottesman 2002) and the RNA chaperon Hfq (Geissmann and Touati 2004) in indirect control of Fur for *sodB* expression in *E. coli.* Thus, the Fur global regulatory system controls the expression of the Fur regulon at the transcriptional, post-transcriptional (Dubrac and Touati

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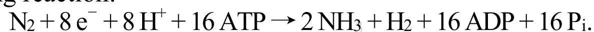
The abbreviations used are: amp, ampicillin; DIG, digoxigenin; Fur (ferric uptake regulation), *fur* gene product; *fur*, *fur* gene; IPTG, isopropyl-β-D-thiogalactoside; ORF, open reading frame; SOD, superoxide dismutase; *sodB*, Fe-SOD gene; tet, tetracycline; UTR, untranslated region; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

2002) and translational (Afonyushkin et al. 2005) levels.

Azotobacter vinelandii is a free-living nitrogen-fixing bacterium and an obligate aerobe. Nitrogen fixing processes require a large amount of reducing equivalent and ATP supplied by respiration according to the following reaction:



$N_2 + 8\,e^- + 8\,H^+ + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$.

Since nitrogenase is extremely oxygen-labile, the concentration of oxygen inside the cells must be extremely low or near zero for nitrogen fixation. The apparent paradox for nitrogen fixation of *A. vinelandii* is that oxygen is essential for energy supply but must be absent inside cells to keep nitrogenase active. This paradox can be solved by high respiratory activity, which results in the removal of oxygen inside cells. However, high respiration activity also inevitably produces a large amount of ROS. To investigate the defense mechanism against ROS in aerobic diazotrophs, we purified Fe-SOD from *A. vinelandii* and found that the contents of SOD in the cell extract was extremely high compared to those of other bacteria, occupying 2% of total protein (Asada et al. 1980). Furthermore, the SOD activity in *A. vinelandii* was not changed in either nitrogen fixing conditions or non-fixing conditions using either nitrate or ammonium as a nitrogen source (Asada et al. 1980). Since the redox state and the production rate of superoxide inside the cells might differ depending on the growth conditions, *A. vinelandii* Fe-SOD seems to be a constitutive enzyme and not to be controlled by redox state or ROS. However, whether this high constitutive synthesis of Fe-SOD was specific to *A. vinelandii* remained to be elucidated.

To investigate further whether the synthesis of Fe-SOD in *A. vinelandii* is inducible, we cloned and sequenced *sodB* from *A. vinelandii*, and analyzed the effect of oxygen concentration inside cells on the *sodB* expression using the facultative anaerobic bacterium *Escherichia coli*. The present results showed that *A. vinelandii sodB* is up-regulated by anaerobiosis and down-regulated by aerobiosis, indicating that oxygen-regulated redox state affects the expression of *A. vinelandii* Fe-SOD.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase (CIAP) were purchased from Takara (Kyoto, Japan). A DNA ligation kit (Ligation high) was obtained from Toyobo (Osaka, Japan). The DNA sequencing kit (BigDye Terminator Cycle Sequencing Ready Reaction) was from Applied Biosystems (Forster city, CA, USA). β-Galactosidase enzyme assay system was from Promega (Madison, WI, USA). GasPak Plus, dry anaerobic indicator strips and GasPak jars were obtained from Becton Dickinson (Cockeysville, MD, USA). Nylon membranes for colony hybridization, positively charged nylon membranes, DIG luminescent detection kit, DIG wash and block buffer set, DIG Easy Hyb and disodium-3-(4-methoxyspiro{1,2-dioxetane-3,2'- (5'-chloro) tricyclo [$3.3.1.1^{3,7}$] decan}-4-yl) phenyl phosphate (CSPD) were obtained from Boehringer Mannheim (Mannheim, Germany). All other reagents were commercial products of the highest grade. Antibody raised against *A. vinelandii* Fe-SOD was prepared as described previously (Kanematsu and Asada 1989).

Bacterial strains, plasmids, media and growth conditions. *Azotobacter vinelandii* IFO12018 was obtained from the Institution for Fermentation, Osaka, Japan. The strain was grown in a modified Burk's nitrogen-free medium at 30℃ for 24 h with vigorous aeration (Burns and Hardy 1972). The medium contained 0.2 g $MgSO_4{\cdot}7H_2O$, 0.08 g $CaCl_2{\cdot}2H_2O$, 0.0025 g $Na_2MoO_4{\cdot}2H_2O$, 0.2 g KH_2PO_4, 0.8 g K_2HPO_4, 0.05 g $FeCl_3{\cdot}6H_2O$ and 20 g sucrose per liter. The pH was adjusted with NaOH to pH 7.0. Where indicated, 1 g KNO_3 was added to the basal medium as a nitrogen source. pBluescript II SK(-) and pBluescript II KS(-) (Stratagene, La Jolla, CA, USA) were used as cloning plasmid vectors. *Escherichia coli* XL1-Blue MRF' was employed as a host strain. Unless otherwise specified, *E. coli* harboring plasmids were grown in Luria-Bertani medium (LB) containing 100 mg/ml ampicillin (amp) at 37℃ for 16-17 h. If necessary, isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were added at 200 μM and 40 μg/ml, respectively.

Hybridization probe. A hybridization probe was designed from the conserved amino acid sequence (DVWEHAYY) of Fe- and Mn-SODs on the basis of the amino acid sequence alignment (Fig. 10.6 in Kanematsu and Asada 1994). A 24-mer DNA probe (5'-GTAATAAGCGTGTTCCCAGACATC-3') was labeled with digoxigenin (DIG) at 5' and 3' ends, and purified by HPLC. This 5', 3' DIG probe (PR492-469) was used for dot blot, colony and Southern hybridization.

Dot blot and Southern blot hybridization. Dot blot analysis was performed using 2 μg genomic DNA from *A. vinelandii* with DIG-labeled DNA probe. Genomic DNA was placed onto a positively charged nylon membrane and the signal was detected by a chemiluminescent method (see below). Southern blotting was conducted with capillary transfer using 20 x SSC as a transfer buffer onto a positively charged nylon membrane overnight. After DNA was fixed on the membrane by UV for 3 min, hybridization was performed in a glass roller bottle using a hybridization incubator (Multi-shaker oven HB, TAITEC). Prehybridization was conducted in a glass roller bottle with the blocking solution Easy Hyb at 42℃ for 1-4 h at 5 rpm. Hybridization was performed overnight with the DIG-labeled DNA probe in DIG Easy Hyb at 5 pmol/ml at 42℃. The membranes were successively washed twice each with 2 x SSC containing 0.1% SDS, then with 0.1 x SSC, containing 0.1% SDS at 42℃. Genomic Southern blot was conducted as follows: the genomic DNA of *A. vinelandii* IFO12018 was digested with *Eco*RI, separated by electrophoresis on a 0.8% agarose gel and capillary-transferred onto a positively charged nylon membrane as described above.

Construction of DNA library in *E. coli*. Genomic DNA (18 μg) of *A. vinelandii* IFO12018 was digested with *Eco*RI, and the resulting DNA fragments were size-fractionated on a 0.8% agarose gel by electrophoresis and fragments ranging 8 - 15 kbp in size were isolated by cutting a gel. The DNA recovered by glass milk method was ligated to pBluescript II SK (-) that had been digested with *Eco*RI and dephosphorylated with CIAP. The ligation was conducted using Ligation high (Toyobo) at 16℃ for 1 h. Transformation was performed by mixing ligation solution with Epicurian Coli XL1-Blue MRF' supercompetent cells (Stratagene).

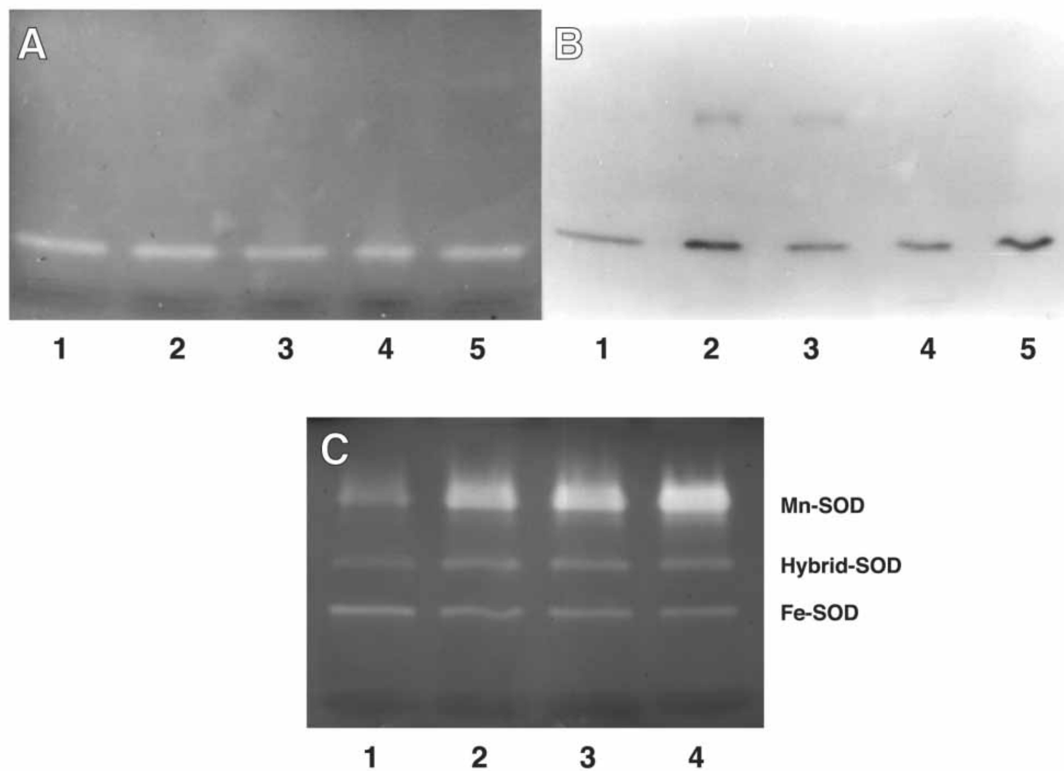


Fig. 1. Effect of methyl viologen on SOD activity in *A. vinelandii* and *E. coli*. Saturated cultures (15 ml) of *A. vinelandii* grown in Burk's medium containing 10 mM KNO_3 at 30℃ for 24 h and of *E. coli* cultured aerobically in LB medium at 37℃ for 16 h were added to each new 50 ml of the same medium in 250 ml culture flask containing 0, 1, 10, 100, and 1,000 μM (only for *A. vinelandii*) methyl viologen, and further incubated for 2 h with agitation. *A. vinelandii* and *E. coli* were incubated at 30℃ and 37℃, respectively. The cells were collected by centrifugation and the pellets were stored at -20℃ until use. Each pellet was suspended in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and disrupted by sonication for total 2 min after digestion with lysozyme at 0.4 mg/ml at 37℃ for 10 min. The cell extracts were obtained by centrifugation at 18,000×*g* for 10 min. For SOD activity staining and Western blotting after native-PAGE, 75 μg and 1.5 μg proteins, respectively, were applied to gel electrophoresis. (A, B) SOD activity staining (A) and Western blotting (B) for *A. vinelandii*. (C) Activity staining for *E. coli*. The cell extracts from MV treatments at 0 μM (lane 1), 1 μM (lane 2), 10 μM (lane 3), 100 μM (lane 4) and 1 mM (lane 5) were used. Activity staining was conducted by the photochemical method using NBT and riboflavin. Immunostaining was done with anti-*A. vinelandii* Fe-SOD serum. MV, methyl viologen; Hybrid-SOD, heterodimer from dimeric Mn- and Fe-SODs.

White transformants were selected on LB agar plates supplemented with amp, X-gal and IPTG and were picked up and stored on LB agar plates containing amp.

Colony hybridization. Arrayed bacterial colonies on LB/amp plates were transferred onto nylon membranes (82 mm in diameter) in duplicate, and the cells were disrupted by putting the filters on a small amount of 0.5 M NaOH containing 1.5 M NaCl for 5 min, and then neutrized with 0.5 M Tris HCl, pH 7.4, containing 1.5 M NaCl for 5 min. The filters were washed with 2 x SSC and dried for several min on 3MM filter papers. After exposure to UV irradiation for 3 min, the filters were washed vigorously with 50 mM NaOH, 0.1% SDS for 15 min. After washed and scribed on the surface of the filters with 2 x SSC, the filters were subjected to hybridization with the DIG-labeled DNA probe. Hybridization was conducted as described in the method for Southern hybridization as above.

Chemiluminescence detection. Chemiluminescence detection was performed by using DIG luminescent detection kit according to the manufacturer's instructions at room temperature unless otherwise stated. Briefly, after hybridization and final washing with 0.1x SSC, 0.1% SDS, the membrane, which was transferred with DNA or colonies, was further rinsed with washing buffer (maleic acid buffer, 0.3% Tween 20) for 5 min, then blocked with blocking solution for 30 min. The membrane was treated with anti-DIG antibody conjugated with alkaline phosphatase (1:10000 dilution) in blocking solution for 30 min, washed three times with washing buffer for 15 min, equilibrated with detection buffer for 5 min, and then excess solution was removed by blotting with 3MM paper filter. The membrane was reacted with CSPD (5 μg per 500 μl detection buffer) in a hybridization bag at 37℃ for 5 min, and then excess solution was removed. After sealing the hybridization bag, the filter was exposed to X-ray film in a cassette for 2h - overnight.

Nucleotide sequencing. Nucleotide sequences were determined using a BigDye terminator cycle sequencing kit with -21M13 forward and M13 reverse universal primers, or sequence specific primers. The cycle sequencing reaction and DNA sequencing analysis were performed with GeneAmp PCR System 9700 and ABI PRISM 377 DNA sequencer, respectively (Applied

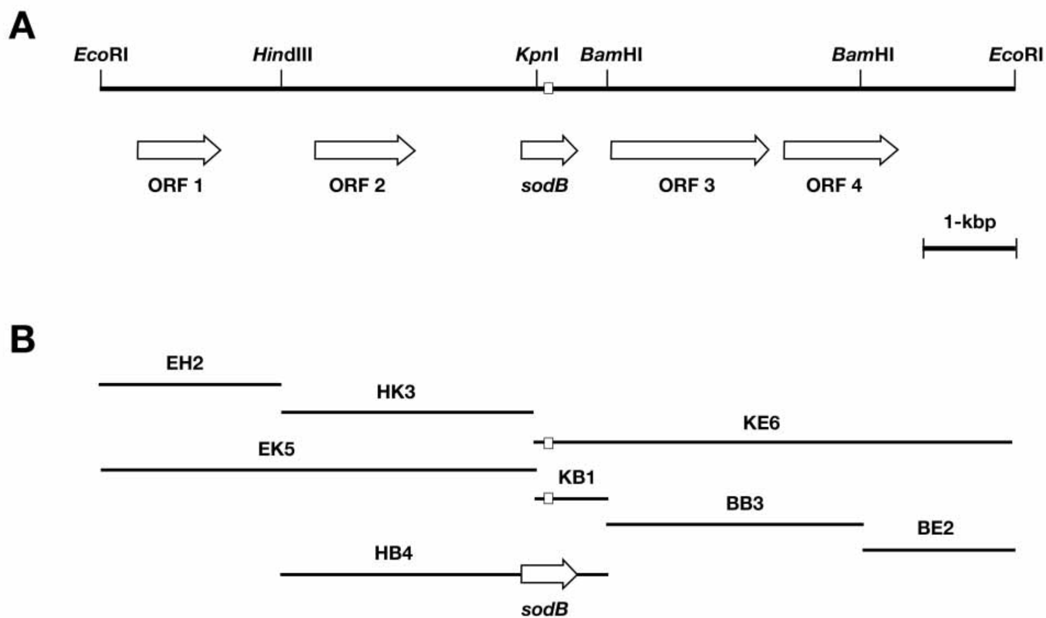


Fig. 2. Restriction map of clone 28 and its subclones. (A) A restriction map and open reading frames (ORFs). The length of clone 28 is 10,102-bp. The ORFs are shown as open arrows with the direction of transcription. An open rectangle indicates the position of the target sequence for the probe PR492-469. Each ORF shows sequence similarity to 1-acyl-*sn*-glycerol-3-phosphate acyltransferase of *Burkholderia pseudomallei* (ORF 1), NAD(P)H-dependent 2-cyclohexen-1-one reductase of *Pseudomonas syringae* (ORF 2), cyclase/phosphodiesterase of *Pseudomonas putida* (ORF 3) and iron-regulated protein of *Pseudomonas mendocina* (ORF 4). *SodB*, a gene encoding *A. vinelandii* Fe-superoxide dismutase. (B) Subclones from clone 28. The name of subclone, e.g. HB4, stands for *Hin*dIII-*Bam*HI DNA fragment having approximate length of 4 kbp. Other clones have the same terminology. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; B, *Bam*HI.

Biosystems).

Other DNA manipulations. Genomic DNA of *A. vinelandii* was isolated by the method of Saito and Miura (1963). DNA was recovered from a gel and purified using Geneclean II (Bio 101, Vista, CA, USA). Ligation was conducted using the kit, Ligation high. Plasmid DNA isolation, restriction endonuclease digestion and agarose gel electrophoresis were done by the procedures described by Sambrook et al. (1989).

Analytical methods. SOD activity was measured as described previously (Kanematsu and Asada 1990) and expressed in McCord and Fridovich units (McCord and Fridovich 1969). Protein contents were determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA) or by the method of Lowry at al. (1951) with bovine serum albumin as a standard. β-Galactosidase activity was assayed by using β-galactosidase enzyme assay system from Promega according to the manufacture's instructions. One unit of the activity was defined as the enzyme hydrolyzes 1 μmol of *o*-nitrophenyl-D-galactopyranoside (ONPG) to *o*-nitrophenol and galactose min^{-1} at pH 7.5 at 37℃. Native-PAGE, SDS-PAGE, SOD activity staining, and immunoblotting were done as before (Kanematsu and Asada 1990, Ueno and Kanematsu 2007).

Nucleotide sequence accession numbers. The nucleotide sequence of the clone HB4 reported in this paper had been submitted (05-APR-1999) to and appeared in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number AB025798 in the year 1999. The remaining sequences of clone 23 were submitted to the databases under accession numbers AB383125 (EH2) and AB383126 (BB3+BE2).

RESULTS

SOD isozymes in *A. vinelandii*. Cell extract of *A. vinelandii* exhibited a single SOD activity band in native-PAGE followed by activity staining (Fig. 1A), which was assigned to Fe-SOD by its purification and characterization (Asada et al. 1980, Kanematsu and Asada 1982, Luo et al. 1987). In addition, it has been reported that *A. vinelandii* grown to stationary phase contained CuZn-SOD in the periplasm with Fe-SOD in the cytoplasm (Genovese et al. 1995). We could not detect any distinct band other than that of Fe-SOD in native-PAGE. This discrepancy may be due to differences in the culture conditions.

Effect of methyl viologen on SOD activity in the cells. Mn-SOD is inducible by methyl viologen (MV) through the stimulation of superoxide production, but Fe-SOD is not (Fridovich 1995). To investigate whether other MV-inducible types of SOD isozyme, i.e. Mn- and/or CuZn-SOD occur in *A. vinelandii*, we analyzed the effect of MV on SOD isozyme pattern using native-PAGE. As expected the activity band of *A. vinelandii* Fe-SOD was not affected, rather decreased slightly by up to 1 mM MV (Fig. 1A). Further, no SOD band was detected except that of Fe-SOD. Western blot analysis using antiserum against *A. vinelandii* Fe-SOD showed no change of position or intensity of the band on the gel (Fig. 1B), indicating that neither protein synthesis nor degradation of Fe-SOD occurred. In *Escherichia coli*, to

Fig. 3. Nucleotide sequence of *A. vinelandii sodB* and deduced amino acid sequence of Fe-SOD. The nucleotide sequence from the position 1420 to 3550 of the HB4 DNA fragment is presented. The coding regions of ORF 2 (3' region) and *sodB*, and the deduced amino acid sequence of Fe-SOD are indicated in blue and green letters, respectively. The green asterisks (*) indicate the translation stop codons. For *sodB*, the putative -35 and -10 regions of a promoter sequence, and the transcription start point (+1) are boxed. The potential ribosome binding site (RBS) is double underlined. Palindromic sequences are indicated with inverted arrows above the nucleotide sequences. The *Kpn*I site is underlined. The target sequence of the probe PR492-469 is shown in bold red letters.

the contrary, MV even at 1 μM strongly induced the synthesis of Mn-SOD, but not that of Fe-SOD or the Fe/Mn hybrid-SOD that consists of each subunit from Mn- and Fe-SODs (Fig. 1C), confirming a previous report (Hassan and Fridovich 1977).

Cloning of a 10-kbp DNA fragment containing *sodB*. To investigate the regulation of Fe-SOD synthesis in *A. vinelandii*, its responsible gene, *sodB*, was cloned. At first, we confirmed the validity of the DIG-labeled DNA probe (PR492-469) by a dot blot analysis visualized

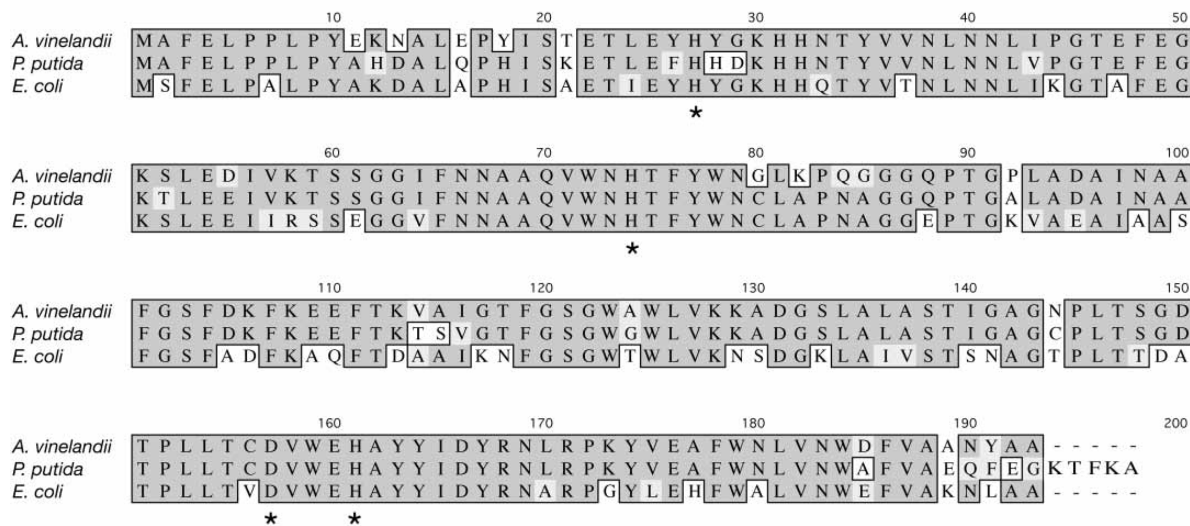


Fig. 4. Comparison of amino acid sequences of Fe-SODs from *A. vinelandii* and other bacteria. The amino acid sequence of Fe-SOD from *A. vinelandii* is compared with those of *E. coli* and *P. putida*. The alignment was conducted with the ClustalW program. The identical and similar amino acid residues are boxed with dark and light shade, respectively. The ligands to iron are indicated with asterisks (*).

with the chemiluminescent method using 2 μg genomic DNA of *A. vinelandii*. Southern blot analysis of the genomic DNA digested with *Eco*RI showed a single positive DNA band of 10 kbp (data not shown) on agarose gel. Under the same conditions, genomic DNA of *E. coli* gave a positive 20-kbp band with the probe PR492-469.

By colony hybridization we screened 630 colonies from the DNA library in *E. coli* constructed with 8−15-kbp *Eco*RI-fragments of *A. vinelandii* genomic DNA, and finally obtained two positive clones (clone 3 and clone 28), both of which contained 10-kbp-inserted DNA. Restriction enzyme analyses of both the clones by digestion with *Eco*RI and with either *Xho*I, *Pst*I, *Bam*HI, *Xba*I, *Hin*dIII or *Sac*I exhibited the same restriction patterns, indicating that the clones 3 and 28 were the same (data not shown).

Construction of a restriction map for the 10-kpb DNA fragment obtained from clone 28. To determine not only the *sodB* nucleotide sequence but also the gene organization around *sodB*, we first constructed a restriction map of the 10-kbp *Eco*RI-DNA fragment, then subcloned smaller DNA fragments generated with restriction enzymes into a vector, pBluescript II SK(-), and finally sequenced the whole region of the 10-kbp DNA fragment.

To map restriction sites on the 10-kbp *Eco*RI-fragment obtained from clone 28, the number and size of the resulting fragments were analyzed by incubation with the following restriction enzymes: *Xho*I, *Pst*I, *Bam*HI, *Xba*I, *Hin*dIII, *Sac*I, *Eco*RV, *Bst*XI, *Hin*cII, *Spe*I, *Sac*II, *Kpn*I, *Apa*I and *Sma*I. Among these enzymes, *Hin*dIII and *Kpn*I each gave two fragments of 9 and 1.9 kbp, and of 6 and 5 kbp, respectively, showing the occurrence of one restriction site for each enzyme in the 10-kbp fragment. Two *Bam*HI sites in the 10-kbp fragment were indicated by the generation of three fragments (6, 3 and 1.8 kbp). By double digestion of the 10-kbp fragment with *Hin*dIII plus *Bam*HI, and with *Hin*dIII plus *Kpn*I, we finally constructed a restriction map (Fig. 2A).

Southern blot analysis with the DNA probe PR492-469 was conducted to locate *sodB* in 10-kbp *Eco*RI-fragment from clone 28. Digestion of the fragment with *Bam*HI, *Hin*dIII and *Kpn*I gave fragments of 6 kbp, 9 kbp and 6 kbp, respectively, all of which hybridized with the DNA probe (data not shown). Thus, it was suggested that the target sequence for the probe in *sodB* was located between the *Kpn*I and *Bam*HI sites. Finally, the entire *sodB* sequence was found between the *Hin*dIII and *Bam*HI sites, encompassing the *Kpn*I site (Fig. 2A).

The direction of the inserted DNA in pBluescript II SK (-) of clones 3 and 28 were determined by digestion with *Hin*dIII. The enzyme cleaves at a site that is localized in either the 5' or 3' side of the insert, and also at a *Hin*dIII site in the multi-cloning site of the vector. The result indicated that the *Hin*dIII site of the clone 28 insert was placed close to *lac* promoter of the vector (Fig. 2). The direction of the clone 3 insert was shown to be the opposite of that of clone 28 (data not shown). Thus, clone 28 was chosen for sequencing.

Subcloning of the fragments derived from the 10-kbp DNA fragment. The DNA fragments of 2, 3 and 6 kbp derived from the 10-kbp *Eco*RI-DNA digested with *Hin*dIII and *Kpn*I were subcloned into pBluescript II SK (-) pre-treated with *Eco*RI and *Hin*dIII, *Hin*dIII and *Kpn*I, or *Kpn*I and *Eco*RI to form the clones EH2, HK3 and KE6, respectively (Fig. 2B). Similarly, digestion of the 10-kbp *Eco*RI-DNA with *Kpn*I and *Bam*HI generated the fragments of 5, 1, 3 and 2 kbp, which were subcloned into the vector previously digested with the proper enzymes to form the clones EK5, KB1, BB3 and BE2, respectively (Fig. 2B). The 4-kbp DNA fragment containing *sodB* from among the fragments of 2, 4, 3 and 2-kbp produced by *Hin*dIII and *Bam*HI digestion was subcloned to the *Hin*dIII-*Bam*HI-cut vector to make clone HB4. We determined the whole sequence of the 10-kbp fragment by sequencing the clones EH2, HB4, BB3 and BE2. The clone EK5 was used to connect EH2 and HB4, and the clone KE6 was used for the connection between HB4 and BB3, and BB3 and BE2 (Fig. 2B). The sequence of the whole 10-kbp DNA fragment was obtained by assembling the sequences of its subclones in

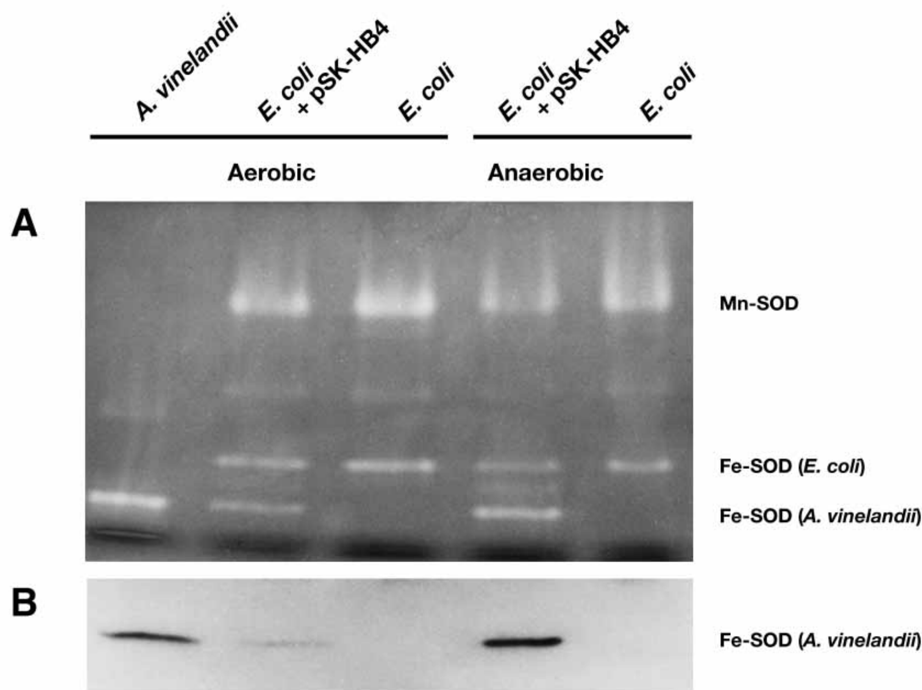


Fig. 5. Effect of anaerobiosis on the expression of *A. vinelandii sodB* in *E. coli*. *A. vinelandii* was cultured (10% inoculum) in 50 ml Burk's medium containing 10 mM KNO_3 at 30℃ for 24 hr. *E. coli* harboring pSK-HB4 and *E. coli* without plasmid were grown (10% inocula) in 50 ml LB/amp and LB medium, respectively, at 37℃ for 16 h under aerobic or for 24 h under anaerobic conditions. Aerobic cultures were obtained with agitating at 170 rpm, whereas anaerobic cultures were stationary cultures with air-tight screw caps. Each cell was collected and disrupted as described in Fig1. For activity staining and Western blotting, 75 μg and 3 μg proteins, respectively, were applied to native-PAGE. SOD activity staining and Western blotting were done as Fig. 1.

pBluescript II SK (-).

Nucleotide sequence of *sodB*. The complete *sodB* was included in the 4-kbp nucleotide sequence of the clone HB4 along with an open reading frame (ORF 2) upstream of *sodB* (Fig 3). Fig. 3 shows the nucleotide sequence of *A. vinelandii sodB* which consists of a 582-bp ORF encoding Fe-SOD of 193 amino acid residues (Kanematsu et al. 1999). The deduced amino acid sequence of Fe-SOD is also shown in Fig 3 and was compared with those of other Fe-SODs in Fig 4. The estimated molecular mass and pI of *A. vinelandii* Fe-SOD were 21,377 Da and 4.98, respectively. The *sodB* nucleotide sequence of *A. vinelandii* IFO 12018 used in this work was consistent with that of *A. vinelandii* CA, reported by Qurollo et al. (2001), although nucleotide substitutions in the third base at four codons do not change amino acid residue.

The upstream sequence of *sodB* showed the presence of a ribosome-binding site (RBS) 7-bp upstream from the ATG initiation codon, -10 region and -35 region as presumed by Qurollo et at. (2001). An inverted repeat sequence, possibly functioning as a transcriptional terminator by forming a stem-loop structure was located at a position of 20 to 45-bp downstream of the stop codon of *sodB* (Fig. 3).

ORFs and their arrangement in 10-kbp DNA fragment. In addition to *sodB*, three ORFs were found in the 10-kpb DNA fragment and designated as ORF 1, ORF 2 and ORF 3 from 5' end. The coding directions of each ORF were the same as that of *sodB* (Fig 2). To elucidate the encoded proteins of the ORFs, BLAST searches in databases using blastn and blastp programs were conducted (Kanematsu et al. 1999). ORF 1 (870 bp) encoded a protein consisting of 289 amino acid residues. The BLAST searches indicated a similarity to 1-acyl-*sn*-glycerol-3-phosphate acyltransferase of *Burkholderia pseudomallei.* ORF 2 (1,050 bp) was located upstream next to *sodB*, which encoded a protein consisting of 349 amino acid residues, and resembled the gene encoding NAD(P)H-dependent 2-cyclohexen-1-one reductase in *Pseudomonas syringae* (Rohde at al. 1999). Downstream of *sodB*, the neighboring ORF 3 (1884 bp) encoded a hypothetical protein consisting of 627 amino acid residues similar to those found in *Bacillus subtilis* and *Synechocystis* sp.

Recently, a 300-kbp contig (ctg 56, accession number NZ_AAAU03000005) of *A. vinelandii* AvOP including the 10-kbp fragment region was obtained and submitted to a database. The sequence of the 10-kbp fragment was almost identical to that of the ctg 56 with a few nucleotide substitutions. The latest BLAST search (Oct 2007) revealed the protein showing the greatest resemblance to ORF 3 was a cyclase/phosphodiesterase of *Pseudomonas putida* (EAX19391), which is a diguanylate cyclase having a GGDEF domain (Ryjenkov et al. 2005). Furthermore, a new ORF 4 of 1,326 bp encoding a protein with 441 amino acid residues homologous to an iron-regulated protein-like protein (ABP8607) was indicated in ctg 56 (Fig. 2).

Expression of *A. vinelandii sodB* in *E. coli*. *E. coli* harboring pBluescript II SK(-) (referred to hereafter as

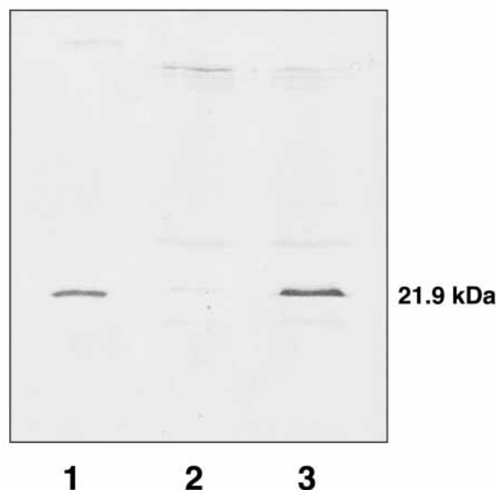


Fig. 6. Western blot analysis after SDS-PAGE of *A. vinelandii* Fe-SOD expressed in *E. coli* cells. *A. vinelandii* was cultured in Burk's medium containing 10 mM KNO_3 at 30℃ for 24 h with agitation. *E. coli* harboring pSK-HB4 and *E. coli* without plasmid were grown in LB/amp and LB/tet, respectively, at 37℃ for 16 h. The cells were disrupted as described in Fig. 1. Protein was denatured with 5% SDS, containing 2% 2-melcaptoethanol at 100℃ for 5 min. About 75 μg protein was applied to SDS-PAGE. Lane 1, *A. vinelandii*; lane 2, *E. coli*; lane 3, *E. coli* + pSK-HB4.

Table 1. Effect of anaerobiosis on copy number of plasmid in *E. coli* monitored by β-galactosidase activity[a]

Plasmid in *E. coli* XL1-Blue MRF'	Aerobiosis[b]		Anaerobiosis[d]	
	-IPTG	+IPTG[c]	-IPTG	+IPTG[e]
None	1	3	nd	nd[f]
pBluescript II SK(-)	48	2466	38	48
pBluescript II KS(-)	23	606	14	21

[a] The cells were disrupted as described in the legend to Fig 1. β-Galactosidase activity was expressed in milli-units/mg protein.
[b] Cells were grown in 50 ml LB/tet (without plasmid) or LB/amp (with pSK or pKS) for 5.5 h with shaking of 160 rpm at 37℃ using each 2 ml of saturated inoculum in the same medium.
[c] Further 2 h incubation at 0.1 mM.
[d] Each 20 ml culture[b] was added to each 20 ml fresh medium containing the antibiotics and cultured in an anaerobic chamber containing GasPak Plus at 37℃ for 15 h.
[e] Further 4 h incubation at 0.1 mM in the chamber with a new GasPak Plus.
[f] Not determined.

pSK) inserted with the 4-kbp *Hin*dIII-*Bam*HI fragment (pSK-HB4) from the 10-kbp DNA fragment expressed *A. vinelandii* Fe-SOD in addition to its own Fe-SOD as revealed by the SOD activity staining after native-PAGE (Fig. 5A). In native-PAGE, *A. vinelandii* Fe-SOD had a higher mobility than *E. coli* Fe-SOD, making them distinguishable. The molecular mass of the expressed *A. vinelandii* Fe-SOD was estimated to be 21.9 kDa, which was in accordance with the deduced molecular mass of 21,377 Da from *sodB*, by Western blot analysis using the anti-*A. vinelandii* Fe-SOD antibody after SDS-PAGE (Fig. 6).

Effect of anaerobiosis on the expression of *A. vinelandii sodB* in *E. coli.* Since the replication of plasmids requires large amount of energy, anaerobic culture conditions may affect the plasmid production in cells. At first, we examined the difference in plasmid copy number of *E. coli* cultured under aerobic or anaerobic conditions by β-galactosidase (Gal) activity, derived from the intrinsic *lacZ* in the plasmids (Table 1). Aerobically grown cells carrying pSK or pKS with IPTG showed much higher Gal activity than anaerobically grown cells with or without IPTG. The activity in the presence of IPTG under anaerobiosis was in the same order as that in the absence of IPTG under aerobiosis, showing no induction effect of IPTG on Gal without oxygen. We were not able to estimate the difference in copy number in the cells cultured under aerobic and anaerobic conditions due to no induction of Gal by IPTG in the anaerobic cells. However, it is likely that the copy number of the plasmid in the aerobic cells is much higher than in the anaerobic cells.

E. coli harboring pSK-HB4 was grown under both aerobiosis and anaerobiosis, and the expression of Fe-SOD was determined. In native-PAGE the intensity of the activity band derived from the cells grown under anaero-

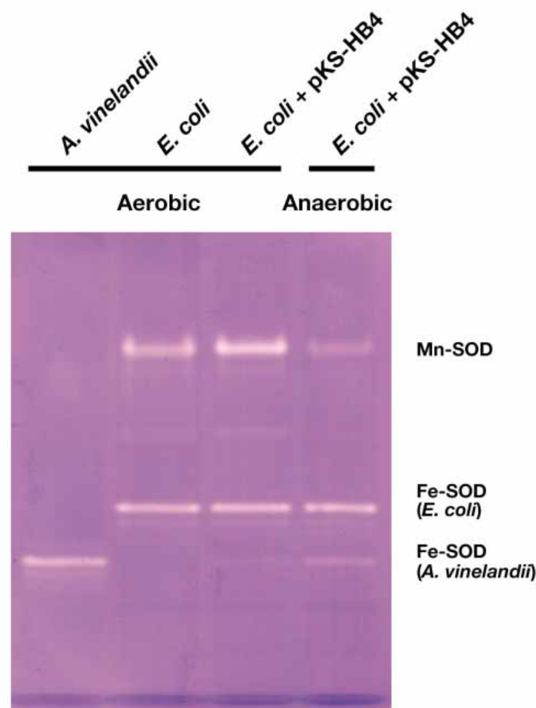


Fig. 7. Effect of *sodB* direction in the plasmid pKS-HB4 on its anaerobic expression in *E. coli.* *A. vinelandii* was grown as in Fig.5. *E. coli* harboring pKS-HB4 and *E. coli* without plasmid were cultured in 50 ml LB/amp and LB/tet, respectively, with shaking of 170 rpm at 37℃ for 16 hr. For aerobiosis cells, an aliquot of the culture was withdrawn and analyzed. For anaerobiosis, the 10 ml culture mentioned above was added to 40 ml fresh medium containing antibiotics, and cultured in an anaerobic chamber containing GasPak Plus for 15 h at 37℃. The absence of oxygen in the chamber was confirmed by an anaerobic indicator (Becton Dickinson). The cells were disrupted as described in Fig 1. For SOD activity staining 75 μg of protein was applied.

biosis was much higher than that under aerobiosis (Fig. 5A). Western blot analysis after native-PAGE confirmed the preferential expression of Fe-SOD under anaerobiosis (Fig. 5B), indicating that anaerobiosis induced the synthesis of *A. vinelandii* Fe-SOD in *E. coli* harboring pSK-HB4.

Similar results were also obtained with pKS-HB4, where the direction of the HB4 fragment was reversed relative to that of pSK-HB4, showing anaerobic expression of *A. vinelandii sodB* in *E. coli* carrying the plasmid (Fig. 7). Thus, the expression of *sodB* in *E. coli* depends on its own promoter and not on the *lac* promoter, because *sodB* in pKS-HB4 was placed in the antisense direction against the *lac* promoter.

DISCUSSION

Methyl viologen does not induce any SOD in *A. vinelandii*. Methyl viologen (MV) mediates electron transfer from the electron transport chain to molecular oxygen, thus enhancing the production of $O_2^{-}\cdot$. *SodA* of *E. coli* is up-regulated by MV treatment through the SoxRS global control system responding to $O_2^{-}\cdot$. The cell extract of *A. vinelandii* exhibited only one activity band of Fe-SOD as revealed by the activity staining on native-PAGE (Fig. 1A), which is in accordance with our previous results (Asada et al. 1980). The MV treatment did not affect the activity band pattern, indicating that *A. vinelandii* contains Fe-SOD and *sodB* expression is not controlled by $O_2^{-}\cdot$. Mn-SOD was not detected in *A. vinelandii* cells, but whether *sodA* is absent in the genome must await the elucidation of its whole genome sequences. It is interesting to note that *P. putida*, which has Fe-SOD that most resembles that of *A. vinelandii* in amino acid sequences (Fig. 4), contains *sodA* and *sodB*, but the transcription of these genes was not enhanced by MV (Kim et al. 1999). Periplasmic CuZn-SOD (Genovese et al. 1995) was not detected in our preparation of *A. vinelandii* cell extract, and it is likely that the expression of its gene, *sodC*, is not induced by MV.

The possibility remains that no MV effect on SOD synthesis in *A. vinelandii* was due to the zero or very low production of $O_2^{-}\cdot$ even in the presence of MV, because the oxygen concentration inside cells, by which MV radical produces $O_2^{-}\cdot$ seems to very low. However, this possibility might be excluded by the fact that nitrogenase activity, measured by acetylene reduction, was inhibited by MV (Kanematsu, unpublished results), indicating the production of $O_2^{-}\cdot$ inside cells. Nevertheless, possible direct interaction of the labile iron-sulfur cluster of nitrogenase with MV radicals, not with $O_2^{-}\cdot$, still cannot be excluded.

Fe-SOD and *sodB* gene of *A. vinelandii*. Fe-SOD of *A. vinelandii* was purified and well characterized (Kanematsu 1986). *A. vinelandii* contains dimeric Fe-SOD constituting 2% of the total protein. Thus, we obtained several grams of the purified enzyme and used it for physicochemical characterization of the enzyme including the Mössbauer spectrum. Since this enzyme seemed to be more stable than Fe-SODs from other sources and contained a stoichiometric amount of iron at the active site, the enzyme was suitable for physicochemical studies. However, one drawback was the unavailability of its amino acid sequence. The present elucidation of the amino acid sequence of *A. vinelandii* Fe-SOD will be beneficial to such studies.

The cloned *A. vinelandii sodB* ORF consisted of 582-bp nucleotides corresponding to 193 amino acid residues. The deduced molecular mass of 21,377 Da from *sodB* was in good agreement with the subunit molecular mass of 21,000 obtained with the purified enzyme (Kanematsu, 1986). Thus, it is clear that *sodB* encodes a typical bacterial dimeric Fe-SOD.

Comparison of the amino acid sequence of *A. vinelandii* Fe-SOD with those of other bacteria reveals 85% homology with *P. putida* (Fig. 4). The sequence homology between *A. vinelandii* and *E. coli* was 70%, which is a reflection of closer phylogenetic distance between *A. vinelandii* and *P. putida* than between *A. vinelandii* and *E. coli*. Both *A. vinelandii* and *P. putida* are gram-negative soil bacteria, while *E. coli* is a gram-negative enteric bacterium.

No synteny between neighboring genes of *sodB* from *A. vinelandii* and *E. coli*. In addition to *sodB*, we found at least four ORFs, annotated in DDBJ/EMBL/GenBank databases, on the 10-kbp DNA fragment determined for the nucleotide sequence. The arrangement of ORFs from the 5' end of the fragment is as follows: 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (ORF 1), NAD(P)H-dependent 2-cyclohexen-1-one reductase (ORF 2), Fe-SOD, diguanylate cyclase (ORF 3) and iron-regulated protein (ORF 4) (Fig. 2). Since Fe-SOD seems not to closely relate with these enzymes in their functions, the cooperative regulation of each gene with *sodB* would be unlikely. It is obvious that *A. vinelandii sodB* is a monocistronic gene on the basis of the analysis of the sequences between ORF 2 and *sodB*, and between *sodB* and ORF 3. To contrast, it has been reported that *sodA* of *P. putida* was a member of an operon consisting of *orfY-fumC-orfX-sodA* and was transcribed to a polycistronic mRNA (Kim et al. 1999).

The gene arrangement around *sodB* of *A. vinelandii* was compared with those of *E. coli* and *P. putida* whose *sodB* most resembles that of *A. vinelandii*. The result showed that the arrangement of *A. vinelandii* resembled that of *P. putida* in the *sodB* downstream region, but not that of *E. coli*. The absence of synteny around *sodB* between *A. vinelandii* and *E. coli* clearly indicates that this arrangement of the genes is not necessarily for the expression of *sodB*.

Expression control of *sodB* of *A. vinelandii*. We investigated the effect of oxygen on *A. vinelandii sodB* expression using a plasmid containing *sodB*, and *E. coli* XL1-Blue MRF' as host cells cultured either aerobically or anaerobically. *E. coli*, a facultative bacterium, is a suitable host since the concentration of oxygen inside cells can be modulated by aerobic or anaerobic cultures.

The control experiments (Table 1) on the plasmid copy number in *E. coli* cells under aerobiosis or anaerobiosis need some comments regarding the inability of IPTG to induce Gal in anaerobiosis. This is probably attributable to catabolite repression under anaerobiosis. Under anaerobic conditions, even the strong inducer IPTG might fail to induce the transcription of *lacZ* due to competition with repression from the catabolic repressor protein CRP. Therefore, the Gal activity measured with the cells under anaerobiosis both with and without IPTG and under aerobiosis without IPTG is the activity escaped from the repression by *lacI* and/or CRP repressors. However, it is not clear whether the measured

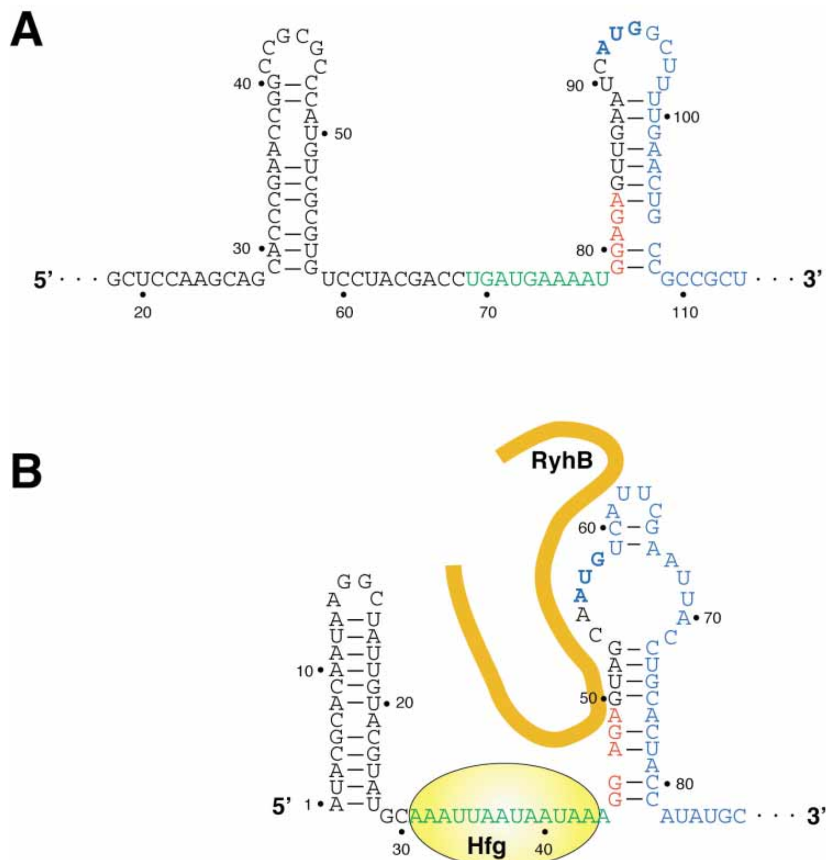


Fig. 8. The stem-loop structures and A/U-rich motif in 5' end of *A. vinelandii sodB* mRNA. (A) Secondary structure of the 5' end of *A. vinelandii sodB* mRNA. (B) A model for the interaction of *E. coli sodB* mRNA with Hfq and RyhB, adapted from Geissmann and Touati (2004) and Afonyushkin et al. (2005) by permission from Oxford University Press and Macmillan Publishers Ltd, respectively. The model is depicted with a slight modification. The change of right stem-loop structure of the *E. coli sodB* mRNA upon Hfq binding is not included in the model. Coding sequences are shown in blue letters. Translation initiation codons are indicated with bold letters. Ribosome binding sites and A/T(U)-rich motifs are shown in red and green letters, respectively.

activity reflects the plasmid copy number. It should be noted that *E. coli* XL1-Blue MRF' possesses the *lac* repressor *lacI*.

Although the expression of *sodB* in *A. vinelandii* did not vary under N-fixing or non N-fixing conditions (Asada et al. 1980), it was clearly demonstrated that *A. vinelandii sodB* is up-regulated under anaerobiosis and down-regulated under aerobiosis in *E. coli* (Fig. 5 and 7). Thus, it seems that *A. vinelandii sodB* has a potential for being controlled inside *A. vinelandii* cells, although the redox level in *A. vinelandii* cells is thought to be very low and constant even in different culture conditions. The presence of *sodB* expression control in *A. vinelandii*, similar to that in *E. coli*, indicates that its gene product, Fe-SOD, plays an important role in the anaerobic environment inside *A. vinelandii* cells. It has been reported that Fe-SOD in *A. vinelandii* is essential for viability (Qurollo et al. 2001). To contrast, it seems that Fe-SOD is not beneficial to the oxygenic cells, because *A. vinelandii sodB* is down-regulated in *E. coli* cells under aerobiosis. The reason why Fe-SOD is not preferred in aerobic cells is an interesting topic to be investigated.

***A. vinelandii sodB* mRNA contains structural features for regulation by the Fur global regulatory system.** The Fur global regulatory system in *E. coli*, which seems to be ubiquitous in bacteria, strictly controls iron level in cells, because excess Fe^{2+} enhances the production of the harmful hydroxyl radical from hydrogen peroxide via the Fenton reaction. With excess iron, Fur becomes active upon binding with Fe^{2+} and down-regulates the expression of genes involving iron metabolism by directly binding to the iron box in the promoter regions of these genes. The transcription of the non-coding small RNA RyhB, which synergistically reduces the stability of RNA transcripts with Hfq by making them accessible to nuclease digestion (Massé et al. 2003), is also down-regulated by Fur-Fe^{2+}. Thus, with the limitation of RyhB, the lifetimes of RNA transcripts from iron metabolism genes are increased and the translations are prolonged, thus exhibiting indirect up-regulation by Fur. It has been indicated that iron-containing proteins necessary for growth or viability are down-regulated and iron-proteins nonessential for survival (i.e. iron storage proteins) are up-regulated (Kadner 2005). In conditions of iron deficiency, the processes described above are reversed. The Fur-Fe^{2+} releases Fe^{2+}, becomes an inactive form, and dissociates from the iron box, permitting the transcription of Fe-protein genes essential for survival and resulting in up-regulation. The abundantly transcribed RyhB binds RNA transcripts for iron storage proteins with Hfq and shortens their lifetimes by accelerat-

ing nuclease attacks, resulting in down-regulation by Fur.

The similarity of the expression control for *sodB* from *A. vinelandii* with that from *E. coli* indicates that *A. vinelandii sodB* is regulated by Fur. In *E. coli*, down-regulation of RyhB, which interacts with *sodB* mRNA through base pairing, results in the accessibility of ribosomes to *sodB* mRNA, exhibiting up-regulation at the translational level (Afonyushkin et al. 2005). The responsible sites of *sodB* mRNA for the interaction with RyhB and/or Hfq have been assigned to two sets of palindromic sequences, which are located near the transcription start and the translation start, respectively, and to 14-bp A/T(U in mRNA)-rich motif located in between the two stem-loops (Dubrac and Touati 2000). In *A. vinelandii sodB*, we found sequences similar to those of *E. coli* (Fig. 8). Two stem-loops are formed between positions 26-58 (based on the +1 of the transcription start) and positions 79-108. The A/T(U)-rich motif localizes in between the two stem-loops. Therefore, it is likely that the *sodB* is controlled by the Fur global regulatory system in *A. vinelandii*.

It was reported that *E. coli sodB* is not regulated by ArcA, SoxRS or OxyR global transcriptional regulators, whereas *sodA* is under control of ArcA and SoxRS (for review, see Kanematsu and Asada 1994). Therefore, it seems that the oxygen molecule itself is not involved in regulation of *A. vinelandii sodB*. However, Zheng et al. (1999) have reported that OxyR and SoxRS up-regulated *fur* expression, indicating a close relationship between oxidative stress and iron stress. Thus, with oxidative stress enhancing the production of $O_2^-\cdot$ and hydrogen peroxide, the OxyR and SoxRS global systems induce many genes in OxyR and SoxRS regulons including *fur*. In turn, $O_2^-\cdot$ generated by oxidative stress reduces Fe^{3+} to Fe^{2+}, which leads to the production of the strong oxidant hydroxyl radical through the Fenton reaction with hydrogen peroxide. Therefore, the Fur induced by the up-regulated *fur* becomes active Fur-Fe^{2+} and then regulates the Fur regulon. As a result, *sodB* is up-regulated. However, since Fe-SOD in neither *E. coli* nor *A. vinelandii* was induced by the $O_2^-\cdot$- generator methyl viologen (Fig. 1), the contribution of the OxyR and/or SoxRS systems to the induction of Fe-SOD seems to be very slight if not absent. Here, we propose a mechanism that explains the anaerobic up-regulation of *A. vinelandii sodB*. Anaerobiosis lowers the redox state inside cells, where Fe^{3+} is reduced to Fe^{2+} by proper reductants, while aerobiosis enhances the production of $O_2^-\cdot$ inside cells, where likewise Fe^{3+} is reduced to Fe^{2+} by $O_2^-\cdot$. Thus, both anaerobiosis and aerobiosis result in the activation of the Fur protein, leading to the up-regulation of *sodB* by the Fur global system.

The present results indicate that *A. vinelandii sodB* is under control of the Fur system in *A. vinelandii*. Obviously, to determine the factor(s) involved in *A. vinelandii sodB* regulation, further investigation is needed.

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