Cloning and Expression of the Catalase Gene from the Anaerobic Bacterium *Desulfovibrio vulgaris* (Miyazaki F)[1]

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We identified a gene encoding a catalase from the anaerobic bacteria *Desulfovibrio vulgaris* (Miyazaki F), and the expression of its gene in *Escherichia coli*. The 3.3-kbp DNA fragment isolated from *D. vulgaris* (Miyazaki F) by double digestion with *Eco*RI and *Sal*I was found to produce a protein that binds protoheme IX as a prosthetic group in *E. coli*. This DNA fragment contained a putative open reading frame (*Kat*) and one part of another open reading frame (ORF-1). The amino acid sequence of the amino terminus of the protein purified from the transformed cells was consistent with that deduced from the nucleotide sequence of *Kat* in the cloned fragment of *D. vulgaris* (Miyazaki F) DNA, which may include promoter and regulatory sequences. The nucleotide sequence of *Kat* indicates that the protein is composed of 479 amino acids per monomer. The recombinant catalase was found to be active in the decomposition of hydrogen peroxide, as are other catalases from aerobic organisms, but its K_m value was much greater. The hydrogen peroxide stress against *D. vulgaris* (Miyazaki F) induced the activity for the decomposition of hydrogen peroxide somewhat, so the catalase gene may not work effectively *in vivo*.

Key words: catalase, gene, oxidative stress, recombinant, sulfate-reducing bacteria.

Sulfate-reducing bacteria (SRB) have many redox proteins that play a role in the dissimilatory reduction of the sulfate system or the electron transport chain, but not all of the steps of the electron flow have been clarified completely (*1*). These bacteria cannot survive under aerobic conditions, so the biosynthetic pathways do not include oxygen. For example, the synthesis of heme has been shown to occur through a pathway involving enzymes different from those used by other aerobes (*2*). In general, strictly anaerobic bacteria cannot survive under aerobic conditions because they lack protective mechanisms against oxygen toxicity that involve catalases or superoxide dismutases (SODs). Although SRB have been classified as strictly anaerobes since their discovery 100 years ago, evidence has been obtained suggesting that they have both enzymes (*3*, *4*). Recent works have shown that SRB can actually tolerate or utilize oxygen; for example, several strains of *Desulfovibrio* and other sulfate reducers have been shown to survive prolonged exposure to oxygen (*5*, *6*), and it has been reported that 0.24–0.48 μM oxygen can support the growth of these strains (*7*).

It has been reported that desulfoferrodoxin from *Desulfoarculus baarsii* can be complementary to a SOD-deficient mutant of *Escherichia coli* (*8*, *9*), and that the deletion of its gene in *D. vulgaris* Hildenborough causes oxygen sensitivity (*10*). These results indicate that desulfoferrodoxin can act as an SOD in SRB (*11*). However, its amino acid sequence is not homologous to those of other SODs, and it has been reported that *D. vulgaris* Hildenborough has an SOD gene homologous to that of aerobes [Shenvi, N.V. and Kurtz, D.M., Jr. (1997) Genbank direct submission, accession number AF034841]. Recently, a desulfoferrodoxin-homologous protein from the hyperthermophilic anaerobe *Pyrococcus furiosus* was shown to catalyze the reduction of superoxide to water without the production of oxygen, and was designated as superoxide reductase (*12*).

The growth of SRB is coupled with the production of large amounts of hydrogen sulfide. This activity is important in the removal of acidic, oxidized forms of sulfur and the immobilization of toxic metal ions, for example, in acid mine drainage effluent; due to their odor, toxicity, and metal-corroding properties, SRB are considered a nuisance in many environments. Oxygen is one of the best and cheapest agents for controlling the growth and activity of SRB. It is, therefore, important to study the survival mechanisms of SRB as to oxidant stress.

We have also carried out a series of genetic studies on the oxygen-related proteins in *D. vulgaris* (Miyazaki F), as well as the previously cloned cytochrome *c* oxidase-like protein gene (*13*) and desulfoferrodoxin gene (*14*). In the pres-

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Abbreviations: SRB, sulfate-reducing bacteria; SOD, superoxide dismutase; ORF, open reading frame; TFA, trifluoroacetic acid; UV/VIS, ultraviolet/visible.

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ent paper, we report the molecular cloning of a gene encoding catalase that binds protoheme IX as a prosthetic group from *D. vulgaris* (Miyazaki F), and its overproduction as a holoprotein in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and Materials—*E. coli* strain JM109, *recA*1, Δ(*lac-proAB*), *end A*1, *gyrA*96, *thi*-1, *hadR*17, *relA*1, *supE*44[F'*traD*36, *proAB*$^+$, *lacI*q ZΔM15] was used throughout. *D. vulgaris* (Miyazaki F) was grown (*15*) and used for DNA isolation. Restriction endonucleases and DNA-modifying enzymes were purchased from Nippon Gene and Takara Shuzo. [γ-^{32}P]ATP (185 TBq/mmol) was obtained from ICN. Catalase from bovine liver and bovine serum albumin were purchased from Wako Pure Chemical, and other marker proteins were bought from Sigma. Antisera for the bovine catalase were purchased from Chemicon International. All other chemicals were of analytical grade for biochemical use.

Cloning and Sequencing—Genomic DNA isolated from *D. vulgaris* (Miyazaki F) was prepared by the method of Saito and Miura (*16*), and digested with *Eco*RI and *Sal*I. The digests were separated into several fractions on an agarose gel according to their size. The separated fragments were ligated into the same site of pUC18, and *E. coli* JM109 was transformed with the resulting ligation mixture. When mini-scale preparation of the plasmid from many colonies of *E. coli* was carried out, some colonies with a brown-colored cell lysate were identified. This colony harbored a plasmid carrying an approximately 3.3-kbp *Eco*RI–*Sal*I fragment of *D. vulgaris* (Miyazaki F) DNA. The nucleotide sequence of the 3.3-kbp *Eco*RI–*Sal*I fragment was determined by sequencing of its restriction fragments cloned into the multicloning site of pUC18 as well as the deletion mutant obtained through the use of exonuclease III and Mung Bean nuclease.

Purification of the Expressed Brown Protein—Transformed *E. coli* cells were cultured for 14 h at 37°C in 10 ml of LB medium containing 50 μg/ml ampicillin. Six flasks containing 167 ml of this medium were inoculated with 1.5 ml of the culture and then incubated overnight with shaking 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 (pH 8.0) and then sonicated with a Model 201M sonicator (KUBOTA) at 9,000 Hz, 200 W for 10 min. After removal of the cellular debris by centrifugation at 10,000 rpm for 20 min, the suspension was centrifuged at 45,000 rpm for 2 h. The green-brown supernatant was dialyzed against 10 mM Tris-HCl (pH 8.0) overnight and then loaded onto a DEAE-cellulose (DE52) column (2.0 × 14.0 cm) equilibrated with 10 mM Tris-HCl (pH 8.0). The column was washed with 200 ml of 50 mM NaCl/10 mM Tris-HCl (pH 8.0), and then developed with a gradient of 50 to 200 mM NaCl in 10 mM Tris-HCl (pH 8.0). The colored fractions were collected, diluted twice, and then loaded onto the DE52 column under the same conditions. Fractions having A_{280}:A_{412} ratios less than or equal to 1.5 were collected and lyophilized. Gel-filtration on a Superose 12 HR 10/30 column (1.0 × 30.0 cm) was carried out using 200 mM NaCl/10 mM Tris-HCl (pH 8.0) as the eluent at the flow rate of 0.4 ml/min.

High-Level Expression of the Brown Protein—We attempted to construct a high-level expression system in *E. coli* through the reduction of another ORF and regulatory region, and through the addition of a strong promoter, the *tac* promoter. First, to produce a restriction enzyme site in the 5'-terminus region we performed site-directed mutagenesis. A deoxyoligonucleotide, Pax-2, was designed to introduce a *Bsp*HI site three nucleotides upstream of the amino-terminal Met codon (ATG); 5'-AGGAGGAACTCATGACGAA-3'. We obtained a mutant plasmid (pAX-200), which is the same as pAX-100 except for having a *Bsp*HI site upstream of the initiation codon of *Kat*, using Inouye's site-directed mutagenesis method (*17*). After digestion of pAX-200 with *Bsp*HI, we extracted an approximately 530-bp fragment. We then blunt-ended this fragment with a klenow fragment and digested it with *Kpn*I. In contrast, pAX-100 was cut with *Eco*RI, blunt-ended with a klenow fragment, and then digested with *Kpn*I. We ligated an approximately 40-bp *Bsp*HI (blunt-ended)–*Kpn*I fragment (nucleotide numbers 1445 to 1488) of pAX-200 and an *Eco*RI (blunt-ended)–*Kpn*I fragment of pAX-100, designated as pAX-300. Finally, pAX-300 was digested with *Eco*RI and *Hin*dIII, and then ligated to expression vector pMK2 previously cut with the same enzyme. Plasmid pMK2 has a high copy number vector similar to pUC18 and has a *tac* promoter (*18*). We therefore named the obtained plasmid pMKAX-300. To compare the amounts of expressed protein, we used the absorbance at 414 and 554 nm of the cell lysate.

In Vitro Activity—Because we thought the brown protein is catalase, its *in vitro* activity was measured according to the method of Aebi (*19*). Various concentrations of hydrogen peroxide (final concentrations, 10 to 30 mM) were used as a substrate in a 50 mM phosphate buffer (pH 7.0). The precise concentration of hydrogen peroxide was determined from the absorbance at 240 nm, and the decomposition of hydrogen peroxide was followed as the decrease in the absorbance at the same wavelength for 60 s. The amount of active protein was estimated from the absorbance of heme. The concentration of heme was also determined from the absorbance at 557 nm under reducing conditions with dithionite in a 10% pyridine/0.2 M NaOH solution (*20*). Absorption spectra were recorded at room temperature with a Hitachi U-2000 spectrophotometer. We determined the concentration of the protein using a Micro BCA Protein Assay Reagent Kit (PIERCE).

Analytical Methods—SDS-PAGE was carried out according to the method of Laemmli (*21*) with a gel concentration of 15.0%. For separation of the prosthetic group and polypeptide, the protein purified by gel-filtration was dissolved in 0.1% TFA and then subjected to reverse-phase HPLC (C8 column: NUCLEOSIL10 C8, 0.46 × 25 cm) with a linear gradient of acetonitrile in 0.1% TFA at the flow rate of 0.8 ml/min. The polypeptide was eluted at 28.4 min (50.5% of acetonitrile). Amino acid sequence analysis of the apoprotein was carried out with an Applied Biosystems model 473A protein sequencer. To identify the prosthetic group,

Fig. 1. **Nucleotide sequence of the cloned 3.3-kbp fragment.** The amino acid sequences deduced from two possible open reading frames are shown as one-letter abbreviations. Putative sequences serving as the regulatory region (−35 region and −10 region) and the ribosome-binding site (SD sequence) are indicated. The direct repeat region (8 times repeats of 11 nt) is also underlined.

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       10        20        30        40        50        60        70        80        90       100       110       120
GAATTCTCCGACCTGGTCTTCGCGCTGGATTCCATCCCCGCCATCTTCGCCGTGTCCACCGACCCATTCATCGTCTACACCTCCAACGTGTTCGCCATCCTGGGCCTGCGGGCGCTGTAC
E  F  S  D  L  V  F  A  L  D  S  I  P  A  I  F  A  V  S  T  D  P  F  I  V  Y  T  S  N  V  F  A  I  L  G  L  R  A  L  Y
      130       140       150       160       170       180       190       200       210       220       230       240
TTCGCCCTGGCGGGGGTCATCCACCGCTTCCGCTACCTCAAGTACGGCCTGTCGCTGGTGCTGGTGTTCATCGGTGCCAAGATGATCGTCAACGCCGCCTTCGACGCCAAGATCATTTCC
F  A  L  A  G  V  I  H  R  F  R  Y  L  K  Y  G  L  S  L  V  L  V  F  I  G  A  K  M  I  V  N  A  A  F  D  A  K  I  I  S
      250       260       270       280       290       300       310       320       330       340       350       360
ACTGGCGCGGCCCTGCTGGTGACCTTCGGCATAATCGCCGGGTCCATGCTGATTTCCATGGCAAAGACACGCGGCGCGGGCGCGGATGCACAGGCCGCTCCGCCCCTGGGCTGGGTGCCG
T  G  A  A  L  L  V  T  F  G  I  I  A  G  S  M  L  I  S  M  A  K  T  R  G  A  G  A  D  A  Q  A  A  P  P  L  G  W  V  P
      370       380       390       400       410       420       430       440       450       460       470       480
GGCACCCCGAGCAAAAGTGAACGGACCACCGACAACACCGGCAAGCCCCCGCGTTGACACCCCCCTTGCCCCGGAATATTCTTTATCAGGAAACAACTGTTCCCCCGTCTGCCCCGCAGT
G  T  P  S  K  S  E  R  T  T  D  N  T  G  K  P  P  R  *
      490       500       510       520       530       540       550       560       570       580       590       600
TGCACCCCAAGGGGGGCCGCACTGGCGGGGCAGCCGCGACTGGACACGACTGTACGCGGCTGGGCACGACTGCACGAACTGATCGGAACTGGTCCGACGGGCCACGACGACGCATCCTCG

      610       620       630       640       650       660       670       680       690       700       710       720
ACAACCGCCCCGGCTCCGGAATGCACCGGCACCCCATGGATACCCGCACCCGCCTCGCCTCCCTGCTGGAACGCCTGCGCGACAACGGACACCGGCTCACCCCCCAGCGGGTGGCCATCG

      730       740       750       760       770       780       790       800       810       820       830       840
TCCGCGCCCTGATCGAGCACGACGGCCACCCCACGGTGGAACAACTGCACCAGTCCATCCTGCCGGACTTTCCCACCACCAGCCTGGCCACGGTGTACAAGACCATCGCCCTGCTGAAGG

      850       860       870       880       890       900       910       920       930       940       950       960
AAGACGGCGAGGTGCTGGAACTGGGCTTTGGCGAACTGGGCAGCCGCTACGACGGCAGGCATCCGGAACCGCACCCCCACCTTATCTGCACCCGCTGCGGGGCCATCGCCGACTATTCGC

      970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
TGCAGGAGCTGGACGAACTGGTGGCGCGCATGGCCGCCGCAACTGGCTTTGCGTGGCCAGCCACCGGTTCGACATGTTCGGGCTGTGCCGACCTGCCGGGTTGCTGCCCACCCAGCCAAC

     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GCGGACCCCCAGCCCGACACGCCAGCCGACACGCCCCCCCCTTCCGGTGCTGAGCGCCCGCACGCCGACGCACACAACACCCCGCCACAACAAGACATCCCGCACGACGACAGTCCAGAC

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
CCGGACCTGATCCGGGCCTGACACGGGCCTGACACGGGCCTGATCCGCGCTCGACCCAAGCCCGACCCAAGCCCCGCCCGCCGGGCCTTTTCATTTTTCGTTGACAGCTGCTCCTGCTTA
                    direct repeat region                                                                  -35
     1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
TGGATAATAATTATTACAAAGTTATTTTTATCCTAAACTATAGCCGTCCCACCGCACTGGCACCCGACTGGAACCCGATCCCCCCCGACCGACACCCACAGTCACCCCCAACAGGAGG
     -10                                                                                                                SD
     1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
AACCCATGACGAAGCACAAGCTCACCACCAACGCAGGCGCCCCGGTACCCGACAACCAGAACGCCATGACCGCCGGGCCGCGCGGCCCCATGCTGCTGCAAGACGTGTGGTTTCTGGAAA
      M  T  K  H  K  L  T  T  N  A  G  A  P  V  P  D  N  Q  N  A  M  T  A  G  P  R  G  P  M  L  L  Q  D  V  W  F  L  E  K
     1570      1580     ·1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
AGCTGGCCCACTTCGACCGCGAGGTGATCCCCGAGCGGCGCATGCATGCCAAGGGGTCCGGCGCGTACGGCACCTTCACCGTCACCCACGACATCACCAGCTACACCAAGGCCGCCCTGT
  L  A  H  F  D  R  E  V  I  P  E  R  R  M  H  A  K  G  S  G  A  Y  G  T  F  T  V  T  H  D  I  T  S  Y  T  K  A  A  L  F
     1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
TCTCAAAAATCGGCAAGAAGACCGATCTGTTCGTGCGCTTTTCCACCGTGGCGGGCGAGCGCGGCGCCGCGACCGCGGAACGCGACATCCGGGGCTTCGCCATCAAGTTCTACACGGAAC
  S  K  I  G  K  K  T  D  L  F  V  R  F  S  T  V  A  G  E  R  G  A  A  T  A  E  R  D  I  R  G  F  A  I  K  F  Y  T  E  Q
     1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920
AGGGCAACTGGGACCTGGTGGGCAACAATACCCCGGTCTTCTTCCTGCGCGACCCGCTGAAGTTCCCCGACCTGAACCACGCCGTGAAGCGCGACCCGCGCACCAACATGCGCAGCGCGA
  G  N  W  D  L  V  G  N  N  T  P  V  F  F  L  R  D  P  L  K  F  P  D  L ·N  H  A  V  K  R  D  P  R  T  N  M  R  S  A  K
     1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040
AGAACAACTGGGACTTCTGGACATCCCTGCCCGAGGCGCTGCATCAGGTCACCGTGGTCATGAGCGACCGGGGCATTCCCGCCAGCTACCGGCACATGCACGGCTTCGGCAGCCACACCT
  N  N  W  D  F  W  T  S  L  P  E  A  L  H  Q  V  T  V  V  M  S  D  R  G  I  P  A  S  Y  R  H  M  H  G  F  G  S  H  T  F
     2050      2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160
TCAGCTTCATCAGCCCGGACAACCAGCGCTACTGGGTGAAGTTCCACCTGCGCACCCAGCAGGGCATCAAGAACCTGACCGACGCCGAGGCCGAGGCCATCGTGGCCAGGGACCGCGAAA
  S  F  I  S  P  D  N  Q  R  Y  W  V  K  F  H  L  R  T  Q  Q  G  I  K  N  L  T  D  A  E  A  E  A  I  V  A  R  D  R  E  S
     2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280
GCCACCAGCGCGACCTGTACGACAGCATCGAACGCGGTGACTTCCCCAGGTGGACCATGTACGTGCAGGTCATGCCTGAAAAGGACGCGGAAAAGCTGCCCTACCACCCCTTCGACCTGA
  H  Q  R  D  L  Y  D  S  I  E  R  G  D  F  P  R  W  T  M  Y  V  Q  V  M  P  E  K  D  A  E  K  L  P  Y  H  P  F  D  L  T
     2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
CCAAGGTGTGGTTCCACAAGGACTGCCCGCTCATCGAGGTGGGCGTGCTGGAACTGAACCGCAACCCGGAAAACTACTTCGCCGAAGTGGAACAGGCCGCGTTCAACCCCGCCAACGTGG
  K  V  W  F  H  K  D  C  P  L  I  E  V  G  V  L  E  L  N  R  N  P  E  N  Y  F  A  E  V  E  Q  A  A  F  N  P  A  N  V  V
     2410      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520
TTCCGGGCATCAGCTTTTCGCCCGACAAGATGCTGCAGGGCCGCCTGTTCTCCTACGGCGACGCGCACCGCTACCGCCTGGGCGTGAACCACCACCTGATCCCGGTCAACGCCGCGCGCT
  P  G  I  S  F  S  P  D  K  M  L  Q  G  R  L  F  S  Y  G  D  A  H  R  Y  R  L  G  V  N  H  H  L  I  P  V  N  A  A  R  C
     2530      2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640
GCCCGGTGCACAGCTACCACCGCGACGGGGCCATGCGCGTTGACGGCAACCACGGCAGCACCCTGGCCTACGAGCCCAACAGCTATGGCGAATGGCAGGAGCAGCCCGACTTCGCGGAGC
  P  V  H  S  Y  H  R  D  G  A  M  R  V  D  G  N  H  G  S  T  L  A  Y  E  P  N  S  Y  G  E  W  Q  E  Q  P  D  F  A  E  P
     2650      2660      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760
CGCCCCTGGCCATCAGGGGCGATGCCGCGCACTGGAACTTCCGCGAGGACGCCGACTACTACGACCAGCCCGGGCGCCTGTTCCGCCTGATGACCCCGCAACAGCAGGACGAGCTGTTCC
  P  L  A  I  R  G  D  A  A  H  W  N  F  R  E  D  A  D  Y  Y  D  Q  P  G  R  L  F  R  L  M  T  P  Q  Q  Q  D  E  L  F  Q
     2770      2780      2790      2800      2810      2820      2830      2840      2850      2860      2870      2880
AGAACACCGCGCGGGCCATGGGCGATGCGCCGGAAGAGATCAAGCGCCGCCACGTGGGCAACTGCGCCAAGGCCGACCCGGCCTATGGCGCGGGCGTGGCCAGGGCCCTGGGCCTGAAGA
  N  T  A  R  A  M  G  D  A  P  E  E  I  K  R  R  H  V  G  N  C  A  K  A  D  P  A  Y  G  A  G  V  A  R  A  L  G  L  K  M
     2890      2900      2910      2920      2930      2940      2950      2960      2970      2980      2990      3000
TGTAGCGCCACCAGCGGCGGGCATCCCCAGCGGAACCGCCCGCCGCCCCCTCACCGCCCCACTCTTCCGCTCCGGCATTCCGGCAACGCGGGCTGCATCCCGCATCTGGCGGAATGCCCG
  *
     3010      3020      3030      3040      3050      3060      3070      3080      3090      3100      3110      3120
GCGGAACCCCCCGTGATGCATCCGTTCCTTGATGCATCCTCCTGTCTGCGGGCCACCCCTGCACGGCCACGCGGAAAGGCCCCCGGCGTCACGCGTGGACGTGCCGGGGGCCTTGTGCGT

     3130      3140      3150      3160      3170      3180      3190      3200      3210      3220      3230      3240
CATGCCATGGAATCCGGGGACCAGGGAACGGGAAGCGAGGAATGGAGACCGGAACACCTTCTCAGGACAAGGCGCCATCCGGGGGGATATCCCCGCCGCCTGAACGCCCGGAAGGCCCCG

     3250      3260      3270
GCCCCGTGGACAGTCCGCCCAAGGGCAGGTCGAC
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we extracted it by an acid–acetone method. The extracted heme was subjected to reverse-phase HPLC on the same column as described above under isocratic conditions with a solution of 70% ethanol/17% acetic acid/13% water at the flow rate of 0.5 ml/min, and monitored as to the absorbance at 400 nm (*20*). Bovine liver catalase was used as a reference.

Immunoblot Analysis—To compare the higher structures of the brown protein and bovine catalase, immunoblot analyses were carried out. Samples were loaded on a 15% polyacrylamide SDS minislab gel. Blotting was carried out electrophoretically with a nitrocellulose filter, which was then blocked by shaking in 3% (w/v) bovine serum albumin in buffer A containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% Tween 20 for 30 min. After a wash with buffer A, it was incubated in buffer A for 30 min with a 1:1,000 dilution of a polyclonal antiserum raised against bovine catalase. The blot was then thoroughly washed with buffer A and incubated with 1 ml of alkaline phosphatase–conjugated goat anti-rabbit IgG in buffer A. Following two washes, the first with buffer A and the second with alkaline phosphatase buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$], the nitrocellulose filter was incubated with nitroblue terazolium and 5-bromo-4-chloro-3-indoyl phosphate in 5 ml of alkaline phosphatase buffer. The blot was finally washed with water and dried.

Hydrogen Peroxide Stress against D. vulgaris (Miyazaki F)—To determine the hydrogen peroxide-inducible responses of *D. vulgaris* (Miyazaki F), we prepared the whole proteins of *D. vulgaris* cells cultured anaerobically or treat-

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                                                 !               !      !  !!
(A)   1 M--------- TKHKLTTNAG APVPDNQNAM TAGPRGPMLL QDVWFLEKLA HFDREVIPER RMHAKGSGAY GTFTVTHDIT
(B)   1 .SSQCPFSHL AAT...MGN. ...A....SL .......L.A ..L.LN.... D.V....... .........F ..........
(C)   1 .--------- E.K....A.. ...V..N.VI .......... .......... .......... .........F ..........
(D)   1 .--------- ENK...AAN. R.IA....SQ .......IM. ..P.LI.... .......... .......... ..........

                            !                    !! !             !!!          !  !
(A)  72 SYTKAALFSK IGKKTDLFVR FSTVAGERGA ATAERDIRGF AIKFYTEQGN WDLVGNNTPV FFLRDPLKFP DLNHAVKRDP
(B)  81 K..R.KI..E V....EM.A. .T........ .D........ .L....E... .......... ......R... ...K......
(C)  72 K..R.KI..E V....EM.A. .......... .D........ .L....E... ..M....... .Y........ ....I.....
(D)  72 K..R..I..Q V..Q.EC.V. .......... .D........ .M....E... ..L....... .......... ..........

                               *      *  *          *  !!!                  *
(A) 152 RTNMRSAKNN WDFWTSLPEA LHQVTVVMSD RGIPASYRHM HGFGSHTFSF ISPDNQRYWV KFHLRTQQGI KNLTDAEAEA
(B) 161 .......T.. .....L.... .......... .......... .......Y.. WNEAGE.F.. ...F...... ........AE
(C) 152 .....NMAYK ...FSH...S ...L.ID... ..L.L...FV .......Y.. .NK..E.F.. ...F.C.... ...M.D....
(D) 152 .N.....N.. .....L.... .....IT..P .......... .......Y.. LNAE.K.I.. ....K.M... .....Q....

                                                          !    * *
(A) 232 IVARDRESHQ RDLYDSIERG DFPRWTMYVQ VMPEKDAEKL PYHPFDLTKV WFHKDCPLIE VGVLELNRNP ENYFAEVEQA
(B) 241 .I.N...... ....EA.... ...K..LF.. I...A....V .......... .SK..Y.... ..EF...... ..F..D...S
(C) 232 L.GK....S. ...FEA.... .Y...KLQI. I....E.STV .......... .P.A.Y..MD ..YF...... D...SD....
(D) 232 .I.K...... ....E..... .F.K.KFQI. L.T.EE.DNY RIN....... .P...F..QD ..I....... .........S

         !                !    !  !!  !!   !
(A) 312 AFNPANVVPG ISFSPDKMLQ GRLFSYGDAH RYRLGVNHHL IPVNAARCPV HSYHRDGAMR VDGNHGSTLA YEPNSYGEWQ
(B) 321 ..A.S.L... .GA...R... A...N.A..Q .......YRQ ....RP.... ..NQ...QG. ....Y..LPH .....FSQ..
(C) 312 ..S...I... .......... .......... .........Q .....PK..F .N........ ....S.NGIT .....G.VF.
(D) 312 ...M.I.E.. .G........ .........Q .......SEQ ....KP...F .AF....... ....Y..AKG ..........

                                         *
(A) 392 EQPDFAEPPL AIRGDAAHWN FREDDADYYD QPGRLFRLMT PQQQDELFQN TARAMGDAPE EIKRRHVGNC AKADPAYGAG
(B) 401 Q......... R.N......D Y.N..N..FS ..RA..N..N AE.KQS..N. ..A......D ...Y..IR.. HWC.A...E.
(C) 392 .....K.... S.E.A.D... H...E-..FS ..RA.YE.LS DDEHQRM.AR I.GELSQ.SK .TQQ.QIDLF T.VH.E....
(D) 392 DS.EKK.... KVH..VFNY. E..Y.D...S ...D.....P ADE.QL..E. ........EL F..Q...R.. Y.......T.

(A) 472 VARALGL--- ---KM*
(B) 481 ..K....TVE DAL.ARDTDP ALGQGGLL*
(C) 471 .EK.IKVLEG KDA.*
(D) 472 .AQ...IDLE EAL.E*
```

Fig. 2. **Comparison of the amino acid sequences of the brown protein and catalase from other organisms.** Lane (A), deduced amino acid sequence of the brown protein (this work); lane (B), amino acid sequence of catalase from *Haemophilus influenzae* (*25*); lane (C), amino acid sequence of catalase from *Proteus mirabilis* (*27*); lane (D), amino acid sequence of catalase from *Bacterioides fragilis* (*26*). . means the same amino acid as in lane (A), and – means a gap. ! and * mean residues which are within 4.0 Å of the heme and 3.4 Å of NADPH in *Proteus mirabilis* catalase, respectively (*31*).

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ed with hydrogen peroxide. The anaerobic cultures were treated with the 10 mM hydrogen peroxide and then cultured for 90 min at 37°C. Cells were collected and suspended in 10 mM Tris-HCl (pH 8.0), and then sonicated for 20 min. An aliquot was then prepared so that SDS-PAGE could be carried out before the immunoblot or activity analysis.

RESULTS

Identification of the Catalase Gene from Desulfovibrio vulgaris (Miyazaki F)—We have carried out a series of genetic studies on the proteins in *D. vulgaris* (Miyazaki F), and cloned the genes of ferredoxins I and II (*22*). In the cloning experiments, we obtained another clone having a 3.3-kbp *Eco*RI–*Sal*I fragment of *D. vulgaris* (Miyazaki F) DNA. Some of these clones exhibited a brown-colored cell lysate, and it was therefore postulated that some brown protein(s) are expressed or that some compound(s), *i.e.* some heme, are produced by the expressed enzymes from the cloned DNA fragment of *D. vulgaris* (Miyazaki F) in *E. coli*.

This clone (pAX-100) was found to possess a plasmid carrying a 3.3-kbp *Eco*RI–*Sal*I fragment of *D. vulgaris* (Miyazaki F) DNA, and the full nucleotide sequence of the *Eco*RI–*Sal*I fragment was determined. The determined sequence is shown in Fig. 1. One open reading frame (ORF) encoding a protein composed of 479 amino acids, including an amino-terminal Met, and preceded by putative ribosome-binding sites (AGGAGG), was observed from nucleotide 1435 to 1440 in the *Eco*RI–*Sal*I fragment (ORF-2). The results of a homology search of the deduced amino acid sequence of the ORF-1 product indicated homology to the COOH-terminal regions of some hypothetical proteins. A product of ORF-3 of the tellurium resistance-region protein from *Alcaligenes* sp., which is thought to be a transmembrane protein with a molecular weight of 38.2 kDa (*23*), exhibits 48% identity and a hypothetical *E. coli* 35.8 kDa protein exhibits 72% identity (*24*). The activities of these products remain unknown. On the other hand, the amino acid sequence of the ORF-2 product is very homologous to those of catalases from various organisms (Fig. 2). The identity of the amino acid sequences between the product of ORF-2, and *Haemophilus influenzae* catalase (*25*), *Bacterioides fragilis* catalase (*26*), and *Proteus mirabilis* catalase (*27*) was 75, 75, and 70%, respectively. So, we named the gene of ORF-2 *Kat*. A possible promoter region exists upstream of the catalase gene in the 3.3-kbp *Eco*RI–*Sal*I fragment. Nucleotides 1301 to 1306, TTGACA, and 1325 to 1331, TAATAAT, resemble the *E. coli* −35 and −10 regions. There were found to be four direct repeat sequences and four similar repeat sequences consisting of 11 nucleotides, (C/T)(C/A)CGG(G/A)CCTGA, from approximately 100 nucleotides upstream of the −35 region. Because these regulatory regions exist between ORF-1 and *Kat*, ORF-1 and *Kat* cannot form an operon.

Purification of the Produced Brown Protein—We purified the brown material from the cell lysate of *E. coli* harboring the 3.3-kbp *Eco*RI–*Sal*I fragment to determine the origin of the brown color, that is, to determine whether the brown material corresponds to the protein produced from the cloned gene or to a reaction product arising through catalysis by the produced enzyme encoded by the ORF in the cloned fragment. Through two steps of chromatography on DE-52 and Superose 12 HR 10/30, approximately 15 mg of a brown-colored protein was purified from 1 liter of LB culture to homogeneity on SDS-PAGE (Fig. 3A). The brown color was thus shown to originate from the clone expressed in *E. coli*. The ORF in the cloned *Eco*RI–*Sal*I fragment appeared to encode a protein capable of binding a brown compound as a prosthetic group.

The molecular weight of the purified protein in the denatured state was estimated to be approximately 59,700 by SDS-PAGE, which is close to the value (54,500) calculated from the amino acid sequence deduced from the nucleotide sequence of *Kat*. On the other hand, we estimated the molecular weight in the native state to be approximately 122,000 on analytical gel-filtration on Superose 12 HR 10/30 (Fig. 3B), indicating that the estimated value was approximately twice that obtained on SDS-PAGE. A minor peak was observed at 25.9 min, was deduced to be a tetramer. However, this protein may primarily form a dimer

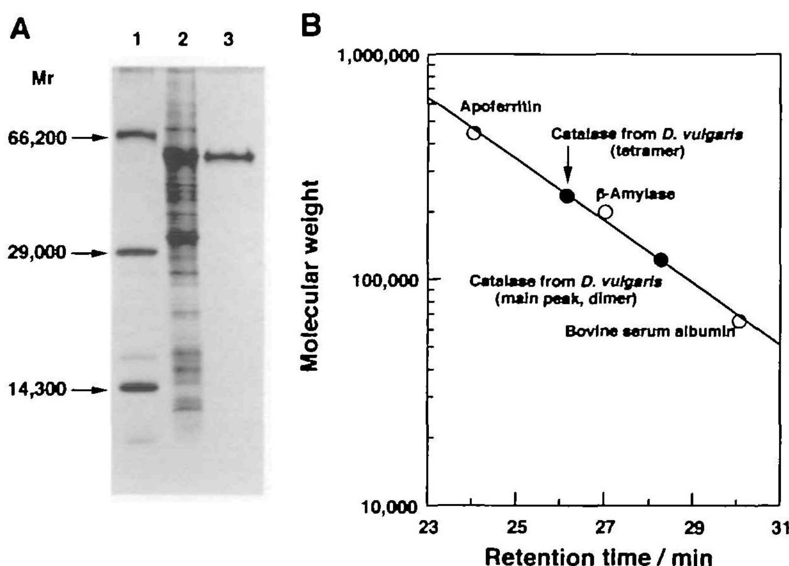

Fig. 3. **Estimation of the molecular weight of the brown protein.** (A) The purified protein was analyzed by SDS-PAGE with a gel concentration of 15.0%. Lane 1, standard proteins: bovine serum albumin (M_r 66,200), carbonic anhydrase (M_r 29,000), and lysozyme (M_r 14,300); lane 2, pAX-100/JM109; lane 3, purified brown protein. The molecular weight of the catalase monomer was estimated to be 59,700. (B) The purified brown protein or molecular weight markers were applied to a Superose 12 HR 10/30 column (1.0 × 30 cm) in an FPLC system (Pharmacia LKB), and elution was performed with 10 mM Tris-HCl/200 mM NaCl (pH 8.0) at the flow rate of 0.4 ml/min. The molecular weight markers were apoferritin from horse spleen (M_r 443,000), β-amylase (M_r 200,000), and bovine serum albumin (M_r 66,200). The retention time of bovine liver catalase was almost the same as that of the brown protein.

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under these conditions.

We produced plasmid pMKAX-300, which has a high copy number and whose promoter is *tac*, and *Kat* closely ligated to the *tac* promoter. Cells transformed with pMKAX-300 showed 0.72-fold expression compared to ones transformed with pAX-100. This ratio, based on the absorbance at 414 and 554 nm of the cell lysate indicates a good correlation with the results of SDS-PAGE (data not shown). It is perhaps important for the expression of *Kat* to use regulatory regions that include a direct repeat region, which may bind some regulated protein (*28*). We therefore used the pAX-100 plasmid to produce this brown protein.

Amino Acid Sequence Analysis of the Expressed Protein—To investigate the correspondence of the protein expressed in *E. coli* to the ORF in the *Eco*RI–*Sal*I fragment, the purified protein was subjected to reverse-phase HPLC. The amino acid sequence of the apoprotein separated by reverse-phase HPLC was analyzed with an automated protein sequencer. The first 15 amino acids were found to be Thr-Lys-His-Lys-Leu-Thr-Thr-Asn-Ala-Gly-Ala-Pro-Val-Pro-Asp, and the amino terminal Met was not detected. It is thus concluded that the brown-colored protein expressed in *E. coli* bearing the 3.3-kbp *Eco*RI–*Sal*I fragment of *D. vulgaris* (Miyazaki F) DNA is a protein composed of 478 amino acids and encoded by *Kat* in the cloned fragment.

Identification of the Prosthetic Group—To identify the prosthetic group bound to the 478-residue protein, an UV/VIS spectrum of the purified holoprotein was measured, as shown in Fig. 4. In the visible region, absorption maxima were observed at 274, 412, 515, 553, 576, and 631 nm, which is characteristic of proteins binding heme. In addition, the UV/VIS spectrum did not change upon reduction with dithionite. Next, the prosthetic group extracted with acid–acetone (*20*) was subjected to reverse-phase HPLC on a C8 column, and the retention time of the obtained prosthetic group was compared with that extracted from bovine liver catalase. As shown in Fig. 5, the retention time of the prosthetic group released from the brown protein was exactly the same as that of protoheme IX (5.03 min). On the basis of the heme content, determined as pyridine hemochrome, and quantitative analysis of the protein, it was estimated that there were 0.91 protoheme IX molecules per monomer.

Thus, *Kat* in the 3.3-kbp *Eco*RI–*Sal*I fragment of *D. vulgaris* (Miyazaki F) DNA is concluded to encode a hemoprotein that is expressed in *E. coli* as a holoprotein binding protoheme IX as a prosthetic group.

In Vitro Activity—Because the deduced amino acid sequence of this protein showed good homology to those of catalases from other organisms, it is likely that this hemoprotein exhibits hydrogen peroxide decomposition activity *in vitro*. Therefore, the hydrogen peroxide decomposition properties of the protein were examined. The results of kinetic analysis with the *Kat* product and hydrogen peroxide shown in Fig. 6 allow K_m and k_{cat} to be estimated to be 133 mM and 1.99×10^6 s^{-1}, respectively. The k_{cat}/K_m value of *D. vulgaris* catalase was similar to the value of horse liver catalase, but the K_m value was approximately 30 times greater than that of horse liver catalase (*29*).

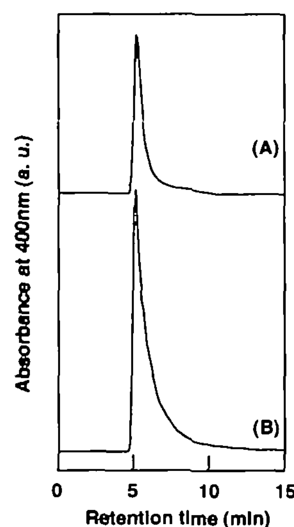

Fig. 5. **HPLC of the prosthetic group bound to the brown protein on a C8 column.** (A) The heme extracted from bovine liver catalase, which was identified as protoheme IX. (B) The heme extracted from the brown protein. The hemes were monitored as to the absorbance at 400 nm. Their retention times were compared and found to be identical.

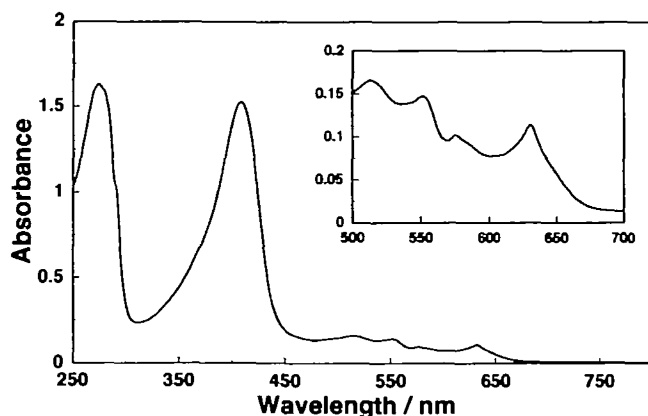

Fig. 4. **UV/VIS spectrum of the purified brown protein.** The absorption spectrum was recorded in 200 mM NaCl/10 mM Tris-HCl (pH 8.0) at room temperature using a Hitachi U-2000 spectrophotometer. The insert shows magnification of between 500 and 700 nm.

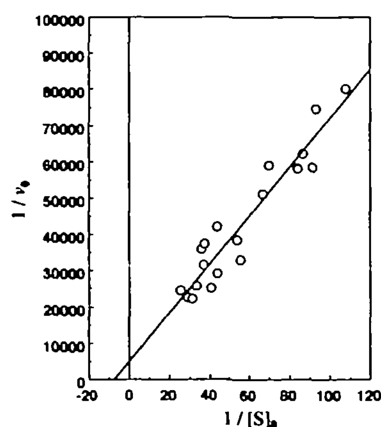

Fig. 6. $1/[S]_0$ *vs.* $1/v_0$ **plot of catalase.** K_m and k_{cat} were estimated to be 133 mM and 1.99×10^6 s^{-1}, respectively.

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Through the above, we have confirmed that the product of *Kat* is a catalase.

Immunoblot Analysis—The recombinant catalase of *D. vulgaris* (Miyazaki F) reacted with a polyclonal antibody raised against bovine catalase 25 times less than with bovine catalase. These results indicate that the surface structures of the two catalases are not similar enough for the bovine catalase antibody to crossreact, although the amino acid sequences of these catalases are very homologous (50% identity), and the amino acid residues are conserved in the heme binding and NADPH binding regions.

Hydrogen Peroxide Stress—The decomposition activity of *D. vulgaris* (Miyazaki F) cells treated with hydrogen peroxide was 2.1 times greater than that under anaerobic growth conditions, while the absorbance of the *b*-type heme of a cell-free extract of hydrogen peroxide-treated cells was only 1.3 times greater than that of anaerobic cells. Using cell-free extracts under both growth conditions, we were not able to detect expression of the gene on the immunoblot analysis described above. The amount of catalase was calculated to be less than 2.3 μg per 1 g wet cells (less than 0.039% per whole protein) in each case. These spectroscopic and immunoblot analysis data suggest that effective expression of the catalase gene is not induced in response to hydrogen peroxide stress.

DISCUSSION

In this study, we isolated a gene encoding a catalase that functions as a protective protein against hydrogen peroxide. This catalase and SOD are among the oxidative stress-starvation response proteins that have been extensively studied in aerobic bacteria (*30*), but much less is known about enzymes in anaerobic organisms. It has been reported, however, that *Bacterioides fragilis*, one of the anaerobic species, expresses catalase as part of the oxidative stress response (*26*). We have shown that *D. vulgaris* (Miyazaki F), also one of the anaerobes, has a gene encoding catalase, but its activity toward hydrogen peroxide is not thought to be sufficient with regard to its K_m value and amount in the cell. In SRB, there are many proteins that bind heme as a prosthetic group (*31, 32*), and it is possible that this catalase is one of the hemoproteins located in the cytoplasm, but its substrate may not be hydrogen peroxide.

The molecular weight of catalase from *B. fragilis* has been estimated to be 124,000 to 130,000, *i.e.* a dimer of a 60,000 Da monomer (*33*), which is similar to in the case of the catalase of *D. vulgaris* (Miyazaki F). However, it is known that bovine liver catalase consists of a tetramer and that its molecular weight is 232,000 (*29*). It has been reported that a diluted solution of catalase is unstable (*34*) because the tetramer may be easily dissociated. From the results of analytical gel filtration, it seems reasonable that catalase from *D. vulgaris* (Miyazaki F) forms a tetramer (*3*). The crystal structure of bovine liver catalase forming a tetramer has been determined (*35, 36*), and the amino acid sequences of *D. vulgaris* catalase are all identical to those of *Proteus mirabilis* PR catalase within 4.0 Å of the heme and within 3.4 Å of NADPH (*37*). The catalase of *D. vulgaris* (Miyazaki F) must form a similar structure, especially in the prosthetic group binding domain, although the addition of NADPH appears to have no influence on the kinetic constants (K_m is 151 mM and k_{cat} is 2.06×10^6 s^{-1}, respectively). The heme content is rather low, but this phenomenon is frequently observed in the case of catalase (*38, 39*). It is possible that the heme-binding capacity is one heme per subunit, as with other catalases (*40*). However, the surface structure of recombinant catalase of *D. vulgaris* (Miyazaki F) was found not to be as similar to bovine catalase that crossreacts with polyclonal antibodies of bovine catalase. This difference should affect the higher structure and the hydrogen peroxide decomposition properties.

The catalase from *D. gigas*, which is more tolerant of oxygen, is thought to be expressed constitutively, and it exhibits unusually low activity. The properties of *D. gigas* catalase are different in terms of both the molecular mass and the uncertain absorption spectrum (*4*). The high K_m and k_{cat} values of *D. vulgaris* catalase may reflect the anaerobic environment in which sulfate-reducing bacteria can survive, that is, an environment with a low oxygen level and even a lower active oxygen concentration. When this bacterium opposes the oxidant conditions, a toxic superoxide anion might be eliminated through dismutation to hydrogen peroxide by SOD, which has a similar structure to others but not desulfoferrodoxin (*41*), and the accumulation of toxic hydrogen peroxide may be prevented by the fast action of catalase. The cytochrome *c* oxidase–like protein can then reduce the oxygen if it is expressed and active like other cytochrome *c* oxidases (*13*). Therefore the genes encoding these proteins must be expressed cooperatively in response to the oxidative stress, although there may be other mechanisms against the active oxygen (*12*).

We identified the catalase gene from a sulfate-reducing bacterium for the first time, and the existence of this gene suggested that there is horizontal transmission of the gene from some aerobic bacterium or that the sulfate-reducing bacterium might at one time have been an aerobic bacterium. Aerobic bacteria are thought to have evolved from anaerobic bacteria, and an essential enzyme of the aerobic organisms may have evolved from that of an anaerobic bacterium.

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