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Purification and Characterization of an Iron Superoxide Dismutase and a Catalase from the Sulfate-Reducing Bacterium *Desulfovibrio gigas*

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The iron-containing superoxide dismutase (FeSOD; EC 1.15.1.1) and catalase (EC 1.11.1.6) enzymes constitutively expressed by the strictly anaerobic bacterium *Desulfovibrio gigas* were purified and characterized. The FeSOD, isolated as a homodimer of 22-kDa subunits, has a specific activity of 1,900 U/mg and exhibits an electron paramagnetic resonance (EPR) spectrum characteristic of high-spin ferric iron in a rhombically distorted ligand field. Like other FeSODs from different organisms, *D. gigas* FeSOD is sensitive to H_2O_2 and azide but not to cyanide. The N-terminal amino acid sequence shows a high degree of homology with other SODs from different sources. On the other hand, *D. gigas* catalase has an estimated molecular mass of 186 ± 8 kDa, consisting of three subunits of 61 kDa, and shows no peroxidase activity. This enzyme is very sensitive to H_2O_2 and cyanide and only slightly sensitive to sulfide. The native enzyme contains one heme per molecule and exhibits a characteristic high-spin ferric-heme EPR spectrum ($g_{y,x}$ = 6.4, 5.4); it has a specific activity of 4,200 U/mg, which is unusually low for this class of enzyme. The importance of these two enzymes in the context of oxygen utilization by this anaerobic organism is discussed.

The sulfate-reducing bacteria *Desulfovibrio* spp. have been classified among the so-called strictly anaerobic organisms. However, there is growing evidence that they can survive exposure to oxygen. One piece of evidence is that these bacteria have been isolated from superficial waters and aerated environments (25, 43, 48), suggesting that these organisms have a mechanism(s) of defense against oxygen radicals and that oxygen may play some physiological role in these bacteria. In fact, *Desulfovibrio gigas* contains a number of potential generators of superoxide anion, including cytochromes, flavodoxins, rubredoxins, and menaquinone.

It was recently discovered that *D. gigas* can utilize polyglucose for the formation of ATP linked to the reduction of O_2 to water (16, 47). The exploration of the system that allows the reduction of O_2 to water has culminated in the discovery of a protein named rubredoxin-oxygen oxidoreductase, which contains Fe-uroporphyrin I as a prosthetic group (51). In addition to providing evidence of a new physiological way of reducing oxygen to water, this discovery has revealed for the first time a role for rubredoxins in sulfate-reducing bacteria. Furthermore, it was demonstrated that these *D. gigas* proteins and another one, characterized as NADH-rubredoxin oxidoreductase (10), are part of an electron transfer chain coupling NADH oxidation to dioxygen reduction. This chain explains the production of ATP via the degradation of polyglucose (16).

It is well known that the partial reduction of oxygen to water during microbial respiration results in intermediate compounds, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), that are potentially toxic to cells. Oxygen radicals are implicated in damage to membrane lipids, proteins, and DNA (18, 29), and their toxicity results when the degree of oxidative stress exceeds the capacity of the cell defense systems. Virtually all aerobic organisms have evolved complex defense and repair mechanisms to overcome the damaging effects of these reactive oxygen species (37, 38). Thus, toxic O_2^- is eliminated by dismutation to H_2O_2 and O_2, a reaction catalyzed by superoxide dismutase (SOD) (29), and accumulation of potentially toxic H_2O_2 is prevented by the action of catalases and peroxidases (24).

Although enzymes like SOD and catalase have been extensively studied in aerobic bacteria, little is known about these enzymes and the mechanisms that regulate their expression in anaerobic organisms. It is believed that in some anaerobic bacteria, as in aerobic organisms, SOD and catalase play a role in the detoxification of oxygen by-products. However, it has been shown that anaerobic bacteria are not uniformly sensitive to oxygen; there is a broad range of oxygen tolerance among these organisms. This difference in sensitivity to oxygen has even been associated with the degree of virulence of some pathogenic anaerobic bacteria (45).

Although SOD activity has been shown to be present in sulfate-reducing bacteria, only the *Desulfomicrobium norvegicum* (formerly known as *Desulfovibrio desulfuricans* strain Norway 4 [19]) and the *Desulfovibrio vulgaris* enzymes have been characterized to some extent (26, 27). Herein we report the purification and characterization of catalase and SOD, which are constitutively expressed during anaerobic growth, from *D. gigas*.

MATERIALS AND METHODS

Preparation of cell extracts. *D. gigas* (ATCC 19364) was grown in a medium described previously (28). Briefly, *D. gigas* cells (900 to 1,500 g) were suspended in 900 ml of 10 mM Tris-HCl buffer, pH 7.6, and ruptured by passing them through a Manton Gaulin press twice. The resulting extract was centrifuged at 18,000 × *g* for 2 h. The supernatant was then centrifuged at 190,000 × *g* for 2 h. The final supernatant was dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.6, and then subjected to procedures for purification of soluble proteins.

Purification of SOD. All purification steps were performed at pH 7.6 and 4°C. Purity was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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TABLE 1. Purification of *D. gigas* SOD and catalase

Enzyme	Purification step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Yield (%)	Purification (fold)
SOD	Crude extract	105,600	3.60×10^5	3.4	100.0	0
	DE52-cellulose	6,980	0.97×10^5	13.9	26.0	4
	Hydroxylapatite	2,000	0.40×10^5	20.0	11.0	41
	DEAE Bio-Gel	45	0.20×10^5	470.0	5.0	138
	Superdex-75	3	0.60×10^4	1,900.0	1.6	559
Catalase	Crude extract	35,862.0	1.9×10^6	52.6	100	0
	DE52-cellulose	12,900.0	4.0×10^5	31.6	21	0.6
	Q-Sepharose	2,200.0	2.3×10^5	104.5	12	2
	DEAE Bio-Gel	328.0	1.2×10^5	384	6	7.3
	Sephacryl S-300	36.8	1.0×10^5	2,728	5.3	52
	Hydroxylapatite	1.6	0.6×10^4	4,200	0.3	80

[a] For SOD, 1 Unit of activity is defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% at 25°C. For catalase, 1 U of activity is defined as the amount of enzyme required for the decomposition of 1 μmol of H_2O_2 per min at 25°C.

(SDS-PAGE). The dialyzed crude extract obtained from 1,500 g of cells was loaded onto a DE-52 cellulose column (10 by 40 cm) equilibrated with 10 mM Tris-HCl buffer. A 4-liter (total volume) linear gradient (0.01 to 0.5 M Tris) was applied. The SOD-containing fraction was not retained on the column, and a major fraction containing a high level of SOD activity was collected, pooled, and then loaded on a hydroxylapatite column (1.6 by 30 cm) equilibrated with 10 mM Tris-HCl. A descending stepwise gradient of 10 to 5 mM Tris-HCl (100 ml per step) was applied, followed by an ascending linear potassium phosphate gradient (0.01 to 0.35 M). SOD, cytochromes, and other minor contaminants eluted together at approximately 100 mM phosphate. This fraction was dialyzed overnight against 3 mM Tris-HCl buffer and then loaded onto a DEAE Bio-Gel column (4.5 by 35 cm) equilibrated with 3 mM Tris-HCl. The partially purified SOD, free of cytochrome *c*, was eluted when the column was washed with the equilibration buffer. The pure SOD was eluted with 50 mM Tris–150 mM NaCl after the preparation was loaded on a high-performance liquid chromatographic Superdex-75 column.

Purification of catalase. The dialyzed crude extract obtained from 900 g of cells was loaded onto a DE-52 cellulose column (10 by 40 cm) equilibrated with 10 mM Tris-HCl. An 8-liter (total volume) linear Tris-HCl gradient (0.01 to 1 M) was applied. A fraction containing catalase activity, eluting at about 0.2 M Tris-HCl, was collected. This fraction was diluted and loaded on a Q-Sepharose column equilibrated with the same buffer. A linear NaCl gradient (0 to 0.4 M) in Tris-HCl buffer was applied. The catalase-containing fraction was eluted at 0.2 M NaCl. This fraction was loaded onto a DEAE Bio-Gel column equilibrated with 5 mM Tris-HCl. A linear gradient (0.005 to 0.5 M Tris) was applied, and the catalase-containing fraction was loaded on a Sephacryl S-300 column equilibrated with 50 mM Tris-HCl–150 mM NaCl. After that step, the catalase-containing fraction was dialyzed overnight against 5 mM phosphate buffer and then loaded on a hydroxylapatite column (1.6 by 10 cm) equilibrated with the same buffer. A linear phosphate gradient (0.005 to 1 M) was applied, and a fraction with high-level catalase activity was collected. This fraction was pure as analyzed by denaturing SDS-PAGE.

Enzymatic assays. SOD activity was determined by using the xanthine oxidase-cytochrome *c* system as described by McCord and Fridovich (36). Catalase activity was determined spectrophotometrically by monitoring the decomposition of H_2O_2, via measurement of the change in absorbance at 240 nm. Activity was calculated assuming that $\varepsilon = 40\ M^{-1} \cdot cm^{-1}$ (40). Inhibition studies were performed by measuring the respective activities after incubation of aliquots of each enzyme for from 30 min to 12 h in buffer containing 10 mM KCN, NaN_3, H_2O_2, or sulfide. Alternatively, different concentrations of inhibitors were added directly to the assay mixture and then the activity was measured.

Activity staining. Nondenaturing PAGE was performed in accordance with the procedure of Hames (23), using riboflavin for photopolymerization of both the resolving and concentrating gels. SOD was located on the gel by the method of Beauchamp and Fridovich (3). The gels were soaked first in 2.45 mM (0.2%) nitroblue tetrazolium for 20 min and then in a solution containing 28 mM tetramethylethylenediamine, 2.8 mM riboflavin, and 36 mM potassium phosphate (pH 7.8) for 15 min. The gels were placed in a glass container and illuminated for 15 min to visualize the bands containing SOD activity.

Analytical methods. Protein contents were measured by the Bradford method (6), using bovine serum albumin as a standard. Molecular masses were determined by SDS-PAGE (32). Protein standards used were lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase *b* (92.5 kDa), bovine catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

N-terminal sequence and amino acid composition analysis. Sequence determination was performed on an Applied Biosystems model 477A sequencer after Western blotting of the proteins onto a polyvinylidene difluoride membrane (Bio-Rad) in accordance with the manufacturer's instructions. Amino acid composition analysis was performed after hydrolysis of proteins at 150°C for 1 h in 6 M HCl–phenol, and the residues were determined with a Pico Tag amino acid analyzer system (Waters-Millipore). Amino acid sequences were compared by using the PILEUP program from the Genetics Computer Group (GCG), Madison, Wisc.

Spectroscopic methods. Optical absorption spectra were recorded on a Shimadzu model UV-1603 spectrophotometer at room temperature. The pyridine hemochrome spectra of catalase were measured in an aqueous alkaline pyridine solution, and the reduced form of the protein was obtained by addition of sodium dithionite. Electron paramagnetic resonance (EPR) spectra were recorded on a model ESP380 spectrometer equipped with a model ESR900 liquid-helium continuous-flow cryostat (Brucker).

Effect of pH and temperature on activity. Purified SOD and catalase were incubated for 30 min at various temperatures and at pHs ranging from 3.0 to 11, and the respective activities were measured as described above. The buffer system used contained 0.2 M boric acid, 0.05 M citrate, and 0.1 M trisodium phosphate.

RESULTS

SOD. (i) Detection, purification, and activity. Measurement of SOD activity, expressed as the amount of enzyme required to inhibit the reduction of cytochrome *c* by 50%, was used to locate the enzyme-containing fractions during the purification steps. The soluble extract obtained after rupture of *D. gigas* cells and ultracentrifugation at 190,000 × *g* for 2 h had a specific activity of $3.4\ U \cdot mg^{-1}$. After the final step of purification, which consisted of loading the fraction containing SOD activity onto a molecular exclusion column, the pure SOD exhibited a specific activity of $1{,}900\ U \cdot mg^{-1}$. A more-detailed description of the purification procedure is provided in Table 1.

(ii) Spectroscopic characterization. The optical absorption spectrum of the purified *D. gigas* SOD (Fig. 1A) shows a protein peak at 280 nm and a broad absorbance between 350 and 600 nm in the visible region. The EPR spectrum of the purified enzyme (Fig. 1B) is similar to those of other microbial iron-containing SODs (13, 31, 35, 54–56) and is indicative of high-spin iron in a low-symmetry environment. A major component is observed, with *g* values at 4.85, 4.0, and 3.65, corresponding to the middle Kramer's doublet ($Ms = | \pm 3/2 >$) of a spin system with $S = 5/2$ and $E/D = 0.25$. A minor component with $g = 4.3$, assigned to a system with $E/D = 1/3$, is also observed.

(iii) Molecular properties. Gel filtration of native purified *D. gigas* SOD on a calibrated Superdex 75 HR column yielded a single peak whose elution volume corresponded to an esti-

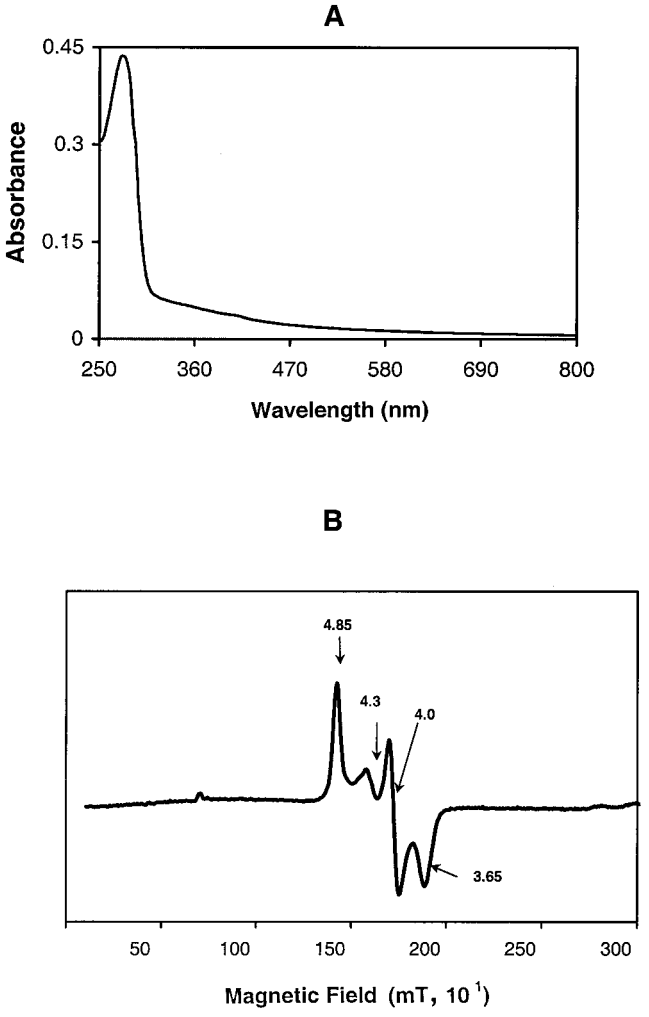

FIG. 1. (A) Absorption spectrum of pure *D. gigas* SOD. The enzyme was present at 0.5 mg/ml in 50 mM Tris-HCl, pH 7.6. (B) EPR spectrum of purified *D. gigas* SOD. The enzyme was in 50 mM Tris, pH 7.6. The spectrum was produced at 4.7 K with a microwave frequency of 9.64 GH2 and a microwave power of 2.4 mW.

mated molecular mass of 43 ± 2 kDa (Fig. 2A). Following SDS-PAGE on a 12.5% denaturing gel, a single band of approximately 22 kDa was observed after Coomassie staining (Fig. 2B). These data demonstrate that *D. gigas* SOD, like most known SODs, is dimeric. Activity staining of a 7.5% native gel loaded with soluble extract and the purified SOD shows a unique major band at the same position (Fig. 2C). However, a band with very low SOD activity could be seen at the top of the gel that had been loaded with the soluble extract; it probably corresponded to the SOD activity recently reported for neelaredoxin (11, 50).

(iv) Amino acid composition and N-terminal sequence. The amino acid composition of *D. gigas* SOD was compared with those of other prokaryotic SODs (Table 2). They all appear to have similar general patterns, although *D. gigas* SOD has a lower content of glutamic acid/glutamine. The N-terminal amino acid sequence of *D. gigas* SOD was also determined and compared with those previously reported for SODs from other organisms (Fig. 3). Using the program PILEUP from the GCG Genetic Computer Group, the N-terminal sequence could be aligned for maximum homology with the known sequences of iron- and manganese-containing enzymes. However, there are four to five additional residues at the N terminus of the *D. gigas* SOD sequence, depending on the compared source. Of the first 20 residues, 7 are totally conserved throughout the sequences. The N-terminal sequence from *D. gigas* SOD shows approximately 80% similarity to *D. vulgaris*, *Bacillus stearothermophilus*, and mitochondrial SOD sequences, 73% similarity to the *Desulfomicrobium norvegicum* SOD N terminus, and 67% similarity to both Fe- and MnSODs from *Escherichia coli*.

Inhibition studies. It is known that CuZnSOD is sensitive to H_2O_2 and cyanide (17) whereas FeSOD is sensitive to H_2O_2 and azide but not to cyanide. In contrast, MnSOD is inhibited by NaN_3 but not cyanide or H_2O_2 (39). *D. gigas* SOD (0.5 μg) was incubated for 12 h in the presence of a 10 mM concentration of each inhibitor, the activity assay was performed, and the results were compared to those for a control (incubated for the same period of time but in the absence of inhibitor). The inhibition pattern shown by *D. gigas* SOD after incubation with H_2O_2, NaN_3, or KCN was the same as that expected for FeSODs. Under these conditions, H_2O_2 was able to inhibit SOD activity by 60% while KCN and NaN_3 showed no inhibition of this activity. Since azide and KCN are instantaneous reversible inhibitors, their effect can be influenced by dilution. Thus, we performed another set of experiments, adding these inhibitors directly into the reaction mixture. This way we confirmed that KCN did not inhibit the SOD activity. However, azide at concentrations of 0.5, 1, 4, and 12.5 mM was able to inhibit 0, 28, 38, and 63% of the SOD activity, respectively. The inhibition of SOD activity was also observed in native gels when azide and H_2O_2 were included in the developing solution used to detect the activity.

Stability to changes in pH and temperature. *D. gigas* FeSOD was stable after 1 h of incubation at 50°C. It has been reported that all SODs other than the CuZnSOD found in *E. coli* (4) are resistant to thermal inactivation. *D. gigas* SOD is as thermostable as other SODs. More than 80% of the activity was maintained after incubation over a broad pH range of 3.0 to 9.0, but at higher pHs the enzyme activity rapidly decreased, as occurs with most of the known FeSODs.

Catalase. (i) Purification. Each step of purification was followed by measurement of the catalase activity as described by Luck (34). The purification scheme was organized to allow the separation of other proteins besides catalase, including cytochromes, flavodoxin, and hydrogenase, important for other studies. A summary of the purification procedures is shown in Table 1. By the final step, an 80-fold purification was obtained. The specific activity of catalase in crude extracts of *D. gigas* was 52.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. After purification of the enzyme, the specific activity was 4,200 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. This activity is approximately 10 times lower than that reported for *D. vulgaris* (27) and about 20 times lower than that reported for *Micrococcus lysodeikticus* (27). Table 3 compares the catalase from *D. gigas* with those from other organisms; differences in heme content, molar extinction coefficient, and number of subunits were observed.

(ii) Molecular properties. Following denaturing SDS-PAGE of the purified enzyme, a major polypeptide band with an estimated molecular mass of 61 kDa was observed (Fig. 4A). The total molecular mass of *D. gigas* catalase was determined both by blue native PAGE (Fig. 4B), as described by Schagger et al. (49), and by molecular exclusion chromatography (data not shown). The size of the native catalase determined by these methods was estimated at 186 ± 8 kDa. This indicates that the *D. gigas* catalase consists of three subunits. It should be noted that in general, catalases have four subunits; however, dimeric and hexameric catalases have been described (Table 3). The

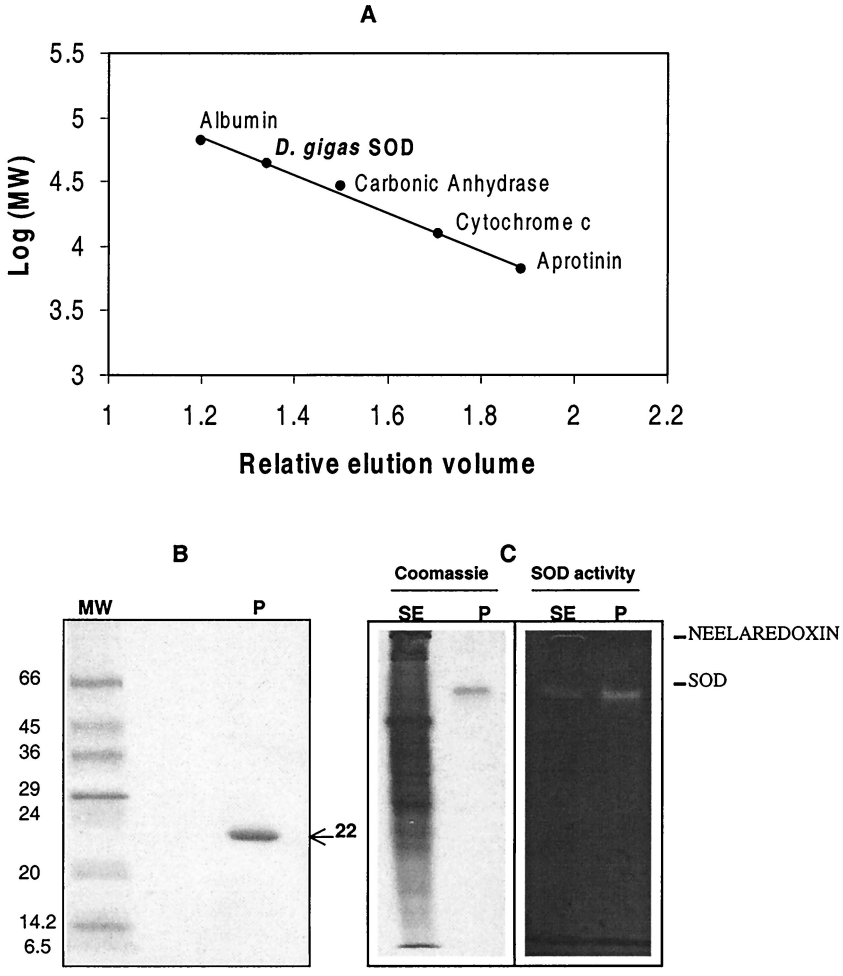

FIG. 2. Molecular weight (MW) determination for *D. gigas* SOD. (A) Gel exclusion high-performance liquid chromatography. (B) Coomassie blue-stained denaturing SDS–12.5% polyacrylamide gel. MW, molecular mass (in kilodaltons). (C) Native 7% polyacrylamide gel stained for SOD activity. SE, soluble extract; P, purified SOD.

purified protein was subjected to N-terminal amino acid analysis by Edman degradation, and the N-terminal sequence was compared with some known catalase sequences obtained from the GenBank, EMBL, PIR-Protein, and Swiss-Prot databases. Some residues are totally conserved among the catalases compared. However, no similarity to bacterial catalase-peroxidase-type enzymes such as *E. coli* HPI and *Salmonella typhimurium* hydroperoxidase I was observed when the N-terminal sequences were compared (Fig. 5). This is consistent with the fact that the enzyme showed no peroxidase activity toward the classic peroxidase substrates 2,4-dichlorophenol-4-aminoantipyrine and pyrogallol (data not shown) and was not reduced by dithionite. However, the *D. gigas* sequence has 74% similarity to the *Bacteroides fragilis* sequence, 70% similarity to the N-terminal *Methanosarcina barkeri* sequence, 68% to the *Bordetella pertussis* and *Proteus mirabilis* sequences, 65% to the

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dgigas            T P A D G P V V L P A L P Y A P N A L E - - - - -   20
dvulgaris         - - - - - - - - M P P L P Y P D T A L E P A I S A   17
ddesulfuricans    - - - - S I F V L P D L P Y A K D A L X P K I S A   21
ecolifesod        - - - - - S F E L P A L P Y A K D A L A P H I S A   20
ecolimnsod        - - - - - S Y T L P S L P Y A Y D A L E P H F D K   20
povalis           - - - - - A F E L P P L P Y A H D A L Q - - - - -   15
stearothermophilus - - - - P F E L P A L P Y P Y D A L E - - - - -   15
mtsod             - - - - M A H V L P D L P Y A Y N A L E P F I S -   20

Consensus         - - - - - - F - L P - L P Y A - D A L E P - - - -   25
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FIG. 3. Comparison of the N-terminal amino acid sequences of *D. gigas* (dgigas) SOD and SODs from other organisms. Sequences were aligned by using the program PILEUP from the Wisconsin Sequence Analysis Package (GCG), applying a gap penalty of 3 and gap extension penalty of 0.10. The compared sequences are from the GenBank, EMBL, Swiss-Prot, and PIR-Protein databases. Residues in boxes are consensus residues. dvulgaris, *D. vulgaris* SOD; ddesulfuricans, *D. desulfuricans* SOD; ecolifesod, *E. coli* FeSOD; ecolimnsod, *E. coli* MnSOD; povalis, *Pseudomonas ovalis* SOD; stearothermophilus, *Bacillus stearothermophilus* SOD; mtsod, *Mycobacterium tuberculosis* SOD.

TABLE 2. Comparison of amino acid compositions of FeSODs from *D. gigas* and other organisms

Amino acid	Residues/mol for SOD from:			
	D. gigas[a]	*D. vulgaris*[b]	*Desulfomicrobium norvegicum*[c]	*Bacteroides fragilis*[d]
Lys	24	34	32	24.5
His	16	10	10	10.2
Arg	8	8	8	7.2
Asp	32	38	54	43.3
Thr	20	28	24	12.8
Ser	11	12	14	17.8
Glu	11	26	36	36.8
Pro	20	28	16	14.3
Gly	31	30	38	32.2
Ala	58	56	44	35.2
Cysteic acid	1	2	4	ND[e]
Val	20	28	16	23.1
Met	8	14	4	4.8
Ile	9	6	14	12.8
Leu	38	34	30	32.1
Tyr	18	18	16	14.5
Phe	7	14	24	17.3
Trp	10	12	12	10.5

[a] Data from this work.
[b] Data from GenBank (accession no. AF034841).
[c] Data from reference 27.
[d] Data from reference 21.
[e] ND, not detected.

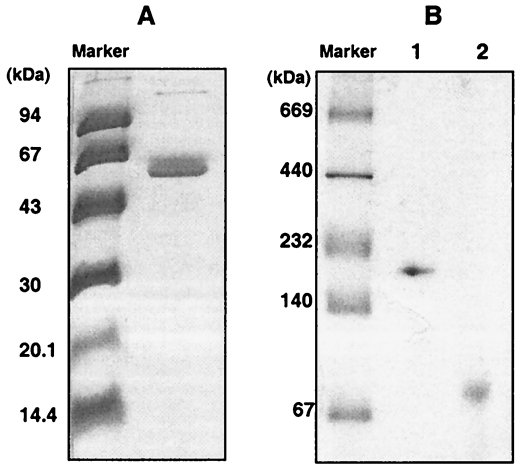

FIG. 4. (A) Molecular mass of denatured *D. gigas* catalase determined by 12.5% SDS-PAGE. (B) Total molecular mass of native *D. gigas* catalase determined by nondenaturing blue native PAGE. Samples were prepared without (lane 1) and with (lane 2) incubation with β-mercaptoethanol and boiling for 5 min.

Bacillus subtilis sequence, and 56% to the *D. vulgaris* and *Helicobacter pylori* sequences. The amino acid composition of the *D. gigas* catalase has some striking differences from other known catalases (Table 4). The contents of proline, alanine, and threonine are higher while aspartic acid/asparagine and glutamic acid/glutamine contents are lower than those of catalases from other organisms.

(iii) Spectroscopic characterization. The optical absorption spectrum of the native catalase from *D. gigas* (5.3×10^{-6} M) shows a Soret band at 405 nm and minor peaks at 500, 535, 589, and 623 nm (Fig. 6A). This spectrum is typical of those reported in the literature for other heme-containing catalases, which exhibit absorption maxima at 404 to 406, 500 to 505, 535 to 540, and 624 to 626 nm (14). Also, *D. gigas* catalase is not reducible by sodium dithionite, a property also typical for other catalases (data not shown). The molar extinction coefficient of *D. gigas* catalase at 405 nm is 12.3×10^4 $M^{-1} \cdot cm^{-1}$, which is approximately half of that reported for the *D. vulgaris* enzyme (Table 3), in agreement with the lower heme content. The EPR spectrum of *D. gigas* catalase (Fig. 6B) is typical of high-spin ferric heme in an almost axial ligand field (E/D = 0.02).

(iv) Pyridine hemochromogen spectrum. The absorption spectrum of the alkaline pyridine hemochromogen of *D. gigas* catalase (0.53×10^{-6} M) is shown in Fig. 7. Again, this spectrum is representative of pyridine hemochromogen spectra of other protoheme IX-containing hemoproteins (α at 556 nm) (9). The iron concentration calculated from these data is 0.46×10^{-6} M. Using this value, the number of hemes per molecule of catalase (molecular mass, 186 kDa) was calculated to be 0.9. This low heme content correlates with the relatively low A_{405}/A_{280} ratio of 0.5. A comparison of the heme contents of different catalases is shown in Table 3.

(v) Sensitivity to inhibitors. Azide and cyanide are known inhibitors of catalase, and the effect on this enzyme after a 30-min incubation in the presence of a 10 mM concentration of each inhibitor was investigated. Under these conditions, azide inhibited the activity of *D. gigas* catalase by 94% while KCN inhibited the activity by 63%. Nicholls (41) reported that when catalase is exposed to hydrogen sulfide in the presence of hydrogen peroxide, an inactive derivative of catalase and sulfur is formed. Since *D. gigas* is a sulfate-reducing bacterium in which oxygenated sulfur compounds are reduced to hydrogen sulfide by specific reductases, it was important to evaluate the effect of sulfide on the activity of catalase. At a sulfide concentration of 10 mM, only a modest inhibition of 18% was observed.

(vi) Stability to changes in pH and temperature. *D. gigas* catalase was incubated for 1 h at various pHs. An optimum pH range of between 7 and 9 was observed. Compared with other catalases (20, 33), which retain activity from pH 4 to 10, this is a narrow optimum pH range. After incubation of the enzyme

TABLE 3. Physicochemical properties of catalases from *D. gigas* and other organisms

Source	Molecular mass (kDa)	No. of subunits	Sp act (U/mg)	Molar extinction coefficient (10^4 $M^{-1} \cdot cm^{-1}$) at Soret band	No. of hemes/molecule	Reference
D. gigas	186	3	4,200	12.3	0.9	This work
Bacteroides fragilis	130	2	8,000	15.0	1.0	45
D. vulgaris	232	4	50,000	20.5	1.8	27
Bovine liver	240	4	36,000	29.0	2.0	14
Equine liver	240	4	45,000	34.0	3.0	14
Micrococcus lysodeikticus	232	4	99,000	41.0	4.0	14

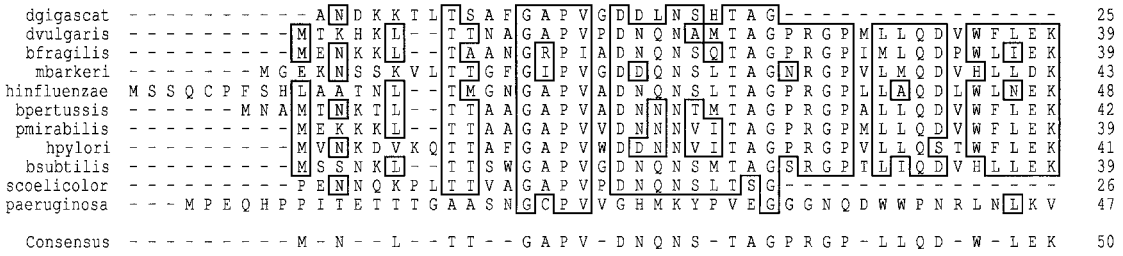

FIG. 5. Comparison of the N-terminal amino acid sequences of *D. gigas* catalase (dgigascat) and enzymes from other organisms. The compared sequences were obtained from the GenBank, EMBL, Swiss-Prot, and PIR-Protein databases. Boxed residues are consensus residues. dvulgaris, *D. vulgaris* catalase; bfragilis, *Bacteroides fragilis* catalase; mbarkeri, *Methanosarcina barkeri* catalase; hinfluenzae, *Haemophilus influenzae* catalase; bpertussis, *Bordetella pertussis* catalase; pmirabilis, *Proteus mirabilis* catalase; hpylori, *Helicobacter pylori* catalase; bsubtilis, *Bacillus subtilis* catalase; scoelicolor, *Streptomyces coelicolor* catalase; paeruginosa, *Pseudomonas aeruginosa* catalase.

at 50°C for 1 h, only 15% of the activity was lost, showing that *D. gigas* catalase is quite thermostable under these conditions.

DISCUSSION

SOD and catalase activities were observed in crude extracts of *D. gigas* cells grown anaerobically. This finding indicates that these enzymes are being constitutively expressed during the growth of this organism in the absence of oxygen. Interestingly, sulfate-reducing bacteria are among a small number of anaerobes whose cell extracts exhibit such activities. McCord et al. (38) surveyed the distribution of SOD and catalase in various microorganisms and concluded that strict anaerobes exhibited no SOD activity and, generally, no catalase activity. However, since that report was published, there has been growing evidence that strictly anaerobic microorganisms in fact have typical SODs (1, 22). The purification and characterization of this enzyme from *D. gigas* grown under anaerobic conditions demonstrate not only that this organism expresses an FeSOD but also that this enzyme is similar to other SODs. In fact, iron-containing SODs isolated from bacteria appear to be either approximately 40-kDa dimers or 90-kDa tetramers. *D. gigas* SOD belongs to the former group of FeSODs, as indicated by the similarity in amino acid composition (Table 2) and the optical absorption and EPR spectra. Moreover, the specific activity of the *D. gigas* SOD is comparable to that of other SODs of prokaryotic organisms. Nevertheless, the possibility that other kinds of SODs are expressed under conditions of

TABLE 4. Amino acid compositions of catalases from *D. gigas* and other organisms

Amino acid	No. of residues/subunit in catalase from:			
	D. gigas[a]	*D. vulgaris*[b]	*Bacteroides fragilis*[c]	*Helicobacter pylori*[d]
Lys	12	22	27	46
His	16	21	15	20
Arg	35	36	33	27
Asp	22	57	64	61
Thr	40	25	21	26
Ser	29	19	19	26
Glu	14	43	59	48
Pro	50	31	29	30
Gly	49	35	34	32
Ala	88	46	38	31
Cysteic acid	5	3	3	2
Val	34	28	21	33
Met	18	14	14	14
Ile	11	15	22	18
Leu	40	30	32	25
Tyr	18	17	19	25
Phe	18	26	28	31
Trp	9	9	8	11

[a] Data from this work.
[b] Data from GenBank (accession no. AB0203411).
[c] Data from SwissProt (accession no. P45737).
[d] Data from SwissProt (accession no. P77872).

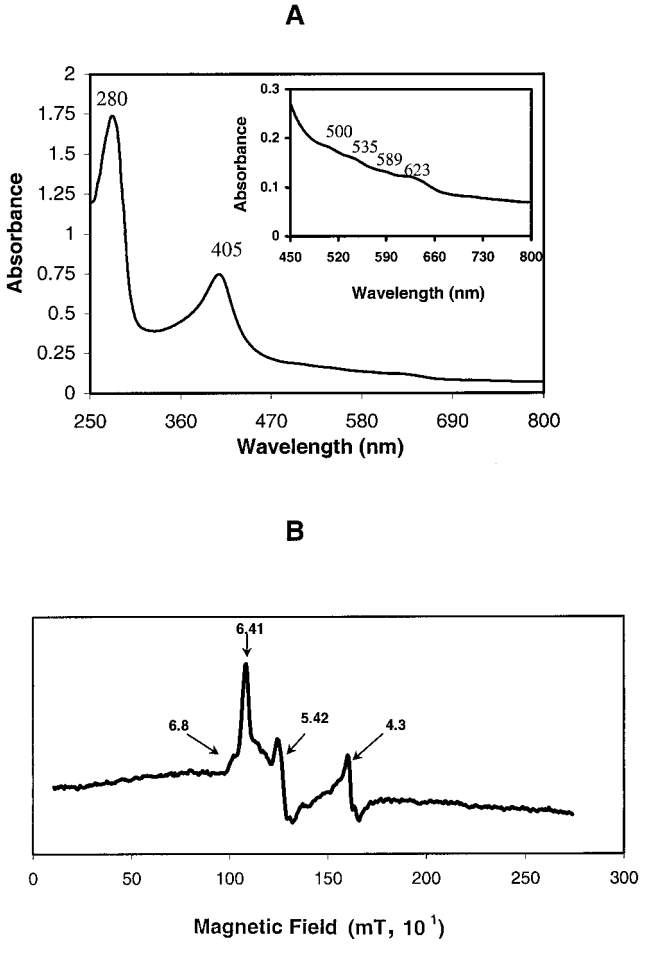

FIG. 6. (A) Absorption spectrum of *D. gigas* catalase. The protein concentration was 1.2 mg/ml; a 1-cm-light-path cuvette was used. (Inset) Detailed visible spectrum. (B) EPR spectrum of purified *D. gigas* catalase. The conditions were as follows: temperature, 4.7 K; microwave frequency, 9.64 GH2 and microwave power, 2.4 mW.

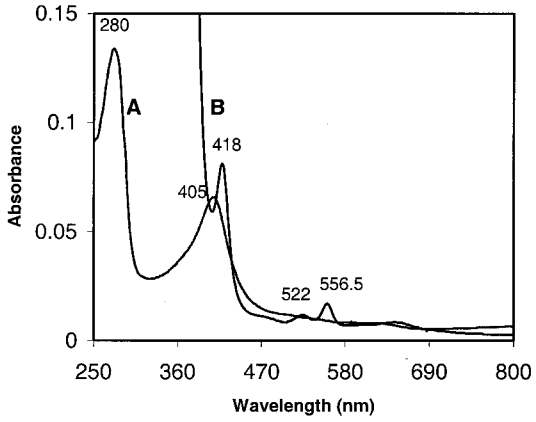

FIG. 7. Pyridine hemochromogen spectrum of *D. gigas* catalase. Curve A, Absorption spectrum of 0.53×10^{-6} M catalase in 50 mM phosphate buffer, pH 7.6; curve B, pyridine hemochromogen spectrum from a reaction mixture containing 0.53×10^{-6} M catalase, 100 mM NaOH, 100 mM pyridine, and 2 mM sodium dithionite.

oxidative stress cannot be ruled out. *E. coli*, for example, constitutively produces an FeSOD but expresses an additional, manganese-containing SOD only under aerobic conditions (22). Therefore, it seems reasonable to consider that SODs serve the same purpose in all of these organisms. It has been shown that *E. coli* mutants devoid of both FeSOD and MnSOD survive under aerobic conditions in rich medium. However, growth is weak in minimal medium and occurs only upon provision of all 20 amino acids, suggesting that there is a drastic effect of oxygen radicals on amino acid biosynthesis (8).

Although catalases containing two subunits have been frequently reported in prokaryotes, such as in *Streptomyces venezuelae* (30), *Comamonas compransoris* (42), *Klebsiella pneumoniae* Kpa (20), *Mycobacterium tuberculosis* (15), and *Bacteroides fragilis* (45), six- and four-subunit catalases have been found in *Haemophilus influenzae* (5) and *E. coli* (53), respectively. Interestingly, *D. gigas* catalase seems to have three subunits and a low content of heme per molecule, which are a remarkable characteristic of this catalase. This may be one explanation for the relatively low specific activity of this enzyme.

Interestingly, catalase activity does not seem to be present in all known *Desulfovibrio* species. While *D. vulgaris*, *D. gigas*, and *Desulfomicrobium norvegicum* are catalase positive, *Desulfovibrio salexigens* and *Desulfovibrio desulfuricans* (strain Essex 6) are catalase negative. Thus, it would be interesting to find out whether the reported absence of catalase in some species is due to an absence of the gene or the expression of the gene only under certain circumstances. So far, there have been no published studies of expression of catalases in *Desulfovibrio* species grown under different conditions.

The spectrum of the hemochrome complex of the *D. gigas* catalase indicates that it is a hemoprotein containing a protoheme IX moiety. A heme/subunit ratio of 1:2 has been found in some bacterial catalases (7, 12, 45). Our data show that *D. gigas* catalase has a 1:3 heme/subunit ratio. This low heme content appears to be due to the loss of heme during the purification process.

It has recently been shown that neelaredoxin, a nonheme blue iron protein isolated from *D. gigas*, also has significant SOD activity (50). As mentioned above, a second, low-activity band evident on the slot of the native gel corresponding to the soluble extract is most probably due to neelaredoxin (Fig. 2C).

Moreover, another protein containing two mononuclear iron centers, desulfoferrodoxin, expressed by some other *Desulfovibrio* species, was shown to complement SOD-deficient mutants of *E. coli* when overexpressed (44). Later, desulfoferrodoxin was shown to have SOD activity as well (46). Thus, it

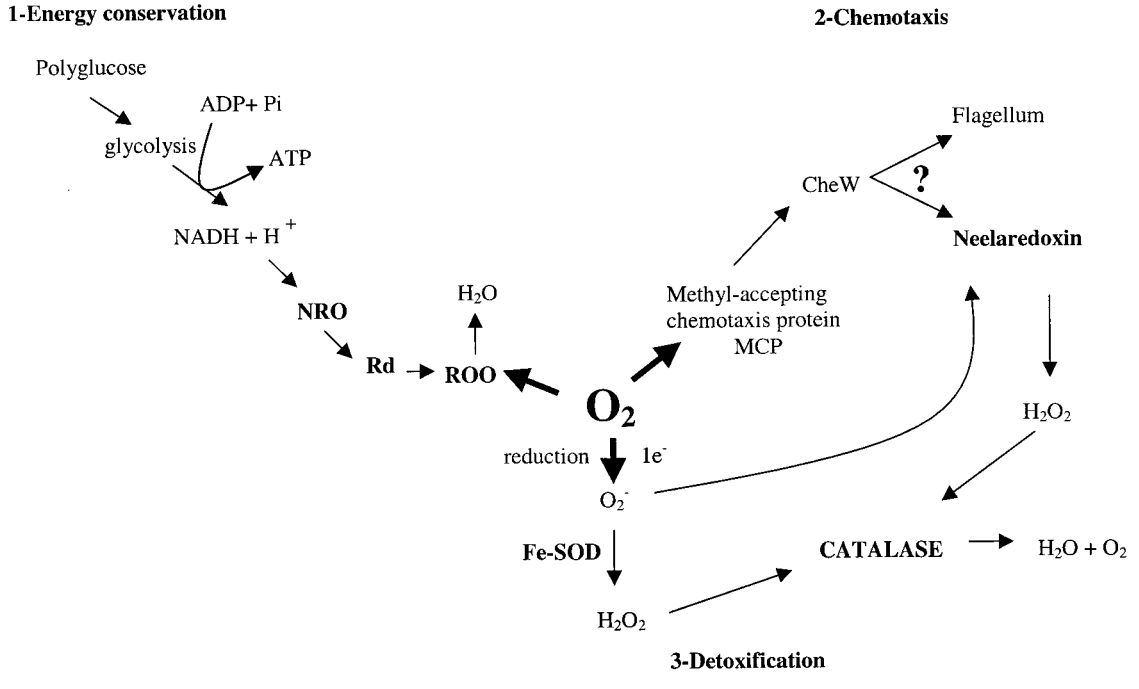

FIG. 8. Oxygen reduction and detoxification pathways of oxygen-reactive species in *D. gigas*. NRO, NADH-rubredoxin oxidoreductase; Rd, rubredoxin; ROO, rubredoxin-oxygen oxidoreductase.

seems that all of these proteins, including the catalase characterized in this work, play a role in scavenging oxygen radicals or in the maintenance of the proper balance of the oxidized and reduced forms of some proteins in the cell. Also, these enzymes may be part of a complex chemotaxis pathway involved in the bacterial response to oxygen, as summarized in Fig. 8. It was recently reported that the neelaredoxin gene is located immediately downstream from two additional open reading frames coding for proteins involved in a chemotaxis-like system. One of them is the methyl-accepting chemotaxis protein, and the other one corresponds to the known CheW protein responsible for activating other proteins involved in chemosensory responses (2, 50). Although it is not certain that neelaredoxin is part of an operon unit, these three proteins may be involved in the oxygen-sensing mechanism in *D. gigas*. Thus, in response to the presence of oxygen or toxic oxygen radicals, the chemotaxis-like system would be activated. The oxygen-activated methyl-accepting chemotaxis protein might transduce a signal through the membrane and activate other proteins of the chemotactic system, namely, CheW, CheA, and CheY, which might lead to changes in the flagellar rotation of the bacteria or even induce the expression of neelaredoxin and SOD. Interestingly, in *Helicobacter pylori*, a SOD gene is located in an oxygen-sensing operon (52). It would not be surprising if the *D. gigas* SOD gene is part of a similar operon.

The expression of SOD and catalase, even under anaerobic conditions, protects the cells against oxygen-reactive species produced following accidental exposure to oxygen. In the case of organisms lacking SOD, other proteins, like neelaredoxin or desulfoferrodoxin, could fulfill its role up to some level. The presence of an electron transfer chain in *D. gigas*, involving NADH-rubredoxin oxidoreductase and rubredoxin, as well as rubredoxin-oxygen oxidoreductase as a terminal oxidase capable of reducing O_2 to water, indicates that oxygen may play a physiological role in this organism. In this case, SOD, catalase, and even neelaredoxin could prevent the damage potentially imposed by the ability of the bacterium to survive in an environment in which oxygen is present. As demonstrated for *D. desulfuricans* (1), oxygen is able to induce the expression of SOD and NADH oxidases as a mechanism of survival under conditions of oxidative stress. For *D. gigas*, the hypothesis that oxygen induces the expression of SOD, catalase, or NADH oxidases is still awaiting confirmation.

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REFERENCES

1. **Abdollahi, H., and J. W. T. Wimpenny.** 1990. Effects of oxygen on the growth of *Desulfovibrio desulfuricans*. J. Gen. Microbiol. **136:**1025–1030.
2. **Armitaje, J. P.** 1997. Behavioral responses of bacteria to light and oxygen. Arch. Microbiol. **168:**249–261.
3. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44:**276–287.
4. **Benov, L. T., and I. Fridovich.** 1994. *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. J. Biol. Chem. **269:**25310–25314.
5. **Bishai, W. R., H. O. Smith, and G. J. Barcak.** 1994. A peroxide/ascorbate-inducible catalase from *Haemophilus influenzae* is homologous to the *Escherichia coli katE* gene product. J. Bacteriol. **176:**2914–2921.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Brown-Peterson, N. J., and M. L. Salin.** 1993. Purification of a catalase-peroxidase from *Halobacterium halobium*: characterization of some unique properties of the halophilic enzyme. J. Bacteriol. **175:**4197–4202.
8. **Carlioz, A., and D. Touati.** 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for life? EMBO J. **5:**623–630.
9. **Chance, B., and D. Hebert.** 1950. The enzyme-substrate compounds of bacterial catalase and peroxides. Biochem. J. **46:**402–413.
10. **Chen, L., M.-Y. Liu, J. LeGall, P. Fareleira, H. Santos, and A. V. Xavier.** 1993. Purification and characterization of an NADH-rubredoxin oxidoreductase involved in the utilization of oxygen by *Desulfovibrio gigas*. Eur. J. Biochem. **216:**443–448.
11. **Chen, L., P. Sharma, J. LeGall, A. M. Mariano, M. Teixeira, and A. V. Xavier.** 1994. A blue non-heme iron protein from *Desulfovibrio gigas*. Eur. J. Biochem. **226:**613–618.
12. **Claiborne, A., and I. Fridovich.** 1979. Purification of the *o*-dianisidine peroxidase from *Escherichia coli* B. Physicochemical characterization and analysis of its dual catalytic and peroxidatic activities. J. Biol. Chem. **254:**4245–4252.
13. **Cseke, C., L. I. Horvath, P. Simon, G. Borbely, L. Keszthelyi, and G. L. Farkas.** 1979. An iron-superoxide dismutase from *Anacystis nidulans*. J. Biochem. **85:**1379–1404.
14. **Deisseroth, A., and A. L. Dounce.** 1970. Catalase: physical and chemical properties, mechanism of catalysis, and physiological role. Physiol. Rev. **50:**319–375.
15. **Dias, G. A., and L. G. Wayne.** 1974. Isolation and characterization of catalase produced by *M. tuberculosis*. Annu. Rev. Respir. Dis. **110:**312–319.
16. **Fareleira, P., J. LeGall, A. V. Xavier, and H. Santos.** 1997. Pathways for utilization of carbon reserves in *Desulfovibrio gigas* under fermentative and respiratory conditions. J. Bacteriol. **179:**3972–3980.
17. **Fridovich, I.** 1975. Superoxide dismutases. Annu. Rev. Biochem. **44:**147–159.
18. **Fridovich, I.** 1978. The biology of oxygen radicals. Science **201:**875–880.
19. **Genthner, B. R. S., S. D. Friedman, and R. Devereux.** 1997. Reclassification of *Desulfovibrio desulfuricans* Norway 4 as *Desulfomicrobium norvegicum* comb. nov. and confirmation of *Desulfomicrobium escambiense* (corrig., formerly "*escambium*") as a new species in the genus *Desulfomicrobium*. Int. J. Syst. Bacteriol. **47:**889–892.
20. **Goldberg, I., and A. Hochman.** 1989. Purification and characterization of a novel type of catalase from the bacterium *Klebsiella pneumoniae*. Biochim. Biophys. Acta **991:**330–336.
21. **Gregory, E. M.** 1985. Characterization of the O_2-induced manganese-containing superoxide dismutase from *Bacteroides fragilis*. Arch. Biochem. Biophys. **238:**83–89.
22. **Gregory, E. M., and I. Fridovich.** 1973. Induction of superoxide dismutase by molecular oxygen. J. Bacteriol. **114:**543–548.
23. **Hames, B. D.** 1990. One-dimensional polyacrylamide gel electrophoresis, p. 30–50. *In* B. D. Hames and D. Rickwood (ed.), Gel electrophoresis of proteins, a practical approach. IRL Press, Oxford, United Kingdom.
24. **Hassan, H. M., and I. Fridovich.** 1978. Regulation of the synthesis of catalase and peroxidase in *Escherichia coli*. J. Biol. Chem. **253:**6445–6450.
25. **Hata, Y., H. Kadota, H. Miyoshi, and M. Kimata.** 1964. Microbial production of sulfides in polluted coastal and estuarine regions, p. 287–295. *In* E. A. Pearson (ed.), Advances in water pollution research, 3rd ed. Pergamon Press, Oxford, United Kingdom.
26. **Hatchikian, E. C., and Y. A. Henry.** 1977. An iron-containing superoxide dismutase from the strict anaerobe *Desulfovibrio desulfuricans* (Norway 4). Biochimie **59:**153–161.
27. **Hatchikian, E. C., J. LeGall, and G. R. Bell.** 1977. Significance of superoxide dismutase and catalase activities in the strict anaerobes, sulfate-reducing bacteria, p. 159–172. *In* A. M. Michelson, J. M. McCord, and I. Fridovich (ed.), Superoxide and superoxide dismutase. Academic Press, London, United Kingdom.
28. **Hatchikian, E. C., J. LeGall, M. Bruschi, and M. Dubourdieu.** 1972. Regulation of the reduction of sulfite and thiosulfate by ferredoxin, flavodoxin and cytochrome c_3 in extracts of the sulfate reducer *Desulfovibrio gigas*. Biochim. Biophys. Acta **258:**701–708.
29. **Imlay, J. A., and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302–1309.
30. **Knoch, M., K. H. Van Pée, L. C. Vinning, and F. Lingens.** 1989. Purification, properties and immunological detection of a bromoperoxidase-catalase from *Streptomyces venezuelae* and from a chloramphenicol non-producing mutant. J. Gen. Microbiol. **135:**2493–2502.
31. **Kusunose, E., K. Ichihara, Y. Noda, and M. Kusunose.** 1976. Superoxide dismutase from *Mycobacterium tuberculosis*. J. Biochem. **80:**1343–1352.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
33. **Loewen, P. C., and J. Switala.** 1986. Purification and characterization of catalase HPII in *Escherichia coli* K12. Biochem. Cell. Biol. **64:**638–646.
34. **Luck, H.** 1963. Catalase, p. 885–898. *In* H. Bergmeyer (ed.), Methods of

enzymatic analysis. Academic Press, New York, N.Y.
35. **Lumsden, J., R. Cammack, and D. O. Hall.** 1976. Purification and physicochemical properties of superoxide dismutase from two photosynthetic microorganisms. Biochim. Biophys. Acta **438:**380–392.
36. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244:**6049–6055.
37. **McCord, J. M., and I. Fridovich.** 1988. Superoxide dismutase: the first twenty years (1968–1988). Free Radic. Biol. Med. **5:**363–369.
38. **McCord, J. M., R. B. Keele, and I. Fridovich.** 1971. An enzyme based theory of obligate anaerobiosis. Proc. Natl. Acad. Sci. USA **68:**1024–1027.
39. **Misra, H. P., and I. Fridovich.** 1978. Inhibition of superoxide dismutases by azide. Arch. Biochem. Biophys. **189:**317–322.
40. **Nelson, D. P., and L. A. Kiesow.** 1972. Enthalpy of decomposition of hydrogen peroxide by catalase at 25°C (with molar extinction coefficients of H_2O_2 solutions in the UV). Anal. Biochem. **49:**474–478.
41. **Nicholls, P.** 1961. The formation and properties of sulphmyoglobin and sulphcatalase. Biochem. J. **81:**374–383.
42. **Nies, D., and H. G. Schlegel.** 1982. Catalase from *Comamonas comprasoris*. J. Gen. Appl. Microbiol. **28:**311–319.
43. **Novozhilova, M. I., and F. S. Berezina.** 1968. Character of the distribution of sulfate reducing and sulfur bacteria in the sediments of Lake Balkhash. Mikrobiologiya **37:**534–539.
44. **Pianzolla, M. J., M. Soubes, and D. Touati.** 1996. Overproduction of the *rbo* gene product from *Desulfovibrio* species suppresses all deleterious effects of lack of superoxide dismutase in *Escherichia coli*. J. Bacteriol. **178:**6736–6742.
45. **Rocha, E. R., and C. J. Smith.** 1995. Biochemical and genetic analyses of a catalase from the anaerobic bacterium *Bacteroides fragilis*. J. Bacteriol. **177:** 3111–3119.
46. **Romão, C. V., M. Y. Liu, J. LeGall, C. M. Gomes, V. Braga, I. Pacheco, A. V. Xavier, and M. Teixeira.** 1999. The superoxide dismutase activity of desulfoferredoxin from *Desulfovibrio desulfuricans* ATCC 27774. Eur. J. Biochem. **261:**438–443.
47. **Santos, H., P. Fareleira, A. V. Xavier, L. Chen, M.-Y. Liu, and J. LeGall.** 1993. Aerobic metabolism of carbon reserves by the "obligate anaerobe" *Desulfovibrio gigas*. Biochem. Biophys. Res. Commun. **195:**551–557.
48. **Sass, H., M. Berchtold, J. Branke, H. Konig, H. Cypionka, and H. D. Babenzien.** 1998. Psychotolerant sulfate-reducing bacteria from an oxic freshwater sediment, description of *Desulfovibrio cuneatus* sp. nov. and *Desulfovibrio litoralis* sp. nov. Syst. Appl. Microbiol. **21:**212–219.
49. **Schagger, H., W. A. Cramer, and G. Von Jagow.** 1994. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal. Biochem. **217:**220–230.
50. **Silva, G., S. Oliveira, C. M. Gomes, I. Pacheco, M. Y. Liu, A. V. Xavier, M. Teixeira, J. LeGall, and C. Rodrigues-Pousada.** 1999. *Desulfovibrio gigas* neelaredoxin: a novel superoxide dismutase integrated in a putative oxygen sensory operon of an anaerobe. Eur. J. Biochem. **259:**235–243.
51. **Timkovich, R., R. S. Burkhalter, A. V. Xavier, L. Chen, and J. LeGall.** 1994. Iron uroporphyrin I and heme c-derivative are prosthetic groups in *Desulfovibrio gigas* rubredoxin oxidase. Bioorg. Chem. **22:**284–293.
52. **Tomb, J. F., O. White, A. R. Kerlavage, et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539–547.
53. **Triggs-Raine, B. L., B. W. Doble, M. R. Mulvey, P. A. Sorby, and P. C. Loewen.** 1988. Nucleotide sequence of *katG*, encoding catalase HPI of *Escherichia coli*. J. Bacteriol. **170:**4415–4419.
54. **Villa Franca, J. J.** 1976. EPR spectra of Fe(III)-superoxide dismutase with special reference to the electron spin relaxation time of Fe(III). FEBS Lett. **62:**230–232.
55. **Yamakura, F.** 1976. Purification, crystallization and properties of iron-containing superoxide dismutase from *Pseudomonas ovalis*. Biochim. Biophys. Acta **422:**280–294.
56. **Yost, F. J., Jr., and I. Fridovich.** 1973. An iron-containing superoxide dismutase from *Escherichia coli*. J. Biol. Chem. **248:**4905–4908.