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Periplasmic Superoxide Dismutase from *Desulfovibrio desulfuricans* 1388 Is an Iron Protein

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Abstract—It is shown that the genome of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* 1388 contains a superoxide dismutase (SOD) gene (*sod*). The gene encodes an export signal peptide characteristic for periplasmic redox proteins. The amino acid sequence showed high homology with iron-containing SODs from other bacteria. Electrophoretically pure SOD was isolated from the periplasmic fraction of bacterial cells by FPLC chromatography. Like other Fe-SODs, *D. desulfuricans* 1388 superoxide dismutase is inhibited by H_2O_2 and azide, but not by cyanide.

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Bacterial cell wall contains many proteins, which unlike cytoplasmic proteins are constantly exposed to the changing conditions in the environment. Exogenous reactive oxygen species can relatively easily penetrate through the cell wall and irreversibly inhibit the activity of many cellular enzymes due to the oxidation of SH-groups, Trp residues, or metals with variable valence in the enzyme active site.

The need for a periplasmic antioxidant system in bacteria of the *Desulfovibrio* genus, which encounter the presence of oxygen *in situ*, is obvious. Reduction of oxygen to water coupled with energy storage in ATP has been found in the periplasm of sulfate-reducing bacteria [1, 2]. However, intracellular oxygen reduction is most probably a protection mechanism [2], since it is only in the symbiosis with aerobic bacteria that sulfate-reducing bacteria display growth in a micro-aerobic environment [3, 4]. Antioxidant protection enzymes (superoxide dismutase (SOD), catalase, thiol peroxidases) are found in the periplasm of Gram-negative bacteria [5].

We have found superoxide dismutase in cells of the sulfate-reducing bacterium *D. desulfuricans* 1388. The activity of the enzyme, which is mostly concentrated in the periplasmic fraction, is determined by growth conditions [6, 7].

The present work reports the isolation of the *sod* gene from the bacterium *D. desulfuricans* 1388 and analysis of its nucleotide sequence. Protein amino acid composition was determined by the conceptual translation method. We also show the results of inhibitory analysis of SOD isolated from the periplasmic fraction.

MATERIALS AND METHODS

Desulfovibrio desulfuricans 1388 strain (VKM 1388) used in this work was obtained from the All-Russian Collection of Microorganisms (Pushchino, Russia). The composition of mineral Postgate B medium containing calcium lactate (3.5 g/liter) as an organic substrate and anaerobic cultivation methods for the sulfate-reducing bacteria were described in our previous work [6].

Cellular fractions were obtained by a modified technique of osmotic lysis of spheroplasts [8].

Superoxide dismutase activity was determined by the xanthine–xanthine oxidase method. One enzyme activity unit corresponded to the amount of protein causing a 50% inhibition of nitrotetrazolium blue reduction [9].

Isolation of DNA from *Desulfovibrio desulfuricans* 1388 was performed by standard phenol–chloroform

Abbreviations: ORF) open reading frame; PCR) polymerase chain reaction; SOD) superoxide dismutase.

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extraction technique followed by ethanol precipitation in the presence of 3 M potassium acetate [10].

Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to complement the nucleotide sequences at the flanks of the *sod* gene from *D. desulfuricans* G20 strain (No. 28877619, GenBank): F1, 5′-TTG TTC ATC TCT GAC GC-3′; R1, 5′-TCA GGT CAG GTG CTT-3′. The reaction mixture for amplification of the *sod* gene contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of *Taq* polymerase (Fermentas, Lithuania), 0.5 ng of each primer, and 100 ng of genomic DNA. PCR was performed using a Thercyc thermocycler (DNA Technology, Russia) in three steps: 95°C for 60 sec (1 cycle); 94°C for 20 sec, 62.5°C for 40 sec, 72°C for 40 sec (35 cycles); 72°C for 10 min (1 cycle).

Sod amplicons were visualized in 2% agarose gel in Tris-acetate buffer system (pH 8.0-8.2) using a DNA Analyzer video system and Cyclotemp-601 UV transilluminator (both from Resurs-pribor, Russia).

DNA fragments from phage λ cleaved with *Hin*dIII and *Eco*RI were used as molecular weight markers.

Analysis of nucleotide sequences and estimation of the amplicon size were performed using Vector NTI 7.1 (InforMax, Inc.) and DNASIS (v. 3.00) software.

Superoxide dismutase was isolated from the periplasmic fraction. Column FPLC chromatography (Pharmacia, Sweden) was applied for purification of SOD from *D. desulfuricans* 1388. Since SOD activity was preserved under aerobic conditions, all manipulations with the enzyme were carried out in air at room temperature. At the first purification step, the enzyme fraction obtained as was described previously [8] from 8 g of cells, was applied on a Q-Sepharose column (1 × 13 cm) equilibrated with 0.05 M Tris-HCl, pH 7.6. The protein was eluted with linear gradient from 0 to 0.5 M NaCl in 0.05 M Tris-HCl buffer, pH 7.6 (elution flow rate 1 ml/min, fraction volume 3 ml). The fractions containing SOD activity (0.12-0.15 M NaCl) were collected together, diluted 2 times, and subjected to chromatofocusing by application onto a Mono P column (0.5 × 20 cm) equilibrated with 0.025 M Tris-HCl, pH 7.6 (elution flow rate 0.2 ml/min, fraction volume 1 ml). After protein loading, the column was washed with two volumes of the starting buffer containing 0.025 M Tris-HCl, pH 7.6. Bound protein was eluted with deaerated Polybuffer 74, diluted with distilled water 10 : 1 and pre-titrated with HCl to the lower value of the pH gradient, pH 4.0. The fractions containing SOD (pH 4.85 ± 0.05) were pooled and dialyzed in a Slide-A-Lyser 3.5K dialysis cassette (Pierce, USA) at 4°C overnight. After dialysis, the protein was concentrated on a Q-Sepharose column (1 × 1.5 cm) equilibrated with 0.05 M Tris-HCl, pH 7.6, and stored at −20°C until use.

Gel electrophoresis was performed in 7% polyacrylamide according to [11]. The gels were stained with Coomassie G-200.

Protein concentration was determined according to the Lowry method [12] using crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

PCR analysis of *D. desulfuricans* 1388 DNA using the primers complementary to the flanking sequences of the *sod* gene from *D. desulfuricans* G20 revealed an amplicon with the molecular weight of 750 bp (Fig. 1).

Further analysis of nucleotide sequence of the obtained PCR fragment determined the presence of a 720 bp open reading frame (ORF), identical to the ORF of the *sod* gene from *D. desulfuricans* G20 strain. The G + C content of the *sod* gene was 57.6 mole %, which is similar to the average G + C content (58.8 mole %) of other *D. desulfuricans* genes (*napA*, *dsrA*, *hydA*, and others). Codon analysis of the nucleotide sequence of *D. desulfuricans* 1388 *sod* gene did not reveal any significant difference from other *D. desulfuricans* genes (codon usage database: http://www.kazusa.or.jp/codon/).

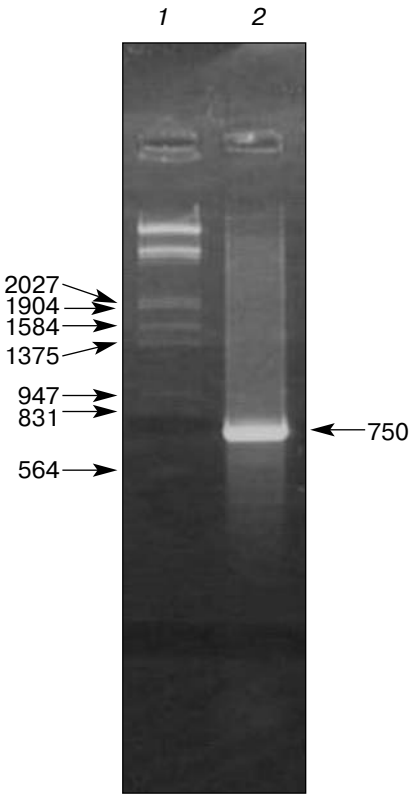

Fig. 1. DNA amplification from *D. desulfuricans* 1388 strain using primers complementary to the flanking sequences of the *sod* gene from *D. desulfuricans* G20 strain: *1*) phage λ DNA cleaved with *Hin*dIII and *Eco*RI; *2*) PCR fragment obtained under amplification of *D. desulfuricans* 1388 DNA.

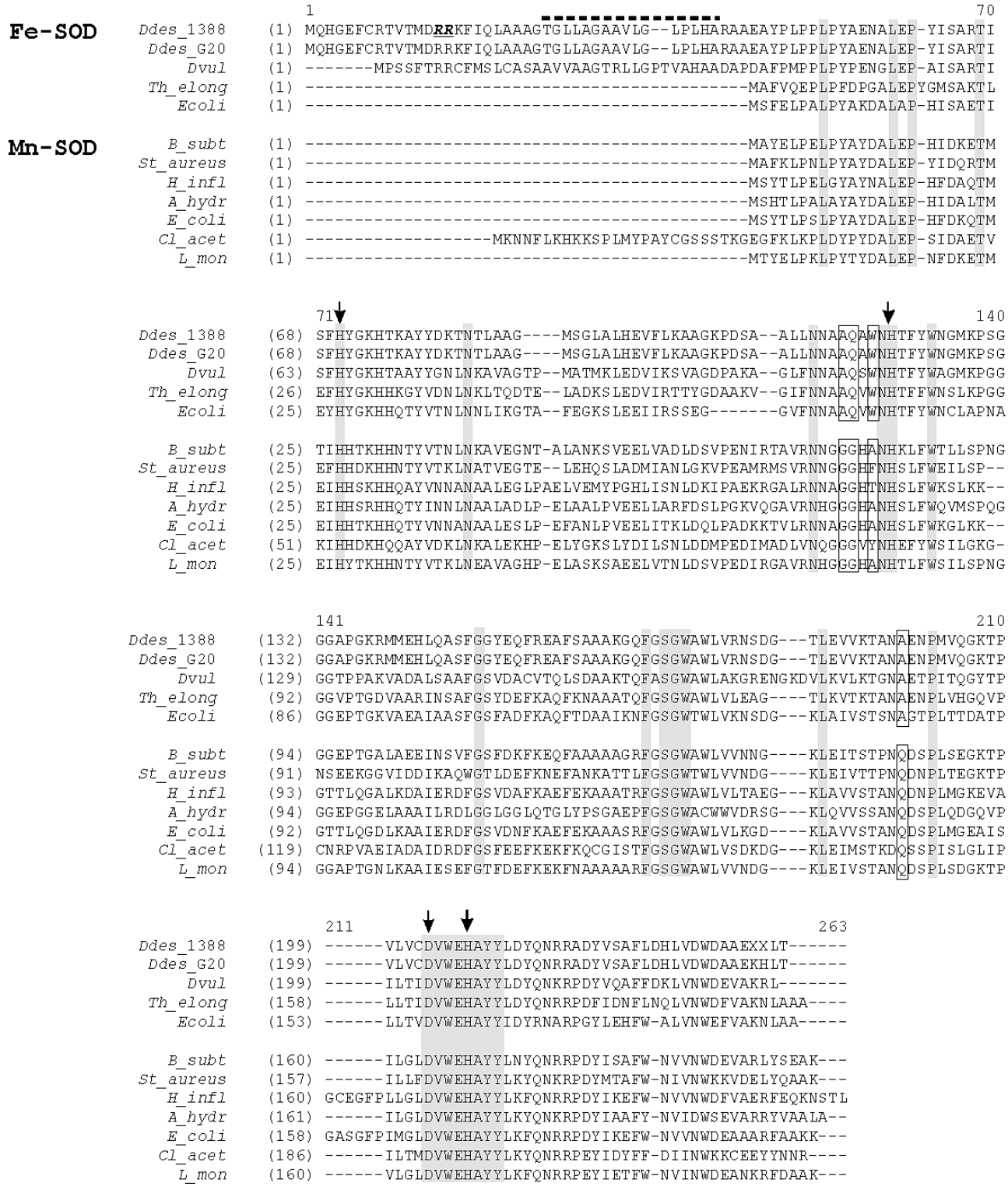

Fig. 2. Comparative analysis of SOD amino acid sequences from various microorganisms (GenBank): *Desulfovibrio desulfuricans* G20 (AABN00000000), *D. vulgaris* (AB093035), *Thermosynechococcus elongatus* BP-1 (NP_682309), *Escherichia coli* (J03511 and X03951), *Bacillus subtilis* (D86856), *Staphylococcus aureus* (AF273269), *Haemophilus influenzae* (X73832), *Aeromonas hydrophila* (AF317226), *Clostridium acetobutylicum* (AE007748), *Listeria monocytogenes* (M80526), and *D. desulfuricans* 1388 laboratory strain. Designations: ↓, amino acid residues involved in metal binding (Fe/Mn); open rectangles, amino acid residues specific for Fe-SOD and Mn-SOD; gray rectangles, conservative amino acid residues; ***RR***, leader peptide; bold dashed line, amino acid sequence involved in the formation of transmembrane helix.

Comparative analysis of amino acid sequences in Fe-SOD and Mn-SOD from several bacteria is presented in Fig. 2. According to the data of Nakanishi et al. [13], amino acid residues at positions 73, 129, 221, 225 (Fig. 2) involved in metal binding and formation of enzyme active site are identical in the SOD sequences and cannot serve as markers for Fe- or Mn-SODs. However, indirect evidence of the presence of iron ions in a protein molecule may be given by amino acids at the positions 124, 125, 127, and 200 (Fig. 2). The comparison between these regions in SOD from *D. desulfuricans* 1388 with the corresponding regions in *sod* genes from other bacteria indicates that the enzyme belongs to the family of Fe-containing superoxide dismutases.

Among all anaerobic bacteria, periplasmic Fe-containing SOD is found only in *Desulfovibrio* spp. [14]. Fe-containing superoxide dismutase is isolated and characterized from cellular extracts of sulfate-reducing bacterium *D. gigas*, but the authors do not report about cellular location of the enzyme [9]. There are data on cloning and sequencing of the gene encoding for Fe-SOD from *D. vulgaris* Hildenborough strain [14, 15], *D. vulgaris* Myazaki F strain [13], and *D. desulfuricans* G20 strain (No. 28877619, GenBank). Based on the identification of export signaling peptide, the periplasmic location of these enzymes was established.

The presence of a leader peptide characteristic for bacterial periplasmic redox proteins [14] as well as amino acid sequence involved in formation of transmembrane helix (PHDhtm transmembrane helices prediction, http://npsa-pbil.ibcp.fr) in the N-terminal region of SOD from *D. desulfuricans* 1388, suggests the periplasmic location of the enzyme (Fig. 2).

Considering previously obtained data regarding the periplasmic SOD location in *D. desulfuricans* 1388 cells [6, 7], as well as the results of genetic analysis presented above, isolation of the enzyme was performed from periplasmic fraction, removing cytoplasmic proteins already at this step. Specific SOD activity in *D. desulfuricans* 1388 periplasmic fraction was 74 units per mg protein. SOD was purified in two steps using an FPLC chromatography system (see "Materials and Methods"). Chromatofocusing using a Mono P column resulted in the enzyme preparation with specific activity of 1000 units per mg protein (table). According to polyacrylamide gel electrophoresis data, the SOD did not contain other enzymes and impurities under non-denaturing conditions.

Purification of periplasmic superoxide dismutase from *D. desulfuricans* 1388

Purification step	Total activity, units	Specific activity, unit/mg protein	Yield	Purification factor
Isolation of periplasmic fraction	3200	74.1	(100)	(1.0)
Q-Sepharose	1680	260	52.5	3.5
Mono P	500	1000	15.6	13.5

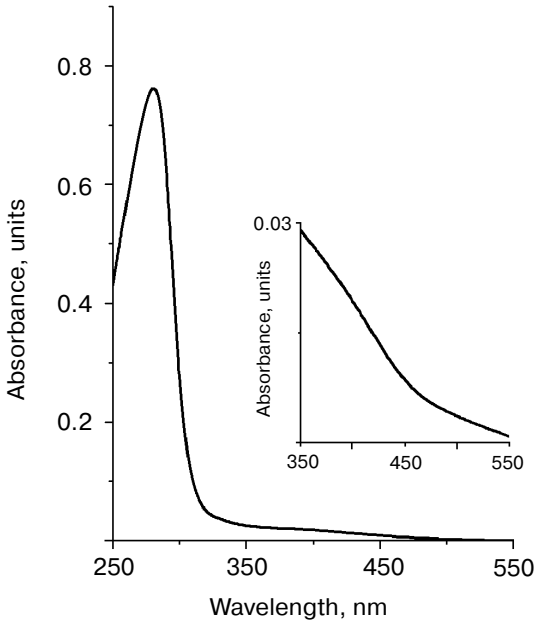

Fig. 3. Absorption spectrum of purified periplasmic SOD from *D. desulfuricans* 1388. The insert shows decrease in absorbance from 350 to 550 nm.

The visible absorption spectrum of SOD (Fig. 3) is similar to Fe-containing SOD from *D. vulgaris* Myazaki F strain. In both cases, a protein peak at 280 nm and gradual decrease in absorbance from 350 to 600 nm is observed. The spectrum does not contain a peak at 473 nm (characteristic for Mn-containing SODs) [13].

Depending on the metal in the SOD catalytic site, the enzyme displays different sensitivity towards classical inhibitors: cyanide, azide, and hydrogen peroxide. It is known that Mn-SODs are sensitive to azide, Cu,Zn-SODs to cyanide and hydrogen peroxide, and Fe-SODs to azide and hydrogen peroxide [9]. SOD isolated from *D. desulfuricans* 1388 was pre-incubated with an inhibitor under investigation (0.01 M) in 0.05 M phosphate buffer, pH 7.6, for 60 min. Then SOD activity was measured under the standard conditions and compared to the control sample (pre-incubated in the absence of inhibitor in 0.05 M phosphate buffer, pH 7.6, for 60 min). In this experiment, hydrogen peroxide inhibited the SOD activity by 70%, azide by 50%, whereas inhibition of enzyme activity by cyanide was not detected. Thus, the results of genetic analysis, indirectly pointing to the presence of Fe ions in the catalytic site of periplasmic SOD from *D.*

desulfuricans 1388, are in agreement with the results of inhibitory analysis, and allow classifying the enzyme as Fe-containing superoxide dismutase.

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