CLONING AND EXPRESSION OF CAMBIALISTIC *BACTEROIDES FRAGILIS* SUPEROXIDE DISMUTASE GENE

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(ABSTRACT)

A gene coding for the cambialistic superoxide dismutase (SOD) was isolated from a LambdaGEM-11 genomic library of *Bacteroides fragilis*. In order to generate a complete genomic library, *B. fragilis* genomic DNA was partially digested with the restriction endonuclease *Sau*3AI and was ligated to cloning vector, LambdaGEM-11. After *in vitro* packaging, DNA was used to infect *E. coli* KW 251. The genomic library was finally established in the plaque population. Recombinant phage DNAs containing the SOD gene were detected by a ^{32}P-labelled synthetic oligonucleotide with 17 bases. The sequence of this oligonucleotide was deduced from the N-terminal amino acid sequence of *B. fragilis* FeSOD. Two recombinant phage DNAs were selected based on the results of plaque hybridization. Further analysis with restriction mapping and DNA sequencing revealed that only one recombinant phage DNA contained the SOD gene. Southern hybridization and restriction mapping located the SOD gene in the *Sal*I-*Bam*HI fragment (2.1 kb). Sequence analysis identified the orientation and open reading frame (ORF) of the gene. Translation of ORF

revealed that SOD consists of 193 amino acid residues. The size of the deduced polypeptide is consistent with the molecular weight of SOD subunit (MW 21,000). The *B. fragilis* SOD sequence was compared with those of other SODs. The amino acid residues contributing metal ligands, the hydrophobic shell of the active site, and amino acids at the subunit contact are almost fully conserved in *B. fragilis* SOD. Expression of *Sal*I-*Bam*HI fragment in *E. coli* SOD double mutant (*sodA*, *sodB*), QC1799, produced an active SOD whose activity zymogram was identical to that of purified *B. fragilis* SOD. In addition, Western analysis of the expressed protein separated on SDS acrylamide gel also displayed a band identical to the subunit of *B. fragilis* SOD. However, a larger molecular weight band was also detected. This band migrated closely to the subunit of *B. fragilis* SOD. This larger peptide may be the product of gene translation from an ATG 21 bases upstream of the ATG start codon of *B. fragilis* gene. The cambialistic feature of SOD gene product was also confirmed from *in vitro* and *in vivo* metal substitution.

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Role of Oxygen Radicals in Cytotoxicity

Ground state O_2 is a diradical whose unpaired electrons have parallel spins. The reduction of O_2 by singlet pairs of electrons is thermodynamically favorable but kinetically slow. Addition of pairs of singlet electrons to triplet O_2 is spin-forbidden. Hence, spin inversion, often facilitated by transition metals, is required. However, addition of a single electron to O_2 produces superoxide radical, an intermediate shown to be cytotoxic. Superoxide radical is one of the partially-reduced metabolites of oxygen (11) that is generated by sequential addition of electrons to molecular O_2 (Rxns 1-4).

(1) $O_2 + 1\ e^- \rightarrow O_2^-$ (Superoxide radical)

(2) $O_2^- + 1\ e^- + 2\ H^+ \rightarrow H_2O_2$ (Hydrogen peroxide)

(3) $H_2O_2 + 1\ e^- \rightarrow OH^-$ (Hydroxide ion) + $OH^{\cdot}$ (Hydroxyl radical)

(4) $OH^{\cdot} + 1\ e^- + H^+ \rightarrow H_2O$

Among these oxygen metabolites, H_2O_2 is an oxidant but not a radical species while O_2^- and $OH^{\cdot}$ are free radical species. O_2^- represents the most well-studied species involved in oxygen toxicity. This radical, formed during cellular metabolism, is produced through enzymatically-catalyzed reaction as well as through spontaneous oxidations. The rate of O_2^- production can be dramatically

increased by application of photosensitizers (31), and redox cycling compounds (37) such as paraquat, plumbagin and menaquinone. O_2^- production has been observed in many biological systems. For example, O_2^- is one of the factors contributing to microbial killing during the phagocytosis process (3). O_2^- radicals are also the products of many enzymatic reactions. Enzymes known to produce O_2^- include xanthine oxidase (24), dihydroorotate dehydrogenase (22), aldehyde oxidase, and the membrane-associated NADPH oxidase (18). O_2^- radicals are also generated in mitochondria upon oxidation of the respiratory chain (23). Although O_2^- is not as reactive as other free radicals, it does inactivate some enzymes. Among them are the *E. coli* dihydroxy-acid dehydratase (43), an enzyme involved in the pathway of biosynthesis of branched-chain amino acids, the selenium-dependent glutathione peroxidase, and catalase (25). O_2^- also attacks cellular targets indirectly by serving as a precursor for OH·. OH· is a highly reactive radical with a short half-life. OH· is formed through the metal-catalyzed Haber-Weiss reaction (Rxn 5-6) (17).

(5) $Me^{n+} + O_2^- \text{----->} Me^{(n-1)+} + O_2$

(6) $Me^{(n-1)} + H_2O_2 \text{----->} Me^{n+} + OH^- + OH\cdot$

The reaction is site-specific when the catalytic metal is bound to polyanionic structures such as DNA or cell membrane. Iron and copper but not manganese serve as catalysts in this process. In this reaction sequence, OH·

is catalytically generated at the metal center liganded to a biological macromolecule and therefore damages the macromolecule by a "multi-hit" effect. Damage to macromolecules includes peroxidation of lipids, modification of amino acids and hydroxylation of nucleotide bases (70, 85). In addition, DNA strands may be cleaved (17).

Various antioxidant enzymes have evolved to remove toxic oxygen species and to protect cells from oxidative damage. These enzymes are superoxide dismutases (SOD)(Rxn 7), catalases (Rxn 8), glutathione peroxidase (Rxn 9) and glutathione reductase (Rxn 10). These enzymes work in concert to give maximum protection to cells against oxidative damages (Rxn 7-10).

(7) $O_2^- + O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$

(8) $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

(9) $H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$

(10) $GSSG + 2\ NAD(P)H \rightarrow 2\ GSH + 2\ NAD(P)$

As one of the antioxidant enzymes, SOD scavenges O_2^- , the singly-reduced product of oxygen metabolism. Without SOD, cells could be subjected to the lethal effects of O_2.

Biochemical Aspect of Superoxide Dismutase

Superoxide dismutase (EC.1.15.1.1) was first isolated from bovine erythrocytes by McCord and Fridovich (46) and has subsequently been isolated from many sources. SODs are divided among three types, depending on the metal found in

the active site: iron- or manganese-containing SOD (FeSOD, MnSOD) and copper, zinc-containing SOD (CuZnSOD) (2). The enzymatic function of SOD is dismutation of superoxide radical to hydrogen peroxide and dioxygen. Catalytic dismutation of O_2^- involves alternate reduction and oxidation of metal ions in a "ping-pong" type mechanism (Rxn 11-12)

(11) $O_2^- + Me^{n+} \rightarrow O_2 + Me^{(n-1)+}$

(12) $O_2^- + Me^{(n-1)+} + 2\ H^+ \rightarrow H_2O_2 + Me^{n+}$

The "turnover" constant (Kcat) is (2.1-2.7) x 10^9 M^{-1} s^{-1} for CuZnSOD and (0.5-1.5) x 10^9 M^{-1} s^{-1} for FeSOD and MnSOD over the pH range 4.8-9.5.

The types of SOD in a cell extract are distinguished by their differential sensitivity to cyanide, hydrogen peroxide and azide (30). Cyanide (1 mM) inhibits CuZnSOD but not FeSOD and MnSOD. FeSOD and CuZnSOD are rapidly inactivated by incubation with low (5 mM) concentrations of hydrogen peroxide whereas MnSOD is stable (38). FeSOD is inhibited 70-90% by 1 mM NaN_3. CuZn and MnSOD retain full activity until subjected to 10-20 mM NaN_3 (49). Hydrogen peroxide inactivates CuZnSOD and FeSOD by reacting with the reduced enzyme form to produce a reactive intermediate that oxidizes essential amino acid residues.

Distribution and Sequences of Superoxide Dismutase

The SODs are systematically distributed among the

biota. Most eukaryotes contain CuZnSODs in the cytoplasma and MnSOD in mitochondria and chloroplasts. FeSOD and MnSOD are commonly found in prokaryotes (5). The distribution of SODs provides a clue to their evolutionary history. One example is the high degree of sequence similarity between mitochondrial MnSOD and the counterpart in prokaryotes providing strong evidence for the postulate of an endosymbiotic origin for mitochondria. Although most frequently formed in cytosol of eukaryotes, CuZnSODs were identified in some bacteria, *Caulobacterium crescentis* (73), and two strains of *Pseudomonas* (74). FeSOD were also found in three families of plants, *Ginkgo biloba* (19), *Brassica campestris* (63), and *Nuphar luteum* (64). These samples are the exceptions to the expected distribution.

Comparison of amino acid sequence composition of SODs revealed that the three types of SODs fall into two distinct phylogenetic families: the CuZnSOD family and Fe/MnSOD family (2). There is little sequence or secondary structure similarity between the two families whereas there is a high level of both amino acid sequence and structural similarity within each family. The remainder of this discussion will focus on the prokaryotic Fe- and MnSODs, especially on the SODs of *E. coli*.

Structure of Iron and Manganese-containing Superoxide Dismutases

The availability of primary sequence of a variety of SODs and X-ray crystallography data from several SODs have provided information for the structural analysis of FeSOD and MnSOD. Application of amino acid sequence to electron-density maps have verified common structural feature of SODs including overall subunit conformation, metal ligands, and the environment of the active site. X-ray crystallographic analysis has been applied to MnSODs from yeast(8), *E. coli* (8), *Thermus thermophilus* (72), and *Bacillus sterothermophilus* (59) and to FeSODs from *Thermoplasma acidophilus* (52), *Pseudomonas ovalis* (61), and *E. coli* (14). These analysis reveal that FeSODs are structural homologues of MnSODs.

The SOD subunit may be divided into two domains: one domain contains α-helices while the other is composed of both α-helices and ß-sheets (Figure 1). The metal binding site is located between these two domains. In the case of *B. stearothermophilus* MnSOD, the α-helix domain is characterized by a pair of long antiparallel α-helices, α-1 and α-3, which cross at a angle of about 35^0. The α-helix domain for MnSOD is characterized by a two-turn helical segment, designated α2, which is not found in FeSODs. It was suggested that α2 in the *T. thermophilius* stabilized the tetrameric structure. However, tetrameric MnSODs of human and mouse have seven residue deletions in the α2 region. It may be that α2 and the basis of tetrameric assembly are

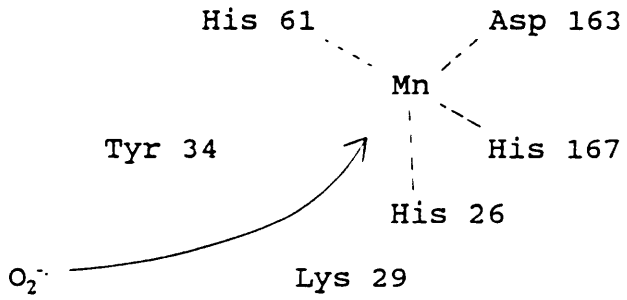

Figure 1. The monomer of *B. stearothermophilus* MnSOD.

The monomer contains two structural domains. The α-domain consists of helices α1 - α3 and the α/β domain includes β-sheet 1-3 and helices α4-α7. Amino acid residues that are ligands to the metal and the metal (Mn) are shown. (Derived from reference 59.)

species-specific or that there is no relationship of α2 to assembly.

High-resolved X-ray studies have allowed the of active site environment to be examined in detail. Metal ligands for FeSOD and MnSOD are three histidines and one aspartate. Iron or manganese at the active site ligates to histidines through an imidazole group and to aspartate through a carboxylate group. The α-helix domain contains two of the histidine ligands and the α/ß domain contributes one histidine and one aspartate. A series of hydrophobic amino acid residues, particularly aromatic residues, surround the active site. Comparison of the three dimensional structure model of *E. coli* FeSOD and *T. thermophilus* MnSOD with the amino acid sequence of a number of SODs reveals that almost every residue which penetrates the metal ligand environment is strongly conserved in MnSOD and FeSOD (14). However, the only exceptions found so far are three residues in *E. coli* FeSOD, Gln^{69}, Tyr^{76}, and Ala^{141} , whereas the corresponding residues in MnSOD are Gly^{69}, Phe^{76}, and Gln^{141}. In both FeSOD and MnSOD, the glutamine (69 for FeSOD, and 141 for MnSOD) is presumed to be a bridge between a tyrosine residue and a tryptophan. This bridge provides an additional interaction to stabilize the ring system around the metal-ligand cluster.

Metal Specificity of Superoxide Dismutase

Although FeSOD and MnSOD are almost identical in terms of structure and of the ligands to the metal, reconstitution studies with the denatured apoprotein have shown that in almost all cases FeSOD and MnSOD are active only with their native intrinsic metal. Each enzyme will bind Mn, Fe or Zn but only the native metal (Fe or Mn) confers catalytic activity. Thus FeSODs are active only with iron at the active site whereas MnSODs are specific for manganese at the active site. This metal specificity may be due to subtle structural difference between FeSOD and MnSOD (9). However, a few SODs, including *Bacteroides fragilis* (29), *Bacteroides gingivalis* (1), *Propionibacterium shermanii* (47), and *Streptococcus mutans* (45), are active with either Fe or Mn at the active site. Both *P. shermanii* and *S. mutans* produce either FeSOD or MnSOD, depending on the relative abundance of these two metals in the culture medium. The term "cambialistic" suggested by Martin was used to describe enzymes capable of being active with more than one prosthetic group. Determination of the sequence of these cambialistic SODs and subsequent site-specific mutagenesis may pinpoint the chemical origins of the observed metal-binding behavior.

Induction of MnSOD

E. coli synthesizes two SODs: FeSOD and MnSOD each of which is a separate gene product. FeSOD is present in cells

maintained anaerobically or under low oxygen tension whereas MnSOD is induced under aerobic growth conditions. The effect of several metabolic perturbations on the MnSOD level in *E. coli* cells has been reported in earlier studies. The amount of MnSOD activity was related to the O_2 tension in the growth medium (27). Application of paraquat and other redox active xenobiotics also induce MnSOD activity when cultures were in the presence of O_2 (36). In addition, the carbon source in the culture medium affected the induction of MnSOD. Glucose, but not succinate or lactate, repressed MnSOD activity (35). Increased manganese in the culture medium also enhanced the level of MnSOD activity (50). The most striking discovery was the induction of MnSOD under anaerobic conditions with the addition of metal chelators, especially iron chelators such as 1,10-phenanthroline, in the culture medium. This observation led to the proposal of negative control for induction of MnSOD (51).

Molecular Basis of Superoxide Dismutase

Genes encoding for SOD from several prokaryotes have been isolated and sequenced. Cloned SOD genes allow the prediction and comparison of amino acid sequences among different SODs. They can be used as probes for cloning of homologous genes and detection of transcriptional levels of corresponding mRNA. They also provide a useful source for production of large quantities of SOD for therapeutic

applications. In addition, they can be used to create microbial strains that are either SOD-deficient or that produce mutant proteins.

Both the FeSOD (*sodB*) and MnSOD (*sodA*) genes in *E. coli* have been cloned and sequenced (62, 77, 79). Each gene constitutes a monocistronic operon. The DNA sequence of FeSOD lacks any recognized regulatory sequence, an observation that is consistent with its being a constitutive enzyme. By contrast, two possible promoters have been identified for *E. coli* MnSOD gene, but apparently only one of them is active during normal aerobic growth. Although the function of the other promoter has not yet been established. It has been postulated that this promoter may be activated under different growth conditions (77). In addition, the DNA sequence of MnSOD gene also shows that there is an almost perfect 19 base palindrome at the -35 region. The position and the size of this palindromic sequence has led to the suggestion that this site could be involved in the control of MnSOD synthesis. Indeed, this sequence is now recognized as an "iron box" which is thought to be a site for repression by *fur* (ferric uptake regulation) protein (58).

SOD-deficient strains have been created in *E. coli* (13), yeast (82), and Drosophila (10) as models for oxidative stress. Examination of these mutants has established that SOD is necessary for aerotolerance. In the

case on *E. coli* mutant devoid of SOD activity, this double mutant (*sodA sodB*) displays a conditional growth, depending on the growth medium. It grows normally in rich medium but is unable to grow aerobically in minimal medium unless supplemented with leucine, valine or isoleucine (13). The conditional auxotrophy may be the result of defective pathway for branched-chain amino acid biosynthesis. The defect is likely caused by the superoxide radical. This suggestion is consistent with the finding that purified dihydroxy-acid dehydratase, a enzyme involved in the biosynthesis of branched-chain amino acids, can be inactivated by superoxide radical (43) .

SOD negative mutants also undergo spontaneous mutation more frequently than do the parent strains (21). All the deleterious effects observed in SOD double mutants are oxygen dependent and can be prevented by expressing plasmid encoded SOD in the *E. coli* mutant. Expression of plasmids carrying either of the sod genes isolated from *E. coli* (FeSOD or MnSOD) (13), or the human CuZnSOD (56), a form not found in *E. coli*, protect the cell against oxidative damages. These studies confirmed the biological role of SOD as a superoxide scavenger.

Although some authors suggest that effects other than SOD deficiency contribute to the conditional auxotrophy of the *E. coli* SOD mutants, there is no direct evidence to support other affects. Pseudorevertants, including those of

E. coli and yeasts, which are able to grow aerobically in minimal medium, have been characterized (39, 82). In *E. coli*, revertants that occurred at a frequency between 10^{-7} and 10^{-6} displayed a mutation at the *ssa* (suppressor of superoxide-dependent auxotrophy) locus located at the 4.0 min position of the chromosome. Treatment of the cells with high concentration of external osmolytes allowed the SOD-deficient strain to mimic pseudoreversion on cell growth (40). Therefore the effect of oxidative injury on the growth of SOD-deficient strain has been confirmed to affect the envelope plasma membranes and suppression of such injury through a mutation at *ssa* locus was suggested. Study of the pseudoreversion of SOD mutant may further reveal the molecular basis of oxidative damage to the plasma membrane.

The Regulation of MnSOD Biosynthesis

Earlier studies on induction of MnSOD led investigators to suggest that superoxide was a direct inducer for MnSOD biosynthesis. This interpretation, however, was challenged by the finding that the presence of high copy numbers of *sodB* coding for FeSOD, did not interfere with the enhanced induction of MnSOD (*sodA*) under aerobic conditions (57). There is further evidence that redox state, but not superoxide itself, signals induction of MnSOD (67).

Regulation of MnSOD biosynthesis in *E. coli* has been

studied with operon and protein fusions between the MnSOD gene (*sodA*) and genes of the lactose operon (80). These fusions were used to explore the effects of various factors on MnSOD expression. It has been clearly demonstrated that MnSOD induction is a transcriptional event and the effect of Mn or Fe concentration on MnSOD activity occurs most likely in posttranscriptional or posttranslational steps. It is note-worthy that the aerobic induction of ß-galactosidase in *sodA-lacZ* strain is inhibited to different extents when plasmids carrying different regions of *sodA* but not *sodB* were introduced into this strain. Two possible explanations have been offered: first, the titration of a transcriptional activator by the multiple copies of the regulatory region carried by the plasmid; second, the direct control of the protein on expression of its own structural gene. Touati *et al.* suggested that biosynthesis of MnSOD is multiregulated.

It is becoming clear that the expression of *E. coli* MnSOD gene (*sodA*) is subjected to several regulatory elements. At least five regulatory loci are involved: *fur* (ferric uptake regulation), *arcA* and *arcB* (aerobic respiratory control), and *soxR* and *soxS* (superoxide regulation).

The primary function of Fur protein in *E. coli* is to regulate about 30 genes involved in the uptake of iron (4). Under Fe sufficiency, Fur binds to ferrous iron (Fe^{+2}). The resulting Fe-Fur complex therefore controls these genes by

serving as a transcriptional repressor which recognizes a particular sequence (iron box) in the promoter region. In the case of iron deficiency, Fe-Fur complex is dissociated and therefore derepresses the relevant genes. The finding that Fur protein was involved in the expression of *sodA* arose from the observation of an "iron box" sequence in the promoter region of MnSOD gene (58). There was also significant derepression of *sodA* in Fur^- cells compared with Fur^+ cells. Furthermore, the mutation in the iron box of *sodA* abolished inducibility of MnSOD by paraquat and by a metal chelator (2-2'-bipyridyl) under both aerobic or anaerobic conditions (53).

The function of *arc* genes in *E. coli* is to control synthesis of proteins involved in aerobic respiration (41). Since the expression of *sodA* and *arc* genes occurs under aerobic conditions, the possible involvement of *arc* genes (*arcA* and *arcB*) in expression of MnSOD has been explored. By measurement of ß-galactosidase activity of fusion operon (*sodA-lacZ*) in different mutant strains, Tardat and Touati (78) found that *arc* genes work cooperatively with *fur* gene to repress *sodA* expression under anaerobic condition and suggested there is a synergy between *fur* and *arc* mutations.

Many proteins, including MnSOD, were induced in aerobically grown *E. coli* when paraquat, menadione, or plumbagin was included in the culture medium (84). Genes in *E. coli*, which respond directly to superoxide radical, are

called the superoxide regulon (*sox*). Two regulatory elements, SoxR and SoxS, are required to activate expression of genes in the *sox* regulon. Genes (*soxR* and *soxS*) coding for these two elements have been localized to a 2.4 kbp DNA fragment (86). The two genes are divergently transcribed and the transcripts overlap. Concentration of *soxS* mRNA, but not *soxR*, increases after induction with paraquat. Both SoxR and SoxS have possible DNA-binding (helix-turn-helix) domains. SoxR, containing a cluster of cysteine residues (CX_2CXCX_5C), may serve as a sensor for superoxide response. Based on the sequence features, Wu and Weiss suggested that SoxR, as a sensor, detects the environmental signal, and transmits the signal to SoxS. The modified SoxS then activates transcription of genes involved in the superoxide regulon. The signal transduction may in fact involve a direct interaction of the two proteins (SoxR and SoxS) while bound to DNA.

Studies of *Bacteroides fragilis* superoxide dismutase

The genus *Bacteroides* are obligately anaerobic, nonsporeforming gram-negative rods that obtain energy by fermenting carbohydrates. They account for 30% of bacteria in the colon and are found in polymicrobial infection sites. Among them, *Bacteroides fragilis* represents the largest group of this kind of pathogen. *B. fragilis* is more aerotolerant than most of the other anaerobes examined.

Some strains can survive under 8% oxygen tension up to several days on blood agar plates. SOD activity has been demonstrated in these cells (15). It is suggested that the presence of SOD may be one virulence factor that allows this pathogenic anaerobe to survive in oxygenated tissue until the proper growth conditions are established. SOD in *B. fragilis* is also inducible under aerobic conditions. A 7-10 fold induction of SOD activity was obtained when cells were exposed to 20% oxygen for 2 hours.

The SOD present under anaerobiosis and the induced SOD were purified to electrophoretic homogeneity (28,29). The SOD, molecular weight 42,000, is a dimer of equally sized subunits joined by noncovalent interactions. Metal analysis of SOD isolated from anaerobically grown cells revealed 1.8-1.9 g-atoms Fe, 0.2 g-atoms Zn, and less than 0.05 g-atoms Mn per mole dimer. Oxygen-induced SOD contained 1.1 g-atoms Mn, 0.3 g-atoms Fe, and 0.2 g-atoms Zn per mole dimer. Comparison of SODs isolated from anaerobic and aerated cells revealed that both SODs were virtually identical except for the metal content. This conclusion was based on the following observations; (1) electrophoretic analysis showed that both SODs and the mixture of them have identical migration patterns in acrylamide gel and on isoelectric focussing. A major protein band (pI 5.3) and a minor band at pI 5.0 are observed on isofocussing gel. (2) the amino acid composition of both SODs are virtually identical. (3)

metal removal and replacement studies *in vitro* showed that activity was restored to the denatured apoSOD of either enzyme by dialysis in either ferrous ammonium sulfate or manganous chloride.

B. fragilis SOD presents a novel model for studies in aspects of molecular biology and protein chemistry because of the cambialistic nature of the enzyme. Interesting questions related to this model will include;

(1) What is the origin basis of this cambialistic enzyme.

(2) How does *B. fragilis* responds to oxidative stress in molecular basis.

(3) What structural features contribute to metal specificity of *B. fragilis* SOD.

Isolation and characterization of the gene for this cambialistic SOD will provide valuable information and tools for subsequent studies and therefore constitute the main focus of this work.

MATERIALS

Ampicillin, kanamycin, polyethylene glycol (MW 8000), tris-(hydroxy methyl)aminomethane (Tris), agarose gel, ethylenediaminetetraacetic acid sodium salt (EDTA), sodium dodecyl sulfate (SDS), sodium citrate, sodium acetate, lysozyme, proteinase K, RNase A, DNase I, isopropanol anhydrous and Kodak X-OMAT AR5 film were purchased from Sigma Chemical Company.

Tryptone, yeast extract, agar, and lambda *Hind*III-digested DNA maker were from Gibco BRL.

Chloroform, phenol, acrylamide, polyvinyl pyrrolidone (MW 400,000), bovine serum albumin (BSA) and ammonium acetate were obtained from Fisher Chemical Company.

DE-53 anion exchange resins are the product of Whatman company. Nitrocellulose membranes were purchased from Schleicher & Schuell Inc. YM10 filters are the product of Amicon.

Lambda GEM-11 *Xho*I Half-Site Arms Cloning System, T4 polynucleotide kinase, and T4 DNA ligase are the product of Promega. Restriction endonucleases were purchased from either Promega or New England Biolabs. [Γ-^{32}P] ATP (6000 Ci/mmole, [α-^{35}S] dATP (1200 Ci/mmole) were purchased from New England Nuclear (NEN). ECL chemiluminescence detection kit was the product of Amersham. DNA sequencing kit (Sequenase version 2), Isopropyl-ß-thiogalactopyranoside

(IPTG), and 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal) were purchased from United States Biochemical (USB). The plasmid pBluescript II KS is the product of Stratagene.

Bacterial strain DH5αF'(F',ϕ80d*lac*Z∆M15, *end*A1, *rec*A1, *hsd*R17(r_k^- m_k^+), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, ∆(*lac*ZYA-*arg*F)U196 is the product of Gibco BRL. KW251 (F^-, *sup*E44, *sup*F58, *gal*K2, *gal*T22, *met*B1, *hsd*R2, *mcr*B1, *mcr*A^-, *arg*A81:*Tn*10, *rec*D1014) were purchased from Promega.

METHODS

Cell Growth of *Bacteroides fragilis*

The *B. fragilis* selected for this study, ATCC 25285 (VPI 2553), is the type strain of the species. Cells were maintained in anaerobic chopped meat medium and were transferred to anaerobically sterilized peptone-yeast-glucose medium (PYG) supplemented with heme and vitamin K (28) and incubated for 18 hr at 37^0C. For aerobic induction of SOD, cells from an overnight culture were diluted in PYG to an absorbance of 0.2 at 600 nm (A_{600}), incubated at 37^0C until A_{600} was between 0.6 and 0.8 and transferred to a sterile 2.8 liter Fernbach flask. The culture (in 280 ml) were aerated at 250 rpm at 37^0C on a platform shaker.

N-terminal sequence of *B. fragilis* SOD

FeSOD was isolated from *B. fragilis* using the method previously published (29). Ten grams of lyophilized *B. fragilis* cells were suspended in 300 ml of 50 mM potassium phosphate, 1 mM EDTA (pH 7.8) (SOD buffer) with a Waring blender. The suspension was then sonically disrupted at 4^0C for 5 min/100 ml using 60 W into the sonifier (microtip) and clarified by centrifugation. The supernatant was brought to 0.2% protamine sulfate (W/V) and stirred 30 minutes. This and all subsequent steps were at 4^0C. After centrifugation, solid ammonium sulfate (0.313 g/ ml, 50% of saturation) was

added to salt out undesired proteins; the SOD remained soluble. The particulate matter was removed by centrifugation and the enzyme was then absorbed onto phenyl-sepharose (2.5 x 15 cm) equilibrated with 50% $(NH_4)_2SO_4$ in water. Under these conditions, the SOD was bound to the column primarily by hydrophobic forces. Proteins were eluted with a gradient of decreasing concentration of $(NH_4)_2SO_4$ in SOD buffer (50% to 0% $(NH_4)_2SO_2$ in SOD buffer, 300 ml each component). The fractions containing SOD activity were pooled, concentrated by ultrafiltration on YM 10 and dialyzed in 10 mM TrisHCl, 1 mM EDTA,(pH 8). The dialyzed protein solution was applied to DE-53 Sephadex column (1.5 x 10 cm) equilibrated with the Tris buffer and eluted with a KCl gradient in Tris buffer (0-100 mM KCl, 175 ml each component). Fractions containing SOD activity were pooled and dialyzed against the Tris-buffer. The enzyme was again applied to a DE-53 column equilibrated with Tris-buffer and was eluted with a gradient of decreasing pH (40 mM MES pH 4.4 mixed with 20 mM Tris, 1 mM EDTA pH 8.0 150 ml each component). Fractions containing SOD activity were pooled and concentrated by ultrafiltration.

N-terminal sequence of the pure *B. fragilis* FeSOD was determined at the Department of Biochemistry and Molecular Biophysics at Virginia Commonwealth University, Richmond, Va, using the dansyl chloride method (26).

DNA Isolation

The procedure used for the isolation of *B. fragilis* genomic DNA was a modification of the method described by Silhavy *et al.* (68). Cells grown overnight in PYG (100 ml) was harvested by centrifugation at 3000 x g for 20 min. The pellet was resuspended in 5 ml of 50 mM Tris-HCl, 50 mM EDTA (pH 8.0) and frozen at -20^{0}C. Lysozyme solution (0.5 ml of solution, 10 mg/ml in 0.25 M Tris.HCl pH 8.0) was added to the frozen cell pellet and the mixture was thawed at room temperature, then stored on ice. Cells were incubated at 50^{0}C in 1 ml of STEP solution (0.5% SDS, 50 mM Tris.HCl, 0.4 M EDTA, 1 mg/ml proteinase K, pH 7.5) for 60 min. Six milliliters of Tris-buffered phenol was added to extract DNA. The aqueous layer, containing DNA, was collected and mixed with 0.1 volume of 3 M sodium acetate and the DNA was precipitated after addition of 2 volume of ethanol to the DNA solution. The precipitate was resuspended in 5 ml of 50 mM Tris.HCl, (pH 7.5), 1 mM EDTA, 200 ug/ml RNase A and incubated 4^{0}C until completely dissolved. An equal volume of chloroform was added to the DNA solution to extract phenol, after which DNA was reprecipitated by adding sodium acetate and ethanol. DNA was dissolved in 50 mM Tris.HCl, 1 mM EDTA (pH 7.5) and was stored at -20^{0}C.

Plasmid DNA was isolated using a method of Maniatis *et al.* (44). All centrifugation steps were performed in a microcentrifuge at room temperature. Overnight cell culture

(1.5 ml) was harvested and the cell pellet was resuspended in 200 ul of suspension buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) and kept at room temperature for 5 min. The cells were mixed with 400 ul of lysing solution (0.2 N NaOH, 1% SDS) and incubated on ice for 5 min. The lysate mixture was neutralized by addition of 300 ul of ammonium acetate (7.5 M), incubated on ice for an additional 10 min and centrifuged for 5 min. The supernatant was mixed with 0.6 volume of isopropanol, incubated at room temperature for 10 min, and plasmid DNA was then pelleted by centrifugation (10 min). The pellet was washed with 70% ethanol, dried and resuspended in 100 ul of TE (10 mM Tris.HCl, 1 mM EDTA pH 8.0) containing 20 ug/ml RNase A. The solution was incubated at 37^0C for 30 min. One half volume of ammonium acetate (7.5 M) was added, and the incubation continued on ice for 30 min. Following centrifugation (5 min), the supernatant was mixed with 2 volume of cold ethanol and allowed to stand at room temperature for 10 min. Plasmid DNA was pelleted by centrifugation (15 min) and was then washed with 70% ethanol. The pelleted DNA was dissolved in TE solution.

Phage DNA was isolated as described by Chisholm (12). Plaques containing phage were harvested from the growth plate using a Pasteur pipette to remove an agar plug. The plug were transferred to 1 ml SM buffer (20 mM Tris.HCl, pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$, 0.1% gelatin), and the phage

was eluted from the plug for at least 2 hr at room temperature. Solution containing the eluted phage (100 ul) was added to 500 ul of prepared host bacteria (*E. coli* KW 251 grown to A_{600} =0.2) and incubated for 30 min at 37^{0}C. Preabsorbed phage suspension was then added to 37 ml of LB in 500 ml Erlenmeyer flasks and grown at 37^{0}C for 15 hr on a platform shaker at 150 rpm, during which the cells lysed. Cell lysate was mixed with 100 ul of chloroform, 370 ul of nuclease solution (5 mg/ml DNase I, 5 mg/ml RNase A, 50% glycerol, 30 mM sodium acetate, pH 6.8) and mixture was incubated 30 min at 37^{0}C. NaCl (2.1 g) was added and mixed until dissolved, after which the solution was clarified by centrifugation (6000 x g, 20 min at 4^{0}C). The supernatant was mixed with polyethylene glycol (MW 8000) (3.7 g), and incubated on ice for 60 min. The solution was centrifuged at 6000 x g (20 min), and the pelleted phage was resuspended in 500 ul of SM buffer. The phage suspension was mixed with 500 ul of chloroform and was centrifuged in a microfuge for 5 min. The supernatant was then mixed with 20 ul 0.5 M EDTA, 5 ul of 20% SDS, and 10 ul proteinase K (2.5 mg/ml) and incubated at 65^{0}C for 30 min. The supernatant was extracted first with phenol (500 ul) and then chloroform (500 ul). The supernatant was added to 170 ul of ammonium acetate (6 M) and precipitated by the addition of 700 ul of isopropanol. After 15 min at 4^{0}C in microcentrifuge, the pellet were washed in 70% ethanol and dried. The pellet was

resuspended in TE and stored at -20^{0}C.

M13 phage single strand DNA was isolated as described by Messing (48). Plaques were transferred to 1.5 ml of 2YT medium (16 g Bacto tryptone, 10 g Yeast extract and 5 g NaCl for per liter) and fresh *E. coli* DH5αF' grown to A_{600}=1.0) (15 ul) was added to medium. The culture was grown at 37^{0}C with vigorous shaking for 12 hr and cells were harvested by centrifugation. Supernatant containing M13 phage was collected and mixed with 200 ul of PEG/NaCl solution (20% polyethylene glycol (MW 6000), 2.5 M NaCl). After incubation on ice for 15 min, M13 phage was pelleted by microcentrifuge (15 min). Supernatant was discarded and phage pellet was dried. Phage was then suspended in 100 ul of TE and was extracted with 50 ul of phenol saturated with TE. Aqueous layer was collected and mixed with 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol. The solution was kept at -20^{0}C overnight. DNA was pelleted at room temperature by microcentrifuge (15 min) and dissolved in 30 ul of TE.

DNA Immobilization

Plaque transfer and DNA immobilization were performed according to the method of Maniatis *et al.*(44). Filters containing plaques were first treated with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min followed by neutralizing solution (0.5 M Tris.Cl pH 7.4, 1.5 M NaCl) for

another 5 min. After rinsing with 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0) filters were placed on paper towels to dry for 20 min at room temperature. DNA was fixed to filters by baking for 2 hr at 80^{0}C in an oven.

Southern transfer procedure as modified by Hardy (33) was used for this study. After DNA fragments were separated in agarose gel (0.5%), the gel was gently shaken in the depurination solution (0.1 M HCl) for 60 min at room temperature. The HCl solution was decanted and the gel rinsed briefly with distilled water. The gel was agitated in denaturation solution (1.5 M NaCl, 0.5 M NaOH) at room temperature for 60 min, rinsed briefly with distilled water and was incubated in cold neutralization solution (2.5 M ammonium acetate) at 4^{0}C for 30 min. The method (44) for gel blotting and DNA transfer was followed except transfer buffer was 1.0 M ammonium acetate and DNA transfer was carried out at 4^{0}C.

DNA Hybridization Techniques

Synthetic oligonucleotides were labelled with Γ-^{32}P dATP by T4 polynucleotide kinase. Labeling reaction was carried out at 37^{0}C for 30 min and free Γ-^{32}P ATP was removed from ^{32}P labelled oligonucleotide by passing the reaction mixture through DE-52 cellulose column saturated with TE. Nitrocellulose membrane bearing the immobilized DNA was prehybridized at 65^{0}C for 5 hr in 6X NET, 5X Denhardt's

solution, 0.1% SDS and salmon sperm DNA (0.2 mg/ ml). 20X NET is 3 M NaCl, 20 mM EDTA, and 0.3 M Tris-HCl, pH 8.0, and 100X Denhardt's solution is 2% ficoll (MW 400,000), 2% polyvinyl pyrrolidone (MW 400,000) and 2% bovine serum albumin (BSA). Hybridization was carried out at 40^{0}C for 17 hr in the hybridization solution with ^{32}P-labelled oligonucleotide probe (5 x 10^7 CPM) replacing the Salmon sperm DNA. Membrane was then washed three times at room temperature by 6X NET, 0.5% SDS with each for 15 min. The stringency washing was performed at 45^{0}C for 5 min in the same washing solution. Membranes were then dried at room temperature and exposed to X-omat AR5 film with intensifying screens for autoradiography.

Transformation

Competent cells were prepared by the Hanahan method (32). Competent cells (200 ul) were mixed with DNA solution (< 20 ul) and incubated on ice for 40 min. The cells were subjected to heat shock at 42^{0}C for 90 sec and subsequent chilling on ice for 2 min. Liquid medium (LB) (800 ul) was added to the mixture and incubated at 37^{0}C for 50 min.

DNA sequencing

The Sanger sequencing method (66) modified by Tabor and Richardson (76) was performed using a commercial sequencing kit (Sequenase version 2). Double stranded template

(plasmid DNA) isolated as described was prepared by denaturing the plasmid in 0.2 M NaOH, 0.2 mM EDTA, followed by neutralization with 0.2 M ammonium acetate (pH 4.6). M13 cloning vector (mp18 and mp19) was used for the preparation of single strand templates. Sequenase (version 2) was used to synthesize complementary strand. DNA was labelled with α-^{35}S-dATP and separated in either 6% or 5% gels containing 48% urea. After electrophoresis, gel was fixed in 10% acetic acid, 10% methanol and dried at 80^{0}C for 1 hr under vacuum.

Expression of SOD Gene

QC1799, an *E. coli* SOD mutant (*sodA, sodB*), was transformed with DNA fragment containing the SOD gene of *B. fragilis*. The transformant, selected for the plasmid I had constructed, was provided by Dr. Malmay of Department of Microbiology and Molecular Biology at Tufts University. A single colony of this transformant was inoculated into 10 ml LB containing ampicillin (500 ug/ml) and kanamycin (40 ug/ml). Cells were grown at 37^{0}C until cell density was between 0.7 and 1.0 A_{600} . Cells were washed twice in PBS (10 ml each) at room temperature and suspended in M63 minimal medium (2g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 0.5 mg $FeSO_4.7H_2O$, 1 mM $MgSO_4.7H_2O$ in per liter of water) supplemented with 0.4% glucose and thiamine (1 ug/ml). The cells were transferred into 200 ml of the same medium.

Cultures were shaken at 200 r.p.m in a rotary shaker at 37^{0}C until the A_{600} was 3.5-4.0. Cells were suspended in SOD buffer and disrupted with ultrasonic treatment for 3 min. The cell suspension was clarified by centrifugation (12000 x g for 20 min) and the supernatant dialyzed against SOD buffer.

Measurement of SOD Activity

Superoxide dismutase activity was measured by inhibition of the superoxide-dependent reduction of cytochrome c as described by McCord and Fridovich (46). In the assay mixture, xanthine oxidase was added to cause a ΔA_{550}/min of 0.025 +/- 0.001 (standard rate). One unit of SOD activity was defined as the amount causing 50% inhibition of the standard rate. For qualitative purposes, SOD activity was visualized on native polyacrylamide gel with the modified method of Beauchamp and Fridovich (7). Gels were soaked in the activity staining solution (16 mg nitrobluetetrazolium, 1 mg riboflavin, 0.2 ml TEMED in 80 ml of SOD buffer) in the dark for 40 min, then rinsed with water and exposed to light. During exposure to light, a blue formazan appears due to the superoxide-mediated reduction of nitrobluetetrazolium. SOD prevents nitrobluetetrazolium reduction by scavenging the superoxide, so that an achromatic band appears at the area of gel containing SOD.

Protein concentration

Protein concentration in the crude extract sample was approximately calculated by the square of absorbance at 280 nm divided by absorbance at 260 nm (83). The method corrects the contamination by nucleic acids at 260 nm. Pure *B. fragilis* SOD was estimated by the absorbance at 280 nm using a extinction coefficient of 1.8 ml mg^{-1} cm^{-1}.

Resolution and Reconstitution of *B. fragilis* SOD

All resolution and reconstitution steps were carried out at 4^0C. SOD was denatured and metal was removed by dialysis of the protein in 5 M guanidinium chloride, 20 mM 8-hydroxyquinoline, for 18 hr. Following dialysis in 5 M guanidinium chloride for 8 hr, denatured SOD was reconstituted by dialysis in 20 mM Tris.HCl (pH 7.0) containing either 1 mM $MnCl_2$ or 1 mM $Fe(NH_4)SO_4$ for 12 hr. Excess metal was subsequently removed by dialysis in SOD buffer for 12 hr.

Immunological Detection

The immunoblotting technique developed by Towbin *et al.* (81) was used with several modifications. Proteins from the SDS gels were electrophoretically transferred to nitrocellulose membrane in transfer buffer (6.25% Tris, 1.4% glycine and 20% methanol). The electrophoretic transfer was allowed to proceed at 4^0C overnight at 20 milliamps. Gel

was stained with 0.5% ponceau S (in 1% acetic acid) to determine transfer efficiency. The membrane was then incubated at room temperature for 2 hrs in 5% nonfat dried milk in PBS solution. Rabbit anti-SOD IgG (0.005 mg/ ml) was added to the membrane and incubated for 1 hr at 4^0C. After washing three times with PBS for 10 min each, membranes were washed once with NT solution (150 mM NaCl, 50 mM TrisHCl, pH 7.5). The membrane was incubated in a 1:3000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate in NT solution for 1 hr. The membrane was washed three times with NT solution (10 min each). A mixture of solution I and II (3.5 ml each) of ECL detection system (20) was incubated with the membrane at room temperature for 1 min. After the detection solution had been drained off, X-ray film was placed on the membrane for 1 min to detect the image.

RESULTS

1. Isolation of *B. fragilis* FeSOD

The scheme for purification of FeSOD from anaerobically grown *B. fragilis* is shown in Table 1. The enzyme was purified to electrophoretic homogeneity as assayed in both 10% native acrylamide gel and 10% acrylamide containing SDS. N-terminal amino acid sequence of the *B. fragilis* FeSOD was determined by the dansyl chloride method. This amino acid sequence (30 residues) and the degenerate 17-mer deoxyoligonucleotide sequence based on the amino acid sequence are shown in Figure 2.

2. Isolation of Superoxide Dismutase Gene from *B. fragilis*

A. Construction of Genomic Library

Genomic DNA isolated from anaerobically grown *B. fragilis* was partially digested with the restriction endonuclease *Sau*3AI. Three reactions, each 1 ml total volume, contained about 100 ug of *B. fragilis* genomic DNA and 0.032, 0.016 or 0.008 units of *Sau*3AI. All reaction mixtures were incubated at 37^{0}C for 30 min. The reactions were stopped by addition of 0.5 M EDTA (2 ul). Ten microliters of each reaction mixture were then loaded into 0.5% agarose gel and electrophoretically separated for 17 hr at 4^{0}C at 20 volts. DNA digested with *Sau*3AI at 0.032 unit

Table 1: Purification Step of *B. fragilis* SOD

Step	TA(U)	TP(mg)	SA(U/mg)	R(%)	nFP
1. Extract	44000	2700	16		1
2. Ammonium Sulfate	35000	1200	30	100	1.9
3. Phenyl Sepharose	32000	61.0	500	90	32
4. DE53 KCl Gradient	20000	15.0	1300	57	83
5. DE53 (pH Gradient)	13000	7.2	1600	32	100

TA: Total Activity

TP: Total Protein

SA: Specific Activity

R: Recovery

nFP: n-Fold Purification

(A)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MET-	THR-	TYR-	GLU-	MET-	PRO-	LYS-	LEU-	PRO-	TYR-	ALA-	ASN-	ASN-	ALA-	LEU-
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
GLU-	PRO-	VAL-	ILE-	SER-	X	-GLN-	THR-	ILE-	ASP-	TYR-	HIS-	TYR-	GLY-	LYS

(B)

5'-ATG AC(C\G) TA(T\C) GAA ATG CC-3'

Figure 2. N-terminal amino acid sequence of *B. fragilis* SOD and the deduced degenerate oligonucleotide sequence.

(A) The N-terminal amino acid residues of *B. fragilis* FeSOD. (B) Four degenerate synthetic oligonucleotides deduced from the first six amino acids of the *B. fragilis* FeSOD N-terminus.

/ug DNA contained the greatest amount of DNA fragments between 9 kb and 23 kb (Figure 3). DNA hydrolyzed by this method from was then purified from three reactions mixtures using phenol-chloroform extraction.

Twenty micrograms of partially digested DNA was filled with dGTP and dATP in a reaction mixture containing 20 units of DNA polymerase (Klenow fragment). The reaction mixture was incubated at 37^0C for 30 min, after which the filled in DNA was purified by phenol-chloroform extraction. One microgram of polymerase-treated DNA was ligated to 2 ug of LambdaGEM-11 *Xho*I half-site arms in a reaction mixture containing 1 Weiss unit of T4 DNA ligase. Another reaction mixture but without polymerase-treated DNA was also prepared as a control. Ligation reactions were incubated at room temperature for 4 hr. *In vitro* packaging reaction was performed at 22^0C for 2 hr. The packaging mixtures included the entire ligation reaction mixture (15 ul) and 50 ul of packaging extract. After adding SM buffer to a final volume of 100 ul, the packaging reaction solution was appropriately diluted (1/10 - 1/1000). One hundred microliters of dilute packaged DNA was used to infect 100 ul of host bacteria *E. coli* KW 251. The number of plaques obtained with each dilution is 300 (1/10), 84 (1/100) and 47 (1/1000). Based on the number of plaque in 1/10 dilution, there were more than 2 X 10^4 plaques in genomic library of *E. coli*. The plaque number for the control experiment of different

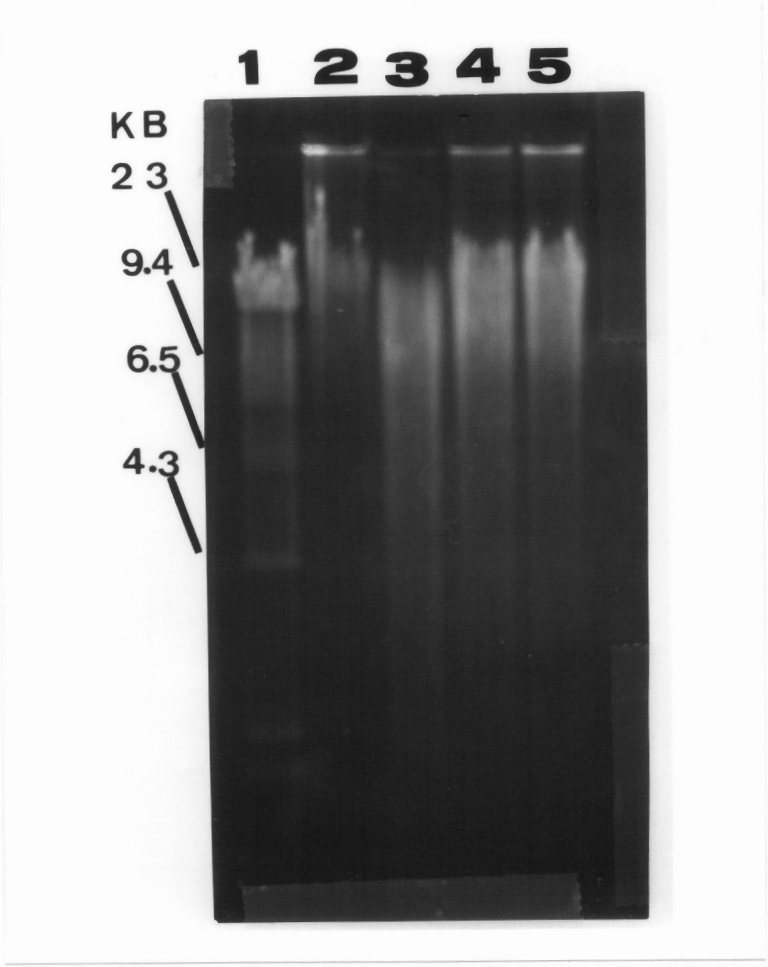

Figure 3. Restriction digestion of *B. fragilis* DNA by different concentration of *Sau*3AI.

DNA was separated by 0.5% agarose gel. Lane 1. HindIII-digested DNA lambda marker. Lane 2-5: DNA digested with different concentration of *Sau*3AI. Lane 2. 0 unit/ug, Lane 3. 0.032 unit/ug, Lane 4. 0.016 unit/ug, Lane 5. 0.008 unit/ug.

dilutions is 0. These results indicates that nearly every plaque contained insert DNA.

B. Screening of Genomic Library

A degenerate synthetic oligonucleotide mixture (17-mer) deduced from the N-terminal sequence of *B. fragilis* FeSOD was used to screen the *B. fragilis* genomic library. Primary screening was performed on the plate containing a high density of plaques. More than 1 x 10^4 plaques were transferred to duplicate nitrocellulose filters. These plaques were hybridized with ^{32}P-labelled oligonucleotide probe and clones exhibiting the strongest autoradiographic signal at identical positions on the duplicate filters were picked up. Since positive clones were chosen from plates with a high density of plaques, it is possible that the plaque was contaminated with other plaques. Therefore positive clones from primary screening were amplified by reinfecting the host cells *E. coli* KW 251 and were titered to 10^2 per plate. Secondary screening was performed as described for primary screening (Figure 4) and twelve positive clones were picked up. Recombinant phage DNAs were isolated as described.

C. Characterization of Positive Clones

Phage DNA isolated from twelve positive clones were digested with restriction endonucleases. The

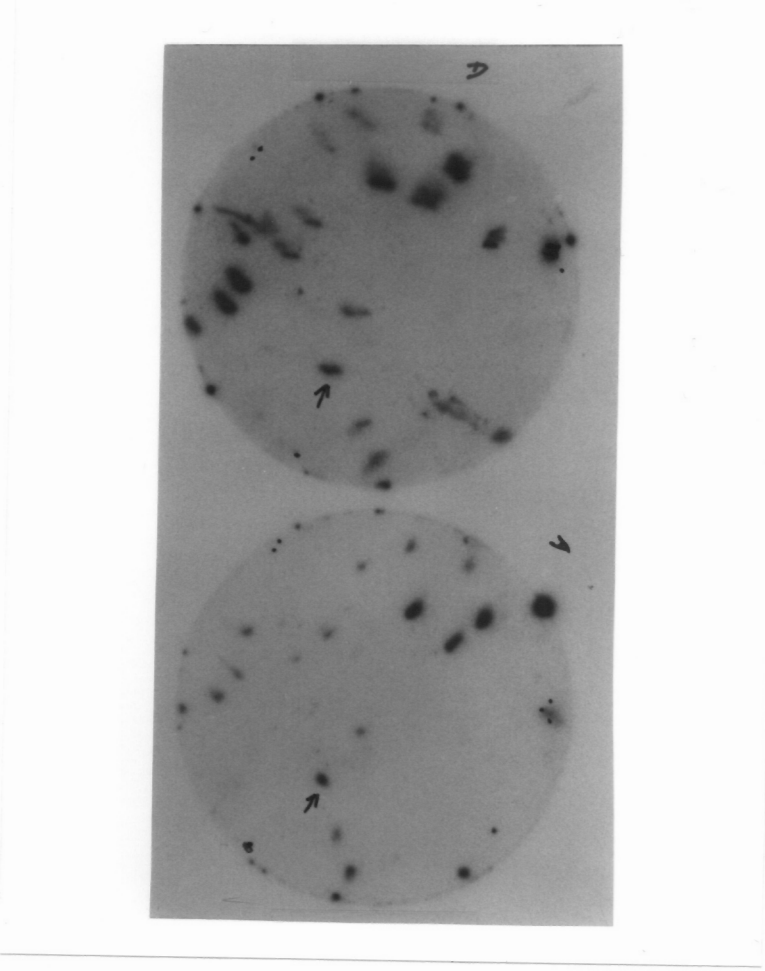

Figure 4. Autoradiography of screening of plaque hybridization by ^{32}P-labeled oligonucleotide probe.

Filters were washed with high stringency at 45^{0}C for 5 minutes after hybridization and then exposed to X-ray film for 48 hours at -70^{0}C. Arrow represents the positive signal which appeared on duplicate filters.

electrophoresis patterns of the endonuclease-treated DNA showed that there were two different DNA populations (#5 and #11) among the twelve clones. Only one DNA clone was different from the others. In order to locate the *B. fragilis* SOD gene in insert DNA fragment (Figure 5), these two DNA clones were subjected to further restriction digestion. After digestion, DNA fragments were electrophoretically separated in 0.5% agarose gel, blotted to nitrocellulose and were probed with ^{32}P-labelled N-terminal oligonucleotide to identify which DNA fragment contains the N-terminal sequence of *B. fragilis* SOD gene. Southern hybridization revealed that both DNA clones hybridized to the oligonucleotide probe but with different efficiency (Figure 6). The DNA clone (#11) that hybridized to the probe with a stronger signal was selected as the one containing *B. fragilis* SOD gene. The probe hybridized with DNA fragments (from #11) digested by *Eco*RI (5.4 kb), *Hind*III (8.3 kb), *Pst*I (9.5 kb), *Sal*I (2.9 kb), and *Eco*RI-*Bam*HI (4.9 kb). Since DNA fragments which hybridized with N-terminal oligonucleotide do not necessarily contain the intact SOD gene, finding the location and orientation of SOD gene is required. Therefore *Hind*III fragment was further digested with restriction endonucleases (Figure 7A). Alignment of DNA fragments that hybridized to the N-terminal oligonucleotide revealed that *Hind*III fragment contained the intact SOD gene and that the N-terminal sequence of the SOD

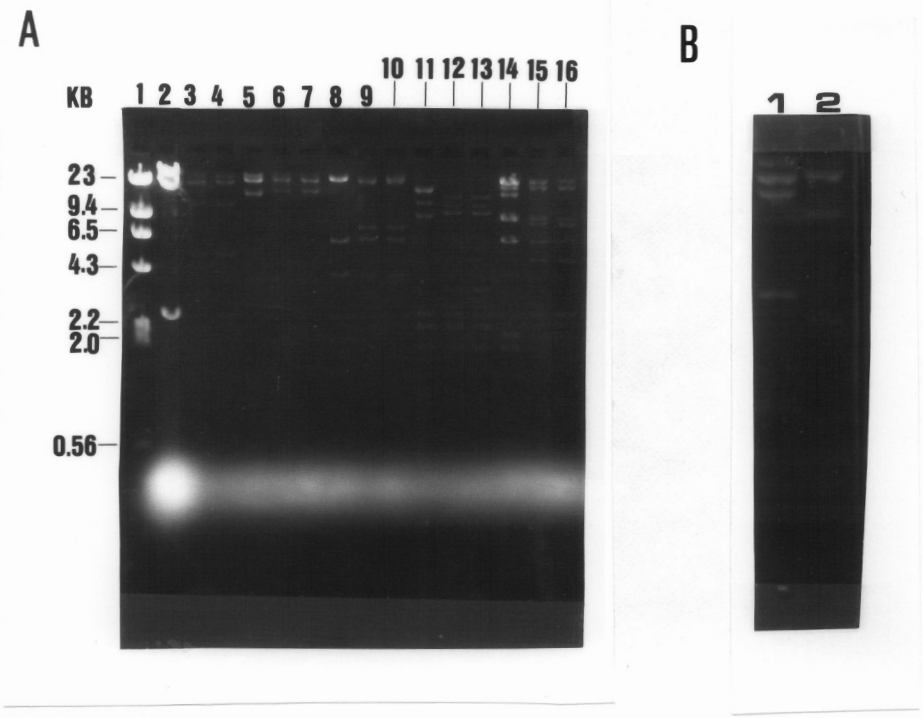

Figure 5. Restriction digestion of the two different recombinant phage DNAs (#5 and #11).

DNAs were separated on 0.5% agarose gel.
(A) Both phage DNAs were subject to digestion by *Eco*RI (Lane 2, 3,and 4), *Bam*HI (Lane 5, 6, and 7), *Hind*III (Lane 8, 9, and 10), *Pst*I (Lane 11, 12, and 13), and *Sal*I (Lane 14, 15, and 16). Phage DNA #5 was placed in Lanes 2, 5, 8, 11, and 14. Phage DNA #11 was loaded in Lanes 3, 4, 6, 7, 9, 10, 12, 13, 15, and 16. Lane 1 contained Lambda *Hind*III digested DNA marker. (B) DNAs were digested by both *Eco*RI and *Bam*HI. Lane 1 (phage DNA #5) and Lane 2 (phage DNA #11).

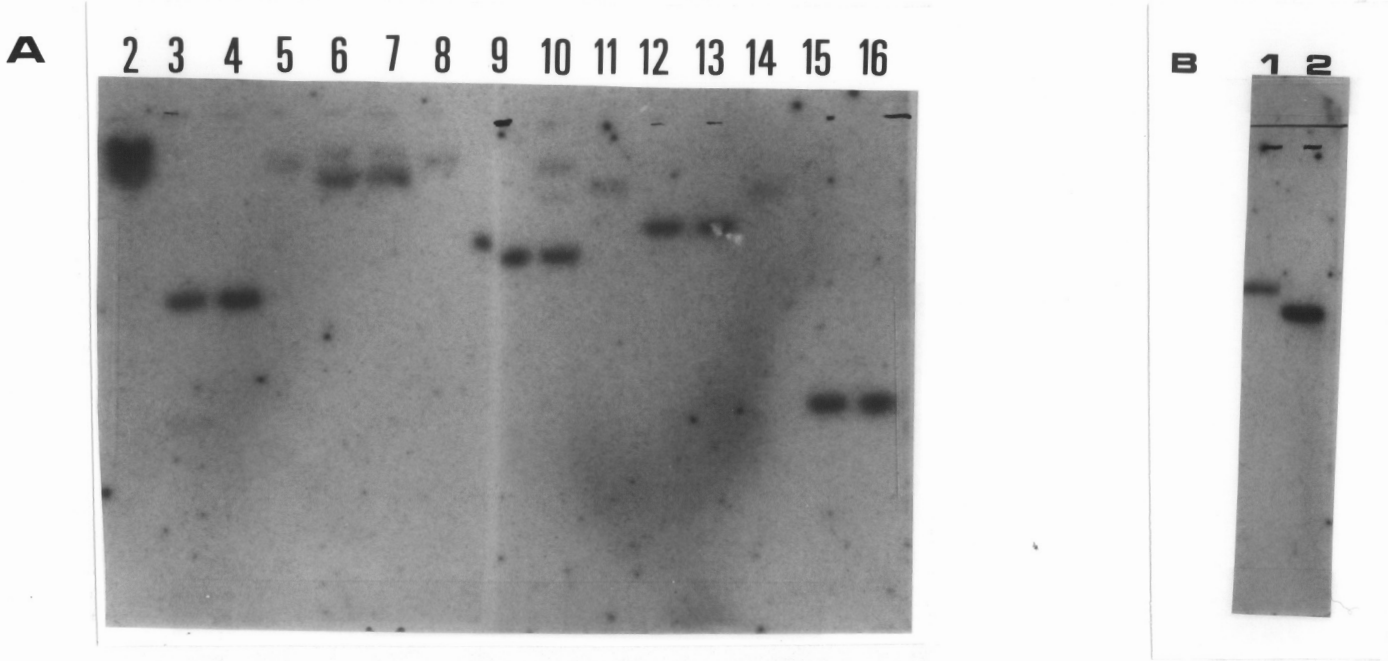

Figure 6. Autoradiography of Southern hybridization.

Phage DNA (#11) was transferred from the gels shown in Figure 5. (A) Probe hybridized to DNA fragments digested by *Eco*RI (lane 3 and 4) (5.4 kb), *Bam*HI (lane 6 and 7) (15 kb), *Hind*III (lane 9 and 10) (8.3 kb), *Pst*I (lane 12 and 13) (9.5 kb), and *Sal*I (lane 15 and 16) (2.9 kb) in Figure 5A. (B) Probe also hybridized to *Eco*RI-*Bam*HI fragment (lane 2) (4.9 kb) in Figure 5B.

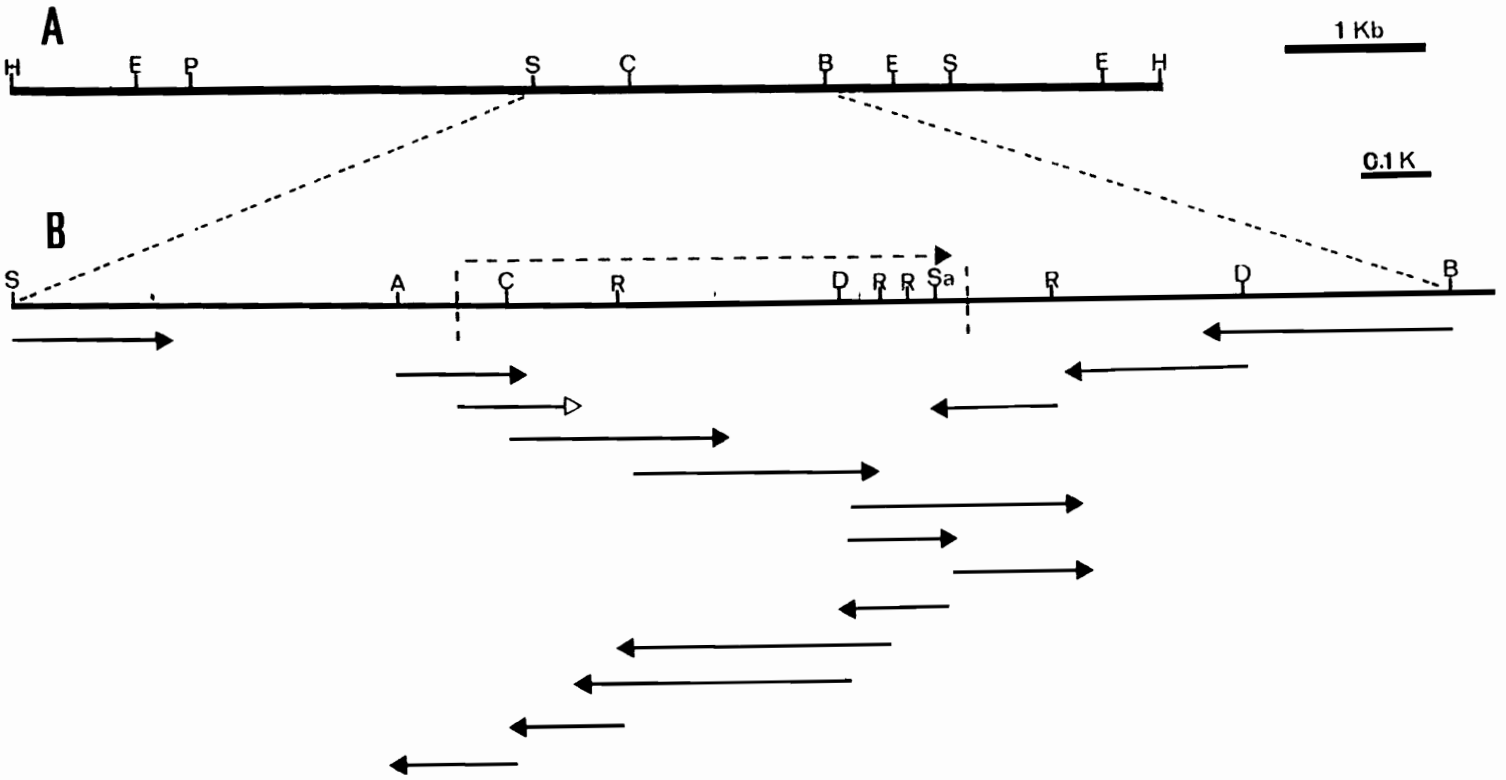

Figure 7. Restriction map of *Hind*III fragment (8.3 kb) and sequencing strategy of *B. fragilis* SOD gene.

(A) Restriction map of *Hind*III fragment digested with six restriction endonucleases. (B) Sequencing was begun from *Sal*I-*Bam*HI fragment (2.1 kb). Solid line with empty arrow indicates that SOD N-terminal oligonucleotide was used as sequencing primer. SOD gene is located between two vertical dashed lines and its orientation is shown by dash line with solid arrow. A: *Apa*I, B: *Bam*HI, C: *Cla*I, D: *Hinc*II, E: *Eco*RI, H: *Hind*III, P: *Pst*I, R: *Rsa*I, S: *Sal*I and Sa: *Sau*3AI.

gene was located in *Sal*I-*Bam*HI fragment (2.1 kb). However these data did not prove that the *Sal*I-*Bam*HI fragment contained the intact SOD gene. Therefore, the N-terminal oligonucleotide was used as a primer to sequence the *Sal*I-*Cla*I fragment and the *Cla*I-*Bam*HI fragment. Sequencing analysis revealed that the oligonucleotide could not hybridize to the *Cla*I-*Bam*HI fragment. The N-terminal sequence of SOD gene must therefore be within *Sal*I-*Cla*I fragment. The sequence showed the orientation of the DNA sequence, therefore confirming that the intact SOD gene was within *Sal*I-*Bam*HI fragment.

3. *B. fragilis* FeSOD gene sequence

DNA sequencing was begun with the *Sal*I-*Bam*HI fragment (2.1 kb). The overall strategy used to sequence the fragment and the complete open reading frame (ORF) of SOD gene is shown at Figure 7B. Translation of the ORF revealed that SOD is composed of 193 amino acid residues (Figure 8). Deduced amino acid sequence has a estimated molecular weight of 21749 through the computional calculation. This value is consistent with the apparent molecular weight (21,000) of SOD subunit on SDS gels. The N-terminal sequence, deduced from the gene, is identical to the chemically determined N-terminal sequence of pure *B. fragilis* FeSOD except for one residue which is ambiguous in the chemically determined sequence. Examination of the nucleotide sequence of regions

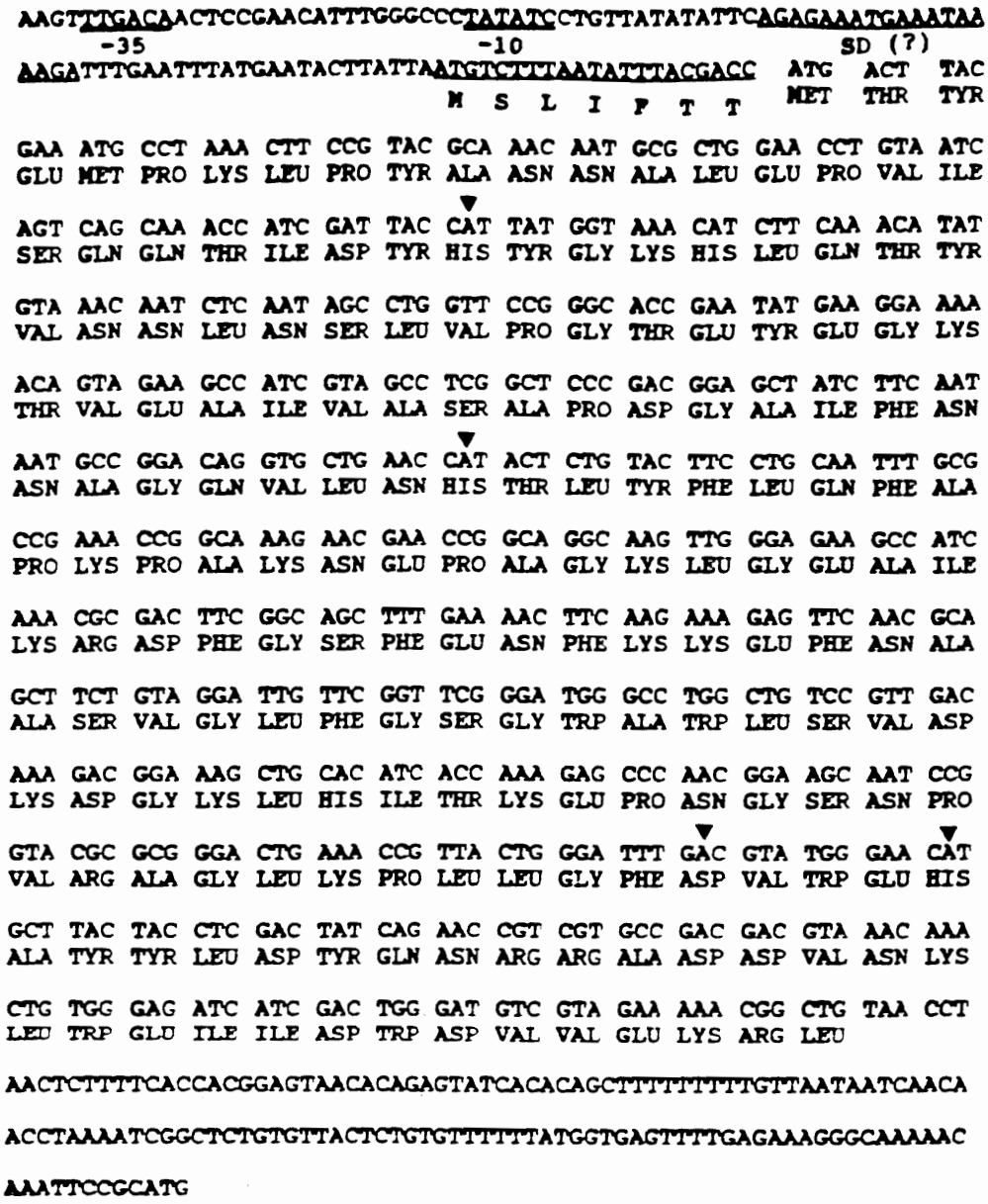

Figure 8. Complete open reading frame (ORF) of *B. fragilis* SOD gene and its flanking region.

The putative promoter region (-10 and -35) is indicated by double underline. The possible Shine-Dalgarno (SD) seqence is shown by underline. Seven amino acid residues beyond translation start codon are also indicated by the abbreviation of amino acid (MSLIFTT). Metal ligands are represented by solid triangles.

flanking the ORF showed that there is an *E. coli*-like promoter region (34) with an interregional spacing of 19 base pair upstream of the 5'-end of the coding strand. The respective hexamer sequence at -35 and -10 region is TTGACA and TATATC, respectively.

Comparison of the deduced amino acid sequence of *B. fragilis* SOD sequence with those of other SODs (1, 6, 14, 54, 55, 59, 61, 65, 72, 75, 77,) reveals that metal ligands present in FeSODs and MnSODs are also conserved in *B. fragilis* SOD (Figure 9). These metal ligands are His^{27}, His^{75}, Asp^{159} and His^{163}. The overall degree of identity among eleven SODs (three cambialistic SODs, four FeSODs, and four MnSODs) is 11.4% with 25 identical residues and the overall similarity among them is 20.5% with 45 similar residues. The paired comparison of identity among eleven SODs shown in Figure 9 (Table 2) indicates that *B. fragilis* SOD shares the highest percentage of identity with *B. gingivalis* SOD, followed by the identity with *E. coli* FeSOD and other FeSODs except *T. pyriformis* FeSOD, and with the lowest percentage of identity with *H. halobium* MnSOD. This result indicates that *B. fragilis* SOD, in term of the overall sequence homology, tends to be more FeSOD-like than MnSOD-like.

4. Expression of SOD gene

*Sal*I-*Bam*HI fragment (2.1 kb) containing the SOD gene and its possible promoter region was subcloned into the

Figure 9. Sequence comparison of *B. fragilis* SOD with other SODs.

B. fragilis SOD was compared with two cambialistic SODs, four FeSODs and four MnSODs. Prefix; CA, FE and MN were used to refer to Cambialistic SOD, FeSOD and MnSOD respectively. BF: *B. fragilis*, BG: *B. gingivalis* (1, 54), BS: *B. stearothermophilus* (59), EC: *E. coli* (14, 77), HH: *H. halobium* (65), PL: *P. leiognathi* (75), PO: *P. ovalis* (61), SM: *S. mutans* (55), TP: *T. pyriformis* (6), TH: *T. thermophilus* (72). "*" indicates the identical residues. "." respresent the similar residues. "▼" indicates metal ligands. "▽" designates conserved residues in the hydrophobic shell of active site. "○" respresents conserved residues involved in the contact interfaces of subunit.

```
CABFSOD     -PAGKLGEAIKRDFGSFENFKKEFNAASVGLFGSGWAWLSVDKDGK-LHI   137
CABGSOD     APKGKLGEAIDKQFGSFEKFKEEFNTAGTTLFGSGWVWLASDANGK-LSI   135
FEECSOD     EPTGKVAEAIAASFGSFADFKAQFTDAAIKNFGSGWTWLVKNSDGK-LAI   135
FEPLSOD     EPTGEVAAAIEKAFGSFAEFKAKFTDSAINNFGSSWTWLVKNANGS-LAI   135
FEPOSOD     QPTGALADAINAAFGSFDKFKEEFTKTSVGTFGSGWAWLVK-ADGS-LAL   136
CASMSOD     KVTAEVAAAINEAFGSFDDFKAAFTAAATTRFGSGWAWLVVDKEGK-LEV   141
MNBSSOD     EPTGELADAINKKFGSFTAFKDEFSKAAAGRFGSGWAWLVVNN-GE-LEI   142
MNTHSOD     EPVGELKKAIDEQFGGFQALKEKLTQAAMGRFGSGWAWLVKDPFGK-LHV   143
MNECSOD     Q--GDLKAAIERDFGSVDNFKAEFEKAAASRFGSGWAWLVLKG-DK-LAV   140
MNHHSOD     EPSGALADRIAADFGSYENWRAEFEVAAGA--ASGWALLVYDPVAKQLRN   136
FETPSOD     DEHSPLTKAIKEKWGSYENFITLFNTRTAAIQGSGWGWLGYDTVSKSLRL   136
                . .   *   .*.       ..  .    .*.*  *      .. *

CABFSOD     TKEPNGSNPV-RAGLK-----PLLGFDVWEHAYYLDYQNRRADDVNKLWE   181
CABGSOD     EKEPNAGNPV-RKGLN-----PLLGFDVWEHAYYLTYQNRRADHLKDLWS   179
FEECSOD     VSTSNAGTPLTTDA-T-----PLLTVDVWEHAYYIDYRNARPGYLEHFWA   179
FEPLSOD     VNTSNAGCPITEEGVT-----PLLTVDLWEHAYYIDYRNLRPSYMDGFWA   180
FEPOSOD     CSTIGAGAPLTSGD-T-----PLLTCDVWEHAYYIDYRNLRPKYVEAFWN   180
CASMSOD     TSTANQDTPISQGLK------PILALDVWEHAYYLNYRNVRPNYIKAFFE   185
MNBSSOD     TSTPNQDSPIMEGKT------PILGLDVWEHAYYLKYQNRRPEYIAAFWN   186
MNTHSOD     LSTPNQDNPVMEGFT------PIVGIDVWEHAYYLKYQNRRADYLQAIWN   187
MNECSOD     VSTANQDSPLMGEAISGASGFPIMGLDVWEHAYYLKFQNRRPDYIKEFWN   190
MNHHSOD     VAVDNHD----EGALWGSH--PILALDVWEHSYYYDYGPDRGSFVDAFFE   180
FETPSOD     FELGNQDMPEWSSIV------PLLTIDVWEHAYYLDYQNLRPKYLTEVWK   180
                    . .              *... *.***.** ..   *   .   .

CABFSOD     IIDWDVVEK------R--L   192
CABGSOD     IVDWDIVES------R--Y   190
FEECSOD     LVNWEFVA----KNLA--A   192
FEPLSOD     LVNWDFVS----KNLA--A   193
FEPOSOD     LVNWAFVAEE-GKTFK--A   196
CASMSOD     VINWNTVARLYAEALT--K   202
MNBSSOD     VVNWDEVAKRYSEAKA--K   203
MNTHSOD     VLNWDVAEEFF---KK--A   201
MNECSOD     VVNWDEAAARFAAKK----   205
MNHHSOD     VIDWDPIAANYDDVVSLFE   199
FETPSOD     IVNWREVEKRY---LQAIE   196
            ...*    .
```

Continued from Figure 9.

Table 2

Degree of amino acid sequence identity between SODs

	CASOD			FeSOD				MnSOD		
	BF	BG	SM	EC	PL	PO	TP	BS	EC	HH
CaSOD										
BF										
BG	62.6									
SM	38.5	42.6								
FeSOD										
EC	50.5	50.5	46.4							
PL	46.9	45.8	43.5	75.5						
PO	50.0	51.1	45.4	69.8	67.4					
TP	38.5	28.4	31.1	25.0	22.3	25.5				
MnSOD										
BS	42.7	47.9	60.4	52.6	52.9	54.6	34.7			
EC	38.0	36.8	49.5	42.7	37.3	41.3	32.1	60.6		
HH	30.2	30.5	33.2	34.9	32.6	34.7	18.4	40.2	26.6	
TH	41.1	43.7	52.7	42.7	39.4	42.9	32.1	62.7	53.7	30.2

1. Values are given as percentage of sequence identities.
2. Abbreviation: CaSOD: cambialistic SOD, BF: *B. fragilis*, BG: *B. gingivalis*, SM: *S. mutans*, EC: *E. coli*, PL: *P. leiognathi*, PO: *P. ovalis*, TP: *T. pyriformis*, BS: *B. stearothermophilus*, HH: *H. halobium*, TH: *T. thermophilus*.

multiple cloning site of the plasmid Bluescript II KS (pBRIIKS). The recombinant plasmid, named pKNSOD (Figure 10), was transformed into DH5αF'. The transformant was then selected on the LB agar plate containing 100 ug/ml ampicillin, 40 ug/ml of each X-Gal and IPTG. The pKNSOD was isolated from the white colony growing on the plate. The pKNSOD was then introduced into *E. coli* SOD double mutant QC1799 and the transformed QC1799 was named QC1799SOD. Expression of SOD in the QC1799SOD was determined by functional complementation analysis. Although the SOD mutant (*sodA sodB*) does not grow aerobically in minimal medium (13), functional SOD conferred the ability of the transformant to do so. Complementation of QC1799 by pKNSOD was observed by the growth in M63 minimal medium (Figure 11). Thus *B. fragilis* SOD gene was able to express its product in QC1799, presumably as active SOD. However, the amount of functional protein expressed depended upon the growth conditions. Significant expression of SOD activity was observed when the culture was grown aerobically in M63 minimal medium. The SOD specific activity ranged between 10 to 16 units/mg when the density of the culture was between 3 and 4 A_{600}. There was no measurable SOD activity found in the culture growing either at M63 medium anaerobically or aerobically in rich medium (LB). Electrophorgrams of the SOD expressed in QC1799 revealed a band of activity that migrated identically with the SOD isolated from *B. fragilis*

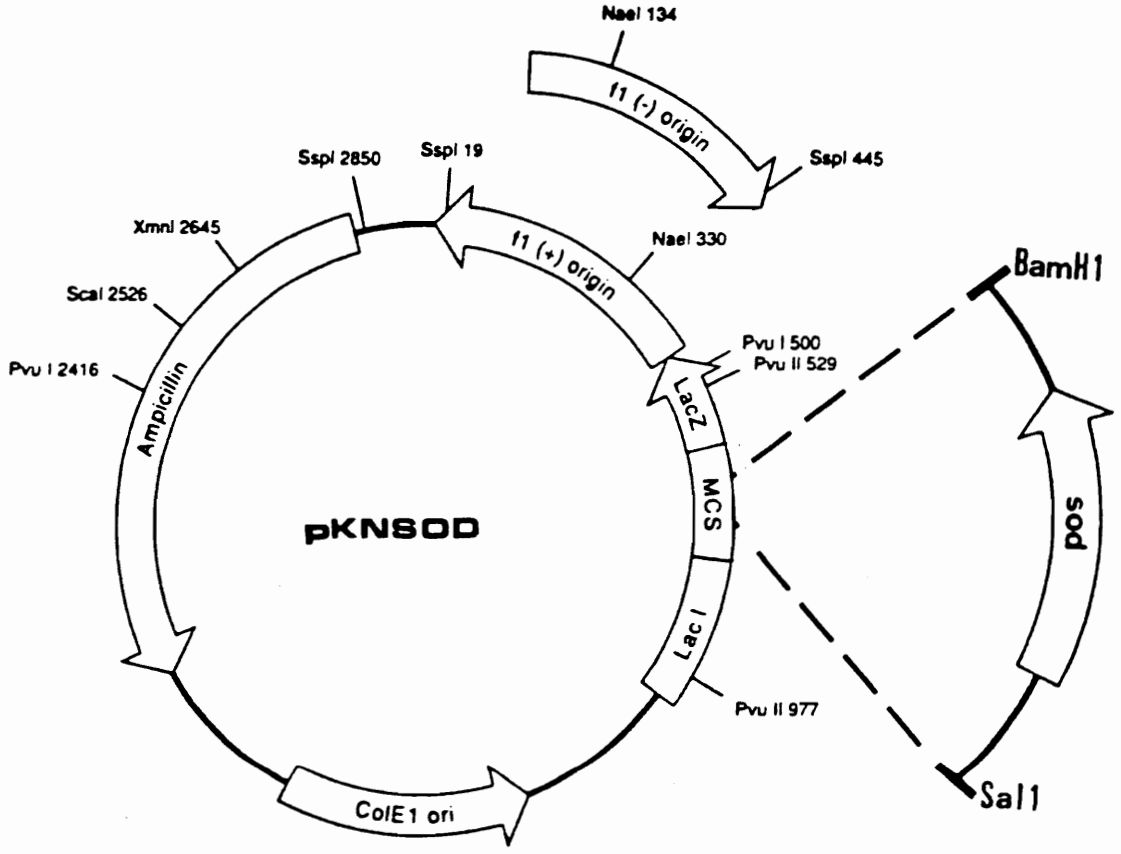

Figure 10. Restriction map of recombinant plasmid, pKNSOD.

pKNSOD was constructed by the insertion of *Sal*I-*Bam*HI fragment (2.1 kb) into the multiple cloning site of the plasmid, bluescript II KS (pBRIIKS).

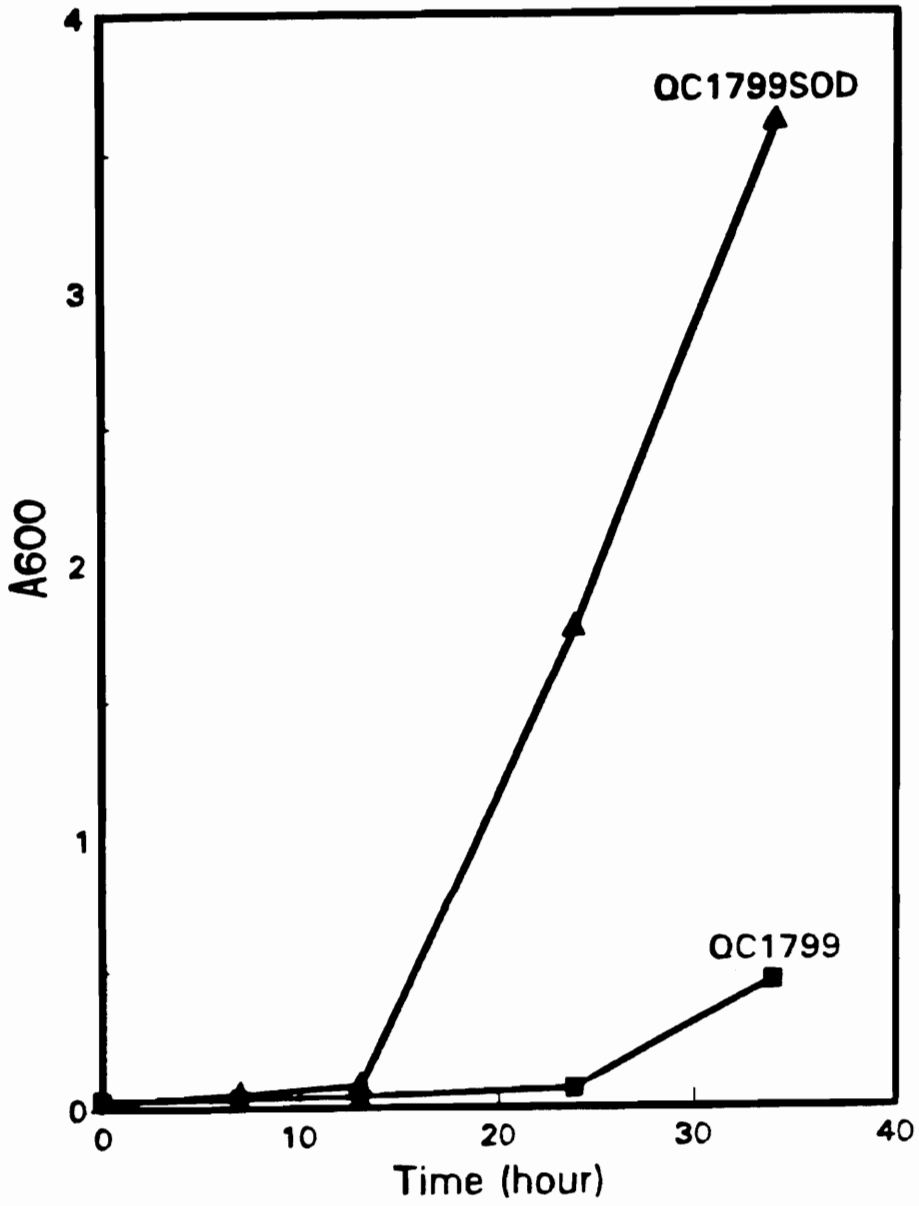

Figure 11. Growth curve of QC1799 and QC1799SOD in aerobic M63 minimal medium.

(Figure 12).

5. Characterization of *B. fragilis* SOD expressed in *E. coli*

QC1799SOD was grown aerobically in M63 minimal medium supplemented with either $FeSO_4$ (100 uM), or $MnCl_2$ (100 uM), or both. The type of metalloprotein thus produced was determined by inhibition with NaN_3. It was reported that 2 mM NaN_3 causes about 90 % of FeSOD activity and 6% of MnSOD activity (28, 29). Therefore the portion of FeSOD and MnSOD in total SOD activity can be calculated by the equation, X + Y =1, where X is the portion of FeSOD and Y is the portion of MnSOD. The percentage inhibition of SOD activity by 2 mM NaN_3 can be equal to 0.9 X + 0.06 Y. By solving the two variables, X and Y, the portion of FeSOD and MnSOD will be obtained. Without the supplement of 100 uM $FeSO_4$ in the medium, the portion of MnSOD in total SOD increased from 59% to 85% when the concentration of $MnCl_2$ was increased to 100 uM. However, with the inclusion of 100 uM $FeSO_4$ in addition to the Mn salt in medium, the portion of MnSOD in total SOD increased from 59% to 72%. Results (Table 3) indicated that the type of SOD (FeSOD or MnSOD) expressed by QC1799SOD seems dependent on the abundance of either Fe or Mn in medium. It also indicated that the presence of Fe in medium could affect the formation of MnSOD and visa verse. *In vitro* reconstitution of the denatured apoprotein also showed that the SOD activity in cell extracts (Table 3) was

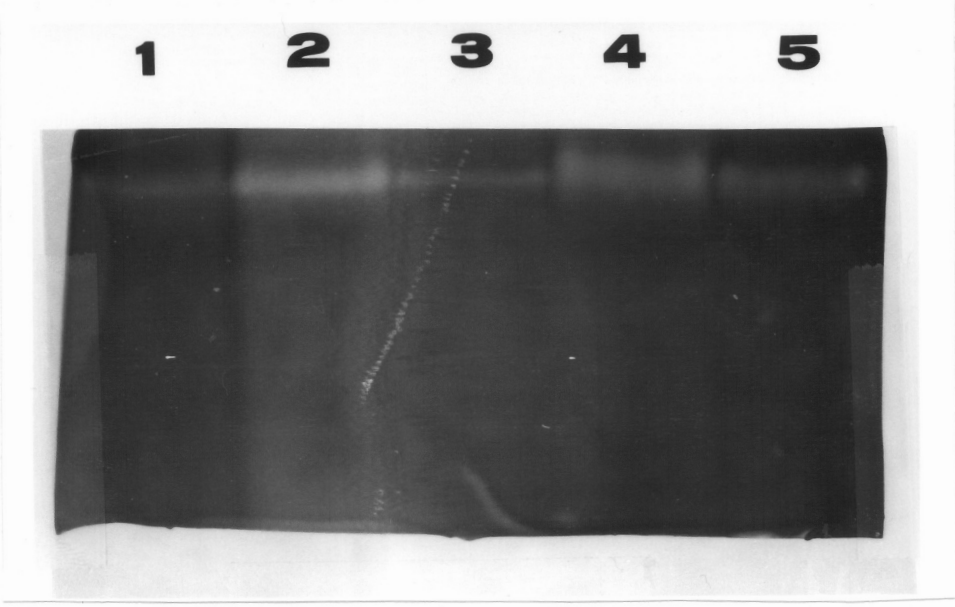

Figure 12. Zymogram of SOD activity.

B. fragilis cell extract were loaded in lane 1 (anaerobic) and lane 2 (aerobic). Lane 3 contained pure *B. fragilis* FeSOD. Lane 4 and 5 contained QC1799SOD cell extract. Samples were separated in 10% native gel.

Table 3

Effect of metal supplement on the type of SOD expressed by QC1799SOD

Metal supplement (uM)[a]	A_{600}	SA[b] (U/mg)	Inhibition[c] (%)	FeSOD[d] (%)	MnSOD[e] (%)
$FeSO_4$	3.6	16.6	40	41	59
$FeSO_4$ + $MnCl_2$	3.6	27	29	28	72
$MnCl_2$	3.3	23.5	18	15	85

a: All metals were supplied at 100 uM in M63 medium.

b: Specific activity.

c: Inhibition of SOD activity by 2 mM NaN_3.

d: The portion of FeSOD in total SOD.

e: The portion of MnSOD in total SOD.

reactivated with either $Fe(NH_4)SO_4$ (Table 4) or $MnCl_2$ (Table 5). This conclusion was based on the observation that the portion of FeSOD or MnSOD after reconstitution depended on the reconstituting metal. After reconstitution with $Fe(NH_4)SO_4$ (1 mM), the percentage of FeSOD portion in total SOD increased from 41% to 83%, from 28% to 100%, and from 15% to 100% in extract of cells grown with Fe, Fe+Mn or Mn alone. The percentage of MnSOD portion increased from 59 to 88, from 72 to 100, and from 85 to 100 in those sample when Mn was the reconstituting metal. The result of the experiments confirm the cambialistic characteristic of *B. fragilis* SOD.

6. Western Analysis

Immunological probing of QC1799SOD cell extract grown aerobically reveals two bands that separated in 15% SDS acrylamide gel (Figure 13) but not in 10% gel. The smaller of the bands corresponds to the molecular weight of *B. fragilis* SOD subunit. The other has a higher molecular weight than *B. fragilis* SOD subunit. It is proposed that the larger protein may be expressed with seven extra amino acid residues before the translation start codon of *B. fragilis* SOD in the *Sal*I-*Bam*HI DNA fragment (Figure 8). *E. coli* QC1799 containing pBRIIKS (named QC1799pBRIIKS) grown anaerobically doesn't contain those two bands as determined by Western analysis. This indicated that protein products

Table 4: Metal reconstitution with $Fe(NH_4)SO_4$ (1 mM)[a]

Metal supplement (uM)	Inhibition[b] (%)	FeSOD[c] (%)	MnSOD[d] (%)
FeSOD[e] (control)	90-93	100	0
$FeSO_4$	72-75	80-83	17-20
$FeSO_4$ + $MnCl_2$	83-91	93-100	0-7
$MnCl_2$	84-89	94-100	0-6

a: Results were from two reconstitution experiments with four inhibition assays in each experiment.

b: Inhibition by 2 mM NaN_3.

c: The portion of FeSOD in total SOD.

d: The portion of MnSOD in total SOD.

e: Pure *B. fragilis* FeSOD.

Table 5: Metal reconstitution with $MnCl_2$ (1 mM)[a]

Metal supplement (uM)	Inhibition[b] (%)	FeSOD[c] (%)	MnSOD[d] (%)
FeSOD[e] (control)	0-4	0	100
$FeSO_4$	16-19	12-16	84-88
$FeSO_4$ + $MnCl_2$	0	0	100
$MnCl_2$	0-21	0-18	82-100

a: Results were from two reconstitution experiments with four inhibition assays in each experiment.

b: Inhibition by 2 mM NaN_3.

c: The portion of FeSOD in total SOD.

d: The portion of MnSOD in total SOD.

e: Pure *B. fragilis* FeSOD.

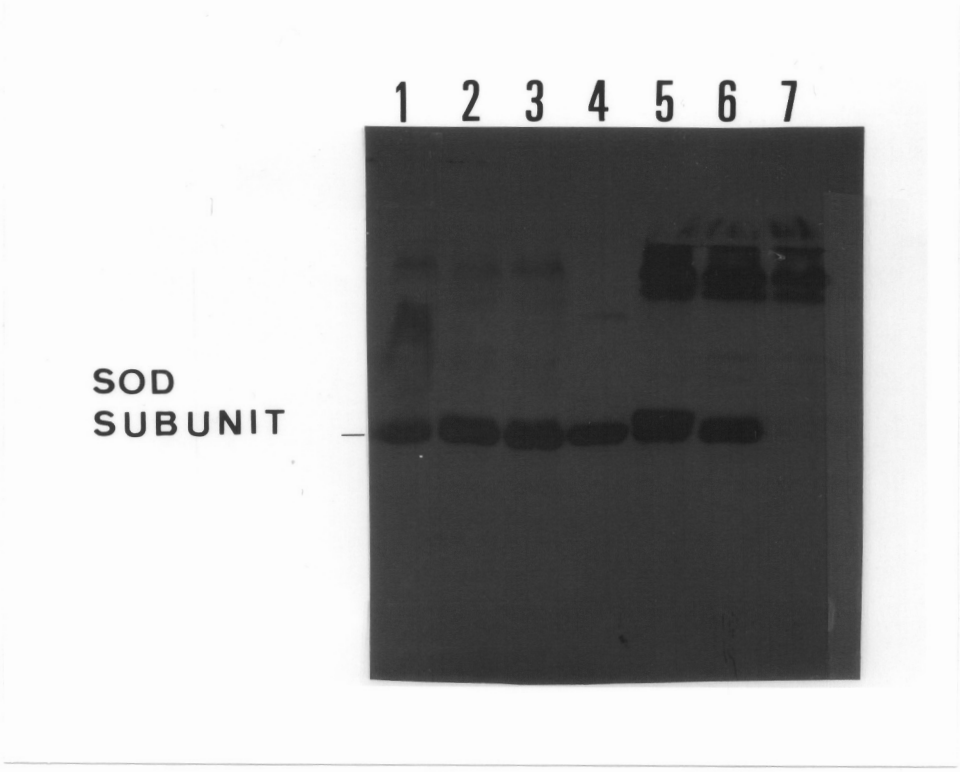

Figure 13. Western analysis of product of QC1799SOD grown in either aerobic or anaerobic M63 minimal medium.

All lanes were loaded with cell extracts except lane 4. Lane 1: BFSOD (anaerobic, 150 ug), Lane 2 and 3 contain aerobic BFSOD with various portion of FeSOD and MnSOD. Lane 2 (50 ug, 90% MnSOD and 10% FeSOD), Lane 3 (80 ug, 50% FeSOD and 50% MnSOD) Lane 4: pure BFSOD (1.5 ug), Lane 5: QC1799SOD (aerobic M63, 50 ug), Lane 6: QC1799SOD (anaerobic M63, 50 ug), Lane 7: QC1799pBRIIKS (anaerobic M63, 50 ug). Samples were separated in 15% SDS gel. BFSOD: *B. fragilis* SOD.

expressed by QC1799SOD are not from pBRIIKS but from *Sal*I-*Bam*HI fragment. In order to determine if *B. fragilis* also expressed the two proteins and if these two proteins contributed to the different types of SOD (FeSOD or MnSOD), cell extract of *B. fragilis* grown either anaerobically or aerobically in PYG medium was included for western analysis in 15% SDS gel. Lane 2 and 3 in Figure 13 contained aerobically induced *B. fragilis* cell extract with various portion of FeSOD and MnSOD. Lane 2 contained 90% MnSOD and lane 3 contain 50% FeSOD and 50% MnSOD in cell extract. Results clearly shows that *B. fragilis* doesn't express the extra band seen in QC1799SOD does. Figure 13 also showed that there was a band with molecular weight of 58000. This band apparently came from the cell extract of QC1799 but not from either pBRIIKS or pKNSOD. It is not clear if this band results from nonspecific binding or from the peptides which may have same epitope as *B. fragilis* SOD. QC1799SOD grown either anaerobically in M63 minimal medium or aerobically in LB medium also showed two bands that were identical to those detected in extract of QC1799SOD grown in aerobic M63 medium (Figure 13 and 14).

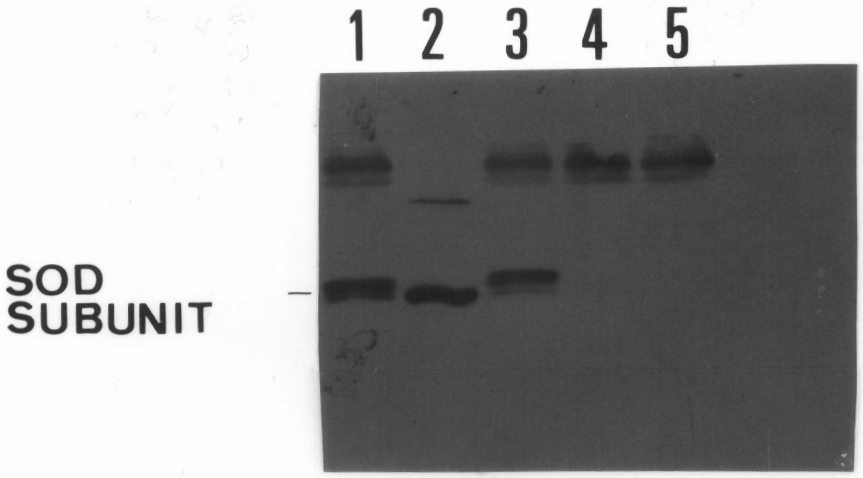

Figure 14. Western analysis of SOD expressed by QC1799SOD in aerobic LB medium.

All lanes were loaded with cell extracts except lane 2. Lane 1: QC1799SOD (aerobic M63, 30 ug), Lane 2: pure BFSOD (1.5 ug), Lane 3: QC1799SOD (aerobic LB, 30 ug), Lane 4: QC1799 (aerobic LB, 30 ug), Lane 5: DH5αF'(aerobic LB, 30 ug). BFSOD: *B. fragilis* SOD.

DISCUSSION

The cloning of *B. fragilis* SOD gene involved using LambdaGEM-11 as cloning vector and fill-in reactions to prevent intramolecular religation, therefore, the cloning efficiency was increased. Although DNA fragments of any size may be cloned into the appropriate arms, it has been reported that only lambdaGEM-11 with insert DNA of 9-23 kb could be efficiently packaged and transfected into bacterial host cells (42). Partial digestion of *B. fragilis* genome by *Sau*3AI therefore provided the restriction fragment size that allowed construction of *B. fragilis* genomic library.

The sequence of *B. fragilis* SOD gene shows that there is one mismatch with the sequence of the synthetic deoxynucleotide probe at position 6, where the gene sequence contains a T instead of C or G. However, the probe was still clearly able to hybridize to clones containing the SOD gene under the conditions of hybridization and of stringent washing. In addition, the probe hybridized with high stringency to another clone (# 5)that has a different restriction map than did the clone (#11) which hybridized with a higher detection signal. This difference suggests that the clones are from different loci on the *B. fragilis* genome. The probe was used as the primer to sequence the two different clones isolated from *B. fragilis* genomic library. The sequence not only identified the location of

B. fragilis SOD gene but also confirmed that the other clone arose as a result of nonspecific binding because the sequence downstream from sequencing primer was not SOD. Therefore, the SOD gene isolated in this study represents the single gene with single copy number in the *B. fragilis* genome.

SOD activity was expressed from the plasmid pKNSOD in *E. coli* QC1799. SOD activity was determined by the xanthine oxidase-cytochrome c inhibition assay and activity was confirmed on a zymogram. It is not clear how the *B. fragilis* SOD gene is expressed in *E. coli* host (QC 1799). There are two possibilities for the SOD gene to be transcribed and translated to protein product. First, *B. fragilis* SOD gene may be expressed through its own promoter that is recognized by RNA polymerase of the host cell. Figure 8 shows that there is an *E. coli*-like promoter region sequence at -35 (TTTGAC) and -10 (TATATC) from the initiation codon. S1 and primer extension studies will be required to determine if this putative promoter region is responsible for the expression of SOD gene in either *E. coli* or *B. fragilis*. Second, SOD product may be translated through the fusion mRNA which is transcribed from the *lac* promoter of pBRIIKS. In either case, 16S rRNA has to recognize a Shine-Dalgarno sequence (SD) (or ribosomal binding site) (69) to translate mRNA into SOD subunit. As is shown in Figure 8, there are no exact *E. coli*-like Shine-

Dalgarno sequence upstream the ORF of *B. fragilis* SOD gene. However, a purine-rich sequence, AGAGAAATGAAATAAAAGA, located approximately 50 bases upstream of the first ATG is a likely candidate for binding of the 16S ribosomal subunit.

Western analysis of the *E. coli* QC1799SOD extract revealed two bands that cross-reacted with the rabbit antiSOD IgG. Both bands are apparently encoded by *Sal*I-*Bam*HI fragment because extracts of QC1799 containing plasmid (pBRIIKS) did not display either of these two bands. Both bands migrated closely in 15% SDS gel. It was possible that there was differential expression of the SOD gene in *B. fragilis* depending on the level of oxygenation. To test this possibility, extracts of anaerobically maintained and aerated *B. fragilis* were separated on SDS-gels containing 15% acrylamide. In either case, only a single protein band in the 20 kilodalton molecular weight range cross-reacted with the antiSOD. The SOD peptide from *B. fragilis* appeared to migrate with the same relative mobility as did the lower molecular weight band from QC1799SOD. SDS-gels containing 15% acrylamide are reportedly able to separate polypeptides differ in molecular weight by 3% in the range from 12,000 to 43,000 (44). The large band observed in the Western analysis of QC1799SOD may contain only a few of amino acid residues in addition to the sequence of the small band. Examination of the deduced sequence (Figure 8) reveals there is potentially an seven extra amino acid residues beyond the

5' end of SOD open reading frame. The seven amino acid residues are N-Met-Ser-Leu-Ile-Phe-Thr-Thr-C. I proposed that these seven residues accounted for the molecular size of the large band. Computer calculation of the isoelectric point showed that the large band has the same pI as the small band. The pI, 5.7, agrees with the experimentally-determined value of 5.3 for the *B. fragilis* SOD. The possibility that these seven amino acids play the role of signal peptide was ruled out because *B. fragilis* SOD is a cytosolic protein. If the large band in fact contains an additional seven amino acid residues as proposed, it is interesting that the 16S rRNA can initiate the translation at area of two AUG codons without losing the sense of distinguishing the AUG codon at the start of a gene and those coding for internal methionines. It is also interesting to point out that if the suspected ribosomal binding site at 50 bases upstream of ORF of SOD gene is responsible for the initiation of translation of *B. fragilis* SOD gene, why is there no large band produced in *B. fragilis*? It is possible that the ribosomal binding site of *B. fragilis* is located within the sequence that codes for extra amino acids and the large protein subunit is an artifact of expression in *E. coli*.

Although QC1799SOD synthesizes active SOD in M63 medium aerobically, there was no measurable SOD activity observed, but detectable signal in Western analysis, in the culture

grown either in LB medium aerobically or in M63 medium anaerobically (Figure 13 and 14). Western analysis revealed that the SOD subunit protein was synthesized. These observations reflect the production of apomonomer of SOD without the active protein. It is not clear if this problem is caused by the limitation of metal cofactors under different growth condition or was due to inappropriate folding of protein.

Martin *et al.* (45) describe SODs that are able to be activated with more than one metal cofactor as "cambialistic SOD". These cambialistic SODs include those isolated from *B. fragilis*, *B. gingivalis*, *P. shermanii* and *S. mutans*. Cambialism of *B. fragilis* SOD had been confirmed previously by *in vivo* and *in vitro* metal substitution into the pure SOD (29 and 28). This work, however, confirms the point of view that *B. fragilis* SOD is the product of a single gene, which is active with binding of either Fe or Mn, by expressing the SOD gene in QC1799. The product of the SOD gene displays two important features of the cambialistic property. First it is reactivated with either Fe or Mn after being subjected to metal reconstitution *in vitro*. Results clearly support this claim (Table 4 and 5). Second, the ratio of FeSOD to MnSOD in the expressed SOD seems dependent on the metal supplements in the medium. This feature is based on the observation that the portion of MnSOD was increased with increasing Mn concentration in the medium (Table 3).

Although there is no evidence indicating that the proportion of FeSOD increases as Fe concentration increase, the results do show that the presence of Fe in the medium could affect the formation of MnSOD and visa verse. This conclusion is based on the comparison of the portion of MnSOD in medium containing 100 uM of $MnCl_2$ to those in medium containing $FeSO_4$ and $MnCl_2$ each at 100 uM. Without $FeSO_4$ in the medium, 85% of the expressed SOD is MnSOD. The figure drops to 72% when $FeSO_4$ was included with $MnCl_2$ in medium.

Comparisons of SOD sequences among FeSODs and MnSODs had shown that there were several invariant amino acid residues distributed throughout the entire sequence. The number of invariant residues could be 39, 33, or 28 depending on SOD source used for comparison (6,16, 60). With the inclusion of three cambialistic SODs for sequence comparison, the number of invariant residues is limited to 25 for 11 SODs. The cambialistic SODs are *B. fragilis* (this work), *B. gingivalis* (1, 54) and *S. mutans* (55). Since the comparison involves three cambialistic SODs, four FeSODs, and four MnSODs, including MnSODs which are from eukaryotes, it is possible that these twenty five invariant amino acids may play a universal role in either binding the metal to apoprotein or shifting the bound metal to the active form of MnSOD or FeSOD.

X-ray crystallographic examination of FeSODs and MnSODs from a few sources combined with protein sequences from a

large number of SODs have remodeled several important structural features of FeSODs and MnSODs. These features include the identification of amino acids serving as metal ligands, those constituting the hydrophobic environment of the active site, and those involving subunit-subunit contact. Metal ligands for MnSODs and FeSODs are apparently identical. This discovery resulted from the the fitting of amino acid sequences to electron-density maps. Metal ligands bind to both Fe and Mn through three histidines and one aspartate. These residues are totally conserved in all FeSODs and MnSODs isolated thus far. According to the sequence alignment (Figure 9) , these metal ligands are also conserved in *B. fragilis* SOD at position 27 (His), 75(His), 159 (Asp) and 163 (His). The residues mentioned in this discussion are based on the position of *B. fragilis* SOD. Residues involved in the stabilization of the dimeric structure are invariant among three FeSODs and six MnSODs (60). This sequence identity suggests that different SODs share very similar contact interfaces between subunits. These residues are nearly completely conserved in *B. fragilis* SOD except at position 32 where leucine replaces histidine. These residues include His 31, Ser 123, Glu 162, His 163 and Tyr 166. In addition, aromatic residues which constitute the hydrophobic shell surrounding the active site are conserved in all FeSODs and MnSODs. The hydrophobic properties of active site may serve to stabilize the metal

at the active site (59). They are almost completely conserved in *B. fragilis* SOD with the exception of two positions (32 and 79). Tryptophan at position 79 is replaced by phenylalanine in *B. fragilis* SOD. although Phe 79 is also a aromatic residues, it is not clear if this phenylalanine may contribute the hydrophobic property to the active site of *B. fragilis* SOD. These conserved residues around the active site include His 31, and the aromatic residues Tyr 35, Trp 125, and Tyr 165.

By examining the aligned sequence of six MnSODs and three FeSODs and the X ray crystallographic data for *B. stearothermophilus* MnSOD, Parker and Blake (60) observed that there are several residues within a sphere of 10 Å from the active site Mn that they suggested to distinguish MnSOD from FeSOD. Among them, corresponding amino acid residues for MnSOD at position 70, 71, 78, 144, and 145 are Gly, Gly, Phe, Gln and Asp while corresponding amino acid residues for FeSODs are Ala, Gln, Tyr, Ala, and Gly (Table 6). Amino acid residues at these positions were considered as primary discriminating candidates because they were found to be totally invariant within each enzyme class. As it can be seen in Figure 9, at least three SODs (*T. pyriformis* FeSOD, *H. halobium* MnSOD, and *B. fragilis* FeSOD) do not totally fit this hypothesis. The summary of comparison of five SODs at these five primary candidates is shown at Table 6. For instance, *T. pyriformis* FeSOD has four out of five

Table 6

Positions where amino acid residues are listed as primary candidates and corresponding residues existed in five SODs

	Residues						
	Consensus SODs		CaSODs			MnSOD	FeSOD
Position	Mn	Fe	B.F.	B.G.	S.M.	H.H.	T.P.
70	Gly	Aln	Gly	Gly	Gly	Cys	Gly
71	Gly	Gln	Gln	Gln	Gly	Gly	Gly
78	Phe	Tyr	Tyr	Tyr	Phe	Phe	Tyr
144	Gln	Ala	Gly	Ala	Gln	His	Gln
145	Asp	Gly	Ser	Gly	Asp	Asp	Asp

Note:

1. Positions are assigned for *B. fragilis* SOD.

2. Abbreviation: CaSODs: cambialistic SODs, B.F.: *B. fragilis*, B.G.: *B.gingvalis*, H.H.: *H. halobium*, T.P.: *T. pyriformis*.

discriminating residues for MnSOD but not for FeSOD. Barra *et al.* (6) suggested that residues of these four positions may not be responsible for conferring the distinguishing properties of the metal binding specificity or that the discriminating rules may hold for either prokaryotic FeSOD or MnSOD, and eukaryotic MnSOD but not for eukaryotic FeSOD. *H. Halobium,* an archaebacterium which evolved independently from eubacteria and eukaryote, however, has two residues which are not listed in the so-called primary candidates. These residues are at position 70(Cys), and 144(His). Three cambialistic SODs, *B. gingivalis* SOD, *B. fragilis* SOD, and *S. mutans* SOD which are active with either Mn or Fe metal cofactor have different residues at these positions. *B. gingivalis* SOD fits into this discriminating rule by having one residue (Gly 70) for MnSOD and four residues for FeSOD. *S. mutans* SOD also fit into this rule with five residues for MnSOD. *B. fragilis* SOD, however, has three out of five residues which fit the rule with two residues (Gly 144 and Ser 145) not listed in the primary candidates. Based on the comparison in Table 6, it is possible that four residues at position 70, 71, 144, and 145, suggested by Parker and Blake as primary candidates are in fact secondary candidates, but only residue at position 78, discriminates FeSOD from MnSOD.

The primary sequences of three cambialistic SODs shown in Figure 9 support the discriminating role of residue at position 78. For instance, both *B. gingvalis* and *B.*

fragilis SODs with the type of FeSOD sequence have the residue Tyr at position 78 while *S. mutans* SOD, having a Phe at position 78, is basically the type of MnSOD. However, there is a discrepancy to explain the cambialism of *S. mutans* SOD which has five primary residues for MnSOD suggested by Parker and Blake. Further study is required to understand the relationship between metal selectivity and the features in the amino acid sequence.

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