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Nucleotide Sequence of *Streptococcus mutans* Superoxide Dismutase Gene and Isolation of Insertion Mutants

KOJI NAKAYAMA

Department of Microbiology, Faculty of Dentistry, Kyushu University, Fukuoka 812, Japan

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A gene (*sod*) encoding superoxide dismutase (SOD) was cloned from *Streptococcus mutans* in *Escherichia coli*, and its nucleotide sequence was determined. The presumptive amino acid sequence of its product revealed that the SOD is basically of Mn type. Insertional inactivation of the *sod* gene resulted in the loss of SOD activity in crude extracts, indicating that the gene represents the only functional gene for SOD in *S. mutans*. Moreover, Southern blot analysis indicated that the *S. mutans* chromosome had no additional gene which was hybridizable with an oligonucleotide probe specific for an SOD motif. The SOD-deficient mutants were able to grow aerobically, albeit more slowly than the parent strains.

Streptococcus mutans, an oral bacterium, plays an important role in the development of dental caries. It grows both aerobically and anaerobically, but its energy metabolism is of anaerobic type. Thus, the ATP synthesis of *S. mutans* depends on glycolysis, and lactic acid is mainly produced as an end product. The lactic acid bacteria such as *S. mutans* lack not only cytochromes but also catalase, suggesting the immaturity of defense against oxidative stress in addition to the lack of aerobic respiration.

S. mutans possesses superoxide dismutase (SOD), one of the major enzymes in the protection against oxidative stress, which dismutates superoxide (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). SOD can be classified in three groups in terms of its metal cofactor: copper-zinc (CuZn-SOD), manganese (Mn-SOD), and iron (Fe-SOD).

CuZn-SOD is found exclusively in peroxisomes of eukaryotes (16). In contrast, Mn-SOD is present in prokaryotes and in mitochondria of eukaryotes, while Fe-SOD is present in prokaryotes and in chloroplasts of eukaryotes. Mn-SOD and Fe-SOD resemble each other with respect to their amino acid sequences, suggesting their common ancestry (11).

Despite the similarity of their primary structures, Mn-SOD is usually activated only by an Mn ion and Fe-SOD is activated by an Fe ion. Several amino acid residues of Mn- and Fe-SODs have been found to be critical residues that distinguish between an Mn-SOD and an Fe-SOD (8, 27). It has been reported that several bacteria possess an SOD which is activated either by Mn or Fe ion, and Hassan (11) pointed out the possibility that this class of SOD may represent an evolutionary intermediate between Fe- and Mn-SODs. Previously, I showed that one of those bacteria, *Porphyromonas* (*Bacteroides*) *gingivalis*, possesses an SOD that is basically of the Fe-SOD type in overall primary structure but intermediate between Fe-SOD and Mn-SOD in amino acid sequence in the vicinity of the second ligand (23), the known site of major difference between the two classes (8, 27). The SOD of *S. sobrinus*, which is genetically and physiologically related to *S. mutans*, was also reported to be enzymatically of this type (18).

In this work, I cloned and sequenced the SOD-encoding gene (*sod*) of *S. mutans* GS-5 to show that the amino acid sequence of *S. mutans* SOD has more features of an Mn-SOD than of an Fe-SOD and of the *P. gingivalis* SOD. Construction of *sod* mutants of *S. mutans* demonstrated that the *sod* gene cloned in this study is the only functional gene for SOD in *S. mutans*. This finding was corroborated by Southern blot analysis with an oligonucleotide probe encoding a motif characteristic of both Fe- and Mn-SODs. Furthermore, I found that SOD-deficient mutants can grow aerobically, albeit more slowly than their parent strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used were *S. mutans* GS-5, *S. mutans* NCTC10449, *S. mutans* MT8148, *S. sobrinus* OMZ176, *S. cricetus* FIL, *Escherichia coli* AB2497 (5), *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.), and *E. coli* QC774 (9). Plasmids pUC18 (36) and pTS19E (3) were used as a plasmid vector for gene cloning and a source of an erythromycin resistance (Em^r) determinant, respectively. Plasmids pKD239, pKD240, and pKD251, derivatives of pUC18, were constructed in this study and are described in Results.

Media and conditions for cell growth. Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) was used for the streptococcal strains. For *E. coli*, L broth (24) and E medium (35) were used as rich medium and minimal medium, respectively. Media were solidified with 1.5% agar for plates. For aerobic growth, cells were cultured in a 200-ml flask containing 20 ml of an appropriate medium with vigorous shaking (100 cycles per min). An anaerobic glove box (Hirasawa Works, Tokyo, Japan) was used under an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide for anaerobic incubation.

Enzymes and oligonucleotides. Restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Hin*cII, *Sal*I, and *Sau*3AI), T4 polynucleotide kinase, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA ligase were purchased from Takara (Kyoto, Japan). Calf thymus alkaline phosphatase was obtained from Boehringer (Mannheim, Germany). These enzymes were used as recommended by the manufacturers. A mixture of synthetic oligonucleotides, 5′-TGGGA(A/G)CA(T/C)GC(A/G/T/C)TA(T/C)TA(T/C)-3′, was purchased from Takara.

Construction of a chromosomal gene library from *S. mutans*. Chromosomal DNA was purified from *S. mutans* GS-5 according to Okahashi et al. (26) and partially digested with *Sau*3AI. The *Sau*3AI fragments were fractionated by agarose gel electrophoresis, and DNA of 4 to 6 kbp purified with GeneClean (Bio 101, La Jolla, Calif.) was ligated with *Bam*HI-linearized and calf thymus alkaline phosphatase-

treated pUC18 DNA. The ligated mixture was used for transformation of *E. coli* DH5α.

Southern hybridization. Southern blotting was performed by using a nylon membrane (NY13N; Schleicher & Schuell, Inc., Keene, N.H.) essentially according to Southern (31). Blots were probed with DNA fragments which were labeled with digoxigenin-dUTP (Boehringer). Hybridization was done by using the Boehringer Nonradioactive DNA Labeling and Detection Kit.

For DNA-DNA hybridization with an oligonucleotide probe, Southern blotting was done by the low-salt electroblotting method (15) by using a DNA transfer apparatus (Nihon Eido, Tokyo, Japan) and a positively charged nylon membrane (GeneScreen Plus; Dupont, NEN Research Products, Boston, Mass.). DNA on the blot was then denatured with an alkaline buffer (0.5 N NaOH, 1.5 M NaCl) for 2 min, soaked in a neutralizing buffer (0.5 M Tris HCl [pH 7.5], 1.5 M NaCl) for 2 min, and baked at 75°C for 30 min. The filter was prehybridized in a hybridization buffer (1% sodium dodecyl sulfate [SDS], 1.5 M NaCl, 10% dextran sulfate) containing 100 μg of salmon sperm DNA (Pharmacia, Uppsala, Sweden) per ml at 65°C for 4 h. The oligonucleotide DNA was 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (222 TBq/mmol; Amersham Japan, Tokyo, Japan). The processed filter was then hybridized with the ^{32}P-labeled oligonucleotide in the hybridization buffer at 46°C for 15 h. The membrane was subsequently washed twice for 5 min at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), twice for 15 min at 46°C with 2× SSC containing 1% SDS, and twice for 15 min at room temperature with 0.1× SSC.

DNA sequencing. Nucleotide sequences were determined by the chain termination method (29) as modified by Hattori and Sakaki (12), using a DNA sequencing kit with Sequenase (United States Biochemical, Cleveland, Ohio) and [α-^{35}S]dCTP (>37 TBq/mmol; Amersham Japan).

Genetic transformation of bacterial strains. Transformation of *E. coli* DH5α and QC774 was carried out according to the procedure of Kushner (17).

Transformation-competent cells of *S. mutans* were prepared by the method described by Hudson and Curtiss (14) except that 1-ml samples of competent cells were mixed with 5 μg of linear DNA in Tris-EDTA buffer.

SOD assays. Cells were harvested, resuspended in buffered salts medium base (25), and agitated for 5 min with 0.1-mm glass beads in a homogenizer (Mini-Beadbeater; Biospec Products, Bartlesville, Okla.). After dialysis against 50 mM phosphate buffer (pH 7.8), SOD activity was measured by a method based on inhibition of O_2^--dependent reduction of cytochrome *c* (21). SOD activity on nondenaturing 8% polyacrylamide slab gels was detected by the method of Beauchamp and Fridovich (6).

Nucleotide sequence accession number. Nucleotide and amino acid sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence data bases under accession number D01037.

RESULTS

Molecular cloning of an SOD-encoding gene of *S. mutans*. Carlioz and Touati (9) constructed a *sodA sodB* double mutant of *E. coli* that shows no SOD activity and is unable to grow aerobically on a minimal medium. Molecular cloning of SOD-encoding genes from several organisms has been successfully done by using genetic complementation of the defect of the mutant (7, 23). I used this genetic method for cloning of the *sod* gene(s) in *S. mutans*.

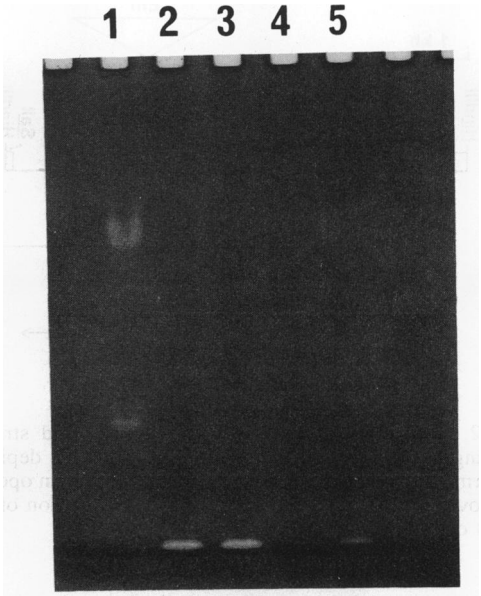

FIG. 1. SOD activity in extracts of *E. coli* transformed with the recombinant plasmids. Samples containing 30 μg of protein were separated on a nondenaturing 8% polyacrylamide gel and stained for SOD activity. Lanes: 1, GC4468 (*sodA*$^+$ *sodB*$^+$)/pUC18; 2, QC774 (*sodA sodB*)/pKD239; 3, QC774/pKD240; 4, QC774/pUC18; 5, SOD activity in an extract (100 μg of protein) of aerobic cells of *S. mutans* GS-5.

A chromosomal gene library from *S. mutans* GS-5 was constructed by using pUC18 and DH5α as a vector plasmid and a host strain, respectively, and about 6,000 recombinant plasmids possessing chromosomal DNA fragments of *S. mutans* were obtained. A mixture of these plasmids was then introduced into cells of the *sodA sodB* mutant (QC774) of *E. coli* by transformation. The cells were spread on minimal medium plates containing 50 μg of ampicillin per ml and incubated at 37°C for 2 days. Two independent clones were obtained. Lysates of the *sodA sodB* cells harboring either of the recombinant plasmids (pKD239 and pKD240) were electrophoresed on a nondenaturing gel and stained for SOD activity (Fig. 1). The cells of the two recombinant clones showed bands of SOD activity which were located at the same position as that of *S. mutans* GS-5. This SOD activity was obviously derived from the cloned chromosomal fragments because no SOD activity was found in the lane for the host strain QC774 harboring pUC18.

Restriction and hybridization analyses. Restriction analysis of these two plasmids (pKD239 and pKD240) revealed that they had the same cloned fragment. Plasmid pKD239 (Fig. 2) was chosen for further characterization. Southern hybridization with the cloned fragment (3.4 kbp) of pKD239 as a probe suggested that DNA rearrangement had not taken place in the course of the gene cloning. It also indicated that the chromosomes of *S. mutans* NCTC10449 and MT8148 had the same DNA region hybridizable to the fragment as that of *S. mutans* GS-5, whereas those of *S. sobrinus* OMZ176 and *S. cricetus* FIL did not (Fig. 3).

Location and DNA sequencing of the *S. mutans sod* gene. To determine the location of the *sod* gene in pKD239 plasmid DNA, the plasmid was partially digested with *Hin*dIII and

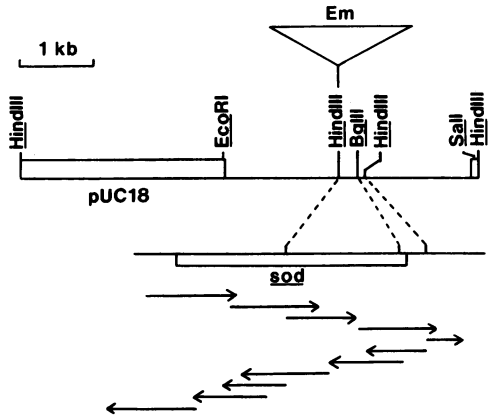

FIG. 2. Restriction map of plasmid pKD239 and strategy for sequencing the *S. mutans sod* gene. The plasmid is depicted in a linear form. The vector plasmid pUC18 is shown as an open box. A triangle over the plasmid indicates the site of insertion of the Em^r fragment of pKD251.

ligated to a 1.8-kbp DNA fragment containing the Em^r gene of pTS19E after their ends were filled with Klenow fragment of *E. coli* DNA polymerase I. A resulting plasmid (pKD251) which contained the Em^r fragment at a *Hin*dIII site of pKD239 did not show SOD activity, indicating that the *Hin*dIII site is located within or near the *sod* gene (Fig. 2). DNA sequencing of the region revealed an open reading frame large enough to encode SOD (Fig. 4).

The amino acid sequence for the presumptive product of the open reading frame had several features of Fe- and Mn-SODs. First, it was composed of 203 amino acid residues, which was in good agreement with the fact that the known Fe- and Mn-SODs consist of about 200 residues. Second, the amino acid residues supposedly serving as metal ligands (His-27, His-81, Asp-163, and His-167 of the *S. mutans* SOD) were found at typical positions. Third, it also

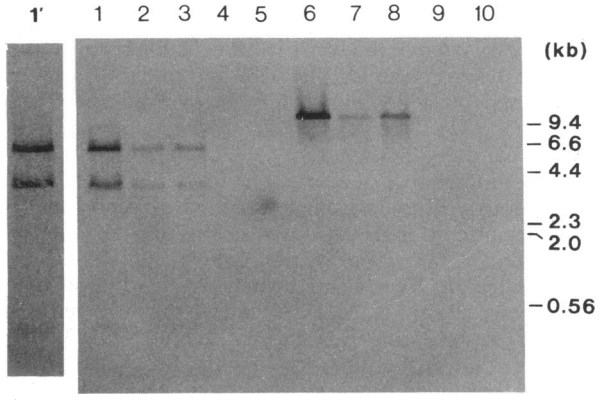

FIG. 3. Southern blot analysis of several streptococci by using an *S. mutans* chromosomal fragment containing the *sod* gene. Chromosomal DNAs of *S. mutans* GS-5 (lanes 1 and 6), *S. mutans* NCTC10449 (lanes 2 and 7), *S. mutans* MT8148 (lanes 3 and 8), *S. sobrinus* OMZ176 (lanes 4 and 9), and *S. cricetus* FIL (lanes 5 and 10) were digested with *Hin*dIII (lanes 1 to 5) and *Eco*RI (lanes 6 to 10) and fractionated by electrophoresis through a 0.8% agarose gel. The Southern blot was probed with the digoxigenin-labeled *Eco*RI-*Sal*I (3.4-kbp) fragment of pKD239. Lane 1' shows an overexposure of lane 1.

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        10         20         30         40         50         60
GGGATGATTTCTGTCAAAGCAAGAATGTATATTTAGAATTATTTTACTAAGACCTCTTTG

        70         80         90        100        110        120
CATGCCGTGTAAAATAAGTAAGCAGAATAGTTGTAAGCTTTGTTTTTTTACGATATGATG
                                  -35                    -10
       130        140        150        160        170        180
AAGATGTATCAAAAAAAGAAAAGAGGAAATGATATGGCTATTCTTTTACCAGATCTTCCA
                        SD        MetAlaIleLeuLeuProAspLeuPro
                                                   5
       190        200        210        220        230        240
TATGCTTATGACGCACTTGAACCATATATTGATGCTGAAACGATGACCCTTCATCATGAT
TyrAlaTyrAspAlaLeuGluProTyrIleAspAlaGluThrMetThrLeuHisHisAsp
10                 15                 20                 25
       250        260        270        280        290        300
AAGCATCATGCAACTTATGTCGCAAACGCTAATGCGGCTCTTGAAAAACATCCAGAAATC
LysHisHisAlaThrTyrValAlaAsnAlaAsnAlaAlaLeuGluLysHisProGluIle
30                 35                 40                 45
       310        320        330        340        350        360
GGAGAAAATTTGGAAGTGCTTTTGGCTGATGTGGAACAAATTCCAGCGGATATTCGTCAG
GlyGluAsnLeuGluValLeuLeuAlaAspValGluGlnIleProAlaAspIleArgGln
50                 55                 60                 65
       370        380        390        400        410        420
TCTTTGATTAATAATGGTGGCGGTCATCTTAATCACGCTCTTTTCTGGGAACTCTTGTCA
SerLeuIleAsnAsnGlyGlyGlyHisLeuAsnHisAlaLeuPheTrpGluLeuLeuSer
70                 75                 80                 85
       430        440        450        460        470        480
CCAGAAAAGACAAAAGTTACTGCGGAAGTAGCCGCAGCAATCAATGAAGCTTTTGGCTCA
ProGluLysThrLysValThrAlaGluValAlaAlaAlaIleAsnGluAlaPheGlySer
90                 95                 100                105
       490        500        510        520        530        540
TTTGATGATTTTAAAGCAGCTTTTACAGCTGCCGCAACGACGCGTTTTGGCTCAGGTTGG
PheAspAspPheLysAlaAlaPheThrAlaAlaAlaThrThrArgPheGlySerGlyTrp
110                115                120                125
       550        560        570        580        590        600
GCTTGGTTAGTAGTTGATAAAGAAGGAAAACTTGAAGTGACCTCAACAGCTAATCAAGAT
AlaTrpLeuValValAspLysGluGlyLysLeuGluValThrSerThrAlaAsnGlnAsp
130                135                140                145
       610        620        630        640        650        660
ACTCCAATTTCACAGGGCTTGAAACCAATTTTAGCACTTGATGTCTGGGAACACGCTTAT
ThrProIleSerGlnGlyLeuLysProIleLeuAlaLeuAspValTrpGluHisAlaTyr
150                155                160                165
       670        680        690        700        710        720
TATCTCAATTATCGTAATGTTCGTCCAAACTATATCAAAGCCTTCTTTGAAGTTATCAAT
TyrLeuAsnTyrArgAsnValArgProAsnTyrIleLysAlaPheGluValIleAsn
170                175                180                185
       730        740        750        760        770        780
TGGAACACTGTTGCTCGTCTTTATGCCGAAGCTCTCACTAAATAAACCTTAAAACCTGTA
TrpAsnThrValAlaArgLeuTyrAlaGluAlaLeuThrLys
190                195                200
       790        800        810        820        830        840
TTTACAGAAACCTTTTTGGTTATGAGATAAGTGCTACTTAGTAAAAGCAACCGTAAGTCG

       850        860        870        880        890        900
CTATACGCTATAAAGGAACAATTTCCGCCGTAGTGCAAAAAATACTGACCATCAGAGGCA

       910        920        930        940        950
AAATTTGAGAAGATTTTCGAGACCTTCAGTTTGACGTTTAAAAGGGAACTGAGG
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FIG. 4. Nucleotide sequence of the *sod* gene and deduced amino acid sequence. A probable Shine-Dalgarno (SD) sequence and a putative promoter (−35 and −10 regions) are indicated by solid lines under the nucleotide sequence. The last digits of the numerals over the nucleotide sequence are aligned with the corresponding nucleotide. Amino acids of the putative gene product are numbered and indicated below the amino acid sequence.

contained several other highly conserved residues of Fe- and Mn-SODs at suitable positions. Moreover, its amino-terminal 22 amino acid residues exclusive of the first methionine were identical to those of the SOD purified from *S. sobrinus* OMZ176 except that Leu-4 of the *S. mutans* SOD was replaced by Thr in the *S. sobrinus* SOD (18).

Several differences are known to exist between amino acid sequences of Fe-SODs and those of Mn-SODs, especially in the vicinity of the second-ligand residue. Comparison of amino acid sequences of the *S. mutans* SOD with those of

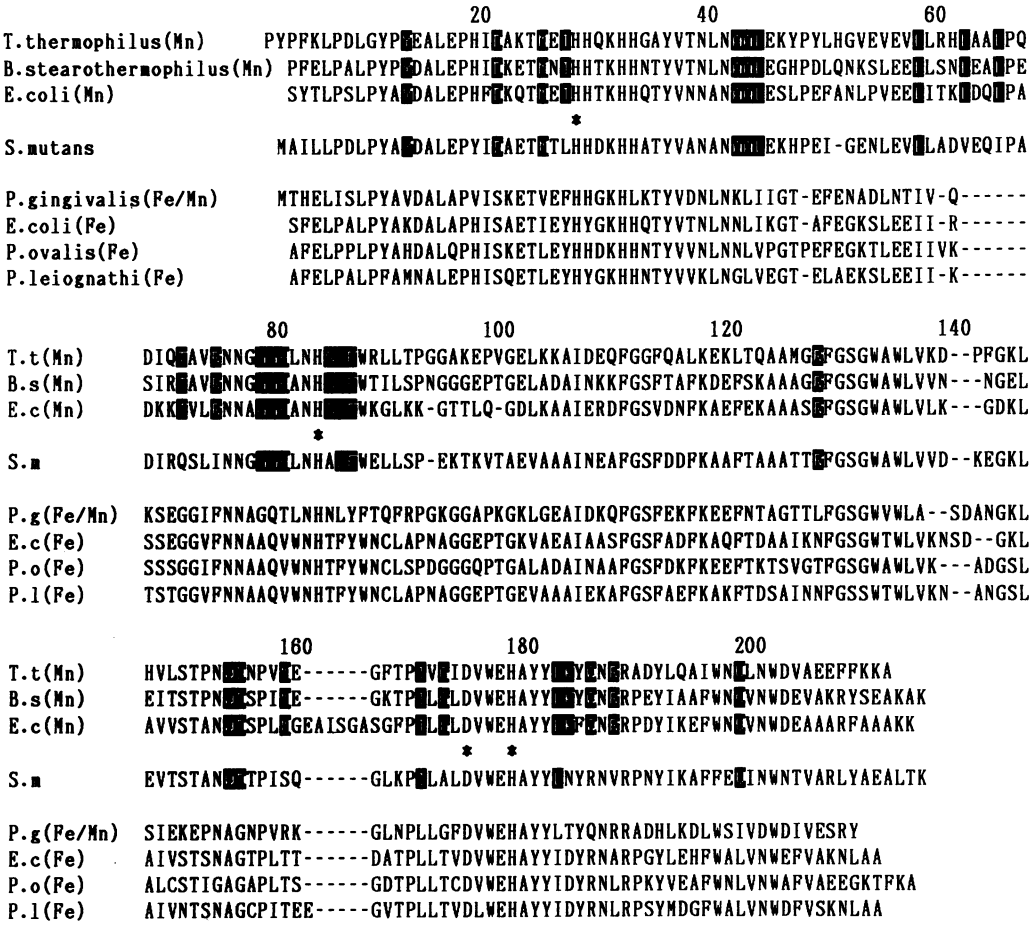

FIG. 5. Amino acid sequence comparison of *S. mutans* SOD with SODs from other eubacteria. Amino acid sequences of SODs from *Thermus thermophilus*, *Bacillus stearothermophilus*, *E. coli*, *Pseudomonas ovalis*, and *Photobacterium leiognathi* are from Parker and Blake (27), and that of *P. gingivalis* is from Nakayama (23). Characteristic residues of Mn-SODs are marked by white letters. Asterisks indicate the metal ligand residues.

Fe- and Mn-SODs showed that the *S. mutans* SOD had many more characteristics of Mn-SOD than of Fe-SOD (Fig. 5).

Construction of *sod* mutants of *S. mutans* by insertional inactivation of the *sod* gene. To determine whether the *sod* gene cloned in this study is the only functional gene encoding SOD, *sod* mutants were constructed from *S. mutans* GS-5, NCTC10449, and MT8148 by insertional inactivation of the gene. The three strains were transformed to Em^r with the 5.2-kbp *Eco*RI-*Sal*I fragment of pKD251 containing the Em^r DNA fragment within the *sod* gene. Each strain gave many Em^r transformants, and representative transformants of GS-5, NCTC10449, and MT8148 were designated KDSM1, KDSM2, and KDSM3, respectively.

The locations of the introduced DNA fragment on the chromosomes of KDSM1, KDSM2, and KDSM3 were determined by Southern hybridization, and the results indicated that their *sod* regions were replaced by the *sod*::Em^r (Fig. 6).

The SOD activity of the *sod* mutants was studied by two different methods (Table 1; Fig. 7). No SOD activity was detected in crude extracts from cells of the *sod* mutants grown either aerobically or anaerobically. These results indicate that the *sod* gene cloned in this study is the only functional gene encoding SOD in *S. mutans*.

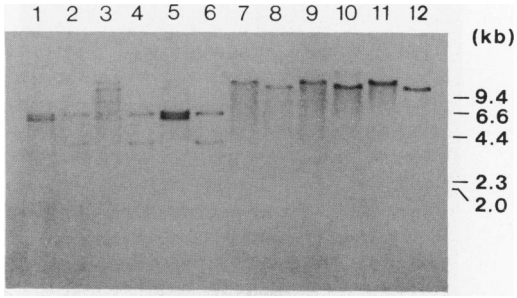

FIG. 6. Southern blot analysis of *S. mutans sod*::Em^r mutants. Chromosomal DNAs of KDSM1 (lanes 1 and 7), GS-5 (lanes 2 and 8), KDSM2 (lanes 3 and 9), NCTC10449 (lanes 4 and 10), KDSM3 (lanes 5 and 11), and MT8148 (lanes 6 and 12) were digested with *Hin*dIII (lanes 1 to 6) and *Eco*RI (lanes 7 to 12). Subsequent procedures were the same as described in the legend to Fig. 3. Intact *sod* is revealed by the presence of 6.4-, 3.7-, and 0.4-kbp *Hin*dIII fragments, although the 0.4-kbp *Hin*dIII fragment is not detectable in this blot. The *sod*::Em^r insertion is revealed by the disappearance of these 3.7- and 0.4-kbp fragments and the appearance of the 6.0-kbp fragment, as can be seen in lanes 1, 3, and 5. The KDSM2 chromosomal DNA digested with *Hin*dIII (lane 3) contains partial digests.

TABLE 1. SOD activity of the *sod*::Em^r mutants

Strain	SOD activity (U/mg of protein)[a]	
	Anaerobic	Aerobic
GS-5	0.4	5.7
KDSM1	ND	ND
NCTC10449	0.4	6.2
KDSM2	ND	ND
MT8148	0.5	4.3
KDSM3	ND	ND

[a] A fresh culture anaerobically grown in Todd-Hewitt broth overnight was diluted 10-fold with prewarmed Todd-Hewitt broth, and anaerobic or aerobic incubation was carried out at 37°C for 140 min. ND, no inhibition of O_2^--dependent reduction of cytochrome *c* was detected when crude extracts containing total protein of more than 7 mg/ml were used.

Aerobic growth of SOD-deficient mutants. As mentioned above, the *sod* mutants were found to be able to grow under aerobiosis. To study this point further, I compared growth of these mutants under aerobic and anaerobic conditions in more quantitative ways. When cells were spread on Todd-Hewitt agar plates and incubated aerobically at 37°C, the mutants formed colonies smaller than those of their respective parent strains. They developed colonies of the same size as those of their parent strains under anaerobic conditions (Table 2). Delay of aerobic growth of the mutants was also seen in liquid cultures (Fig. 8).

Absence of silent *sod* genes in the *S. mutans* chromosome. The chromosomes of several bacteria, including *E. coli*, possess two *sod* genes, one encoding an Mn-SOD and the other encoding an Fe-SOD. In *E. coli*, the Mn-SOD-encoding gene (*sodA*) is repressed under the anaerobic condition and induced by exposure to O_2, whereas the Fe-SOD-encoding gene (*sodB*) is constitutively expressed. As described earlier, I was unable to find any other SOD in the crude extract of *S. mutans* GS-5 than the SOD encoded by the *sod* gene cloned here. However, other researchers found

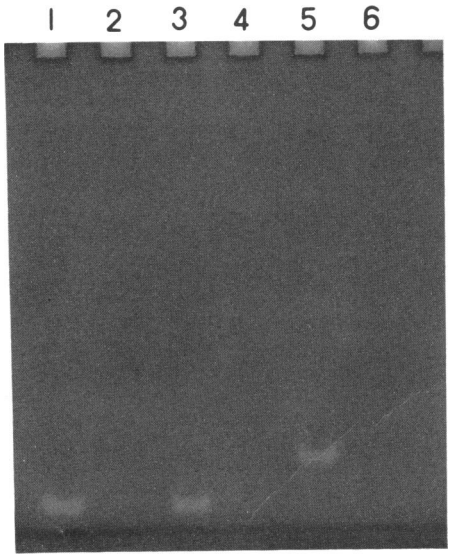

FIG. 7. Absence of SOD activity in extracts of *S. mutans sod* strains. Samples containing 100 μg of protein from aerobically grown cells were separated on a nondenaturing 8% polyacrylamide gel and stained for SOD activity. Lanes: 1, GS-5; 2, KDSM1; 3, NCTC10449; 4, KDSM2; 5, MT8148; 6, KDSM3.

TABLE 2. Aerobic and anaerobic growth of the SOD-deficient mutants on solid media

Strain[a]	Colony diam (mm)[b]	
	38 h	62 h
Aerobic		
GS-5	0.467 ± 0.118	1.006 ± 0.111
KDSM1	0.098 ± 0.013	0.430 ± 0.040
NCTC10449	0.546 ± 0.068	0.936 ± 0.067
KDSM2	0.112 ± 0.023	0.514 ± 0.081
MT8148	0.640 ± 0.095	1.058 ± 0.107
KDSM3	0.114 ± 0.020	0.479 ± 0.051
Anaerobic		
GS-5	0.823 ± 0.063	1.150 ± 0.076
KDSM1	0.833 ± 0.069	1.196 ± 0.086
NCTC10449	0.652 ± 0.096	0.923 ± 0.071
KDSM2	0.760 ± 0.039	0.969 ± 0.071
MT8148	1.053 ± 0.079	1.370 ± 0.146
KDSM3	1.103 ± 0.056	1.455 ± 0.122

[a] Cells were grown on Todd-Hewitt agar plate at 37°C.
[b] Measured under a microscope 38 and 62 h after the start of incubation. Values represent means of at least six determinations ± standard deviation.

a minor SOD as well as a major one in crude extracts of *S. sobrinus* strains at separate positions on a nondenaturing polyacrylamide gel (18, 19, 34). As *S. mutans* is physiologically and genetically related to *S. sobrinus*, it is possible that a minor SOD in *S. mutans* escaped detection under the experimental conditions used. I explored this possibility by genetic means as follows.

The sequence Trp-Glu-His-Ala-Tyr-Tyr is highly conserved in both Fe-SODs and Mn-SODs. The oligonucleotides capable of encoding this amino acid sequence were synthesized, and a mixture of the oligonucleotides was used

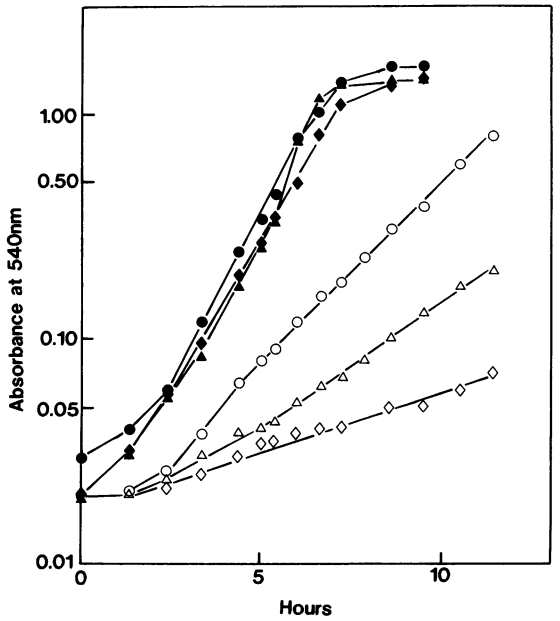

FIG. 8. Aerobic growth of the SOD-deficient mutants in liquid medium. Anaerobically grown cells (0.2 ml) were added to 20 ml of Todd-Hewitt broth and incubated at 37°C with shaking. Growth was monitored by measuring the optical density at 540 nm. Symbols: ●, GS-5; ○, KDSM1; ▲, NCTC10449; △, KDSM2; ◆, MT8148; ◇, KDSM3.

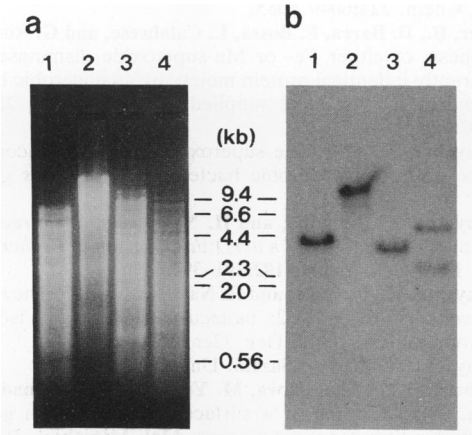

FIG. 9. Hybridization of the oligonucleotide probe derived from the common SOD motif to chromosomal DNA purified from *S. mutans* GS-5. The chromosomal DNA was digested with the restriction endonucleases *Hin*cII (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3), and the fragments were separated by electrophoresis through a 0.8% agarose gel (a). The DNA was then transferred to a nylon membrane and hybridized to the oligonucleotide probe as described in Materials and Methods (b). Lane 4 contains *E. coli* AB2497 chromosomal DNA digested with *Hin*cII.

as a probe for hybridization. The chromosomal DNA of *S. mutans* GS-5 was digested with several restriction enzymes and subjected to Southern hybridization with the probe (Fig. 9). The oligonucleotide probe was hybridized to one DNA fragment which has the same length as the fragment hybridized to the *sod* probe. Thus, it is unlikely that *S. mutans* GS-5 possesses another *sod* gene encoding either Mn- or Fe-SOD on its chromosome.

DISCUSSION

Martin et al. (18) purified an SOD from *S. sobrinus*, which is taxonomically related with *S. mutans*, and found that the SOD can be activated by either Fe or Mn ion. Judging from the amino-terminal sequences of the *S. mutans* SOD, the gene for which was cloned in this study, and the *S. sobrinus* SOD, these SODs may be similar to each other. Similarity in their electrophoretic mobilities (18) also supports this notion. The SOD which can accept either Fe or Mn ion as a cofactor has been found from *Propionibacterium shermanii* (22), *Bacteroides fragilis* (10), *B. thetaiotaomicron* (28), *P. gingivalis* (1), and *Methylomonas* strain J (20) in addition to *S. sobrinus*. Cloning and DNA sequencing of this type of SOD of *P. gingivalis* (23) and purification and amino acid sequencing of the SOD (2) revealed that its amino acid sequence around the second ligand, where the major difference between Fe-SOD and Mn-SOD is seen, shows an unusual feature distinct from that of either Fe- or Mn-SOD (8, 27). This feature is also seen in the SOD of *B. fragilis* (25a).

On the other hand, the *S. mutans* SOD is considered to be an Mn-SOD on the basis of the amino acid sequence, particularly that around the second ligand. If one assumes that the *S. mutans* SOD can be activated with either Fe or Mn ion, which is likely because of the similarity of the SODs of *S. mutans* and *S. sobrinus*, it follows that perhaps not all SODs of this type share one common feature in their amino acid sequences. In this connection, the SODs of *Methylomonas* strain J and *Mycobacterium tuberculosis* are of Mn type with respect to amino acid sequence but can be activated by Fe ion (20, 37). Further study is needed to clarify the relationship between the selectivity for metal ions and features in the amino acid sequence.

There is evidence indicating the importance of SOD for aerobic growth. A *sodA sodB* mutant of *E. coli* grows more slowly than its parent and shows hypersensitivity to O_2 (9). A mutation in the gene either for CuZn-SOD or for Mn-SOD of *Saccharomyces cerevisiae* renders cells O_2 sensitive (33). The retardation of aerobic growth in the SOD-deficient mutants of *S. mutans* represents another line of evidence for the involvement of SOD in the mechanism for protection against oxidative stress. However, it might be surprising that the SOD-deficient mutants could grow under aerobic conditions because *S. mutans* does not possess catalase. It is probable that other enzymes, including peroxidases and oxidases, can contribute to the capacity for aerobic growth of SOD-deficient mutants (13, 32). Incidentally, an *E. coli* mutant deficient both in SODs and in catalases also grows as well as an SOD-deficient mutant in aerobic conditions (30).

S. mutans has a single chromosomal gene for SOD. On the other hand, the *E. coli* chromosome has two *sod* genes, one (*sodA*) for Mn-SOD and the other (*sodB*) for Fe-SOD. It is generally thought that a common ancestor of the present-day genes encoding Mn- and Fe-SODs was duplicated at some time during the bacterial evolution, giving rise to a gene for Mn-SOD and one for Fe-SOD. There are at least two possibilities to explain the presence of a single *sod* gene (Mn type) as opposed to two on the *S. mutans* chromosome. One is that Mn-SOD is the prototype and Fe-SOD is its derivative, and *S. mutans* remains evolutionally at the stage before the *sod* gene duplication. The other is that a gene encoding Fe-SOD in *S. mutans* has been lost in the process of evolution. The former explanation appears to be incompatible with the abundance of Fe ions on the ancient earth (4), but it may be argued that the prototype is the one that can accept Fe as a cofactor. More inductive study should lead to resolution of this question.

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