Cloning, Nucleotide Sequence, and Disruption of *Streptococcus mutans* Glutathione Reductase Gene (gor)

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We cloned and sequenced the glutathione reductase gene (gor) of an oxygen-tolerant *Streptococcus mutans*, and constructed a gor-disruption mutant by homologous recombination. The gor gene consisted of 1,350 bp, coding for a protein of 450 amino acid residues. The deduced amino acid sequence of the S. mutans gor gene product showed extensive similarity with those of glutathione reductases from prokaryotes and eukaryotes. Although the mutant could grow aerobically, it showed no growth in the presence of 2 mM diamide, a thiol-specific oxidant. In contrast, growth of the wild-type strain was not significantly inhibited by 2 mM diamide, and glutathione reductase activity was increased 2.2-fold under these conditions. In addition, the level of glutathione reductase activity in the wild-type strain was increased 3.6-fold upon exposure to air, and the elevated level of the enzyme was retained throughout the aerobic growth. Thus, glutathione reductase may be important in protection of *S. mutans* against oxidative stress.

Key words: *Streptococcus mutans*; glutathione reductase; gor gene; diamide; oxidative stress

The sensitivity to O$_2$ varied among *Streptococcus mutans* strains and was inversely correlated with the level of NADH oxidase activity and superoxide dismutase.$^3$ An O$_2$-tolerant *Streptococcus mutans* has two distinct NADH oxidases (Nox-1, H$_2$O$_2$-forming oxidase; and Nox-2, H$_2$O-forming oxidase).$^5$ Nox-1 acts as an NADH oxidase-deficient mutant in combination with the *S. mutans* AhpC, one component of alkyl hydroperoxide reductase (AhpR).$^5$ AhpR is a two-component enzyme, involving in defense against oxidative stress.$^5$ We constructed NADH oxidase-deficient mutants of *S. mutans*. The triple mutant with disrupted Nox-1, Nox-2, and AhpC was still able to grow fairly well on glucose under highly aerobic conditions, suggesting that the organism has at least one antioxidant against oxidative stress (Higuchi, et al., under submission).

Nox-1 and AhpC were identified as homologues of the two components of AhpR, AhpF and AhpC, respectively.$^5$ The expression of AhpR in *S. typhimurium* is controlled by the positive regulator OxyR.$^4$ To explore the OxyR-like regulon, we tried to amplify the oxyR gene by PCR using primers that were constructed according to the consensus sequence of OxyR. Here, we happened to obtain a DNA fragment with sequence similarity to glutathione reductase (GR; NAD(P)H: oxidized-glutathione oxidoreductase; EC 1.6.4.2) instead of OxyR.

In this paper, we describe the nucleotide sequence of the gene encoding GR from an oxygen-tolerant *S. mutans* and the isolation and some properties of the gor disruption mutant. Our results suggest that *S. mutans* GR is important in protection of the bacterium against oxidative damage.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. *S. mutans* GS-5, an oxygen-tolerant strain, and *E. coli* DH5α were routinely used. Plasmids pUC118 (TaKaRa Biochemicals, Tokyo) and pSPC1(a gift from Sato) were used as the plasmid vector for gene cloning and the source of a spectinomycin resistance (Spc$^r$) determinant, respectively. *S. mutans* were grown at 37°C in Todd-Hewitt Broth (THB, Difco Laboratories, Detroit, Mich.), with or without spectinomycin (250 μg/ml) under vigorously shaking aerobic or strictly anaerobic conditions as described previously.$^3$ *E. coli* were grown aerobically at 37°C in Luria broth (LB) with or without spectinomycin (50 μg/ml). Solid media contained 1.5% agar.

Degenerate PCR and isolation of amplified fragments. Genomic DNA was prepared from *S. mutans* GS-5 as described previously.$^6$ On the basis of multiple alignments of OxyR homologues, a sense primer (oxyR1) containing a sequence in the beginning region of an α-helix near the N-terminus and antisense primer (oxyR2) containing a sequence at the redox center were constructed for degenerate PCR; 5'-CA(C/T)TT(T/C) (C/G)G(T/C/G)CGTGCC(G/T)GC-3' and 5'-CA(C/G) CGCAI(A/G)CAITGICCC(A/G)TC(A/T)TC(T/C/A) A(A/G)GC-3', respectively. By using the genomic DNA of *S. mutans* GS-5, DNA fragments were amplified with primers oxyR1 and oxyR2 by the PCR at 94°C for 1 min, at 50°C for 1 min, and at 72°C for 1 min, with 30 cycles. The deduced size of the amplified fragment for oxyR is 550 bp. PCR-generated fragments approximate-

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Abbreviations: GR, glutathione reductase; gor, glutathione reductase gene; NBT, nitro blue tetrazonium; PMS, phenazine methosulfate; PAGE, polyacrylamide gel electrophoresis; Spc$^r$, spectinomycin resistance gene; PCR, polymerase chain reaction; I, inosine; ORF, open reading frame
ly comprising 550 bp were eluted from a 1.5% agarose gel using EASYTRAP™ Ver.2 (TaKaRa, Tokyo), then cloned to analyze the nucleotide sequence.

Identification and isolation of the gor gene. The DNA fragment encoding a partial ORF similar to GR was used as a Southern hybridization probe. A 2.4-kbp PstI hybridized fragment was screened by colony hybridization, and was isolated and cloned into pUC118 to obtain pUGP131. Then pUGP131 was subeloned to analyze its nucleotide sequence. Southern hybridization and colony hybridization were done with ECL nucleic acids labeling and detection system (Amasham) according to the manufacturer's instructions.

DNA sequencing. The cycle-sequence reaction was done with a Thermo Sequenase dye terminator cycle sequencing kit (Amersham Life Science, Cleveland, OH) with M13 forward and reverse IRD-41-labeled dye primers (Aloka, Tokyo, Japan). A DNA sequencing system (Model 4000, Li-Cor, Lincon, NB) was used for sequencing.

Construction of a plasmid for disruption of the target gene. Plasmids containing the gor gene inactivated by insertion of spc gene were constructed as follows. A Spc' determinant was obtained by digestion of pSPC1 with EcoRI. A plasmid containing the gor gene, pUGP131, was digested with EcoT22I, a restriction enzyme that cuts in the middle of gor at two sites but does not cut pUC118. Both DNA fragments were filled in with the Klenow fragment of DNA polymerase I before ligation, to obtain blunt ends. The newly constructed plasmid was designated pGES (Fig. 2B).

Transformation of S. mutans and homologous recombination. Genetic transformation of DNA fragments into S. mutans was done as described by Perry and Kuramitsu90 with some modifications. S. mutans GS-5 was transformed to Spc' with the 2.9-kbp Pst1-PstI fragment of pGES. Transformants were selected on THB agar containing spectinomycin.

Screening of gor disruptants by PCR. Colonies on spectinomycin plates were isolated and analyzed for the insertion of the Spc' marker into the genomic DNA by direct PCR using gor 1 (5'-AAGCTTTCTTTCTCGTGACATCTGAG-3') and gor 2 (5'-AACGTTGAA-TAGACTTAGCAGCGG-3').

Cell growth and preparation of cell-free extracts. Anaerobic cultures in late log phase were taken out from the anaerobic box to air, vortexed, and kept standing at 37°C for 20 min, then 0.25 ml of the culture was put in to a 100 ml flask containing 5 ml medium and incubated with vigorously shaking under aerobic conditions (150 cycles per minute). For enzyme induction, the cells were grown under the same conditions as described above, excepting that 2.5 ml of the culture was transferred to a 500 ml flask containing 50 ml of the appropriate medium. Harvested cells were washed twice with ice-cold 50 mm potassium phosphate buffer (PPB, pH 7.0) containing 0.2 mm EDTA. Cells were disrupted by sonication (on ice) 6 times for a 30 sec with 30 sec cooling interval. After cell debris was removed by centrifugation at 25,000 × g for 30 min, the clear lysates were used immediately as a source of GR. Protein concentration was measured by the dye-binding method.90

Assay of glutathione reductase activity. GR activity in extracts was measured at 25°C by monitoring the oxidation of NADPH in the reaction mixture (3 ml) at 340 nm. The reaction mixture contained 100 mm PPB (pH 7.5), 1 mm EDTA, 0.14 mm NADPH, 1.2 mm GSSG, and extracts (10–50 μg/ml protein). The reaction was started by adding GSSG solution. GR activity on a gel after native PAGE91 was detected by staining the gel with NBT and PMS after dipping in the above buffer solution containing NADPH with or without GSSG.

Disk sensitivity assay of cells to killing by oxidants. A portion (0.1 ml) of overnight anaerobic culture was added to 10 ml of fresh THB and cultured with standing under air to late log phase, and portions (0.2 ml) of cultures were then added to soft agar and plated on THB. Oxidants (H2O2, Santoku Chemical Industry, Co. Ltd., Tokyo; CHP, cumene hydroperoxide, Sigma; diamide, 1,1'-Azobis (N,N-dimethylformamide), Tokyo Kasei Co., Ltd., Tokyo, and menadione, 2-methyl-1,4-naphthoquinone, Wako Pure Chemicals Co., Ltd., Osaka) were then put on 8-mm paper disks (Toyoyoshi Co., Ltd., Tokyo), and the disks were placed on the center of the agar. The sensitivities of cells were measured as the diameter of the zone of killing after 36 h at 37°C, except for CHP for 24 h incubation.

**oxyR1**

CACTCTGCTTGTTGCGCTCTGCGCAATACAAAAAGGTTTTTATCTGGACATCTGCTGTCCATTTA 60
HAVFRRNAANTKGFNLVETTVGT

CATGATAGATTTTATAGCCATGCTACATTTAAAATCTTACGAGGTTGTAT 120
DSRGFLAPDAFDTNENETVLEGY

GCTCTTCGGAGTCAAGGAGGAATAGTACACCGTACATGACAGCTGCGGT 180
AIDGVNGLKELTPVAVKAGR

CAACTGCTGAAACGGCTTTTTTAACCATAGAAGGCTGGCCTAGATGTTAAATAGGATGT 240
QLSRFLFNHHPQAKMDYKDV

GCTACGGTTTTACAGCAGCTACGTTTTATCTGCTACATGCCGCTGCTGCTG 300
365
ATVIFSPHVPSGIGSLGSEAA

TTAGCATGATGATGCTGGAAGAAAAAGGACCTTTATATGCTTACCTTTACTACCTGGATAT 420
LDQYGEENVTYVYRFTFTSMY

AGGCCACATACCCACGAGTACGTGACAGCTTGGATTGCCAGTTCTATCAGGAT 480
450
TAVTSWHRQSCMKLKLTVGED

GAAAGAGCTTTGCTTGCTAGTATGTATGTTGTTTGAAGGTCTCGCTGCGGT 600
EJKVLHGGIYGVRWPILSA

**oxyR2**

Fig. 1. Nucleotide Sequence of the PCR Product Amplified Using Primers (oxyR1, oxyR2).

The sequence was identified as a partial ORF homologous to the structural gene of GR of E. coli. Each arrow indicates the sequence corresponding to either oxyR1 or oxyR2. The underline shows the sequence corresponding to the second part of the FAD domain.90
Fig. 2.  (A) Nucleotide Sequence of the gor Gene from S. mutans and the Deduced Amino Acid Sequence.

The complete nucleotide sequence comprising the ORF of the gor gene, and 427 bases upstream and 672 bases downstream of ORF are shown. The predicted amino acid sequence is designated in single-letter amino acid code. A putative –35 and –10 promoter region and a probable Shine-Dalgarno (S.D.) sequence are denoted by dotted lines. An inserted repeat sequence is indicated by arrows and is probably used as a transcription terminator for gor. The sequence of the second part of the FAD domain is indicated by an underline. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB019579.

(B) Restriction Map of Plasmid pUG131 and Strategy for Inactivating the S. mutans gor Gene, Indicating the Location of the Inserted spc Gene.
Results and Discussion

Presence of a partial open reading frame of a gor homologue

Three DNA fragments approximately comprising 550 bp which were amplified by degenerate PCR using the primers oxyR1 and oxyR2, were cloned and sequenced. One of them which consists of a 480-bp nucleotide sequence starting from oxyR1 was a partial ORF homologous with the structural gene for E. coli GR (Fig. 1), while the other two fragments did not show any homologue as far as we searched. Consequently, we happened to obtain a fragment coding for a partial amino acids sequence which shows 60% identity to the C-terminal region of E. coli GR including the second part of the FAD domain (Fig. 1). Accordingly, we examined whether or not this fragment is a part of gene for GR of S. mutans, we tried to clone the full length of the gene.

Nucleotide sequence of the gor homologue and deduced amino acid sequence

Southern blotting was done for the digested genome of S. mutans using the 480-bp fragment as a hybridization probe. A 2.4-kbp PstI hybridized fragment was obtained and isolated, cloned to obtain pUGP131. The sequence analysis of pUGP131 indicated one complete ORF, possibly corresponding to the gor of S. mutans. The ORF consisted of 1,350 bp, coding for a protein of 450 amino acids (Fig. 2A). This gor homologue had putative -35 and -10 promoter regions upstream of the initiation codon, and a palindromic sequence downstream from the termination codon (Fig. 2A). A putative Shine-Dalgarno sequence is also found at the -16-bp position from the initiation codon (Fig. 2A). The deduced amino acid sequence of the gor homologue of S. mutans was compared with that of GRs from other organisms (Fig. 3). The predicted amino acids sequence of the protein was 76.9%, 63.3%, 49.7%, and 50.1% identical with GRs of Streptococcus thermophilus, 11 E. coli, 9 Saccharomyces cerevisiae, 12 and Homo sapiens (X15722), respectively. The GR from S. mutans also indicated a higher similarity to partial amino acid sequence at the N-terminus of the GR from Enterococcus faecalis. 13 Analysis of the GR sequence alignment (Fig. 3) showed that the sequence around the disulfide reductase center was completely conserved in all the five organisms whether they are Gram-positive or Gram-negative bacteria, prokaryotes or eukaryotes. The GR proteins from prokaryotes, including S. mutans, S. thermophilus and E. coli, consisted of 450 amino acid residues, and match each other quite well in the N-terminal and C-terminal regions.

Properties of GR and the GR deficient mutant

To identify the physiological function of GR in S. mutans, a gor disruption mutant of S. mutans was con-

![Fig. 3. Amino Acid Sequence Comparison of the S. mutans gor Gene Product with GRs from Other Organisms.](attachment:image.png)

Amino acid sequences of GRs from S. thermophilus, 11 E. coli, 9 Saccharomyces cerevisiae, 12 and Homo sapiens (X15722) are shown. Residues with identity to GR of S. mutans are shown on a black background, while gaps are indicated by dash (–). Asterisks indicate identical amino acids.
structured using plasmid pGES (Fig. 2B). One spectinomycin-resistant transformant was isolated and analyzed by direct PCR, for the location of the introduced DNA fragment on the chromosome of the transformant. The shift of PCR products from 1.4-kbp to 1.8-kbp by electrophoresis in the transformant compared with wild-type (Fig. 4A) verified that the transformant is the mutant which has gor:spc<sup>c</sup>.

The GR activity in extracts from the mutant and wild-type cells cultured aerobically was studied by activity staining. No band from the extract of the mutant cells on the gel corresponding to NADPH-dependent GSSG reductase activity was detected, but a band with the activity from extract of wild-type cells was clearly appeared (Fig. 4B). With this method, SOD activity is also detectable (Fig. 4B).<sup>15</sup> But this SOD activity appeared independently with or without NADPH or GSSG in the reaction medium (data not shown). The enzyme assay of extracts measured spectrophotometrically also showed no GR activity in the mutant cells. These results indicate that the mutant is a GR mutant and the cloned gor homologue actually encodes GR.

Since the principal thiol-disulfide redox buffer in the cytoplasm of bacteria is thought to be GSH,<sup>15</sup> GR of <i>S. mutans</i> is probably important for keeping the redox potential at a low level for protecting thiol-containing proteins against oxidative stress. To clarify the protective role of GR against oxidative stress in <i>S. mutans</i>, the sensitivities of GR mutant and wild-type strains to oxidants, such as H<sub>2</sub>O<sub>2</sub>, cumene hydroperoxide (CHP, organic hydroperoxide),<sup>16</sup> diamide (thiol-specific oxidant),<sup>15</sup> and menadione (superoxide anions generator)<sup>17</sup> were studied using a disk sensitivity assay (see Materials and Methods). The results in Table 1 indicated that the GR mutant was more sensitive to killing by diamide than the wild-type strain, but slightly sensitive to H<sub>2</sub>O<sub>2</sub> and similar to wild-type treated with CHP or menadione. Thus, it is suggested that the function of GR was not an antioxidant against active oxygen, such as organic hydroperoxide and superoxide anion in <i>S. mutans</i>.

To analyze the effects of diamide on the cell growth, the aerobic growth of GR mutant and the wild-type strain was then studied with or without 2 mM diamide. The results in Fig. 5A indicated that the GR mutant grew no more in the presence of 2 mM diamide, which retarded the growth rate of wild-type strain, while no significant difference between the aerobic growth rate of the GR mutant and wild-type strain was observed. As diamide is known to be a membrane-permeant thiol-specific oxidizing agent,<sup>15</sup> the growth inhibition of the GR mutant by diamide seemed to be due to the defect in the disulfide reduction system for GSH. Furthermore, by the addition of 2 mM diamide, the GR activity in

### Table 1. Sensitivities of GR Deficient Mutant and Wild-Type Strain to Killing by Oxidants

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Zone of Killing (diameter, mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-Type</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>16.0±2.0</td>
</tr>
<tr>
<td>CHP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.1±0.8</td>
</tr>
<tr>
<td>Diamide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.8±0.3</td>
</tr>
<tr>
<td>Menadione&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.5±0.5</td>
</tr>
</tbody>
</table>

The amount of oxidant was used: 0.5 mM H<sub>2</sub>O<sub>2</sub>, 10 μl; 0.5 mM CHP, 2.5 μl; 0.5 mM Diamide, 2.0 μl; 0.02 mM Menadione, 2.0 μl. The results are the means±SD for triplicate.

<sup>a</sup> dissolved in water.
<sup>b</sup> dissolved in dimethyl sulfoxide.
<sup>c</sup> dissolved in ethanol.

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**Fig. 4.** Analysis of the gor Inactivated Mutant (GR-Deficient Mutant) of <i>S. mutans</i>.

(A) Analysis of the Inserted gor:spc<sup>c</sup> DNA Fragment into Genomic DNA by Direct PCR.

The PCR products were separated by 1% agarose gel and stained by ethidium bromide. Lane 1, marker; 2, wild-type; 3, GR mutant.

(B) Analysis of the Protein Bands Showing GR Activity in Cell Extracts.

Samples containing 10 μg of protein were separated by a non-denaturating 10% polyacrylamide gel. Lane 1, purified GR from yeast (Boehringer-Mannheim); 2, anaerobically grown wild-type; 3, aerobically grown wild-type; 4, aerobically grown GR mutant.

**Fig. 5.** Effects of Diamide on Cell Growth and GR Activity.

(A) Inhibitory Effects of Diamide on the Aerobic Growth of the GR Mutant and Wild-Type Strain of <i>S. mutans</i>.

Symbols: (○), wild-type as control; (●), wild-type plus diamide; (◇), GR mutant as control; (◆), GR mutant plus diamide. An arrow indicates the timing for the addition of 2 mM diamide (final concentration). The data shown are the mean of duplicates.

(B) Effects of Diamide for Induction of GR.

Under the same conditions of Fig. 5A, GR activities were measured at the time after 2 hrs adding with or without (control) 2 mM diamide. The data shown is the means of duplicates.
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