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Role of the *dpr* Product in Oxygen Tolerance in *Streptococcus mutans*

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We have previously identified and characterized the alkyl hydroperoxide reductase of *Streptococcus mutans*, which consists of two components, Nox-1 and AhpC. Deletion of both *nox-1* and *ahpC* had no effect on the sensitivity of *S. mutans* to cumene hydroperoxide or H_2O_2, implying that the existence of another antioxidant system(s) independent of the Nox-1–AhpC system compensates for the deficiency. Here, a new antioxidant gene (*dpr* for Dps-like peroxide resistance gene) was isolated from the *S. mutans* chromosome by its ability to complement an *ahpCF* deletion mutant of *Escherichia coli* with a *tert*-butyl hydroperoxide-hypersensitive phenotype. The *dpr* gene complemented the defect in peroxidase activity caused by the deletion of *nox-1* and *ahpC* in *S. mutans*. Under aerobic conditions, the *dpr* disruption mutant carrying a spectinomycin resistance gene (*dpr*::Spc^r mutant) grew as well as wild-type *S. mutans* in liquid medium. However, the *dpr*::Spc^r mutant could not form colonies on an agar plate under air. In addition, neither the *dpr*::Spc^r *ahpC*::Em^r::*nox-1* triple mutant nor the *dpr*::Spc^r *sod*::Em^r double mutant was able to grow aerobically in liquid medium. The 20-kDa *dpr* gene product Dpr is an iron-binding protein. Synthesis of Dpr was induced by exposure of *S. mutans* cells to air. We propose a mechanism by which Dpr confers aerotolerance on *S. mutans*.

Bacteria contain certain enzymes capable of reacting with oxygen, and they cannot avoid confronting harmful reactive oxygen species, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and organic hydroperoxide, if they are exposed to air. To live in the presence of oxygen, they have to convert these reactive oxygen species to nontoxic molecules. Therefore, enzymes such as superoxide dismutases (SOD), catalases, and peroxidases are ubiquitously distributed in aerotolerant bacteria.

Lactic acid bacteria, including *Streptococcus mutans*, lack cytochromes and other heme-containing proteins. Most lactic acid bacteria, except several lactobacilli which acquire catalase activity if a source of heme is added to their growth medium (42), also lack catalase. However, they can grow in the presence of air. In view of the defense against oxygen toxicity, the lack of catalase in lactic acid bacteria is inconsistent with their aerotolerance. Mechanisms by which lactic acid bacteria cope with peroxide-mediated stress are therefore an area under active investigation. We previously identified H_2O_2-forming NADH oxidase (Nox-1) in *S. mutans* and found that Nox-1 is homologous with a flavoprotein component, AhpF, of *Salmonella typhimurium* alkyl hydroperoxide reductase (AhpR), consisting of AhpF and AhpC (16, 17, 30). We also identified *ahpC*, which is homologous with *ahpC* of *S. typhimurium*, upstream of *nox-1* in *S. mutans* (30). Analyses of purified AhpC together with Nox-1 have verified that these proteins act as a bicomponent peroxidase system in *S. mutans*, catalyzing the NADH-dependent reduction of organic hydroperoxides or H_2O_2 to their respective alcohols and/or H_2O (31). Furthermore, the bicomponent system restored the resistance of *Escherichia coli* TA4315 (*ahpCF*-defective mutant) (36) to cumene

hydroperoxide (18). However, the *nox-1 ahpC* double deletion mutant of *S. mutans* still showed the same level of peroxide tolerance as did the wild-type strain (18). These results suggested the existence of another antioxidant system(s) in addition to Nox-1 and AhpC in *S. mutans*. Here we identified a new gene, *dpr* (for Dps-like peroxide resistance gene); its product, Dpr, as an iron-binding protein responsible for peroxide tolerance in *S. mutans*. In this study, we also propose a mechanism by which Dpr confers aerotolerance on *S. mutans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and cell growth. The bacterial strains and plasmids used in this study are described in Table 1. *S. mutans* was grown aerobically or anaerobically at 37°C in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.), TY medium containing 1% glucose (TYG) (15), or THB supplemented with 5% horse serum for generation of component cells. Overnight cultures of wild-type and mutant *S. mutans* strains were incubated in an anaerobic glove box (Hirasawa Works, Tokyo, Japan) under an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. For aerobic growth, overnight cultures were diluted 50-fold and incubated to late exponential phase (an A_{660} of 1.0) under aerobic conditions without shaking. The cultures were then diluted again to an A_{660} of 0.020 in a 100-ml flask containing 5 ml of TYG medium and incubated with vigorous shaking under aerobic conditions (150 cycles/min). Fifty units of bovine liver catalase (Sigma) per ml, with or without pretreatment at 80°C for 10 min, or 100 nmol of deferoxamine mesylate (deferoxamine) (ICN Pharmaceuticals, K. K. Tokyo, Japan) per ml was added to the growth medium when indicated. Cell density was monitored by determination of A_{660} during growth. For measurement of viable cells, aliquots of cultures were diluted and spread onto TYG plates during growth. After 48 h of incubation in air at 37°C, the CFU on plates were counted. For anaerobic growth, 10 ml of fresh medium was inoculated with 0.1 ml of the late exponential phase (A_{660} of 1.0) anaerobic subculture and incubated without shaking in an anaerobic glove box. *E. coli* was grown aerobically at 37°C in LB broth (25). Solid media were supplemented with 1.5% agar. When present in selective plates, antibiotics were used at the following concentrations: for *S. mutans*, erythromycin at 10 µg/ml and/or spectinomycin at 250 µg/ml; for *E. coli*, ampicillin at 100 µg/ml, erythromycin at 300 µg/ml, chloramphenicol at 25 µg/ml, and spectinomycin at 50 µg/ml. Plasmids pUC118 (TaKaRa Biochemicals, Tokyo, Japan) and pACYC184 (Nippon Gene Co., Ltd., Tokyo, Japan) were used as vectors for gene cloning, and pSPC1 (23) was the source of a spectinomycin resistance (Spc^r) determinant.

Construction of a chromosomal gene library from the *nox-1* and *ahpC* double deletion mutant (AhpR deletion mutant) of *S. mutans*. Chromosomal DNA prepared from bacterial cells as described previously (17) was partially digested

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with *Sau*3AI and separated by agarose gel electrophoresis. DNA fragments comprising 4 to 6 kb were eluted from the gel using EASYTRAP Ver.2 (TaKaRa) and ligated into the *Bam*HI site of plasmid vector pUC118 DNA. *E. coli* strain TA4315 (Δ*ahpC* Δ*ahpF*), which is hypersensitive to *tert*-butyl hydroperoxide (tBHP; Katayama Chemical Co., Ltd., Osaka, Japan), was transformed with the *S. mutans*-derived library and subjected to selection by the method of Mongkolsuk et al. (26) on LB agar plates containing ampicillin and 0.5 mM tBHP to identify transformants in which the organic hydroperoxide-hypersensitive phenotype was suppressed.

Primers used for amplification of *dpr* and *sod* by PCR. Five primers, dpr1 (5′-CAAAGAAAGAGCCAAGAGAAGC-3′), dpr2 (5′-ACTGGAATTCTAA TCCAACGTCTGGGA-3′), dpr3 (5′-TTTCTATTCTGTGCTCCCTGCG-3′), sod1 (5′-TCCCCCGGGGATGATTTCTGT CAAAGC-3′), and sod2 (5′-TTGA ATTCTAGCGTATAGCGACTTACGG-3′), were used to amplify *dpr* and *sod* by PCR.

Construction of the plasmid for knockout of *dpr*. An Spc^r determinant was obtained by digestion of plasmid pSPC1 with *Eco*RI, and its cohesive ends were blunted. Knockout plasmid pS23B1ES was constructed by introducing the Spc^r-encoding gene into the *Eco*RV site of pS23B1. The plasmid had a *dpr*::Spc^r fusion gene and did not confer tBHP tolerance on *E. coli* TA4315.

Transformation of *S. mutans* and homologous recombination. Genetic transformation of *S. mutans* with DNA fragments was performed as described previously (18).

Measurement of plating efficiency of *S. mutans*. Overnight anaerobic cultures of *S. mutans* were diluted 100-fold and incubated while standing under air. At the exponential phase (A_{660} of 0.6), the cultures were spread onto TYG plates and incubated at 37°C anaerobically (A) and aerobically (B). After 48 h of incubation, the CFU on both plates were counted. Plating efficiency under air was defined as the ratio of CFU in B relative to those in A. Five hundreds units of catalase was spread on top of the plate immediately before the cells were spread when indicated.

Preparation of cell extracts. Cells grown aerobically or anaerobically were harvested by centrifugation at $12,000 \times g$ for 10 min, washed twice with 50 mM potassium phosphate buffer containing 0.2 mM EDTA (pH 7.0), and disrupted by sonication. After unbroken cells and cell debris were removed by centrifugation at $25,000 \times g$ for 30 min, the clear lysate was collected.

Assay for SOD. SOD activity was measured by the xanthine oxidase-cytochrome *c* method (39). One unit of SOD activity was defined as the amount of protein that gave a 50% decrease in the rate of reduction of cytochrome *c*.

Analytical procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (22). Protein was measured by the method of Bradford (4). The N-terminal amino acid sequence was determined by a gas-phase protein sequencer (model ABI 491; Applied Biosystems) equipped with an on-line amino acid analyzer (model 610A; Applied Biosystems). For staining of iron-binding proteins, $Fe(NH_4)_2(SO_4) \cdot 6H_2O$ was added to the crude extract at a final concentration of 1 mM and the mixture was incubated on ice for 30 min. The crude extract was then resolved by nondenaturing PAGE (native PAGE) (16). The gel was stained with 3-(2-pyridyl)-5,6-bis(2-[5-furyl sulfonic acid])-1,2,4-triazine (Ferene S; Sigma) by the method of Chung (8).

Nucleotide sequencing. The cloned DNA was fragmented by digestion with various restriction enzymes, subcloned into plasmid pUC118, and sequenced. The cycle sequencing reaction was done with a Thermo Sequenase cycle sequencing kit (Amersham) with M13 forward and reverse IRD-41-labeled dye primers (Aloka, Ltd., Tokyo, Japan). A DNA sequencing system (model 4000; Li-Cor, Lincoln, Nebr.) was used for sequencing.

Homology search, ORF identification, and alignment of multiple nucleotide and amino acid sequences. Protein and nucleotide sequences were compared with database sequences using the BLAST (version 1.49) programs implemented at the EMBL, GenBank, and DDBJ nucleotide sequence databases and the SWISSPROT and NBRF-PIR protein sequence databases. Open reading frame (ORF) identification and multiple-sequence alignment were performed using the GENETYX program (Software Development Co., Tokyo, Japan) and multiple-sequence alignment with hierarchical clustering (Laboratoire de Genetique Cellulaire, Toulouse, France). Secondary-structure prediction was performed by the method of Rost and Sander (32).

Nucleotide sequence accession number. The sequence of the cloned DNA described here has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB036428.

RESULTS

Identification of a peroxide resistance gene from *S. mutans*. We started with the cloning of a potential peroxide tolerance gene(s) from a *Sau*3AI genomic library of *S. mutans* strain BEE (*ahpC*::Em^r::*nox-1*) ligated into the *Bam*HI site of pUC118 and transformed into *E. coli* TA4315 (Δ*ahpC* Δ*ahpF*) with a tBHP-hypersensitive phenotype. One positive transformant was obtained from approximately 10,000 colonies plated on LB broth plates containing ampicillin (100 μg/ml) and 0.5

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
Strains		
S. mutans		
GS-5	Wild type, serotype c	41
BEE	*ahpC*::Em^r::*nox-1*	18
DES	*dpr*::Spc^r	This study
KD251	*sod*::Em^r	This study
BEE-DES	*dpr*::Spc^r *ahpC*::Em^r::*nox-1*	This study
KD251-DES	*dpr*::Spc^r *sod*::Em^r	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (φ80lacZΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	14
TA4315	Δ*ahpC* Δ*ahpF*	36
Plasmids		
pUC118	Ap^r *lacPOZ'*	44
pACYC184	Tet^r Cm^r	6
pSPC1	Spc^r 1.1-kb *Eco*RI fragment	23
pS23	5.3-kb fragment containing *dpr* in pUC118	This study
pS23B1	2.6-kb *Bam*HI-*Bam*HI fragment containing *dpr* from pS23 in pUC118	This study
pS23E12	1.8-kb *Eco*RI-*Eco*RI fragment containing *dpr* from pS23 in pUC118	This study
pS23E121	0.94-kb *Eco*RI-*Eco*RI fragment from pS23E12	This study
pS23E122	0.86-kb *Eco*RI-*Eco*RI fragment from pS23E12	This study
pD13184	*dpr* in pACYC184	This study
pS23B1ES	*dpr*::Spc^r from pS23B1	This study
pS23E12ES	*dpr*::Spc^r from pS23E12	This study
pKD251	*sod*::Em^r	27

mM tBHP. The positive clone contained a DNA insert of about 5.3 kbp. The plasmid carrying the 5.3-kbp fragment was designated pS23 (Fig. 1A, lane 1). pS23 DNA was digested with *Bam*HI or *Eco*RI, and the fragments were ligated into the *Bam*HI and *Eco*RI sites of pUC118, respectively, and transformed into *E. coli* TA4315. After selection for colony growth on LB broth-ampicillin plates containing tBHP, positive clones with 2.6-kbp *Bam*HI and 1.8-kbp *Eco*RI fragments were obtained (pS23B1 and pS23E12, respectively; Fig. 1A). Sequence analysis revealed that the 1.8-kbp fragment of pS23E12 included two ORFs (ORFs 1 and 2 in Fig. 1B) and that an *Eco*RI site was present within ORF 1.

To examine whether ORF 1 and/or 2 is responsible for peroxide tolerance in plasmid pS23E12, the 1.8-kbp fragment was digested with *Eco*RI to obtain 0.94- and 0.86-kbp fragments. The 0.94- and 0.86-kbp fragments were subcloned into the *Eco*RI site of pUC118 to obtain plasmids pS23E121 and pS23E122, respectively (Fig. 1A). Neither plasmid could confer peroxide resistance on *E. coli* TA4315, suggesting that ORF 1 is responsible for peroxide tolerance in *E. coli* TA4315. ORF 1, including its putative promoter region, was then amplified by PCR using primers dpr1 and dpr2, ligated into the *Eco*RV site of pACYC184 to obtain plasmid pD13184 (Fig. 1A), and then transformed into *E. coli* TA4315. The recombinant *E. coli* generated in this manner exhibited a peroxide resistance phenotype. In addition, ORF 1 was disrupted by introducing an Spc^r-encoding gene into the *Eco*RV site in ORF 1 in pS23E12

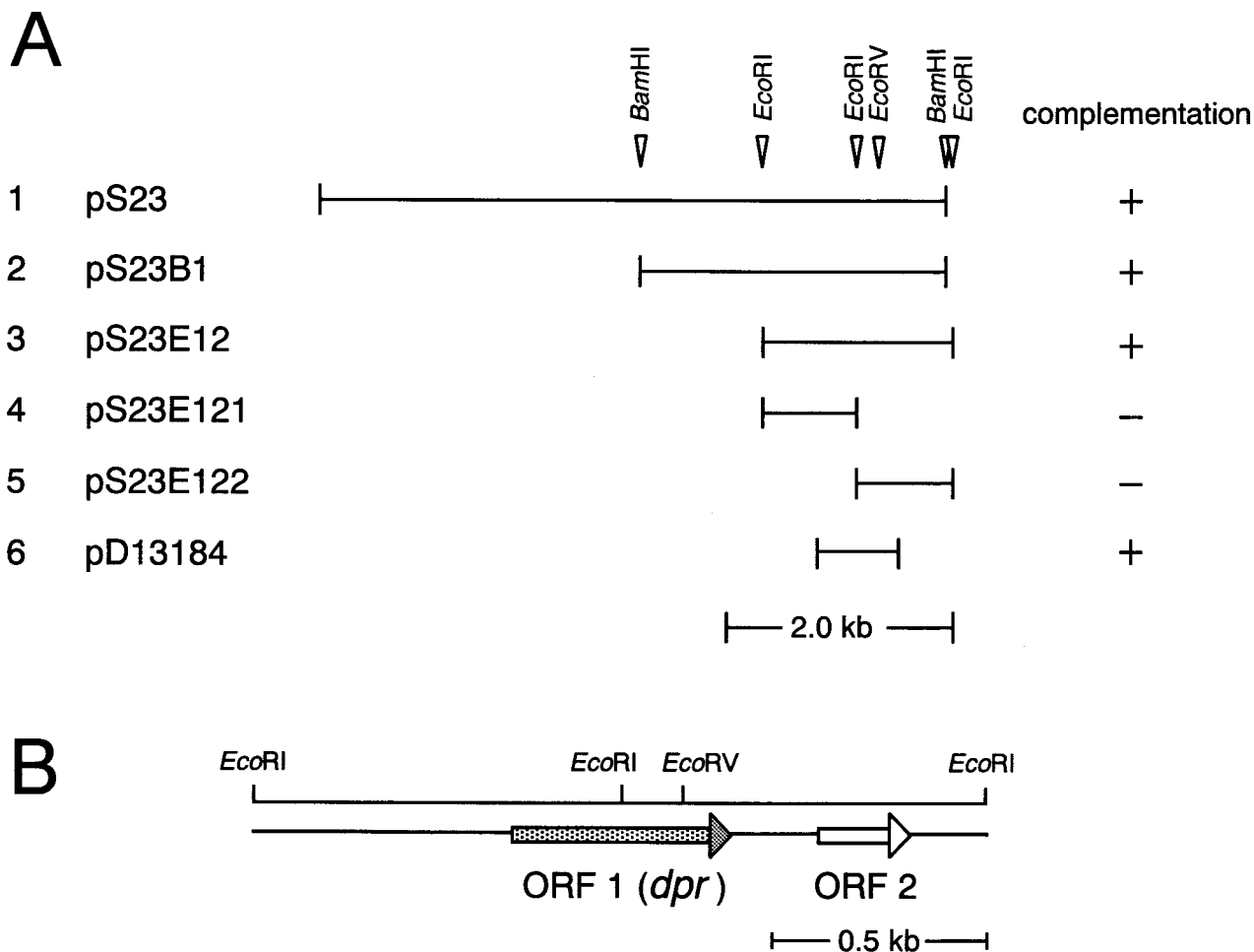FIG. 1. DNA segments from *S. mutans* which suppressed the tBHP-hypersensitive phenotype of *E. coli* TA4315 (Δ*ahpC* Δ*ahpF*) (A) and physical map of the 1.8-kbp segment of pS23E12 (B). The solid lines in panel A indicate the regions of the chromosomal DNA of *S. mutans* (lanes 1 to 5) and the DNA amplified by PCR (lane 6). In panel B, the arrows indicate the locations of ORFs.

to obtain plasmid pS23E12ES containing a *dpr*::Spc^r fusion gene and the intact ORF 2 gene. *E. coli* TA4315(pS23E12ES) was sensitive to tBHP (data not shown). Thus, we concluded that ORF 1 is the tBHP resistance gene.

ORF 1 consisted of 525 bp encoding a protein of 19,617 Da (20-kDa protein) with 175 amino acid residues. This ORF had putative −35 (5′-TAGAAT-3′) and −10 (5′-TATAAA-3′) promoter regions upstream of the translation start codon and palindromic sequences downstream from the translation stop codon. A putative Shine-Dalgarno sequence (5′-AGGAG-3′) was also found 13 bp upstream of the start codon. The deduced amino acid sequence of the 20-kDa protein showed low homology with that of the Dps (DNA-binding protein from starved cells) (1) family of proteins (Table 2). The secondary structure of the 20-kDa protein was also predicted to be similar to that of Dps (as described later). Accordingly, the gene encoding ORF 1 was designated *dpr* (*dps*-like peroxide resistance gene). On the other hand, ORF 2, 204 nucleotides long, encoded a protein of 8,332 Da comprising 68 amino acid residues. The deduced amino acid sequence of the putative 8.3-kDa protein showed 52% identity with that of a hypothetical protein from *Bacillus megaterium* (35).

Abortive colony formation of *dpr* disruption mutants of *S. mutans* under aerobic conditions. Three *dpr* disruption mu-

tants of *S. mutans*, *dpr*::Spc^r, *dpr*::Spc^r *ahpC*::Em^r::*nox-1*, and *dpr*::Spc^r *sod*::Em^r, were constructed, and their ability to form colonies was examined under both anaerobic and aerobic conditions. For disruption of *dpr* on the *S. mutans* chromosome, homologous recombination was performed between a linearized DNA fragment carrying a mutated *dpr* gene and the functional genomic gene. Plasmid pS23B1ES (Table 1), containing *dpr*::Spc^r, was digested with both *Hin*dIII and *Sac*I to obtain a 3.8-kbp linearized DNA fragment. The linearized DNA fragment was transformed into *S. mutans* GS-5 (wild type), BEE (*ahpC*::Em^r::*nox-1* mutant), and KD251 (*sod* disruption mutant). Transformants were selected on a THB-agar plate containing spectinomycin (250 μg/ml) under anaerobic conditions in an anaerobic glove box. The insertion of the Spc^r-encoding gene into the *dpr* gene on the chromosomal DNA of the transformants was confirmed by direct PCR using a pair of primers, dpr1 and dpr3. *S. mutans* KD251 was constructed as described above, using plasmid pKD251 (27) containing *sod* interrupted by an Em^r-encoding gene, and also confirmed by direct PCR using primers sod1 and sod2 (data not shown). Absence of SOD activity in crude extracts of strain KD251 cells was confirmed (data not shown). From the results obtained for the colony-forming ability of a series of *dpr* disruption mutants of *S. mutans*, the following findings became evident. (i) All *dpr*

TABLE 2. Pairwise identities of amino acid sequences between *S. mutans* Dpr and Dps family proteins

Organism and protein	% Sequence identity with:						
	S. mutans Dpr	*L. innocua* Fer[a]	*B. subtilis* MrgA[b]	*E. coli* Dps[c]	*B. subtilis* Dps[d]	*H. pylori* NapA[e]	*H. ducreyi* pilin[f]
L. innocua Fer[a]	40.0						
B. subtilis MrgA[b]	30.9	36.0					
E. coli Dps[c]	27.6	25.6	22.4				
B. subtilis Dps[d]	26.3	43.0	45.5	20.0			
H. pylori NapA[e]	26.1	32.1	29.5	22.4	34.2		
H. ducreyi pilin[f]	23.0	21.5	21.2	35.1	21.6	23.6	
Synechococcus DpsA[g]	21.5	26.3	26.8	22.6	22.7	20.5	21.9

[a] Nonheme ferritin of *L. innocua* (accession no. P80725).
[b] MrgA of *B. subtilis* (accession no. P37960).
[c] Dps of *E. coli* (accession no. P27430).
[d] Dps of *B. subtilis* (accession no. AF008220).
[e] Neutrophil-activating protein of *H. pylori* (accession no. AE000543).
[f] Fine-tangled pilus major subunit of *H. ducreyi* (accession no. Q47953).
[g] DpsA of *Synechococcus* sp. strain PCC7492 (accession no. Q55024).

disruption mutants, including those generated in the *ahpC*::Em^r::*nox-1*, *sod*::Em^r, and wild-type backgrounds, were equally capable of forming colonies of normal numbers and size under anaerobic conditions (data not shown). (ii) As shown previously (18, 27), the *ahpC*::Em^r::*nox-1* double mutant and the *sod*::Em^r mutant with an intact *dpr* gene showed the same level of aerobic plating efficiency as did the wild-type strain (Fig. 2), except that the colony size of the *sod* mutant is significantly smaller than that of the wild type in the presence of air. (iii) In contrast, all *dpr* disruption mutants showed a decrease of about 10^4-fold in the number of colonies formed compared to the wild-type strain in the presence of air (Fig. 2). Thus, we concluded that *dpr* is an essential gene for colony formation by *S. mutans* in the presence of air. The defective colony formation of the *dpr* mutants in the presence of air was completely reversed by addition of catalase to the plate (Fig. 2).

Growth of *dpr* disruption mutants in liquid medium. To examine *dpr* function in liquid cultures of *S. mutans*, the growth of *dpr*::Spc^r, *dpr*::Spc^r *ahpC*::Em^r::*nox-1*, and *dpr*::Spc^r *sod*::Em^r mutants was measured under aerobic and anaerobic conditions and compared to that of the wild-type parent and the *ahpC*::Em^r::*nox-1* and *sod*::Em^r mutants of *S. mutans*. The following findings became evident. (i) All mutants grew as well as the wild-type strain under anaerobic conditions (data not shown). (ii) Under aerobic conditions, the *dpr*::Spc^r mutant grew as well as the wild-type strain. However, viable cells of the *dpr*::Spc^r mutant at 21 h of incubation were reduced to approximately 40% of the wild-type level (data not shown). The growth of the *ahpC*::Em^r::*nox-1* mutant was partially inhibited (Fig. 3A). However, the *dpr*::Spc^r *ahpC*::Em^r::*nox-1* triple mutant was severely hampered in its ability to grow aerobically (Fig. 3A). (iii) Under aerobic conditions, growth inhibition of the *sod*::Em^r mutant was observed in the first few hours but the *sod*::Em^r mutant grew to almost the same level as the wild type after 10 h of incubation (Fig. 3B). In contrast, the growth of the *dpr*::Spc^r *sod*::Em^r double mutant stopped after 10 h (Fig. 3B). (iv) Addition of catalase or deferoxamine (100 μM), which is a cell-permeating iron chelator, to the growth medium compensated for the growth defect of both the *dpr*::Spc^r *ahpC*::Em^r::*nox-1* and *dpr*::Spc^r *sod*::Em^r mutants (Fig. 3C and D). Heat-inactivated catalase did not compensate for the growth defect of these mutants (Fig. 3C and D).

Identification and iron-binding ability of Dpr. We identified the *dpr* gene product, Dpr, from a crude extract of wild-type *S. mutans*. The wild-type and *dpr* disruption mutant *S. mutans*

strains were grown aerobically to early stationary phase (A_{660} of approximately 3.0). The crude extracts obtained from the cells by sonication were analyzed for Dpr by SDS-PAGE and native PAGE. A protein band of 20 kDa, which corresponds to the size of Dpr, was detected in wild-type cells but not in the *dpr* mutant (Fig. 4A). The 20-kDa protein in the SDS-poly-acrylamide gel was blotted onto a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan), and its amino acid sequence was determined. The N-terminal 14 residues of the
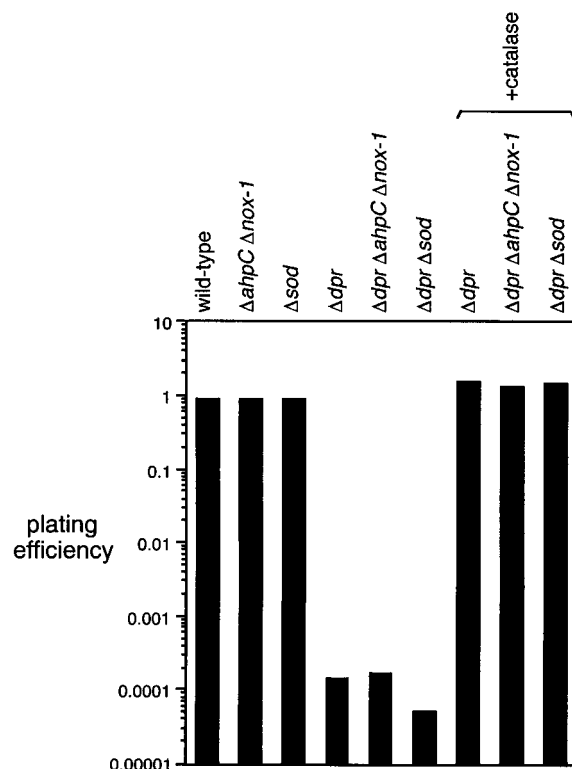

FIG. 2. Aerobic plating efficiency of wild-type and mutant *S. mutans*. Wild-type and mutant cells of *S. mutans* were grown without shaking in TYG at 37°C. At exponential phase, cells were spread onto TYG plates and incubated at 37°C in an anaerobic glove box or under air. After 48 h of incubation, plating efficiency was measured as described in Materials and Methods. The data shown are the means of triplicate determinations.
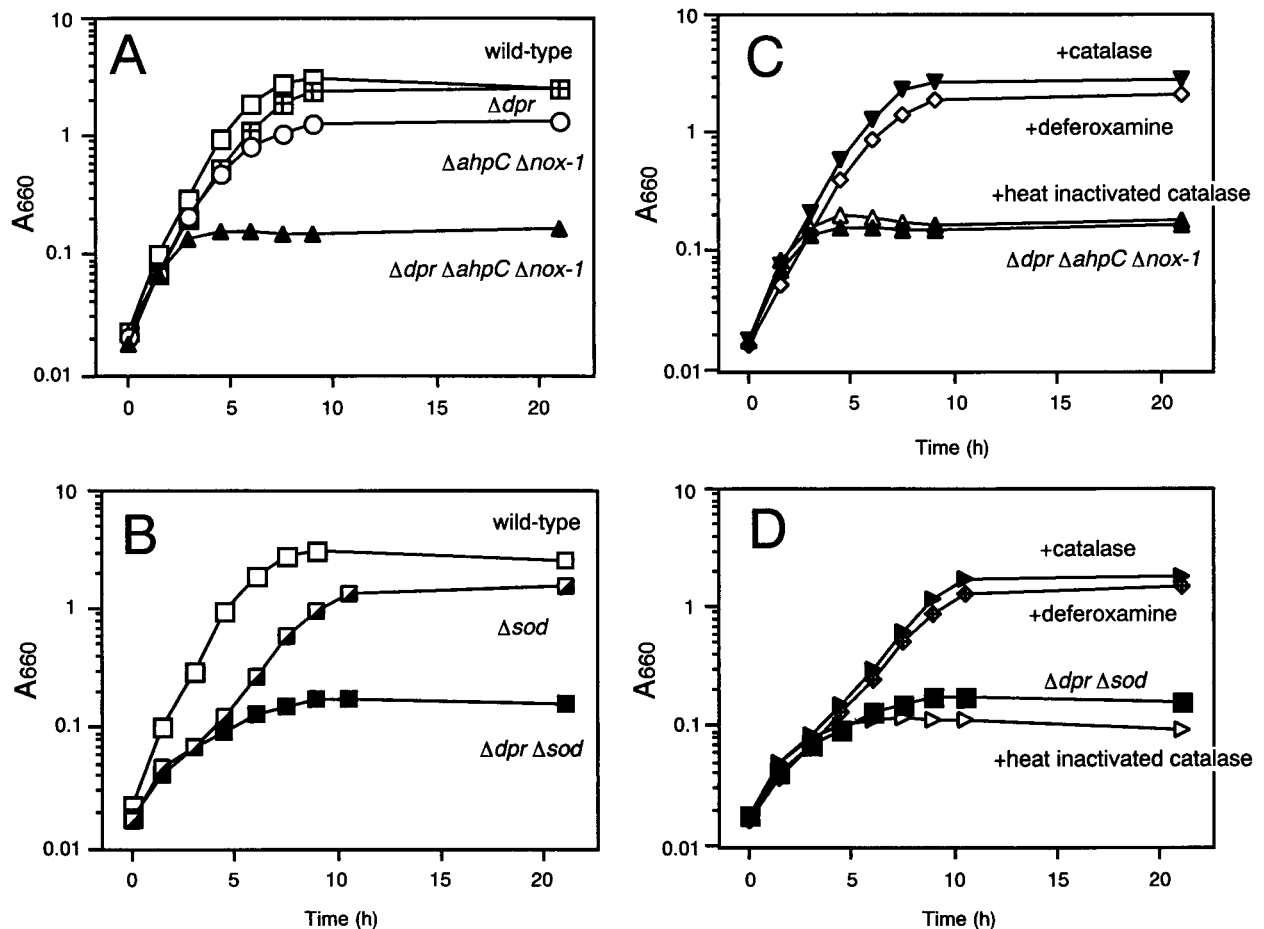
FIG. 3. Aerobic growth of wild-type and mutant *S. mutans* in liquid medium. Wild-type and mutant cells of *S. mutans* were grown in TYG at 37°C without shaking. At late exponential phase, cultures were added to fresh TYG medium with or without catalase or deferoxamine and incubated aerobically at 37°C with shaking. Growth was monitored by measuring A_{660}. Panels A and C emphasize the growth characteristics of *dpr*::Spc[r], *ahpC*::Em[r]::*nox-1*, and combined mutants in the absence or presence, respectively, of catalase or deferoxamine, while panels B and D emphasize the growth characteristics of the *sod*::Em[r] and *dpr*::Spc[r] mutants in the absence or presence, respectively, of catalase or deferoxamine. The data shown are the means of triplicate determinations.

protein were identical to those of the deduced amino sequence of codons 2 to 15 of Dpr. Thus, the 20-kDa protein identified by SDS-PAGE in wild-type *S. mutans* was found to be the *dpr* gene product, Dpr. Native PAGE analysis also revealed the presence of a protein band in the wild-type *S. mutans* crude extract whose N-terminal 11 residues were identical to those of Dpr (Fig. 4B).

It is known that the Dps family of proteins form spherical complexes like ferritin and that some of them bind iron (3, 37). Therefore, the iron-binding ability of Dpr was examined. Native PAGE gels were stained with Ferene S, which is an iron-specific staining reagent. Protein bands corresponding to Dpr and ferritin (used as a positive control) were stained with Ferene S (Fig. 4C). Thus, it was demonstrated that Dpr can bind iron.

Aerobic induction of Dpr. The aerobic induction of Dpr in the wild-type GS-5 strain of *S. mutans* was examined. At early log phase (A_{660} of 0.3), 50 ml of an anaerobic culture (time zero) in an anaerobic box was transferred to a 500-ml flask and incubated with shaking under air. At the times indicated in Fig. 5, samples were taken and the cells were harvested by centrifugation. After cells were disrupted, crude extracts were prepared and analyzed for the 20-kDa protein by SDS-PAGE

(Fig. 5A). Dpr was not detected in cells grown anaerobically (time zero in Fig. 5B). In contrast, cells which were exposed to air started to synthesize Dpr and its relative amount per unit number of cells reached a maximum at 4 h of exposure (Fig. 5B). Thus, it was concluded that the synthesis of Dpr in GS-5 is induced by air.

Comparison of amino acid sequences of Dpr, Dps, and Dps homologues. Dps was found to be a nonspecific DNA-binding protein which accumulates in stationary-phase cells of *E. coli* (1). To date, three family members, including *E. coli* Dps, have been shown to bind to DNA for protection from oxidative stress (1, 7, 28). On the other hand, functional divergence of other Dps family proteins has also been reported, i.e., in the nonheme ferritin of *Listeria innocua* (3), the fine-tangled pilus major subunit of *Haemophilus ducreyi* (5), and the neutrophil-activating protein of *Helicobacter pylori* (37).

The amino acid sequence alignment of *S. mutans* Dpr and proteins of the Dps family which were previously reported was examined. Seven amino acid residues, H[50], G[55], F[58], H[62], E[81], R[82], and D[144], in Dpr were conserved in Dps homologue proteins, suggesting that Dpr is a member of the Dps family. Dps family proteins commonly form multimers of a size smaller than that of ferritin (1, 3, 7, 28, 37). The multimer of *E. coli*
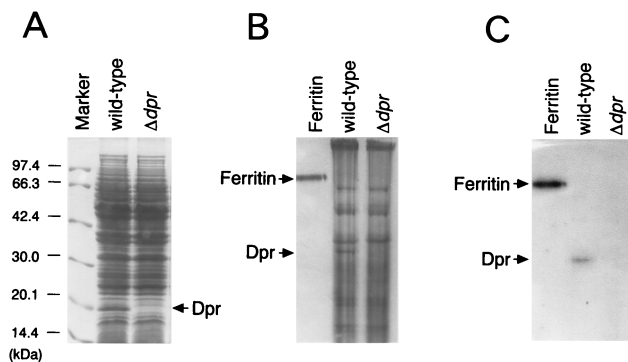
FIG. 4. Identification of Dpr and its iron-binding activity. Crude extracts from cells of wild-type and *dpr*::Spc[r] mutant *S. mutans* grown to early stationary phase were analyzed. (A) SDS-PAGE and staining with Coomassie brilliant blue R250. (B) Native PAGE and staining with Coomassie brilliant blue R250. (C) Native PAGE and staining with Ferene S. Forty micrograms of total protein of wild-type *S. mutans* or the *dpr*::Spc[r] mutant was loaded into each well. Molecular mass markers (A) and 2 μg of ferritin (B and C) were used as standards.

Dps is a dodecamer of four-α-helix bundle monomers (12). The primary structure of Dpr showed only 27.6% identity with that of *E. coli* Dps, but computer prediction of the secondary structure of Dpr indicated that Dpr may also have α-helical segments similar to those of Dps, except for the presence of one additional α-helical segment (E^7-S^{13}) in the N terminus of Dpr. These results suggested that Dpr also forms spherical oligomers like ferritin. Recently, Ilari et al. solved the X-ray crystal structure of *L. innocua* ferritin at 2.35 Å resolution and identified five iron-binding sites, H^{31}, H^{43}, D^{47}, D^{58}, and E^{62} (18a). These amino acid residues were also conserved in Dpr (H^{50}, H^{62}, D^{66}, D^{77}, and E^{81}).

DISCUSSION

Iron has toxic properties in the presence of oxygen. Iron ions stimulate the generation of highly reactive and toxic oxygen species such as hydroxyl radicals. In vitro experiments have
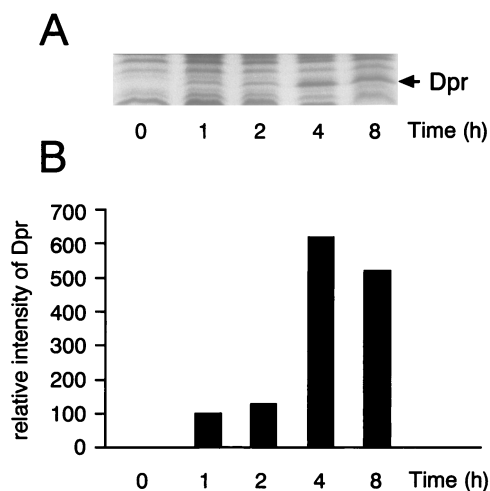

FIG. 5. Quantitative analysis of Dpr in *S. mutans* after exposure of cells to air. Crude extracts of *S. mutans* GS-5 (wild type) were analyzed before and after exposure to air for 1, 2, 4, and 8 h in liquid TYG medium. Forty micrograms of protein from the corresponding extracts was subjected to SDS-PAGE, and the gel was stained with Coomassie brilliant blue R250 (A). Bands corresponding to Dpr (20 kDa) were scanned by a Bio-Rad 620 video densitometer (B).

shown that Fe^{2+} catalyzes nonenzymatic hydroxyl radical synthesis from H_2O_2 via the Fenton reaction (13), but H_2O_2 remained intact in the absence of iron ions at physiological pH (13). Therefore, tight regulation of the intracellular free iron ion concentration is believed to be a significant factor in organisms survival under aerobic conditions.

In this study, we have identified a new antioxidant gene, *dpr*, in the chromosomal DNA of *S. mutans*. After constructing a series of *dpr*::Spc[r] mutants which lost the ability to grow under air, we demonstrated that Dpr plays a vital role in the aerobic survival of *S. mutans*. Dpr was found to be a member of the Dps family of proteins, which form spherical oligomers like ferritin. In fact, Dpr was found to have iron-binding ability (Fig. 4C). Thus, a possible protective role of Dpr might be to sequester iron, thereby protecting cells from peroxides and conferring oxygen tolerance.

Dpr was induced by exposure of *S. mutans* to air and accumulated abundantly in *S. mutans* (Fig. 5). The existence of a high concentration of Dpr in *S. mutans* might enable intracellular free iron ion concentrations to be kept low. The ability of the *ahpC*::Em[r]:*nox-1* mutant of *S. mutans* to grow aerobically might be due to the titration of intracellular free iron ions by Dpr (Fig. 3A), resulting in nonconversion of H_2O_2 to hydroxyl radicals. Neither the *dpr*::Spc[r] *ahpC*::Em[r]:*nox-1* mutant nor the *dpr*::Spc[r] *sod*::Em[r] mutant could grow under air (Fig. 3A and B). In the former case, the high concentrations of intracellular H_2O_2 and free intracellular iron ions might prompt the conversion of H_2O_2 to hydroxyl radicals via the Fenton reaction and lead to cell death. Addition of catalase or deferoxamine to the growth medium complemented the defective growth of not only the former mutant but also the latter mutant (Fig. 3C and D), indicating that the defective growth of the latter mutant may also have been caused by hydroxyl radical formation. It has been reported that O_2^-, derived from SOD deficiency, enhances the Fenton reaction by releasing Fe^{2+} from iron-containing proteins (19, 24). In the *dpr*::Spc[r] *sod*::Em[r] mutant, an increase in the amount of hydroxyl radicals caused by Fe^{2+} would be enough to kill the cell. Wai et al. reported that a ferritin-deficient mutant of *Campylobacter jejuni* shows H_2O_2 and O_2^- sensitivity, indicating that sequestration of iron by ferritin contributes to oxygen tolerance (40). Touati et al. reported that nonheme ferritin, FtnA, which is overexpressed by a multicopy plasmid carrying *ftnA*, suppressed the iron-mediated oxygen sensitivity of *E. coli* (Δ*fur* Δ*recA*) (38). These findings agree well with the results we obtained with *S. mutans*.

One more significant role of protection by Dps and Dps family proteins was considered to be DNA-binding ability. Wolf and Hammes have shown that *E. coli* Dps forms an extensive crystalline lattice in the presence of DNA in vitro and in vivo, proposing that the DNA-Dps cocrystallization mode provides protection of DNA by sequestration (42). On the other hand, it was reported that two members of the Dps family of proteins, *L. innocua* ferritin and *H. pylori* HP-NAP, function as authentic ferritin and cannot bind DNA (3, 37). To elucidate the details of the mechanism of the aerotolerance conferred by Dpr, the molecular properties of Dpr, including its DNA-binding ability, should be explored.

All three mutants of *S. mutans*, *dpr*::Spc[r], *dpr*::Spc[r] *ahpC*::Em[r]:*nox-1*, and *dpr*::Spc[r] *sod*::Em[r], were unable to form colonies on solid media under air (Fig. 2). Unlike the results obtained with liquid cultures, the ability to form colonies was absolutely dependent on Dpr. Addition of catalase to agar plates compensated for the growth defect (Fig. 2), indicating that there exists a significant amount of H_2O_2 in the cell in spite of the presence of *ahpC* and *nox-1*. Our previous study

indicated that the level of expression of AhpC, which is the peroxidatic protein of the bicomponent peroxidase system, was low during the early growth phase under air (18). At an early growth phase of *S. mutans* on solid medium, the cells would also contain a small amount of AhpC protein. Therefore, hydroxyl radicals could be generated from the remaining H_2O_2 to cause cell death in the absence of Dpr on the solid medium. In contrast, the *dpr*::Spc^r mutant could grow aerobically in liquid medium. As H_2O_2 is a highly diffusible oxidant in liquid medium, endogenously synthesized H_2O_2 might permeate the cell membrane and be released easily from the cells until its intracellular and extracellular concentrations reached equilibrium (11). Consequently, at early exponential phase, when both cell density and AhpC levels are low, the intracellular concentration of H_2O_2 might be kept low enough to enable *dpr*::Spc^r mutants to grow (Fig. 3A). From mid-log phase (A_{660} of 0.2), the increase in cell mass would lead to increased production of H_2O_2, causing both internal and external accumulation of H_2O_2. At this stage, the *dpr*::Spc^r *ahpC*::Em^r::*nox-1* mutant could not grow, as mentioned above.

Dpr homologues were also present in other streptococci, including *Streptococcus pyogenes* (B. A. Roe, S. Clifton, M. McShan, and J. Ferretti, Streptococcal Genome Sequencing Project at the University of Oklahoma) and *Streptococcus pneumoniae* (29), and their deduced amino acid sequences showed 75.4 and 57.1% identity, respectively, with that of *S. mutans* Dpr. To date, nonheme peroxidases (30, 31), a pseudocatalase (20), and oxidases (2, 10, 16, 34) of lactic acid bacteria, including streptococci, have been reported to substitute for catalase, which is present in other aerotolerant bacteria but not lactic acid bacteria, and some of them, including SOD, were proven to play a role in aerotolerance (9, 10, 21, 27, 33). Unlike them, Dpr did not directly react with oxygen and reactive oxygen species. The iron-binding ability of Dpr might indirectly contribute oxygen tolerance in *S. mutans*. This mechanism of aerotolerance conferred by Dpr is rational for lactic acid bacteria that are known to lack catalase and to require only low levels of iron for growth.

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