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Regulation of the Intracellular Free Iron Pool by Dpr Provides Oxygen Tolerance to *Streptococcus mutans*

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Dpr is an iron-binding protein required for oxygen tolerance in *Streptococcus mutans*. We previously proposed that Dpr could confer oxygen tolerance to the bacterium by sequestering intracellular free iron ions that catalyze generation of highly toxic radicals (Y. Yamamoto, M. Higuchi, L. B. Poole, and Y. Kamio, J. Bacteriol. 182:3740-3747, 2000; Y. Yamamoto, L. B. Poole, R. R. Hantgan, and Y. Kamio, J. Bacteriol. 184:2931-2939, 2002). Here, we examined the intracellular free iron status of wild-type (WT) and *dpr* mutant strains of *S. mutans*, before and after exposure to air, by using electron spin resonance spectrometry. Under anaerobic conditions, free iron ion concentrations of WT and *dpr* strains were 225.9 ± 2.6 and 333.0 ± 61.3 μM, respectively. Exposure of WT cells to air for 1 h induced Dpr expression and reduced intracellular free iron ion concentrations to 22.5 ± 5.3 μM; under these conditions, *dpr* mutant cells maintained intracellular iron concentration at 230.3 ± 28.8 μM. A decrease in cell viability and genomic DNA degradation was observed in the *dpr* mutant exposed to air. These data indicate that regulation of the intracellular free iron pool by Dpr is required for oxygen tolerance in *S. mutans*.

Iron is an essential and beneficial nutrient for most organisms but has toxic properties in the presence of oxygen (4, 15, 31). Iron ions stimulate the generation of highly reactive and toxic oxygen species such as hydroxyl radicals (11, 15). In vitro experiments have shown that Fe(II) catalyzes nonenzymatic hydroxyl radical formation from hydrogen peroxide via the Fenton reaction, whereas hydrogen peroxide remained intact in the absence of iron ions at physiological pH (11). Intracellular iron source for the Fenton reaction is considered to be a free iron pool in cells (15, 17). Tight regulation of iron metabolism, especially the intracellular free iron pool, is therefore regarded as a determining factor for survival of an organism in air (11, 15, 17, 31).

Methods to investigate the intracellular free iron pool in intact cells were recently developed, and the presence of several factors affecting the free iron status was reported in both prokaryotes and eukaryotes (17, 19). It has been reported that, in *Escherichia coli* and *Saccharomyces cerevisiae*, accumulation of intracellular superoxide, owing to a superoxide dismutase deficiency, increases the level of free iron pool by releasing iron ions from proteins containing iron-sulfur clusters (19, 29). In an *E. coli fur* mutant, aberrant regulation of iron uptake was associated with an increase in the level of free iron (19). In mammalian cells, repression of ferritin H subunit expression increased the level of intracellular free iron (17). In all reported cases, an increase in the free iron pool correlated with an increase in oxidative stress (17, 19, 29, 31).

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Streptococcus mutans, a principal causative agent of human dental caries, cannot synthesize heme and lacks both a respiratory chain and catalase, which are required for elimination of hydrogen peroxide in most aerobic organisms. However, *S. mutans* grows under aerobic conditions and induces several antioxidant proteins when cells are exposed to air (12, 13, 24, 27, 35–37). We previously identified *dpr* (for *dps*-like peroxide resistance) as a potential peroxide resistance gene from *S. mutans*. Studies of a series of *dpr*-deficient strains led us to conclude that *dpr* plays a vital role in aerobic survival of *S. mutans* (36). Our further studies on the purified *dpr* gene product showed that Dpr forms ferritin-like spherical dodecamers and binds up to 480 iron atoms per complex (37). Primary amino acid sequence homologies indicate that Dpr is a member of the Dps (for DNA-binding protein from starved cells) (3) protein family (36). Dps is a nonspecific DNA-binding protein that is induced by oxidative or nutrient stress in *E. coli* (3). Stable Dps-DNA complex formation is believed to protect DNA from hydrogen peroxide action (3, 21, 33). However, in the case of *S. mutans*, Dpr could not bind DNA (37). We therefore proposed another mode of cell protection from oxidative stress by Dpr, based on its sequestration of intracellular iron ions. We demonstrated in vitro that Dpr prevents iron-dependent hydroxyl radical formation (36). At almost the same time, Zhao et al. reported iron-binding and iron-detoxifying properties of *E. coli* Dps (38). It was also reported that some Dps family proteins having iron binding, but not DNA-binding ability, were involved in oxidative stress resistance (8, 16, 28). The crystal structure of Dps family proteins, including *Streptococcus suis* Dpr homologue, revealed a ferritin-like structure of the proteins, indicating that this class of proteins could incorporate iron ions as ferritin does (8, 10, 14, 18, 38). Taken together with our data on Dpr properties, it was suggested that Dps family proteins could affect the cellular free

iron ion status, thereby conferring oxygen tolerance. In the present study, we measured the intracellular free iron pool of wild-type (WT) and *dpr* strains of *S. mutans* and clarified the role of Dpr in regulating the intracellular free iron pool and on bacterial survival in air.

MATERIALS AND METHODS

Strains, media, and growth conditions. *S. mutans* GS-5 (WT strain) and DES (*dpr*-deficient mutant) (36) were used in the present study. Cells were prepared for analysis by electron spin resonance (ESR) spectrometry as follows. A 10-ml preculture of *S. mutans*, prepared in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) under anaerobic conditions (in an anaerobic glove box [Hirasawa Works, Tokyo, Japan] in an atmosphere of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen), was added to a 300-ml culture in the same medium. The culture was incubated at 37°C for 3.5 h (A_{660} = ~0.8) under anaerobic conditions. At this time, part of the culture (100 ml) was removed and used as the zero time sample. The remaining culture (200 ml) was centrifuged at 7,800 × *g* for 5 min, resuspended in the same volume of fresh THB medium, transferred to 500-ml flasks, and then incubated at 37°C with shaking (120 cycles/min). After 30 min of incubation, 100 ml of the culture was removed as the 30-min sample. The rest of the culture was incubated for another 30 min and used as the 60-min sample.

ESR spectrometry sample preparation. Portions (100 ml) of the cultures described above were centrifuged at 7,800 × *g* for 5 min. Pellets were resuspended in 5 ml of THB medium with or without 20 mM deferoxamine (Sigma) and then incubated at 37°C with shaking (170 cycles/min) for 10 min under aerobic conditions. Cells were collected by centrifugation at 7,800 × *g* for 5 min, washed with ice-cold 20 mM Tris-HCl buffer at pH 7.0, and resuspended in 0.3 ml of the same buffer containing 10% (vol/vol) glycerol. An aliquot of each sample was taken to measure the optical density at 660 nm. Then, 200 μl of each cell suspension was transferred to a quartz ESR tube, immediately frozen, and stored at −80°C until ESR measurements were carried out.

ESR spectrometry. ESR spectra were recorded on an RE-3X ESR spectrometer (JEOL, Ltd., Tokyo, Japan). Samples were maintained at −196°C by using a finger Dewar vessel filled with liquid nitrogen. Experimental conditions used for low-temperature Fe(III) electron paramagnetic resonance (EPR) were as follows: center field, 250 mT; sweep width, 150 mT (250 mT for wider sweep); frequency, 9.21 GHz; microwave power, 5 mW; modulation amplitude, 1 mT; modulation frequency, 100 kHz; receiver gain, 1×100; sweep time, 4 min; and time constant, 0.03 s. The *g* value was calculated by using the standard formula $g = h\nu/\beta H$, where *h* is Planck's constant, *v* is the frequency, β is the Bohr magneton, and H is the external magnetic field at resonance.

Calculation of intracellular free iron concentration. The double-integrated intensities of the $g = 4.3$ signal of each sample were converted to intracellular free iron ion concentrations as follows. The amount of deferoxamine-Fe(III) in the ESR sample was quantified by using the EPR signals of deferoxamine-Fe(III) of known concentrations. First, 1 ml of cell suspension (optical density at 600 nm of 1.0) was calculated to contain 0.58 μl of intracellular water volume, based on (i) the reported internal water content in *S. mutans* cells of 1.6 μl per mg (dry weight) (25) and (ii) the fact that 1 ml of cell suspension (A_{660} = 1.0) contained 0.365 ± 0.034 mg (dry weight). We used this value, along with the ESR signal from an external Fe(III) standard and the optical density of the ESR sample, to quantify intracellular free iron concentrations.

Measurement of total iron. *S. mutans* cells were collected by centrifugation at 7,800 × *g* for 10 min. Cells were washed once with phosphate-buffered saline (pH 7.0) and twice with Milli-Q water (Millipore Corp., Tokyo, Japan). Washed cells were resuspended in 1 ml of Milli-Q water and then transferred to a Teflon container. Water was removed from cells by incubation at 90°C for 20 h, and the bacterial dry weight was measured. Next, 2 ml of concentrated nitric acid (Ultratrace analysis grade; Wako Pure Chemical Industries, Osaka, Japan) and 0.2 ml of concentrated perchloric acid (Ultrapure AA-100; Tama Chemicals, Kanagawa, Japan) were added to about 100 mg of dried bacterial cells in a Teflon container, and the cells were dissolved into liquid by microwave treatment as described previously (23). After the cells were dissolved, the containers were heated on a hot plate at 160°C to near dryness and then dissolved in 5 ml of 5% nitric acid solution for analysis by atomic absorption spectrometry with an atomic absorption spectrometer (170-30; Hitachi, Tokyo, Japan). The iron content and bacterial cell dry weight of samples, coupled with the reported internal water content in *S. mutans* cells of 1.6 μl per mg (dry weight) (25), allowed us to quantify the total iron concentration in the cell.

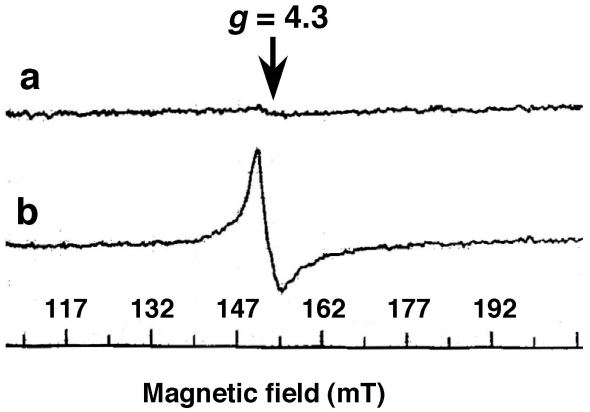

FIG. 1. ESR results of nontreated and deferoxamine-treated WT *S. mutans* cells. Anaerobically grown *S. mutans* WT cells (late exponential phase) were used to obtain whole-cell ESR spectra. ESR spectra of *S. mutans* cells not treated (a) or treated (b) with deferoxamine are shown.

Monitoring survival, genomic DNA degradation, and expression of Dpr. For viable cell determinations, culture dilutions were plated on solid THB medium supplemented with 500 U of bovine liver catalase (Sigma). After 48 h of incubation in an anaerobic box at 37°C, the CFU were counted. Genomic DNA of *S. mutans* was prepared as described previously (35), with some modifications. Cells were treated with both mutanolysin (200 U/ml; Sigma) and acromopeptidase (1,000 U/ml; Wako) for 15 min at 37°C in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA prior to lysis by sodium dodecyl sulfate. DNA samples (500 ng) were electrophoresed on a 1% Tris-acetate agarose gel and then visualized by ethidium bromide staining. For Western blot analyses, cell lysates were prepared as described previously (36) and separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. Protein bands corresponding to Dpr were identified as described by using anti-Dpr antibody (37).

RESULTS AND DISCUSSION

Pool size of intracellular free iron in WT and *dpr*-deficient mutant of *S. mutans*. The intracellular free iron pool was measured by ESR spectrometry essentially according to the method of Woodmansee and Imlay (34). Anaerobically grown *S. mutans* cells were treated with deferoxamine, a cell-permeable iron chelator, that converts "free iron" to the Fe(III) species. This species gives an intense ESR signal at $g = 4.3$ (34). Representative low-temperature Fe(III) ESR spectra of whole *S. mutans* cells are shown in Fig. 1. A strong signal at $g = 4.3$ was obtained for deferoxamine-treated *S. mutans* cells. The Fe(III) ESR signal at $g = 4.3$ is characteristic of ferric iron in a high spin complex (34). In contrast, the ESR spectrum of iron-loaded Dpr, which was prepared as previously described (37), gave a very broad ESR signal at a *g* value of ca. 2 but did not give an ESR signal at a *g* value of 4.3 (data not shown). Thus, the signal at $g = 4.3$, obtained for *S. mutans* cells treated with deferoxamine, is distinguishable from the signal corresponding to Dpr-bound iron.

We used ESR spectrometry to compare intracellular free iron concentrations in WT and *dpr* strains before and after exposure to air (Fig. 2). Under anaerobic conditions, similar intensities of signal at $g = 4.3$ were detected for both strains. Upon exposure to air, the intensity of signal obtained for WT cells decreased. In contrast, the intensity of signal for the *dpr*-deficient cells remained high (Fig. 2).

The double-integrated intensities of ESR signals were then

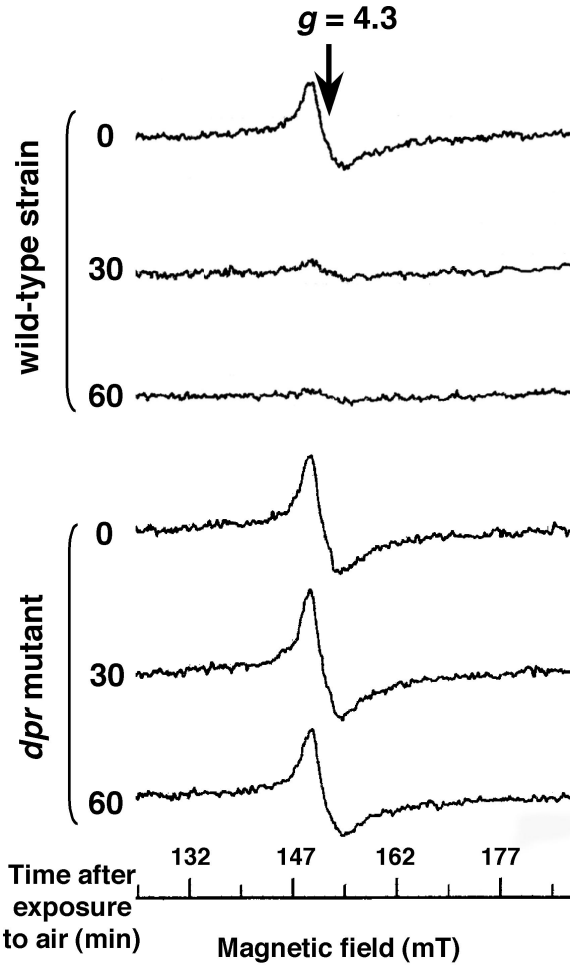

FIG. 2. ESR results of WT and *dpr S. mutans* cells. ESR spectra of deferoxamine-treated WT and *dpr* mutant strains before (0 min) or after (30 and 60 min) exposure to air are shown. The zero time samples were processed in the presence of 50 μg of chloramphenicol/ml to prevent protein synthesis during preparation. ESR results typical of those obtained in three independent experiments performed for each strain and condition tested are shown.

converted to intracellular free iron ion concentrations (see Materials and Methods). Under anaerobic conditions, WT and *dpr* strains had intracellular free iron concentrations of 225.9 ± 2.6 and 333.0 ± 61.3 μM, respectively (Fig. 3A and B). After cell exposure to air for 1 h, the values of the WT strain dropped to 22.5 ± 5.3 μM; in contrast, values for the *dpr* mutant remained high, at 230.3 ± 28.8 μM (Fig. 3A and B). Total iron content in cells, determined by atomic absorption spectrometry, is also shown (Fig. 3A and B). In contrast to the rapid decrease of intracellular free iron in the WT strain, total iron content decreased by only 13% after 1 h of exposure to air, indicating that the decrease of free iron concentrations did not result from the change of total iron content but rather from the change of iron status inside the cell. Western blot analysis of Dpr expression under the different growth conditions showed that Dpr synthesis was induced by exposing cells to air (Fig. 3C). In WT cells, the decrease in intracellular free iron concentrations correlated with increased Dpr expression (Fig. 3). In order to examine whether Dpr synthesis is really responsible for attenuating intracellular free iron concentrations, chloramphenicol (50 μg/ml) was added to the WT culture prior to aerobic incubation. After 1 h of incubation in air, cells were analyzed by Western blotting and ESR spectrometry. Chloramphenicol treatment of WT cells prevented Dpr expression (data not shown) and resulted in higher intracellular free iron concentrations (468.5 ± 15.6 μM). These results strongly indicate that, in *S. mutans*, Dpr incorporates free iron ions in vivo and contributes to lowering the intracellular free iron ion concentration.

In the *dpr* mutant, total iron amounts decreased more rapidly than in the WT strain after exposure of the cells to air (Fig. 3A and B). After 1 h of incubation of the *dpr* mutant in air, both total and free iron concentrations decreased by ca. 30%. This result implies that decreased iron uptake in the *dpr* mutant, due to cell death as described below, might reduce the intracellular free iron concentrations in the *dpr* mutant by reducing the total iron content.

Effects of high intracellular free iron concentrations on growth, survival, and DNA degradation of *S. mutans*. In the presence of oxygen, excess amounts of intracellular free iron ions may catalyze the generation of reactive oxygen species that degrade cellular components and cause cell death (15, 31). We explored the effects of high intracellular free iron concentrations on growth and survival of the *dpr* mutant (Fig. 4A). Cell densities of both WT and *dpr* mutant strains slightly increased during the incubation period in air. However, the number of *dpr* mutant CFU decreased 100-fold after 1 h of exposure of the cells to air, whereas the number of WT strain CFU remained constant (Fig. 4A). A main target of oxygen-induced cellular damage is DNA (15, 31). The effect of aeration on genomic DNA extracted from WT and *dpr* mutant strains was examined by gel electrophoresis (Fig. 4B). Under anaerobic conditions, no significant differences in electrophoretic mobility were observed between genomic DNA from WT and *dpr* mutant strains. After exposure to air, however, marked degradation of DNA was observed in the *dpr* mutant extract (Fig. 4B). DNA integrity and cell survival of the *dpr* mutant were restored by the addition of catalase or deferoxamine, each of which removes a substrate for the Fenton reaction, to growth medium during cell exposure to air (Fig. 5). These results strongly indicate that iron-mediated generation of hydroxyl radicals via the Fenton reaction degraded cellular components such as DNA and caused cell death in the *dpr* mutant.

The present study provided the first direct evidence that Dps family proteins can affect the intracellular free iron pool. Pulliainen et al. recently identified a Dpr homologue in *Streptococcus suis* as an iron-binding protein and demonstrated, by introducing the site-mutated alleles of Dpr into an *S. suis dpr* mutant, that the iron-binding ability of the protein is required for hydrogen peroxide resistance (28). The result is in agreement with our present study with *S. mutans*. It is now evident that ferritin-like iron binding is a common feature of Dps family proteins (6, 8, 16, 28, 30, 32, 37, 38). It is therefore not surprising that Dps family proteins could potentially participate in the regulation and detoxification of intracellular free iron pool, as we described here. The presence of Dpr and Dps family proteins may prevent an iron-catalyzed Fenton reaction and, as a consequence, play an important role in oxygen tol-

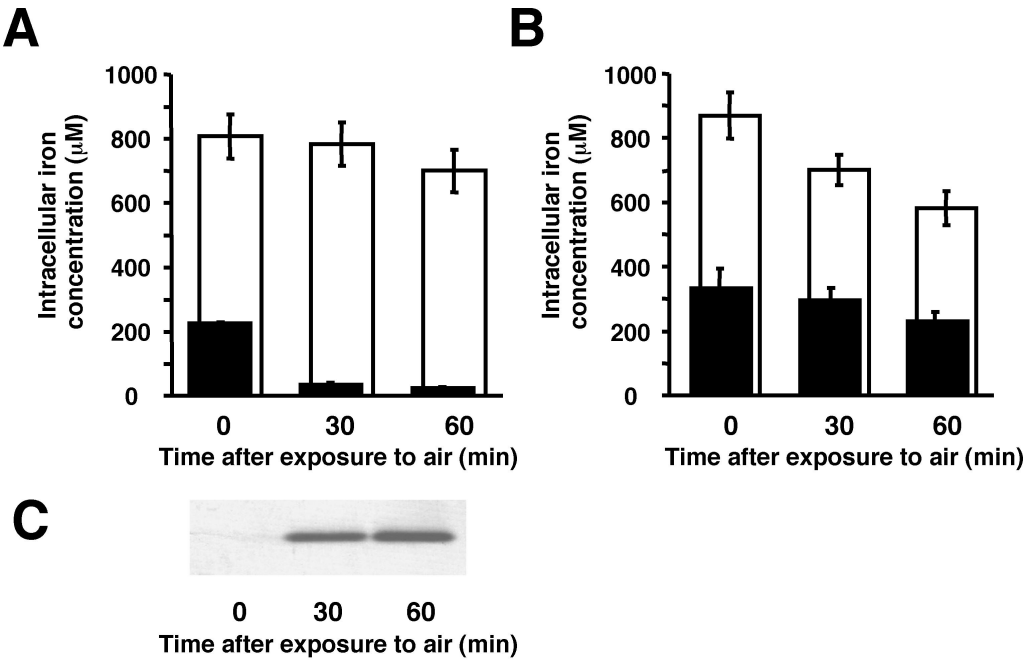

FIG. 3. Intracellular free iron ion concentrations of WT and *dpr* strains of *S. mutans*. Total iron content (□) and intracellular free iron ion concentrations (■) of the *S. mutans* WT strain (A) and *dpr* mutant (B) before (0 min) or after (30 and 60 min) exposure of cells to air are shown. The total iron content and intracellular free iron concentrations were calculated as described in Materials and Methods. The results are the means ± standard deviations for triplicate determinations. (C) Expression of Dpr in *S. mutans* WT strain upon exposure to air. Expression of Dpr was analyzed by immunoblotting with Dpr-specific antibody for detection. Each lane was loaded with 1.25 μg of protein of the corresponding extract. A result typical of three independent experiments is shown.

erance, especially in catalase-deficient bacteria such as *S. mutans* and *S. suis*.

The addition of catalase to the medium restored the survival of the *dpr* mutant under air (Fig. 5), indicating the presence of hydrogen peroxide in the cells under this condition. Several lactic acid bacteria are known to accumulate hydrogen peroxide in the medium via the action of hydrogen peroxide-generating enzymes such as pyruvate oxidase and NADH oxidase (7, 22, 26). *S. mutans* lacks pyruvate oxidase but has hydrogen peroxide-forming NADH oxidase (Nox-1) (2, 13). Although Nox-1 functions as a component of bicomponents, peroxidase and the AhpC component (27), the expression of only Nox-1

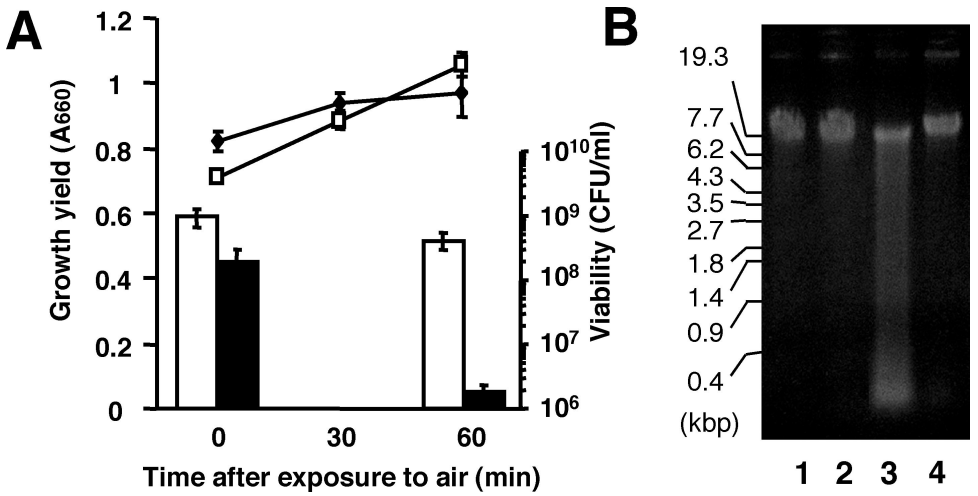

FIG. 4. Growth, survival, and genomic DNA degradation of *S. mutans* WT and *dpr* strains before or after cell exposure to air. (A) Growth of WT strain (□) and *dpr* mutant (●) were monitored by measuring optical density at 660 nm. Respective CFU values are shown as white and black bars. The results presented are the means ± standard deviations for triplicate determinations. (B) Genomic DNAs extracted from WT and *dpr* mutant strains before or after exposure to air were analyzed by gel electrophoresis. Genomic DNAs of the *dpr* mutant at 0 min (lane 1), WT strain at 0 min (lane 2), *dpr* mutant after 60 min (lane 3), and WT strain after 60 min (lane 4) are shown. A result typical of three independent experiments is shown.

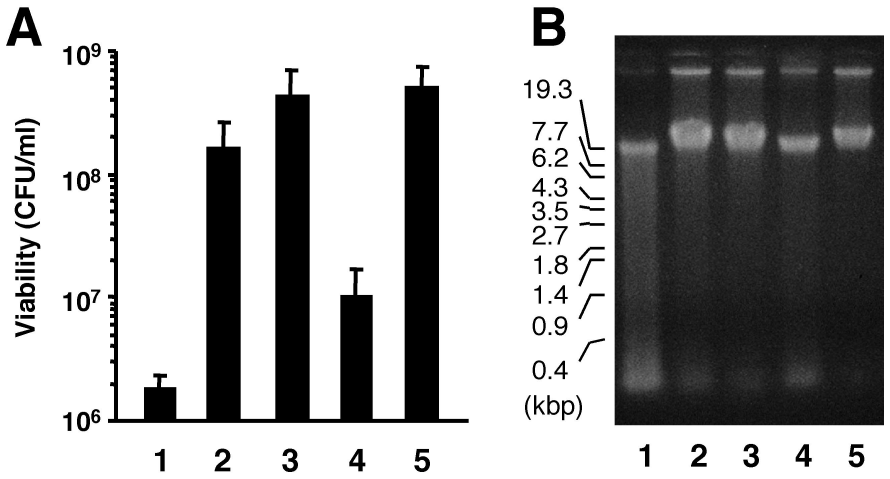

FIG. 5. Effects of catalase and deferoxamine on survival and DNA degradation of *dpr* mutant. Either catalase or deferoxamine was added to an anaerobic culture of the *dpr* strain, which was further incubated at 37°C with aeration by shaking (120 cycles/min) for 60 min. CFU (A) and electrophoretic profiles of genomic DNA (B) were analyzed. *dpr* mutant (lane 1), *dpr* mutant supplemented with 100 or 1,000 U of bovine liver catalase (lanes 2 and 3)/ml and *dpr* mutant supplemented with 0.1 or 1 mM deferoxamine (lanes 4 and 5) are shown. In panel A, the results are the means ± standard deviations for triplicate determinations. In panel B, results typical of three independent experiments are shown.

(absence of AhpC) under conditions described previously (13) might allow the bacterium to produce hydrogen peroxide.

An interesting finding of the present study is that *S. mutans* cells contained significant amounts of intracellular iron (Fig. 3), particularly since lactic acid bacteria including streptococci are believed to require little or no iron for growth (5). The total iron contents in *S. mutans* (from 0.005 to 0.008% in dry weight) were some 2.5- to 4-fold less than that in *E. coli* grown in rich medium (1). Although the iron requirement in *S. mutans* reportedly depends on growth conditions (20), iron assimilation could facilitate metabolism, e.g., for amino acid biosynthesis utilizing the iron-containing protein aconitase (9) or potentially for activating iron-requiring ribonucleotide reductases identified in the genome sequence (2).

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