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Purification, Gene Cloning, Gene Expression, and Mutants of Dps from the Obligate Anaerobe *Porphyromonas gingivalis*

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The periodontopathogen *Porphyromonas gingivalis* is an obligate anaerobe that is devoid of catalase but exhibits a relatively high degree of resistance to peroxide stress. In the present study, we demonstrate that *P. gingivalis* contains a Dps homologue that plays an important role in the protection of cells from peroxide stress. The Dps protein isolated from *P. gingivalis* displayed a ferritin-like spherical polymer consisting of 19-kDa subunits. Molecular cloning and sequencing of the gene encoding this protein revealed that it had a high similarity in nucleotide and amino acid sequences to Dps proteins from other species. The expression of Dps was significantly increased by exposure of *P. gingivalis* to atmospheric oxygen in an OxyR-dependent manner, indicating that it is regulated by the reactive oxygen species-regulating gene *oxyR*. The Dps-deficient mutants, including the *dps* single mutant and the *ftn dps* double mutant, showed no viability loss upon exposure to atmospheric oxygen for 6 h. In contrast to the wild type, however, these mutants exhibited the high susceptibility to hydrogen peroxide, thereby disrupting the viability. On the other hand, no significant difference in sensitivity to mitomycin C and metronidazole was observed between the wild type and the mutants. Furthermore, the *dps* single mutant, compared with the wild type, showed a lower viability in infected human umbilical vein endothelial cells.

Atmospheric oxygen is metabolically converted to reactive oxygen species (ROS), including superoxide anion radical, hydrogen peroxide, hydroxy radical, and singlet oxygen, in bacterial cells. ROS are also generated by phagocytic host cells such as polymorphonuclear leukocytes and macrophages and attack invading bacterial cells. It is widely recognized that two cellular systems function to protect organisms from oxidative stresses (15, 33). One is regulated by antioxidant enzymes in which molecular oxygen and ROS are diminished or eliminated (42). Superoxide dismutase (SOD), catalase, peroxidase, and oxidase are involved in this reaction. The other is catalyzed by endonucleases by which oxidatively damaged nucleic acids are repaired. This includes *Escherichia coli* exonuclease III and endonuclease IV (51). These two systems cooperatively function to minimize the detrimental effects of ROS upon cells, as evidenced by the presence of common regulatory genes such as *oxyR* (43).

Porphyromonas gingivalis is a gram-negative obligate anaerobe belonging to the division *Cytophagales* (23). This bacterium is one of the organisms that is most strongly associated with chronic adult periodontitis and expresses numerous potential virulence factors, such as fimbriae, hemagglutinins, lipopolysaccharides, and various proteases that are capable of hydrolyzing collagen, immunoglobulins, iron-binding proteins, and complement factors (21, 27). *P. gingivalis*, by definition, cannot grow in aerobic conditions but exhibits a high degree of aerotolerance. This aerotolerance enables the organism to survive in periodontal pockets that are occasionally exposed to aerobic conditions.

P. gingivalis posseses SOD that is essential for tolerance to atmospheric oxygen, as revealed by the finding that *P. gingivalis sod* mutant shows a rapid viability loss by exposure to atmospheric oxygen (34), although it exhibits a marked resistance to peroxide stress. It has been demonstrated that the Dps (DNA-binding protein from starved cells) protein in *E. coli* plays an important role in the protection of cells from peroxide stress (1, 2). This protein is produced primarily in the stationary-phase cells, and its expression is regulated by σ^{38}, σ^{70}, and OxyR. Structurally, the Dps protein forms a ferritin-like spherical oligomeric structure. In addition, the Dps monomer displays essentially the same protein fold (four-helix bundle) as the ferritin monomer (17). It is of special importance that *E. coli* Dps exhibits DNA- and iron-binding activities by which the cells probably gain the resistance to oxidative stresses. Recent studies have demonstrated that a diverse group of Dps homologues are found in various prokaryotes, including *Synechococcus* sp., *Bacillus subtilis*, *Listeria innocua*, *Streptococcus mutans*, and *Bacteroides fragilis* (5, 6, 36, 38, 49), and are related to the ferritin-bacterioferritin-rubrerythrin superfamily (3, 17).

In the present study, we provide the first evidence indicating the presence of a Dps homologue in *P. gingivalis*. We have also constructed the Dps-deficient mutants to analyze the function of this protein in the organism. The results clearly indicate that the Dps protein is implicated in the protection of the organism

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
P. gingivalis		
ATCC 33277	Wild type	ATCC
KDP139	*ftn*::Em^r Em^r	37
KDP141	*dps*::Tc^r Tc^r	This study
KDP142	*ftn*::Em^r *dps*::Tc^r Em^r Tc^r	This study
KDP143	*oxyR*::Tc^r Tc^r	This study
KDP146	*dps*$^+$ *dps′-′lacZ* Em^r	This study
KDP148	*dps*$^+$ *dps′-′lacZ oxyR*::Tc^r Em^r Tc^r	This study
E. coli		
BL21 (DE3)	*ompT hsdSB* with a λ prophage carrying the T7 RNA polymerase gene	44
DH5α	*supE44* Δ*lacU169*(ϕ80*lacZ*Δ*M15*) *hsdR17 relA1 endA1 gyrA96 thi-1*	18
TA4112	*araD139* Δ(*argF-lac*)*205 flbB5301 non-9 gyrA219 relA1 rpsL150 metE70* Δ(*oxyR btuB*)*3*	8
Plasmids		
pET11a	Ap^r, overexpression plasmid	Novagen
pUC19	Ap^r, cloning vector plasmid	50
pCR2.1	Ap^r, PCR TA cloning vector plasmid	Invitrogen
pKD355	Ap^r Em^r, contains the *ermF ermAM* DNA block of pVA2198 (16) between *Eco*RI and *Bam*HI of pUC18	This study
pKD375	Ap^r Tc^r, contains the *tetQ* DNA block	40
pKD386	Ap^r *dps*$^+$, contains 2.6-kb *Sau*3AI chromosomal fragment (*dps* region) of *P. gingivalis* in pUC19	This study
pKD387	Ap^r *dps* (deletion), contains 4 bp deletion at *Bst*XI within *dps* of pKD386	This study
pKD388	Ap^r *dps*::Tc^r, contains the *tetQ* DNA block at *Bst*XI within *dps* of pKD386	This study
pKD390	Ap^r, pET11a containing *P. gingivalis dps*	This study
pKD391	Ap^r, contains 3.1-kb *lacZ* fragment PCR-amplified from pRS414 (40) in pCR2.1	This study
pKD392	Ap^r Em^r, contains 3.1-kb *Eco*RI *lacZ* fragment of pKD39 in *Eco*RI site of pKD355	This study
pKD393	Ap^r Em^r, *lacZ* reporter suicide/integration plasmid, contains unique *Eco*RI and *Bam*HI at *lacZ* fusion sites	This study
pKD394	Ap^r Em^r *dps′-′lacZ*, contains promoter and 5′-terminal region of *dps* in pKD393	This study
pKD396	Ap^r *oxyR*$^+$, contains *P. gingivalis oxyR*$^+$ in pUC19	This study
pKD397	Ap^r *oxyR*::Tc^r, contains the *tetQ* DNA block at *Bsp*EI within *oxyR* of pKD396	This study

from peroxide stress, thereby contributing to its survival in periodontal pockets and host cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in the present study are listed in Table 1.

Media and growth conditions. Unless otherwise specified, *P. gingivalis* was grown in an anaerobic atmosphere (10% CO_2, 10% H_2, 80% N_2) at 37°C. Enriched brain heart infusion (BHI) medium (containing 37 g of BHI [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract (Difco), 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K_1/liter), enriched tryptic soy (TS) agar (containing 40 g of Trypto-Soya agar [Nissui, Tokyo, Japan], 5 g of BHI, 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K_1/liter), and blood agar prepared by adding hemolyzed defibrinated sheep blood to enriched TS agar at 5% were used for *P. gingivalis*. *E. coli* was grown at 37°C in L broth or on L agar (L broth solidified with 1.5% agar). Erythromycin (10 μg/ml), tetracycline (0.5 μg/ml), and ampicillin (50 μg/ml) were added as required for selection and maintenance of the strains.

Purification of *P. gingivalis* ferritin-like protein and determination of its N-terminal amino acid sequence. *P. gingivalis* ATCC 33277 was grown in enriched BHI broth for 48 h. The cells were harvested by centrifugation at 15,000 × *g* for 20 min at 4°C. The pellet was suspended in 30 ml of buffer A (10 mM Tris-HCl buffer [pH 7.5]), and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (Sigma Chemical Co., St. Louis, Mo.) and leupeptin (Peptide Institute, Osaka, Japan) were added to final concentrations of 0.1 and 1 mM, respectively. The cells were broken by sonication (25 W; 30 pulses/min; 1-s pulse length) in a Branson sonicator with 1-min intervals for 10 min on ice. The sonicate was shaken for 15 min at 37°C and centrifuged to remove the unbroken cells. The supernatant was saved, and CsCl was added to a final concentration of 40% (wt/vol), followed by centrifugation at 80,000 × *g* for 24 h at room temperature. The solution was carefully separated into fractions, and each fraction was dialyzed overnight against buffer A by changing the buffer at 6-h intervals. The fractions were then examined by electron microscopy. The fraction containing ferritin-like particles was concentrated by ultrafiltration with a Microcon YM-10 (Millipore Corp., Bedford, Mass.). For further purification, the concentrated ferritin-like particles were subjected to gel filtration on a Sephadex FPLC column (FPLC column TM200; Pharmacia, Uppsala, Sweden). Proteins were eluted with buffer A at a flow rate of 0.5 ml/min. Protein elution was monitored by measuring the absorbance at 280 nm. Each fraction was examined by electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the presence of ferritin-like particles.

To determine the amino-terminal amino acid sequence of this ferritin-like protein, the protein on the polyacrylamide gel was electrophretically transferred onto polyvinylidene difluoride membrane and stained with Coomassie brilliant blue R-250. The stained protein band was cut out and analyzed in an automatic protein sequencer (Applied Biosystems model 476A; Perkin-Elmer Cetus, Norwalk, Conn.).

Molecular cloning of *P. gingivalis dps* and construction of recombinant plasmids and bacterial strains. *Sau*3AI plasmid library (pUC19) of *P. gingivalis* ATCC 33277 chromosomal DNA was introduced to *E. coli* TA4112 (*oxyR*) after amplification of the library in DH5α and a recombinant plasmid clone (pKD386) that conferred resistance to *t*-butyl hydroperoxide on the *oxyR* strain was obtained. The *Bst*XI site within *dps* of pKD386 was eliminated with T4 DNA polymerase to yield pKD387. A *tetQ* DNA block of pKD375 (40) was inserted into the *Bst*XI site of pKD386 to yield pKD388. The *dps* gene DNA was PCR amplified from pKD386 with primers (an upper primer [CCATATGAAAAAG ATTCTTGAAGTAACGGG] and a lower primer [GGGATCCTTACTTGGC AGCGTAGGCAGACA]) and introduced to pET11a, resulting in pKD390.

For construction of the *lacZ* reporter suicide-integration plasmid, a 3.1-kb *lacZ* region DNA was PCR amplified from pRS414 DNA (41) with PCR primers (an upper primer [CGGAATTCCCGGGGATCCCGTCGTT] and a lower primer [AAGATCTTATTTTTGACACCAGACCAACTGG]) and ligated to the linear pCR2.1 DNA by using the TA cloning method, resulting in pKD391. *E. coli* DH5α harboring pKD391 showed β-galactosidase activity, indicating that the subcloned *lacZ* gene was functional. A 3.1-kb *Eco*RI fragment of pKD391 containing the *lacZ* gene was then inserted to the *Eco*RI site of pKD355, resulting in pKD392. *Eco*RI and *Bam*HI sites other than the *Eco*RI-*Sma*I-*Bam*HI multiple cloning sites were eliminated in pKD392 by partial restriction digestion and Klenow filling, giving rise to pKD393.

For construction of a *dps′-′lacZ* fusion plasmid, a DNA fragment comprising the 5′-terminal region of *dps* and its upstream region was PCR amplified from

the chromosomal DNA of *P. gingivalis* ATCC 33277 with primers (an upper primer [CGAATTCCTCTAGAGGATCTTCTTC] and a lower primer [GGGA TCCAAACCCGTTACTTCAAGA]). The upper primer can hybridize to the chromosomal DNA 1.6-kb upstream of the start codon of *dps* and generate an *Eco*RI site at one end of the PCR product, whereas the lower primer can hybridize to the chromosomal DNA within the *dps* gene and generate a *Bam*HI site at the other end of the PCR product. The amplified DNA fragment was cloned into pCR2.1, sequenced, excised by double digestion with *Eco*RI and *Bam*HI, and ligated to the *Eco*RI-*Bam*HI region of pKD393. The resulting plasmid pKD394 produced the *dps'-'lacZ* fusion protein with the N-terminal 10 amino acids of Dps.

P. gingivalis oxyR preliminary sequence data was obtained from The Institute for Genomic Research website (http://www.tigr.org). The *P. gingivalis oxyR* gene region (927 bp) was PCR amplified from the chromosomal DNA of ATCC 33277 with PCR primers (an upper primer [CCATATGAATATACAGCAGCTCG AA] and a lower primer [CGGATCCTCAAGCCAAATGCTGCCCTGT]), cloned into pCR2.1, sequenced, excised with *Eco*RI, and ligated to the *Eco*RI site of pUC19, resulting in pKD396. The *Bsp*EI site within the *oxyR* gene of pKD396 was converted to *Bgl*II by using a *Bgl*II linker DNA and the *tetQ* DNA block was inserted into the *Bgl*II site, resulting in pKD397. ATCC 33277 and KDP146 (*dps*$^+$ *dps'-'lacZ*) were transformed to tetracycline resistance by electrotransformation with the *Pst*I-linearized pKD397 DNA to yield KDP143 (*oxyR*::Tc^r) and KDP148 (*oxyR*::Tc^r *dps*$^+$ *dps'-'lacZ*), respectively.

For construction of *P. gingivalis dps* mutants, ATCC 33277 and KDP139 were transformed to tetracycline resistance with linearized pKD388 (*dps*::Tc^r) DNA to yield KDP141 (*dps*::Tc^r) and KDP142 (*ftn*::Em^r *dps*::Tc^r), respectively.

Purification of Dps from a *dps*-overexpressing *E. coli*. *E. coli* BL21(DE3) harboring pKD390 was grown to an optical density at 540 nm (OD_{540}) of 0.5 in 300 ml of L broth containing 50 μg of ampicillin/ml. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture at 1 mM, followed by incubation for 2 h to overproduce the recombinant Dps protein. The cells were collected by centrifugation at 9,000 × *g* for 20 min, resuspended in 10 ml of 20 mM phosphate buffer (pH 7.0), and disrupted by sonic oscillation (1 min each, 10 times). After centrifugation at 10,000 × *g* for 15 min, the supernatant was saved, to which ammonium sulfate was added at 85% saturation, and the precipitate was dissolved in 3 ml of phosphate buffer. After centrifugation to remove insoluble materials, the supernatant was dialyzed against the same phosphate buffer with a seamless cellulose tube (UC36-32-100; Sankohjunyaku Co., Tokyo, Japan) for 24 h and applied to a column (2.0 by 25 cm) of DEAE-Sepharose (Pharmacia) equilibrated with the same buffer. Proteins were eluted with 180 ml of a 0 to 1,000 mM linear NaCl gradient in the same buffer. The recombinant Dps protein was usually eluted at 400 mM NaCl. After concentration of the protein with a Microcon YM-10, the protein sample was applied to Mono-Q column (Pharmacia) and eluted with 180 ml of a 0 to 1,000 mM linear NaCl gradient in the same buffer. The Dps protein fraction that was eluted at 400 mM NaCl was then subjected to gel filtration on Superdex 200 column (Pharmacia). The system was equilibrated with the same buffer, proteins were eluted at a flow rate of 0.3 ml/min and 2-ml fractions were collected. The fractions constituting the prominent protein peak were pooled and concentrated with a Microcon YM-10. The iron-loaded Dps was obtained by incubating 50 μg of the recombinant Dps with 1 mM ferrous ammonium sulfate in 20 mM morpholinepropanesulfonic acid-NaOH (pH 7.0) at 4°C for 1 h (45). To remove excess iron, the mixture was extensively dialyzed against the same morpholinepropanesulfonic acid buffer by using the seamless cellulose tube (UC36-32-100). The iron-loaded Dps was concentrated by using the Microcon YM-10.

DNA-binding activity. Recombinant *P. gingivalis* Dps (10 μg) treated with or without ferrous ammonium sulfate was mixed with 500 ng of linear DNA (1-kb DNA Ladder; Promega, Madison, Wis.) and kept at 4°C for 1 h. The mixture was subjected to agarose gel electrophoresis (1%; Tris-acetate buffer).

Iron starvation. To determine the ability to grow under iron starvation, cells of test strains were first grown in the presence of hemin and then deprived of the iron source. The initial inoculum that had been prepared by growing the strains in hemin-containing enriched BHI medium overnight was diluted 10-fold with hemin-free enriched BHI medium and incubated. Every 24 h, the OD_{540} of the cultures was measured, and a 10-fold dilution of the cultures with hemin-free enriched BHI medium was repeated.

Agar diffusion assay. *P. gingivalis* cells were anaerobically grown in enriched BHI medium and spread on enriched TS plates, and a sterile disk containing 4 μl of 6% hydrogen peroxide, 15% hydrogen peroxide, 1 mg of mitomycin C/ml, or 0.5 mg of metronidazole/ml was placed at the center of each plate and incubated anaerobically at 37°C for 4 days.

Sensitivity of *P. gingivalis* to hydrogen peroxide in aerobic conditions. *P. gingivalis* cells grown in enriched BHI medium for 48 h were diluted twofold with fresh enriched BHI medium with or without 1.0 mM hydrogen peroxide and incubated aerobically at 37°C with shaking (125 cpm). To determine the number of survivals, cultures were withdrawn at intervals and plated on enriched TS plates after adequate dilution. The plates were anaerobically incubated for 7 days at 37°C

Cell culture and infection of HUVEC with *P. gingivalis*. Human ambilical vein endothelial cells (HUVEC) obtained from Cell Applications, Inc. (San Diego, Calif.) was maintained in MCDB151 medium (Sigma) containing 15% fetal calf serum, acidic fibroblast growth factor (Sigma), heparin (Sigma), and kanamycin sulfate. For infection, cells were seeded at 5×10^5 cells per well in six-well tissue culture dishes with 2 ml of the same medium. *P. gingivalis* cells were grown in enriched BHI medium to a mid-logarithmic phase, washed with phosphate-buffered saline (PBS), and suspended into kanamycin sulfate- and fetal bovine serum-free MCDB151 medium. The bacterial suspension was then added to the HUVEC monolayer at a multiplicity of infection of 10,000. After incubation at 37°C for the indicated period, the infected cells were collected, washed with the same medium containing 300 μg of gentamicin and 400 μg of metronidazole/ml, suspended in the antibiotic-containing medium, and incubated at 37°C for 1 h to kill extracellular bacterial cells. The infected HUVEC were collected, washed with PBS, and suspended in MCDB151 medium. After freezing and thawing of the HUVEC suspension, enriched BHI medium was added to the suspension. The mixture was vortexed for 15 s and serially diluted, and each dilution was plated on enriched TS agar and then incubated anaerobically at 37°C for 5 days to determine the survival of the intracellular bacterial cells. All assays were conducted independently at least five times. The results are expressed as the means ± the standard deviations (SD) of multiple experiments.

Confocal laser scanning microscopy. *P. gingivalis* cells of a fresh overnight culture were precipitated by centrifugation at 6,000 rpm for 10 min, washed, and suspended in PBS. The cell suspension was supplemented with 2,7-*bis*-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein (BCECF; 10 mM stock solution; Molecular Probes, Inc., Eugene, Oreg.) at a final concentration of 5 μM and incubated anaerobically at 37°C for 30 min. After centrifugation at 6,000 rpm for 5 min, the precipitated bacterial cells were suspended in MCDB151 medium without antibiotics. HUVEC that had been grown on a glass coverslip in a six-well tissue culture plate were washed twice with PBS and infected with the BCECF-labeled *P. gingivalis* cells at a multiplicity of infection of 10,000. After a 10-min incubation at 37°C, the infected HUVEC were washed twice with PBS and fixed in 10% formaldehyde in PBS at room temperature for 15 min. The fixed cells were washed twice with PBS and treated with PBS containing 50 mM NH_4Cl and 0.3% Tween 20 for 10 min at room temperature. After being washed with PBS twice, the HUVEC were subjected to confocal laser scanning microscopy (DMIRB/E; Leica Microsystems, Wetzlar, Germany). From confocal laser scanning images, incorporated bacterial cells were counted in a depth of 1.0 μm at the central section of a single HUVEC. Data were expressed as the means of more than 50 determinations ± the SD.

Other methods. Iron staining with Ferene S and heme staining with 3,3′,5,5′-tetramethylbenzidine (TMBZ) were performed as described previously (37). Electrotransformation of *P. gingivalis* cells was done as previously described (35). SDS-PAGE was performed under reducing conditions on 15% gels essentially as described previously (25). The gels were stained with 0.1% Coomassie blue R-250. For immunoblot analysis, proteins on SDS-PAGE gels were electrophretically transferred to nitrocellulose membranes as previously described (46). The membranes were immunostained with a 5,000-fold dilution of an anti-*P. gingivalis* Dps antiserum. The antiserum was prepared from a rabbit immunized with *P. gingivalis* Dps purified from the *E. coli* strain overproducing *P. gingivalis* Dps. The reacting proteins were detected by using the ECL Western blotting system (Amasham).

DNA sequencing was carried out by using a dideoxy sequencing kit (Auto Read Sequencing kit; Pharmacia) with plasmid templates and an automated DNA sequencer (ALF DNA Sequencer; Pharmacia). The sequence data were analyzed with the GeneWorks software program (IntelliGenetics, Mountain View, Calif.).

Colony hybridization and Southern blot hybridization were performed by standard methods (39, 47).

Statistical analysis. The Student *t* test was used to compare differences in CFU numbers among bacterial strains by using StatView J4.5 software (Abacus Concepts, Inc., Berkeley, Calif.).

Nucleotide sequence accession number. The nucleotide sequence reported in the present study was deposited in the DDBJ/EMBL/GenBank database under accession no. AB025779.

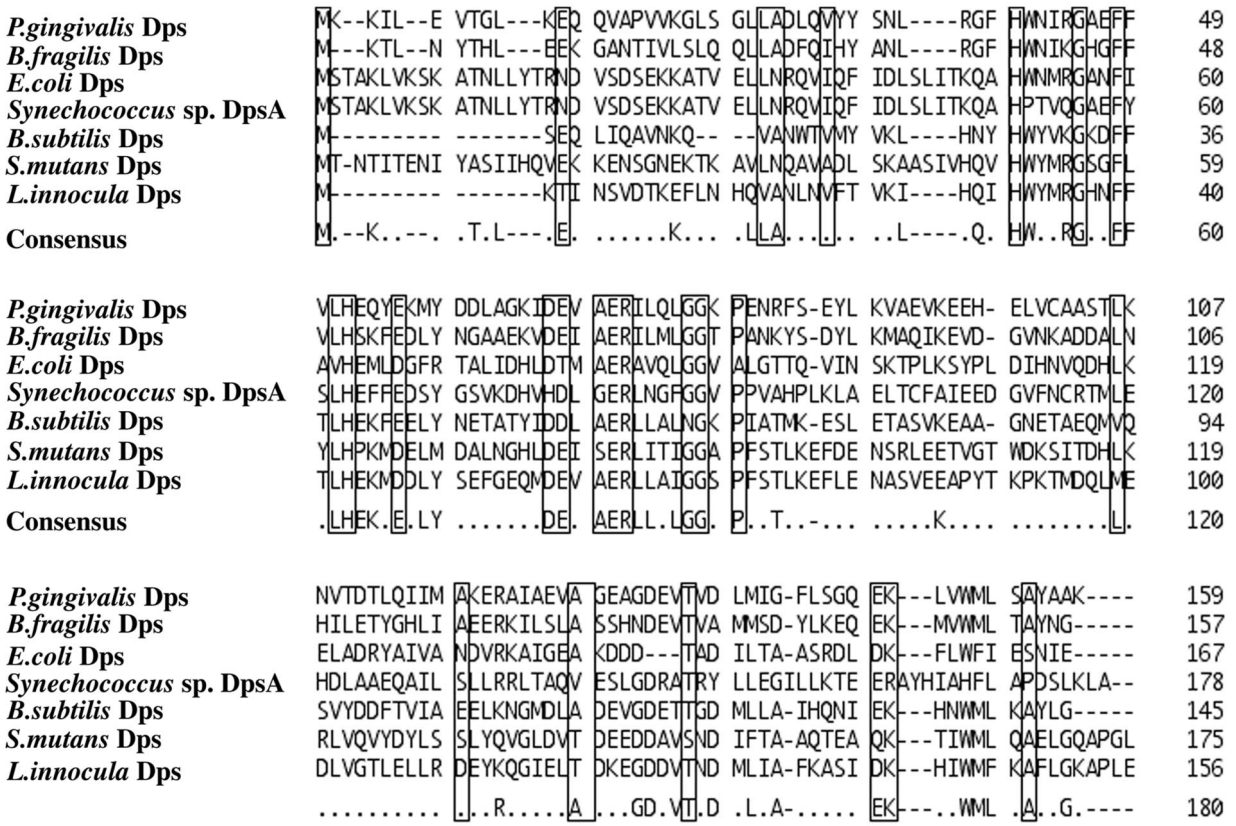

FIG. 1. Alignment of the amino acid sequence of *P. gingivalis* Dps with those of the Dps previously isolated and determined in other prokaryotes.

RESULTS

Isolation and characterization of a Dps homologue from *P. gingivalis*. In the process of purification of ferritin from *P. gingivalis* (37), we found that the organism contained other ferritin-like particles in the cell extracts. The fraction containing ferritin-like particles was separated from the ferritin fraction by CsCl density gradient centrifugation, followed by gel filtration on Superdex 200. The final preparation of ferritin-like particles gave a single protein band with an apparent molecular mass of 19 kDa when examined by SDS-PAGE under reducing conditions (data not shown). Since the apparent molecular mass of the protein was estimated to be more than 200 kDa, the protein appeared to be a dodecamer consisting of the 19-kDa protein. The N-terminal amino acid sequence of the protein was found to start with MKKILEVTG LKEQQV, which was highly homologous to those of Dps from other bacterial species.

Molecular cloning of a *P. gingivalis* gene conferring peroxide resistance on the *E. coli oxyR* mutant. Apart from the biochemical characterization of the ferritin-like protein, we tried to isolate *P. gingivalis* genes responsible for peroxide resistance. *P. gingivalis* chromosomal DNA library was introduced into the *E. coli oxyR* mutant and *t*-butyl hydroperoxide-resistant transformants were isolated. All of the transformants harbored recombinant plasmids containing the same chromosomal DNA region, revealed by restriction enzyme analysis (data not shown). One of the recombinant plasmids (pKD386) contained a 2.6-kb chromosomal DNA fragment. Subcloning analysis located the peroxide-resistant gene on a 1.1-kb DNA region within the 2.6-kb fragment. Unexpectedly, one open reading frame in the 1.1-kb region encoded a protein with 159 amino acid residues, the N-terminal sequence of which was the same as that of the ferritin-like particle protein. As shown in Fig. 1, the protein had a marked resemblance of the amino acid sequence to Dps proteins from other bacterial species. Therefore, this protein was referred to as *P. gingivalis* Dps.

Verification of *P. gingivalis dps* gene. To determine whether the *dps* gene is responsible for protection against peroxide stress, we constructed a derivative (pKD387) of pKD386 in which 4 bp were deleted at the *Bst*XI site in the *dps* gene and examined the *oxyR* mutant harboring pKD387 for sensitivity to *t*-butyl hydroperoxide by spreading these cells on L plates containing *t*-butyl hydroperoxide (60 ng/ml). The plating efficiencies of the *oxyR* mutants harboring pUC19 (vector), pKD386 (*dps*$^+$), and pKD387 (*dps* with deletion) were 1.8×10^{-4}, 4.5×10^{-1}, and 5.7×10^{-4}, respectively, indicating that *P. gingivalis* *dps* gene has the ability to confer peroxide resistance on the *E. coli oxyR* mutant.

P. gingivalis Dps purified from the recombinant *E. coli* cells was mixed with ferrous ammonium sulfate, electrophoresed through nondenaturing gels, and stained with Ferene S and TMBZ. Ferene S but not TMBZ stained *P. gingivalis* Dps,

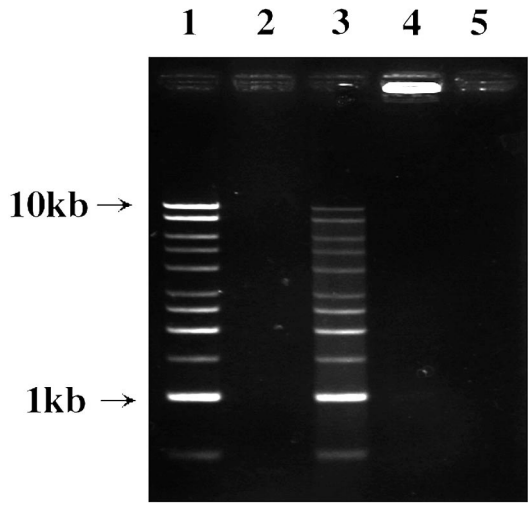

FIG. 2. DNA-binding activity of *P. gingivalis* Dps. Recombinant *P. gingivalis* Dps purified from the *E. coli* overexpressing *P. gingivalis dps* or the recombinant *P. gingivalis* Dps treated with ferrous ammonium sulfate was incubated with linear DNA (1-kb DNA ladder) at 4°C for 1 h. The mixture was then subjected to agarose gel electrophoresis. DNA on the gel was stained with ethidium bromide. Lanes: 1, DNA (500 ng) alone; 2, recombinant Dps (10 μg) alone; 3, recombinant Dps (10 μg) and DNA (500 ng); 4, iron-loaded recombinant Dps (10 μg) and DNA (500 ng); 5, iron-loaded recombinant Dps (10 μg) alone.

suggesting that *P. gingivalis* Dps is able to bind nonheme iron (data not shown). The DNA-binding activity of Dps was assayed by using linear plasmid DNA as described previously (1). The linear DNA mixed with the iron-loaded Dps did not migrate into an agarose gel, indicating the DNA-binding activity of Dps (Fig. 2).

Expression of the *dps* gene. To investigate the *dps* expression in *P. gingivalis*, we first constructed the *lacZ* reporter suicide-integration plasmid (pKD393) for *P. gingivalis* and introduced the promoter region of *dps* to pKD393. The resulting plasmid (pKD394) containing the *dps'-'lacZ* protein fusion gene was then introduced to *P. gingivalis* ATCC 33277 (wild type) to yield KDP146 (*dps*$^+$ *dps'-'lacZ*). An *oxyR*::Tc^r mutation was then introduced to ATCC 33277 and KDP146, resulting in KDP143 (*oxyR*::Tc^r) and KDP148 (*oxyR*::Tc^r *dps*$^+$ *dps'-'lacZ*), respectively. Aerobic incubation caused a slight increase of the *dps* expression in the wild-type background, and such an increase was not observed in the *oxyR* background, indicating that the *dps* expression was partially controlled by *oxyR* (Fig. 3).

Construction of a *dps* mutant of *P. gingivalis*. To gain insight into the biological significance of Dps in *P. gingivalis* cells, a Dps-deficient mutant was constructed. The *dps* gene DNA disrupted by insertion of the *tetQ* cartridge DNA was introduced into cells of *P. gingivalis* wild-type strain (ATCC 33277) and the *ftn* mutant (KDP139) by electroporation. A number of tetracycline-resistant colonies were obtained in both strains, and KDP141 (*dps*::Tc^r) and KDP142 (*ftn*::Em^r *dps*::Tc^r) were chosen for further characterization. Southern blot hybridization and immunoblot analyses revealed the proper construction of KDP141 and KDP142 (Fig. 4). A gene encoding a putative transporter was located downstream of the *dps* gene. However, expression of the putative transporter gene would not be affected by insertion of the drug resistance cartridge into the *dps* gene since the direction of transcription of the gene was opposite to that of *dps*.

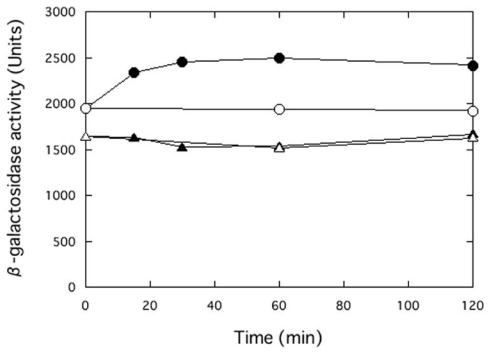

FIG. 3. Time course of induction of β-galactosidase activity in the *dps'-'lacZ* fusion strains. *P. gingivalis* KDP146 (*dps*$^+$ *dps'-'lacZ*) (circles) and KDP148 (*oxyR dps*$^+$ *dps'-'lacZ*) (triangles) were grown anaerobically in enriched BHI medium at 37°C. At an A_{600} of 0.3, the cultures were shifted under aerobic conditions (solid symbols) or kept under anaerobic conditions (open symbols). Samples were withdrawn after the indicated time, and the activity of β-galactosidase was determined by the method of Miller (32). The β-galactosidase activity of the wild-type parent strain ATCC 33277 was <4 U under both aerobic and anaerobic conditions.

Characterization of the Dps-deficient mutants of *P. gingivalis*. (i) Growth under iron depletion. To determine the contribution of Dps to intracellular iron storage, the wild-type (ATCC 33277), *ftn* (KD139), *dps* (KD141), and *ftn dps*

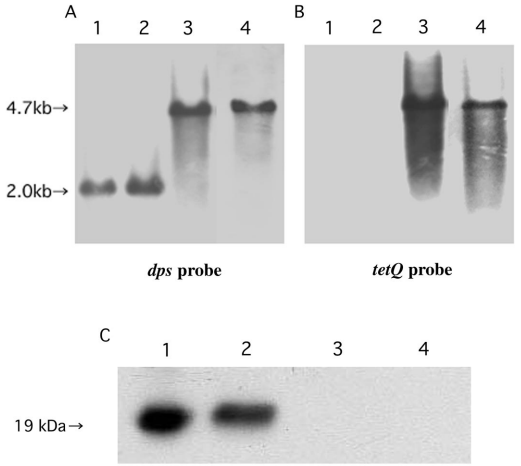

FIG. 4. Proof of authenticity of the *P. gingivalis dps* mutant KDP141 and the *ftn dps* double mutant KDP142. (A and B) Southern blot analyses of the chromosomal DNA. The chromosomal DNAs of the wild-type ATCC 33277 (lane 1) and the *ftn* mutants KDP139 (lane 2), KDP141 (lane 3), and KDP142 (lane 4) were digested with *Nco*I. The resulting DNA fragments were subjected to agarose gel electrophresis, followed by blotting. Hybridization was performed by using the 0.6-kb *Nde*I-*Bgl*II fragment of pKD390 as a *dps* probe (A) and the 2.7-kb *Bam*HI-*Bgl*II fragment of pKD375 as a *tetQ*probe (B). (C) Immunoblot analysis. After purified *P. gingivalis* Dps (lane 1) and the cell extracts of ATCC 33277 (lane 2), KDP141 (lane 3), and KDP142 (lane 4) were electrophoresed through an SDS-polyacrylamide gel, the proteins were transferred to a nitrocellulose membrane and immunoreacted with antiserum against *P. gingivalis* Dps.

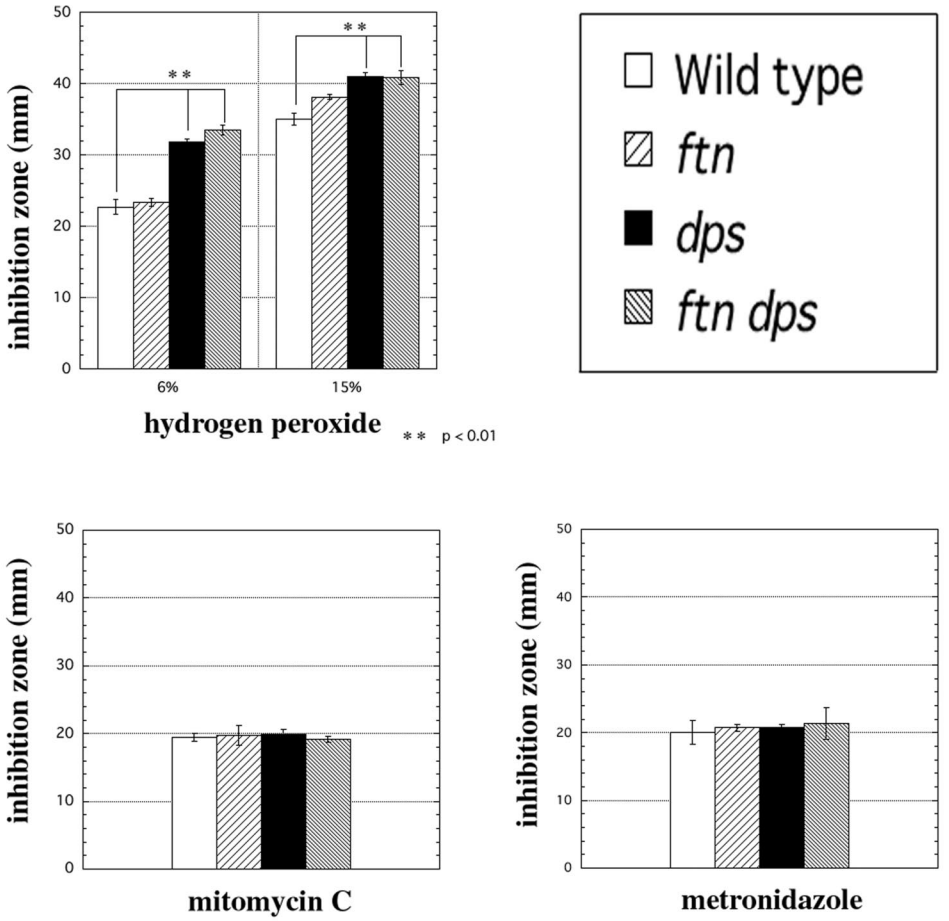

FIG. 5. Sensitivity of *P. gingivalis* cells to hydrogen peroxide, mitomycin C, and metronidazole. *P. gingivalis* ATCC 33277 (wild type), KDP139 (*ftn*), KDP141 (*dps*), and KDP142 (*ftn dps*) were anaerobically grown in enriched BHI medium for 48 h. The cells were spread on enriched TS plates, and a paper disk containing hydrogen peroxide (A), mitomycin C (B), or metronidazole (C) was placed at the centers of the plates, followed by incubation at 37°C anaerobically for 7 days. The diameters of the clear zones next to the disks were measured (in millimeters). The data shown are the means and SD of triplicate experiments.

(KD142) strains were iron starved after growth in enriched BHI broth containing hemin as the iron source. The *dps* mutant showed the same growth depression as the wild type, whereas the *ftn* mutant showed earlier depression than the wild type, as previously reported (37). There was no difference in growth depression between the *ftn* and *ftn dps* mutants. These results suggested that Dps made no contribution to intracellular iron storage (data not shown).

(ii) Sensitivity to hydrogen peroxide, mitomycin C, and metronidazole. To determine the sensitivity of the *dps* mutants to hydrogen peroxide, mitomycin C, and metronidazole, we used agar diffusion assays under anaerobic conditions (Fig. 5). The *dps* and *ftn dps* mutants were more sensitive to hydrogen peroxide than were the wild-type and *ftn* strains (Fig. 5A). There was no difference in sensitivity to mitomycin C and metronidazole among these strains (Fig. 5B and C). These results indicated that Dps contributed to protection against peroxide in *P. gingivalis* cells.

(iii) Sensitivity to atmospheric oxygen with or without hydrogen peroxide. Although *P. gingivalis* is an obligate anaerobe, it exhibits a relatively high degree of aerotolerance. In order to determine whether Dps contributes to the aerotolerance of this organism and protection against hydrogen peroxide in aerobic condition, bacterial cells that had been grown anaerobically in enriched BHI medium overnight were diluted twice with fresh enriched BHI with or without hydrogen peroxide (final concentration, 0.5 mM) and aerobically incubated. It was shown that the *dps*, *ftn*, and *ftn dps* mutants were as tolerant to atomospheric oxygen as was the wild-type parent (Fig. 6A). Compared to the wild-type and *ftn* strains, the *dps* and *ftn dps* mutants were very sensitive to hydrogen peroxide under aerobic conditions (Fig. 6B). These results clearly indicated that the *dps* gene had an important role in protecting the cells from hydrogen peroxide.

(iv) Survival in HUVEC. *P. gingivalis* can invade endothelial cells, including HUVEC (11–13). To investigate the role of Dps in survival of *P. gingivalis* cells in HUVEC, we determined intracellular viability of the *P. gingivalis dps* mutant and the wild-type parent strain in HUVEC upon treatment with metronidazole and gentamicin after infection (Fig. 7). Although the wild-type parent strain and the *dps* mutant showed decrease of viability in HUVEC in a time-dependent manner, the *dps* mutant showed fewer surviving cells than did the wild-type parent strain. Since this difference in the number of intracel-

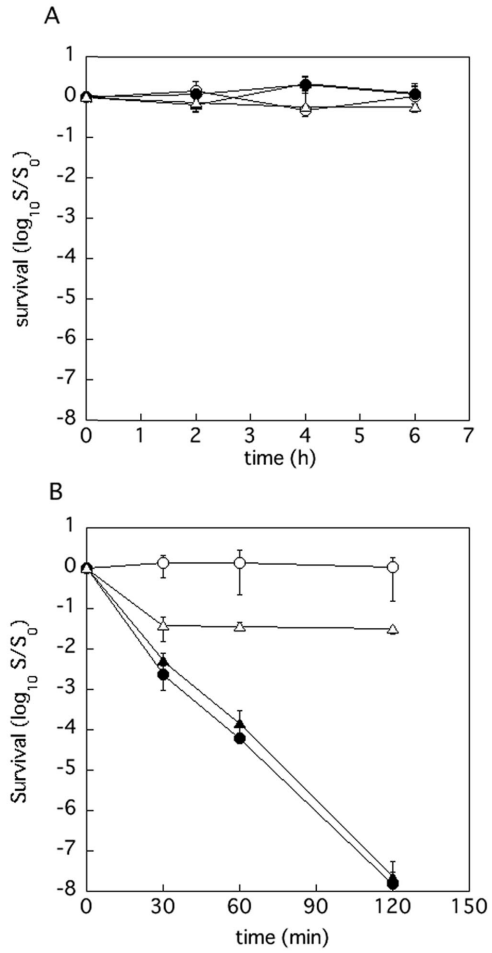

FIG. 6. Sensitivity of *P. gingivalis* mutants to air (A) and air with hydrogen peroxide (B). *P. gingivalis* cells that had been anaerobically cultured in enriched BHI medium overnight were diluted twice with fresh enriched BHI medium (A) or enriched BHI medium containing hydrogen peroxide (final concentration, 0.5 mM) (B) and then incubated aerobically with vigorous shaking. Samples were withdrawn at intervals and plated, after dilution in enriched BHI medium, on enriched TS plates. The plates were incubated anaerobically at 37°C for 7 days. Symbols: ○, ATCC 33277 (wild type); △, KDP139 (*ftn*); ●, KDP141 (*dps*); ▲, KDP142 (*ftn dps*).

lular survivals might result from a difference in invasion, we counted the intracellular bacterial cells in a block within a single HUVEC after infection by confocal laser scanning microscopy. The numbers of bacterial cells of the wild-type parent and the *dps* mutant incorporated into a depth of 1.0 μm at the central section of a single HUVEC were 48.4 ± 12.2 and 46.6 ± 15.2, indicating that there was no significant difference in invasion between these strains. These results suggested that Dps contributed to survival of *P. gingivalis* cells in HUVEC.

DISCUSSION

We purified ferritin-like particles from *P. gingivalis* cell extracts and determined the N-terminal amino acid sequence of the ferritin-like particle protein. On the other hand, we cloned a *P. gingivalis* gene conferring peroxide resistance on the *E. coli oxyR* mutant and found that the gene product had the same molecular mass and N-terminal sequence as the ferritin-like particle protein, indicating that the peroxide resistance gene encodes the ferritin-like particle protein of *P. gingivalis.* Database analysis of the deduced amino acid sequence of the protein indicated that the *P. gingivalis* ferritin-like protein belonged to the Dps protein family, resulting in the gene designation *dps*. An *E. coli* catalase-null (*katG katE*) mutant was also used as a host strain for cloning of *P. gingivalis* peroxide resistance genes. All of the chromosomal DNA fragments obtained contained *dps* (unpublished data), suggesting that in *P. gingivalis* genes *dps* might be the only gene which could suppress increased peroxide sensitivity of *E. coli oxyR* and *katG katE* mutants.

Expression of Dps homologue genes of various microorganisms is upregulated by exposure to oxidative stress (1, 2, 6, 22, 38). In *E. coli* and *B. fragilis*, the stress response of their *dps* genes is regulated by OxyR (2, 38). In the present study, we constructed the *lacZ* reporter suicide-integration plasmid for analysis of gene expression of *P. gingivalis*. Using this *lacZ* reporter system, we found that the *P. gingivalis dps* gene was constitutively expressed but was upregulated by exposure to atmospheric oxygen and that this induction was totally dependent on OxyR. There was an approximately 10-fold increase in *B. fragilis dps* expression upon exposure to atmospheric oxygen (38), whereas *P. gingivalis dps* expression increased by only 25%, indicating that the degree of constitutive expression of *P. gingivalis dps* was much greater than that of *B. fragilis dps*. *B. fragilis* possesses catalase, whereas *P. gingivalis* does not possess catalase (31, 38). The high degree of constitutive expression of Dps in *P. gingivalis* might compensate for the absence of catalase in this organism.

Analysis of the *P. gingivalis dps* mutant revealed that *P. gingivalis* Dps was responsible for protection against peroxides, especially against hydrogen peroxide. Previous studies suggest that the ability of Dps to protect cells from oxidative damage may be derived from DNA condensation and masking with the Dps protein and from sequestration of iron ions that might otherwise generate detrimental free radicals (17, 24, 29, 48,

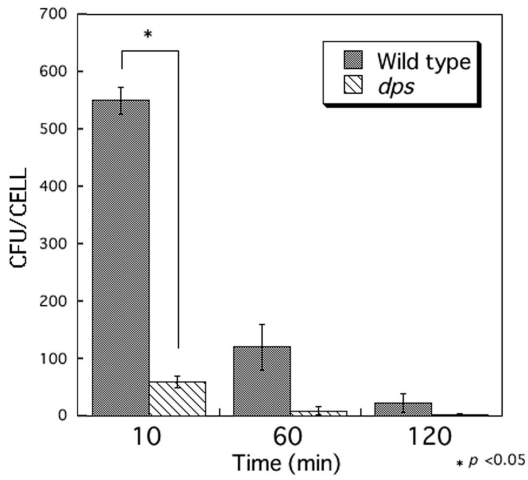

FIG. 7. Survival of *P. gingivalis* cells in HUVEC. Monolayers of HUVEC were infected by *P. gingivalis* ATCC 33277 (wild type) and KDP141 (*dps*). Experiments were done at least five times, and the data are presented as the mean and the SD of the CFU per HUVEC.

49). If DNA condensation and mechanical masking with Dps contributes to the protection of chromosomal DNA from peroxides, Dps-deficient mutants might show sensitivity to other DNA-damaging agents as well. However, the *P. gingivalis dps* mutant had no sensitivity to mitomycin C or metronidazole that can damage DNA. Nondenaturing PAGE profiles showed that *P. gingivalis* Dps contained nonheme iron. However, *P. gingivalis* Dps may not contribute to the iron storage of this organism, as revealed by the iron deprivation experiment. In addition, the *ftn dps* double mutant showed the same sensitivity to hydrogen peroxide as did the *dps* single mutant. These results suggest that, in *P. gingivalis* Dps, the sequestration of iron ions might not be plausible to explain the ability to protect cells from peroxides. We found in a previous study (34) that *P. gingivalis sod* mutant shows a rapid viability loss upon exposure to atmospheric oxygen. In contrast, we found in the present study that the *dps* mutant showed no viability loss upon exposure to atmospheric oxygen for 6 h. On the other hand, the *dps* mutant showed a viability loss by the treatment of hydrogen peroxide in aerobic conditions and that this viability loss was not enhanced by the addition of the *ftn* mutation. These results strongly suggest that *P. gingivalis* Dps is responsible for protection against specific ROS such as hydrogen peroxide. In this context, Dunkan and Touati (14) have shown that the *E. coli dps* mutant as well as a catalase-null mutant shows increased sensitivity to both hypochlorous acid and a catalase-null mutant. Hassett et al. (20) reported hat overproduction of *Pseudomonas aeruginosa* Dps provides protection against hydrogen peroxide but increases sensitivity to cumen hydroperoxide in *P. aeruginosa oxyR* mutant. *Synechococcus* sp. DpsA has catalase activity (36). In addition, rubrerythrin belonging to the ferritin-bacterioferritin-rubrerythrin superfamily, which is in turn related to the Dps family, has been found to have NADH peroxidase activity (9). *P. gingivalis* Dps might have peroxide-reducing activity, although we have not yet found this activity. To elucidate the mechanism of the peroxide resistance conferred by *P. gingivalis* Dps, the molecular and catalytic properties of the Dps should be explored.

Recently, a number of epidemiological studies have revealed a positive correlation between periodontal disease and coronary heart disease (4, 30). Patients with periodontal disease are more likely to experience transient bacteremias produced by flossing, mastication, and toothbrushing, which can occasionally let periodontal bacteria localize to endothelial cells (10). In fact, *P. gingivalis* has been immunolocalized in the shoulders of atherosclerotic plaques (7), and *P. gingivalis* DNA has been found in endarterectomy samples of patients with carotid stenosis by PCR analysis with primers specific to *P. gingivalis* DNA (19). The microorganism can invade various host cells, including epithelial cells and endothelial cells (11, 13, 26, 28). Survival of *P. gingivalis* cells in these host cells is important for the development of infection. The present study has provided the finding that Dps contributed to the intracellular survival of *P. gingivalis* cells. As far as we know, the present study is the first description of the significance of Dps in the survival of microorganisms in host cells.

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