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Rapid Viability Loss on Exposure to Air in a Superoxide Dismutase-Deficient Mutant of *Porphyromonas gingivalis*

KOJI NAKAYAMA*

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

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***Porphyromonas gingivalis*, an obligate anaerobe, exhibits a relatively high degree of aerotolerance and possesses superoxide dismutase (SOD) which is induced by exposure to air. To clarify roles for SOD in this organism, the gene encoding SOD (*sod*) on the *P. gingivalis* chromosome was disrupted in a gene-directed way by use of a suicide plasmid containing a mutated *sod*. A *sod* mutant thus obtained showed no SOD activity in crude extracts and exhibited a rapid viability loss immediately after exposure to air, whereas the wild-type parent showed no decrease in viability for at least 5 h under aerobic conditions. These results clearly indicate that SOD is essential for aerotolerance in *P. gingivalis*.**

Although oxygen is beneficially used by many organisms in respiration and other functions, oxygen is basically toxic and mutagenic because of its reactive derivatives (5). Most living organisms, therefore, acquired defense systems to protect themselves against reactive oxygen species, probably after the appearance of molecular oxygen in the earth's atmosphere. Two cellular systems have been developed for protection against oxidative stress (16). One system, which includes superoxide dismutase (SOD), catalase, peroxidase, and oxidase, can diminish or eliminate in cells molecular oxygen and the consecutive univalent reductants of oxygen. The other system, including DNA repair enzymes, such as *Escherichia coli* exonuclease III and endonuclease IV, repairs the oxidative damage to cell components. These two systems cooperatively function to minimize the detrimental effects of reactive oxygen species upon cells, as evidenced by the presence of common regulatory systems that control the expression of certain genes of the two systems (16).

SOD is one of the major factors in the defense systems; it catalyzes the disproportion of superoxide arising from the univalent reduction of oxygen (5). There have been numerous reports showing that SOD plays a significant role in protection against oxidative stress (6). One of the classical approaches to proving the importance of SOD in this protection is to construct a SOD-deficient mutant and determine the sensitivity of the mutant to oxygen. Among prokaryotes, SOD-deficient mutants have been constructed for *E. coli* and *Streptococcus mutans* (3, 13). Unexpectedly, these mutant strains were able to grow aerobically in rich medium, albeit much more slowly than their parent strains, indicating that the presence of SOD in these microorganisms is important but not essential for aerobic growth.

Porphyromonas (*Bacteroides*) *gingivalis*, which is implicated in periodontal disease, is an obligate anaerobe. By definition, the bacterium cannot grow under aerobic conditions but exhibits a high degree of aerotolerance. Also, it possesses a particular SOD, which is basically an Fe-SOD, according to its amino acid sequence, but which is activated by either Fe or Mn (1, 2, 12). A survey of SOD in obligately anaerobic bacteria has revealed that its activity seems to be correlated with the aerotolerance of the bacteria (7, 17).

In this study, I constructed an SOD-deficient mutant of *P. gingivalis* via replacement of the *sod* allele by use of a suicide plasmid system. The SOD-deficient mutant strain showed a rapid viability loss immediately after exposure to air, whereas the parent strain survived, with no decrease in cell viability, for at least 5 h under aerobic incubation conditions, clearly indicating that the presence of SOD in this organism is essential for survival under aerobic conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *P. gingivalis* ATCC 33277, *E. coli* DH5, *E. coli* JM109, and *E. coli* QC774 (a *sodA sodB* double mutant; 3). Plasmids pVAL-1 (18), pACYC184 (4), and pUC18 (19) were used for the construction of a suicide plasmid vector (pKDCMZ) for *P. gingivalis*. Plasmid pKD210 (Fig. 1), which possesses a *Sau*3AI DNA fragment (3.1 kbp) containing the *sod* gene of *P. gingivalis* ATCC 33277, was used as a source of the *sod* gene. Plasmids pKDCM, pKDCMZ, pKD264, and pKD265 and *P. gingivalis* mutants KDP99, KDP100, KDP101, KDP102, KDP103, and KDP104 were obtained in this study.

Media and conditions for cell growth. Enriched BHI broth (containing, per liter, brain heart infusion [Difco Laboratories, Detroit, Mich.], 37 g; yeast extract [Difco], 5 g; cysteine, 1 g; hemin, 5 mg; and vitamin K_1, 1 mg) and enriched tryptic soy agar (containing, per liter, Trypto-Soya agar [Nissui, Tokyo, Japan], 40 g; brain heart infusion, 5 g; cysteine, 1 g; hemin, 5 mg; and vitamin K_1, 1 mg) were used as the liquid and solid media, respectively, for the growth of *P. gingivalis* cells. L broth (containing, per liter, tryptone [Difco], 10 g; yeast extract, 5 g; and sodium chloride, 5 g) was used for growing *E. coli* cells. An anaerobic glove box (Hirasawa Works, Tokyo, Japan) was used with an atmosphere of 80% nitrogen–10% hydrogen–10% carbon dioxide for anaerobic incubation.

Enzymes and oligonucleotides. Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, and T4 DNA ligase were purchased from Takara (Kyoto, Japan) and Toyobo (Osaka, Japan). Calf thymus alkaline phosphatase was obtained from Boehringer GmbH (Mannheim, Germany). A mixture of synthetic oligonucleotides, 5′-TGGGA(A/G)CA(T/C)GC(A/G/T/C)TA(T/C)TA(T/C)-3′, was obtained from Takara.

* Mailing address: Department of Microbiology, Faculty of Dentistry, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan. Phone: 81-92-641-1151, ext. 4253. Fax: 81-92-641-3770.

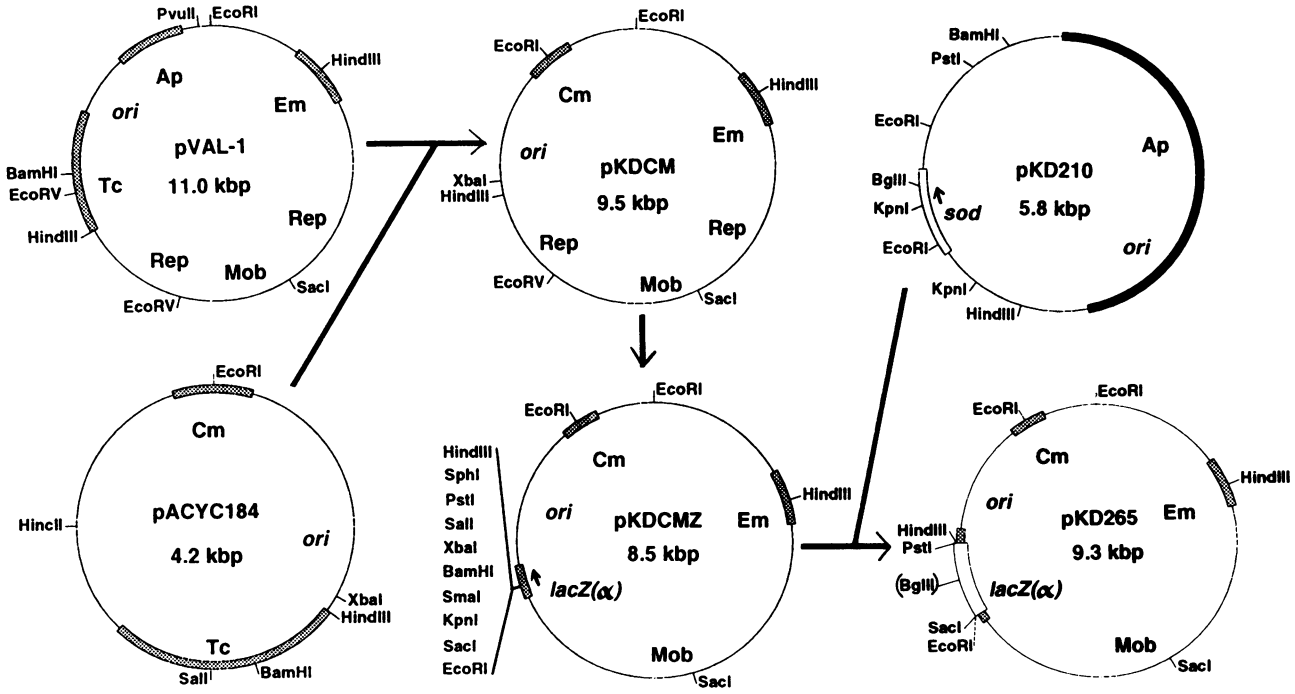

FIG. 1. Construction of the suicide plasmid vector (pKDCMZ) and its derivatives. Plasmid pVAL-1, a shuttle plasmid for *Bacteroides* spp. and *E. coli*, was partially digested with restriction enzymes *Pvu*II and *Hin*dIII, and ligated with a *Hin*cII-*Hin*dIII fragment of pACYC184, resulting in plasmid pKDCM. To remove a region responsible for plasmid replication in *Bacteroides* or *Porphyromonas* spp. and introduce restriction sites useful for DNA cloning, pKDCM was digested with *Eco*RV and *Xba*I and ligated with the *Hae*II fragment of pUC18, containing the *lacZ*(α) region, after the ends of both fragments were blunted with T4 DNA polymerase, giving rise to plasmid pKDCMZ. The *Eco*RI fragment (0.8 kbp) of pKD210 was treated with T4 DNA polymerase and ligated with an *Sma*I digest of pKDCMZ. The resulting plasmid, pKD264, was then digested with *Bgl*II and self-ligated after end filling with T4 DNA polymerase to yield plasmid pKD265. Thin and thick lines of pKD210 represent the DNA regions from the *P. gingivalis* chromosome and plasmid pUC18, respectively. Ap, ampicillin resistance; Cm, chloramphenicol resistance; Em, erythromycin resistance; Tc, tetracycline resistance; Mob, region for plasmid mobilization; Rep, region for plasmid replication; *ori*, replication origin.

DNA manipulations. Chromosomal DNA was isolated from *P. gingivalis* cells by the guanidine isothiocyanate method (10) with an IsoQuick DNA extraction kit (MicroProbe, Garden Grove, Calif.). An *Eco*RI fragment (0.8 kbp) containing the 3′ portion of *P. gingivalis sod* in pKD210 (Fig. 1) was used as a probe for *sod*. The labeling of probe DNA and Southern hybridization with the *sod* and oligonucleotide probes were carried out as previously described (13).

Plasmid construction. The construction of a suicide plasmid vector (pKDCMZ) and its derivatives is depicted in Fig. 1.

Mobilization of pKDCMZ derivatives from *E. coli* to *P. gingivalis*. Bacterial mating between *E. coli* and *P. gingivalis* was performed essentially as described by Hoover et al. (8). Cells of *E. coli* DH5 harboring a pKDCMZ derivative and R751 were aerobically grown to 2×10^8/ml at 37°C in L broth containing 20 μg of chloramphenicol per ml. Cells of *P. gingivalis* ATCC 33277 were anaerobically grown to 6×10^8/ml at 37°C in enriched BHI broth. Five milliliters each of the donor and recipient cultures were then mixed, and the cultures were harvested by centrifugation. The cell pellet was resuspended in 1 ml of prewarmed enriched BHI broth, and 0.2-ml portions were spotted on enriched tryptic soy agar. The plates were aerobically incubated at 37°C for 4 h prior to anaerobic incubation at 37°C for 36 h. Bacterial cells on the plates were collected with a cotton swab, resuspended in enriched BHI broth, spread on enriched tryptic soy agar containing 20 μg of erythromycin and 100 μg of gentamicin per ml, and incubated anaerobically at 37°C for 7 days.

Assay for SOD activity. Cells were harvested, resuspended in buffered salts medium base (14) containing 100 μg of leupeptin (Peptide Institute, Minoh, Japan) per ml, and sonicated with a supersonic vibrator (UR-150P; Tominaga Works, Tokyo, Japan). Extracts were assayed for SOD activity by the cytochrome *c*-xanthine oxidase method (11).

RESULTS AND DISCUSSION

Southern blot hybridization analyses with an oligonucleotide probe specific for a SOD motif. The *P. gingivalis sod* gene was cloned and sequenced previously (12). The amino acid sequence deduced from the nucleotide sequence of the *sod* gene was consistent with the amino acid sequence chemically determined, except for one residue at position 29 (2, 12). To determine whether the *P. gingivalis* chromosome possesses another gene(s) encoding SOD, Southern blot hybridization was carried out with an oligonucleotide probe specific for a SOD motif. A SOD belonging to the Fe-SOD/Mn-SOD family contains a highly conserved sequence (Try-Glu-His-Ala-Tyr-Tyr) near its carboxy terminus (15), and the oligonucleotide capable of encoding this amino acid sequence was successfully used for the detection of *sod* genes in my previous study (13). The chromosomal DNA of *P. gingivalis* ATCC 33277 was digested with restriction enzymes and subjected to Southern blot hybridization with this probe. The probe hybridized to one DNA fragment in each digestion, and these restriction fragments were of the same lengths as those that hybridized to the

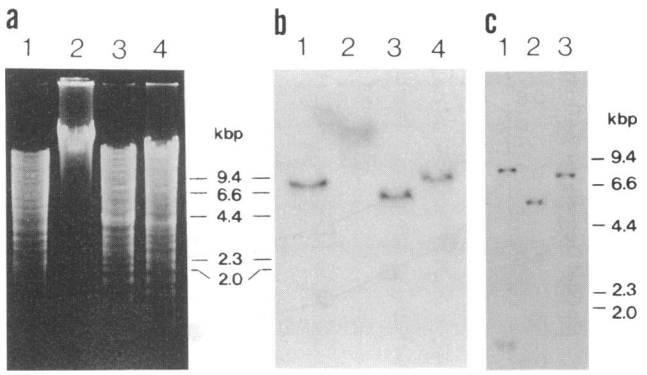

FIG. 2. Hybridization of the oligonucleotide probe derived from the common SOD motif to chromosomal DNA of *P. gingivalis* ATCC 33277. (a) The chromosomal DNA was digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), *Bam*HI (lane 3), and *Hin*cII (lane 4), and the fragments were separated by electrophoresis in an 0.8% agarose gel. (b) The DNA was then transferred to a nylon membrane and hybridized to the oligonucleotide probe. The chromosomal DNA of *P. gingivalis* ATCC 33277 was resistant to *Eco*RI digestion, probably because of modification at *Eco*RI restriction sites. (c) Chromosomal DNA digested with *Hin*cII (lane 1), *Bam*HI (lane 2), and *Hin*dIII (lane 3) was also electrophoresed in a different gel and hybridized to the *sod* probe.

sod probe (the 0.8-kbp *Eco*RI fragment in the *sod* region), indicating that the *sod* gene was the unique one for SOD in this organism (Fig. 2).

Construction of a *sod* mutant of *P. gingivalis* by use of a suicide plasmid. To determine whether SOD plays a significant role in protection against oxidative stress in *P. gingivalis*, a *sod* mutant was constructed by the introduction of a mutated *sod* gene into the chromosome through integration of a derivative of suicide plasmid pKDCMZ (pKD265; Fig. 1) possessing the mutated *Bgl*II site in the *sod* gene (Fig. 3). Mating between *E. coli* DH5 harboring pKD265 and R751 and *P. gingivalis* ATCC 33277 yielded Em^r transconjugants of *P. gingivalis*, and Southern blot hybridization analysis revealed that the chromosomal DNA from all of the transconjugants examined possessed full-length plasmid pKD265 DNA inserted at the *sod* region (Fig. 3b). It was also shown that the transconjugants were classified into four types with respect to the location of the mutated *sod* gene (Fig. 3a and c). Of these, structures I and II could be accounted for by reciprocal recombination with a single crossover event between the homologous DNA regions of the chromosome and the plasmid. However, the generation of structures III and IV would require multiple reciprocal recombination or nonreciprocal recombination (gene conversion). Representative transconjugants exhibiting structures I, II, III, and IV were designated KDP100, KDP101, KDP102, and KDP103, respectively. A similar mating was carried out with plasmid pKD264, which had the same structure as pKD265 except for the possession of an intact *Bgl*II site, and an Em^r transconjugant (KDP99) was obtained.

The SOD activity of the transconjugants was determined with cells grown anaerobically and exposed or not exposed to aerobic conditions. No SOD activity was detected in crude extracts from cells of KDP100 and KDP102, whereas KDP101 and KDP103 exhibited almost the same SOD activity as the wild-type parent (Table 1). These results confirmed that *sod* is the only functional gene encoding SOD in *P. gingivalis*.

Rapid viability loss and cell lysis of SOD-deficient mutants on exposure to air. Although *P. gingivalis* is an obligate anaerobe, it exhibits a relatively high degree of aerotolerance (1). To determine whether SOD contributes to the aerotolerance of this organism, the viability of the SOD-deficient mutants was determined under aerobic conditions. The SOD-sufficient transconjugants (KDP101 and KDP103) as well as the wild-type parent showed no decrease in viability for at least 5 h after exposure to air. In contrast, the SOD-deficient transconjugants (KDP100 and KDP102) showed a rapid viability loss immediately after exposure to air, although the culture of KDP100 contained an aerotolerant subpopulation (Fig. 4). On the other hand, the SOD-deficient transconjugants (KDP100 and KDP102) grew

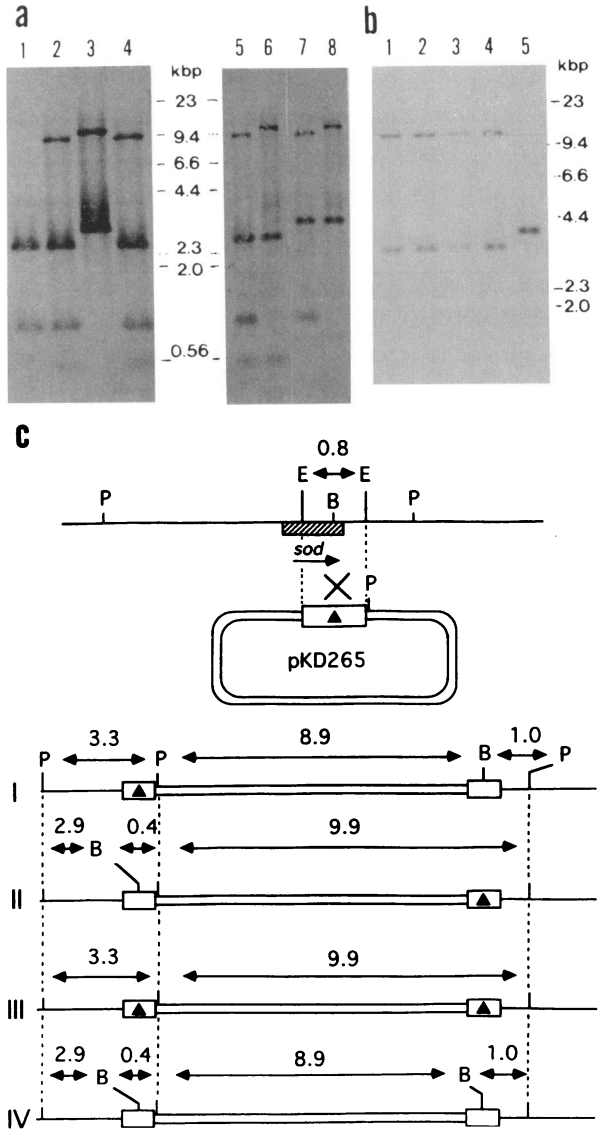

FIG. 3. Southern blot analyses of chromosomal DNA of Em^r transconjugants. The chromosomal DNA was digested with *Bgl*II and *Pst*I (a) and with *Pst*I (b) and subjected to agarose gel electrophoresis and then Southern blot hybridization with *sod* probe DNA. (a) Lanes: 1, ATCC 33277; 2, KDP99; 3 and 8, KDP102; 4 and 5, KDP103; 6, KDP101; 7, KDP100. (b) Lanes: 1, KDP100; 2, KDP101; 3, KDP102; 4, KDP103; 5, ATCC 33277. (c) Schematic structures of the *sod* regions of KDP100 (I), KDP101 (II), KDP102 (III), and KDP103 (IV). Restriction sites: B, *Bgl*II; E, *Eco*RI; P, *Pst*I. Closed triangles represent the mutated *Bgl*II site.

TABLE 1. SOD activity of Em^r transconjugants

Strain	SOD activity (U/mg of protein) under the following conditions[a]:	
	Anaerobic	Aerobic
KDP100	ND	ND
KDP101	2.8	12.1
KDP102	ND	ND
KDP103	3.2	13.3
ATCC 33277	3.0	12.5

[a] A fresh culture anaerobically grown in enriched BHI broth overnight was diluted twofold with enriched BHI broth and incubated aerobically at 37°C for 2 h. The overnight culture (anaerobic) and the 2-h aerated culture (aerobic) were used for the determination of SOD activity. ND, no inhibition of O_2^--dependent reduction of cytochrome *c* was detected when undiluted crude extracts (about 7 mg of protein per ml) were used.

under anaerobic conditions as well as did the SOD-sufficient ones (KDP101 and KDP103) (data not shown). These results clearly indicate that SOD activity is essential for tolerance of oxidative stress in this organism.

The turbidity of cultures of the SOD-deficient mutants decreased on exposure to air, showing that oxidative stress caused cell lysis in the mutants (Fig. 5). This result may strengthen the idea inferred by Imlay and Fridovich (9) that the absence of SOD can cause structural instability of the cell envelope. It is conceivable that oxidative damage to the cell envelope, as well as damage to DNA and aerosensitive proteins, may account for cell death.

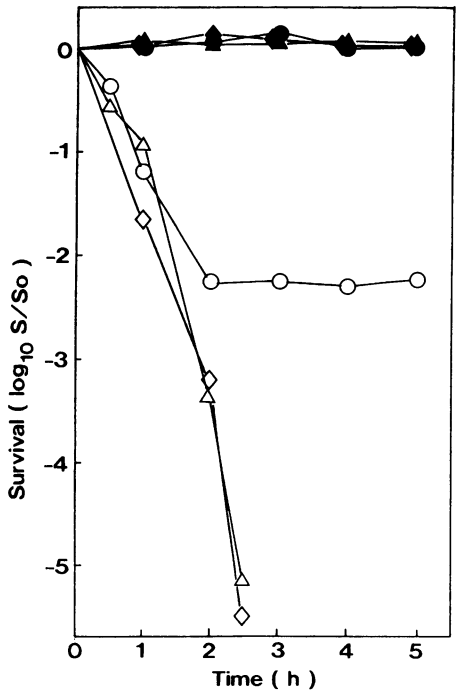

FIG. 4. Sensitivity of *P. gingivalis* mutants to air. Cells of *P. gingivalis* mutants and their wild-type parent (ATCC 33277) were anaerobically grown in enriched BHI broth at 37°C with vigorous shaking (100 cpm). Samples were withdrawn at intervals and plated, after dilution in enriched BHI broth, on enriched tryptic soy agar plates. The plates were anaerobically incubated for 7 days at 37°C. Symbols: ○, KDP100; ●, KDP101; △, KDP102; ▲, KDP103; ◇, KDP104; ◆, ATCC 33277. S, number of surviving cells; So, number of viable cells at time 0.

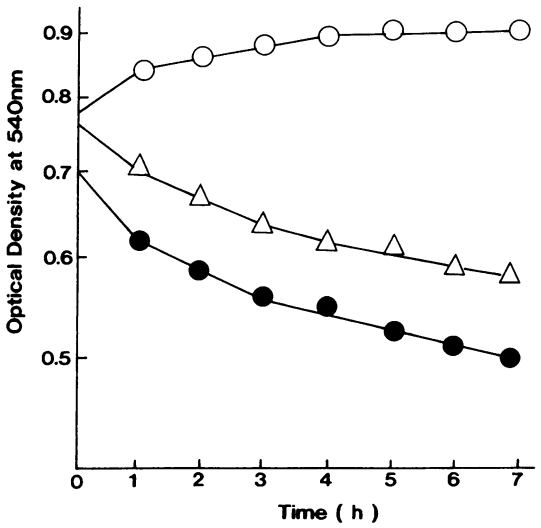

FIG. 5. Cell lysis of SOD-deficient mutants on exposure to air. Cells of SOD-deficient mutants and their parent strain were aerobically incubated as described in the legend to Fig. 4. The optical density at 540 nm of the cultures was periodically measured. Symbols: ○, ATCC 33277; ●, KDP100; △, KDP102.

The aerotolerant survivors of KDP100 are *sod*$^+$. The aerotolerant subpopulation of KDP100 could be *sod*$^+$ because the wild-type *sod* sequence corresponding to the mutated site exists on the chromosome of KDP100. To check this possibility, SOD activity, Em^s, and the chromosomal structure in the *sod* region were studied with aerotolerant cells. Of eight aerotolerant survivors examined, three were Em^s and the others were Em^r, but crude extracts from all of the survivors contained SOD activity at almost the same level as that in the wild-type parent (data not shown). Southern blot analyses revealed that the chromosomes of the Em^s survivors had the same structure as that of the parent strain, with plasmid pKD265 DNA being lost, while all of the Em^r survivors had the type IV chromosomal structure (Fig. 6a). This result indicates that the *sod*$^+$ gene has been restored to the chromosomes of both Em^s and Em^r survivors.

Isolation of an Em^s segregant from SOD-deficient mutant KDP102. The presence of an Em^s aerotolerant subpopulation in the culture of KDP100 indicated that the integrated plasmid DNA was segregated from the chromosome of *P. gingivalis* at a frequency of about 10^{-3} (the number of Em^s aerotolerant cells times two divided by the total number of cells). Isolation of an Em^s segregant from KDP102 was done by replica plating on erythromycin-containing enriched tryptic soy agar. One Em^s segregant (KDP104) was obtained from approximately 1,700 colonies of KDP102. Southern blot hybridization revealed that the chromosome of KDP104 did not possess plasmid pKD265 DNA and that the mutated site remained in the *sod* gene (Fig. 6b). Cells of KDP104 as well as KDP102 had no SOD activity in crude extracts and exhibited hypersensitivity to oxygen (Fig. 4), demonstrating that the integration of the plasmid itself did not affect the aerotolerance of the organism and that the hypersensitivity to oxygen was totally due to the *sod* mutation.

The necessity of SOD for the aerotolerance of *P. gingivalis* suggests that the acquisition of SOD was a critical step in the evolution of primordial anaerobic organisms for adaptation to an environment containing oxygen. Also, it is possible that cells

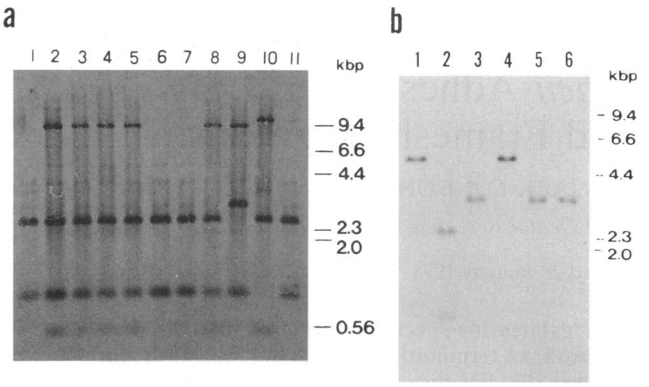

FIG. 6. Southern blot analyses of aerotolerant mutants of KDP100 and an Em^s segregant of KDP102. (a) The chromosomal DNA of aerotolerant Em^s mutants (lanes 1, 6, and 7), aerotolerant Em^r mutants (lanes 2, 3, 4, 5, and 8), KDP100 (lane 9), KDP101 (lane 10), and ATCC 33277 (lane 11) was digested with *Pst*I and *Bgl*II. (b) The chromosomal DNA of ATCC 33277 (lanes 1, 2, and 3) and an Em^s segregant (KDP104) of KDP102 (lanes 4, 5, and 6) was digested with *Bam*HI (lanes 1 and 4), *Pst*I and *Bgl*II (lanes 2 and 5), and *Pst*I (lanes 3 and 6). The digested DNA was subjected to agarose gel electrophoresis and then Southern blot hybridization with *sod* probe DNA.

of *E. coli* and *S. mutans* have a repair system for oxidative damage efficient enough to make their aerobic growth possible without SOD activity.

P. gingivalis is frequently found in the subgingival microflora of patients with periodontal disease and is considered to be one of the causative agents of the disease. Subgingival microflora is often exposed to air. Furthermore, tissue invasion may place the invading microorganism more or less under oxidative stress. Hence, aerotolerance could be an important attribute for periodontopathogens. Thus, SOD, which makes cells of *P. gingivalis* aerotolerant, may be a critical factor that qualifies *P. gingivalis* as a pathogen in periodontal disease.

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