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Effects of Temperature Stress on Expression of Fimbriae and Superoxide Dismutase by *Porphyromonas gingivalis*

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We examined the biosynthesis of fimbriae and superoxide dismutase (SOD) produced by the periodontopathic bacterium *Porphyromonas gingivalis* in response to elevated temperature. *P. gingivalis* 2561, grown at 37°C to mid-logarithmic phase, was subsequently incubated at 39, 41, and 43°C, respectively, to stationary phase. There was no difference in the growth of cells at 37 and 39°C. However, at 39°C there was a 54% reduction in the amount of fimbrillin (fimbriae) as well as decreased expression of mRNA for *fimA*. On the other hand, under the same conditions, a more than twofold increase in the amount of SOD activity, as well as in the levels of SOD mRNA, was observed. Moreover, cells cultured for 20 h at 39°C showed an 86% decrease of fimbrillin protein and a threefold increase in SOD activity. These observations suggest that *P. gingivalis* may undergo alterations in its virulence and susceptibility to host immune responses as a result of the elevated temperatures found in inflamed periodontal pockets.

Porphyromonas gingivalis is a gram-negative black-pigmented anaerobe associated with several periodontal diseases including adult periodontitis, generalized juvenile periodontitis, periodontal abscesses, and refractory periodontitis (31). *P. gingivalis* fimbriae or fimbrillin (FimA; structural subunit of fimbriae) is involved in interactions of this organism with host cells (8, 9, 11, 23, 31). Superoxide dismutase (SOD) is an enzyme of *P. gingivalis* presumed to be involved in protecting the cells against neutrophils (1). Fimbriae are specific antigens of *P. gingivalis* (11), and immunization of germfree rats with fimbriae protects against *P. gingivalis*-induced periodontitis (9). Fimbriae likely mediate adherence and colonization of this organism in periodontal lesions (14, 16). Recently, it has been reported that a *fimA* gene-inactivated *P. gingivalis* mutant was significantly less able to bind to saliva-coated hydroxyapatite and to cause periodontal bone loss in a gnotobiotic rat model of periodontal disease relative to wild-type *P. gingivalis* 381 (20).

Generally, aerobic and many anaerobic bacteria express enzymes such as SOD, catalase, and peroxidase which neutralize toxic oxygen metabolites (5). However, *P. gingivalis* expresses SOD activity but not catalase or peroxidase activities (2). *P. gingivalis* has higher SOD activity than other gram-negative anaerobic rods, such as *Prevotella loescheii*, *Prevotella intermedia*, *Prevotella denticola*, *Prevotella melaninogenica*, *Porphyromonas levii*, *Fusobacterium nucleatum*, and *Capnocytophaga gingivalis* (2, 3). This higher activity of SOD in *P. gingivalis* is considered an important factor in protecting the cells against bactericidal superoxide anion (O_2^-) generated from neutrophils (1) and occasional exposure to oxygen in the air (3). A *sod* gene-inactivated *P. gingivalis* mutant exhibited a rapid viability loss immediately after exposure to the air, whereas the wild-type parent showed no decrease in viability following 5 h of exposure to the air (22). Therefore, fimbriae and SOD have been suggested to be virulence factors that contribute to the pathogenicity of *P. gingivalis* (21).

Recently, temperature-sensitive probes have been developed to detect the temperature of periodontal pockets, and utilization of such probes revealed a higher temperature in pockets with periodontal disease than in anatomically equivalent healthy sulci (10). The mean temperature in the healthy sulci was reported to be 36.8°C, and a significant temperature elevation of up to 2°C was observed in periodontal pockets (4, 10). This elevated temperature stress might influence the pathogenicity of *P. gingivalis* in periodontal pockets. In this study, we examined the biosynthesis of fimbriae and SOD in response to elevated temperature (temperature stress).

P. gingivalis 2561 was cultured in 10 ml of prereduced brain heart infusion broth (18 mg/ml; Difco Laboratories, Detroit, Mich.) supplemented with 5 mg of yeast extract per ml, 5 μg of hemin per ml, and 0.2 μg of menadione per ml and buffered at pH 7.4 and then was incubated to late-logarithmic growth phase (A_{580} = 1.1) at 37°C in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2) as previously described (16). Two types of experiments were carried out to assess the temperature stress effects on this organism. For studies of the temperature stress effect for less than 12 h, a 2% (vol/vol) inoculum from a culture grown to late-logarithmic growth phase (A_{580} = 1.1) was transferred into 10 ml of fresh broth equilibrated anaerobically. The inoculated cells were grown to mid-logarithmic phase (A_{580} = 0.7) at 37°C and subsequently grown to stationary phase at 37, 39, 41, and 43°C, respectively. Alternatively, to examine changes following temperature stress for 20 h, a 2% (vol/vol) inoculum of a late-logarithmic phase 37°C culture was transferred to 10 ml of fresh broth which was then incubated to late-logarithmic phase (A_{580} = 1.1) at 37, 39, and 41°C, respectively. The cells were centrifuged at 4,000 × *g* for 15 min at 4°C, washed three times with 50 mM phosphate buffer containing 0.15 M NaCl (PBS; pH 7.2), and suspended in the same buffer to adjust the cell numbers (A_{580} = 0.5).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed on a 10% gel according to the method of Lugtenberg et al. (18). Coomassie blue-stained gels were scanned with a densitometer (UltroScan XL; Pharmacia

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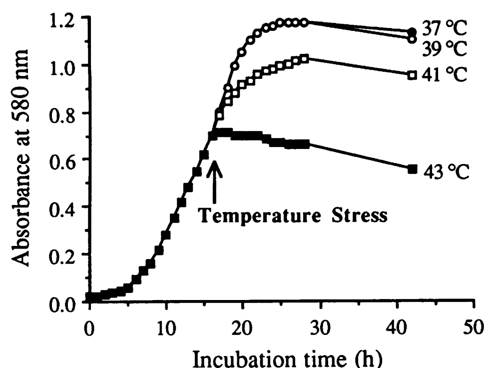

FIG. 1. Time course of the growth of *P. gingivalis* 2561 at various temperatures. The cells grown to mid-logarithmic phase (A_{580} = 0.7) at 37°C were subsequently transferred and cultured to late-stationary growth phase at 37, 39, 41, and 43°C, respectively.

LKB Biotechnology Inc., Piscataway, N.J.). For immunoblot analysis, the proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by Western transfer (immunotransfer) with a semidry transfer system (Semi-Phore TE-77; Hoefer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's instructions. The membrane was probed with antibodies to *P. gingivalis* fimbriae as described previously (28, 29).

Total RNA for Northern (RNA) blot analysis was isolated from *P. gingivalis* cells at the indicated growth conditions as described previously (26). The same amounts of total RNA isolated from cells at various conditions were separated by electrophoresis on a 2.2 M formaldehyde–1.0% agarose gel and transferred onto Zetabind membrane (Cuno Inc., Meriden, Conn.) by capillary elution (26). The 0.85-kb *Hin*cII *fimA* DNA fragment was isolated from pETfim (28) in which the *fimA* gene of *P. gingivalis* 2561 was subcloned. A 0.5-kb *Eco*RI-*Bgl*II DNA fragment was isolated from pCC19 (6) in which the *sod* gene of *P. gingivalis* ATCC 53977 was subcloned. Hybridization was performed with ^{32}P-labeled *sod* and *fimA* DNA fragments constructed by the random primer DNA-labeling method (26). The same blot was used for hybridization with *fimA* and *sod* probe. For reprobing, the hybridized radiolabeled probe was removed from the blot by incubation for 2 h in 5 mM Tris-HCl (pH 8.0) containing 0.2 mM EDTA, 0.5% sodium PP_i, and 0.1% Denhardt solution at 65°C (26).

The SOD activity was assayed by a modification of the method of MacCord and Fridovich (19) as described previously (3). Cells suspended in PBS were lysed by five 1-min sonication treatments. The supernatant fluids of disrupted cells were dialyzed against PBS overnight at 4°C and used to assay for SOD activity (3). Protein content was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.), and the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) was used according to the manufacturer's instructions when the sample solution contained SDS. The RNA content of samples was determined on the basis of the A_{260} (26).

Figure 1 shows cell growth after inoculation of *P. gingivalis* into fresh medium from a culture in late-logarithmic phase. The cells grown at 37°C to mid-logarithmic phase (A_{580} = 0.7) were subsequently cultured at 39, 41, and 43°C to stationary phase in an anaerobic environment. There was no difference in the growth curves of cells at 37 and 39°C. However, the amount of fimbrillin gradually decreased after incubation at 39°C (Fig. 2). Increasing the time of incubation at 39°C resulted in a progressive decrease of fimbrillin protein in the cells: 25% at 3 h, 52% at 6 h, and 54% at 12 h. There was also no difference in the cell growth curves of control cells grown at 37°C and bacterial cells grown for 20 h at 39°C. However, an 86% decrease of fimbrillin protein was observed in the temperature-stressed cells. Immunoblot analysis also revealed the gradual decrease of fimbrillin protein that reacted with fimbria-specific antibodies (Fig. 2B). A 75-kDa major immunodominant surface protein (30) also decreased in intensity along with the decrease of fimbrillin. Following 41°C incubation, the decrease of fimbrillin and 75-kDa protein was also observed to be identical to that at 39°C (data not shown).

Under the same growth conditions, a more than twofold increase was observed in SOD specific activity after incubation for 3 h at 39°C (Fig. 3). Moreover, SOD activity of cells grown at 39°C for 20 h reached 36.7 U/mg of protein, which is threefold higher than in the control cells grown for 20 h at 37°C. The temperature-stressed cells at 41°C showed lower SOD activity than at 39°C.

To confirm the alteration of expression of fimbrillin and SOD by temperature stress, changes in the levels of mRNA for *fimA* and *sod* genes were examined by Northern blot (Fig. 4).

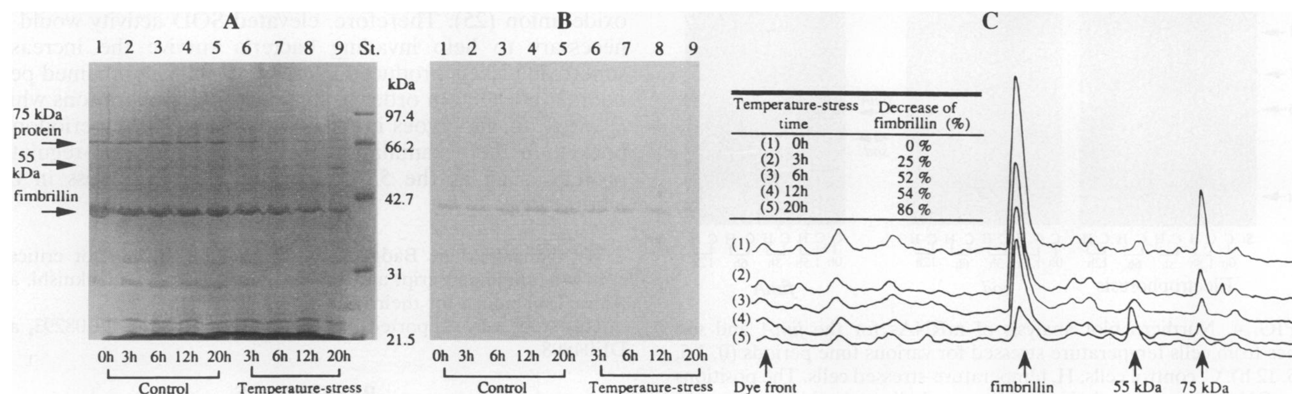

FIG. 2. (A) SDS-PAGE of extracts of *P. gingivalis* 2561 cells. The same amount of protein (50 μg) from various cell lysates was subjected to SDS-PAGE. (B) Immunoblot analysis of fimbriae of *P. gingivalis* 2561. Lanes 1 to 5, cells incubated at 37°C for various times (0, 3, 6, 12, 20 h) as controls. Lanes 6 to 9, temperature-stressed cells incubated at 39°C for various time periods. Lane St., standard molecular mass marker. (C) A comparison of fimbrillin protein levels on a gel by densitometry.

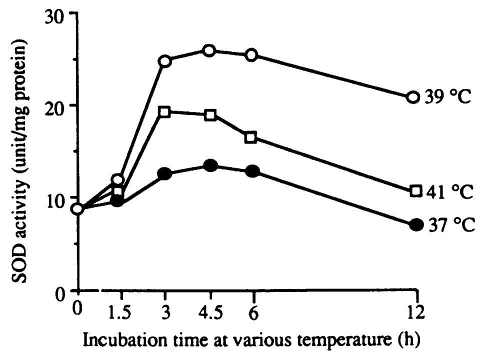

FIG. 3. The induction of SOD activity in *P. gingivalis* 2561 by temperature stress. Cells suspended in PBS were lysed by five 1-min sonication treatments. The supernatant fluids of disrupted cells were dialyzed against PBS overnight at 4°C and used to assay for SOD activity.

The blots were hybridized with *sod* probe and then probed with the *fimA* gene. The sizes of the transcripts for the *fimA* and *sod* genes were approximately 1.3 and 1.1 kb, respectively (Fig. 4). The concentration of mRNA for *fimA* was decreased while that for *sod* was increased relative to that of control cells following growth at 39°C (Fig. 4). These observations were consistent with the alteration of fimbrillin and SOD protein expression. The SOD activity induced by temperature stress was confirmed to be an iron-containing SOD on the basis of its inactivation by H_2O_2 and its inhibition by NaN_3 (data not shown).

It has been reported that heat shock responses in various pathogenic bacteria result in an increase in the synthesis of certain proteins called heat shock proteins (HSPs) (7, 24). These HSPs have many important physiological functions (7, 24). Periodontal pathogens including *P. gingivalis* and *Actinobacillus actinomycetemcomitans* also express HSPs, likely to correspond to a family of conserved immunodominant DnaK- and GroEL-like proteins (17, 27). However, the significance of heat shock responses in the pathogenesis of periodontal bacterial infections is not fully understood, and little is known about the regulatory mechanisms responsible for heat shock gene expression. Many bacteria examined previously in heat shock experiments have been reported to survive elevated temperatures of more than 5°C above their normal growth temperatures (7, 17, 24, 27). Recently, it has been reported that *P. gingivalis* W50, a nonfimbriated strain, increased the expression of 92-, 80-, 74-, 62-, 45-, and 12-kDa proteins and decreased 50- and 19-kDa protein expression following a 5°C heat stress of less than 4 h (17). Although *P. gingivalis* 2561 failed to grow at 43°C, a 2°C elevation of temperature was enough to cause a temperature stress response involving the regulation of fimbrillin and SOD expression in *P. gingivalis* 2561. The expression of classical known HSPs was not detected on an SDS-PAGE gel under these conditions. In Fig. 2A, cells temperature stressed for 20 h showed only one induced protein band (55 kDa) which might be related to HSP 60. Also, this 55-kDa protein might be similar to a 53-kDa antigenic protein previously reported (15). The amount of low-molecular-mass proteins of less than 20 kDa was also likely to be induced. However, HSPs other than the 55-kDa protein were not clearly detected on an SDS-PAGE gel. Further experiments are necessary to characterize temperature stress-regulated proteins.

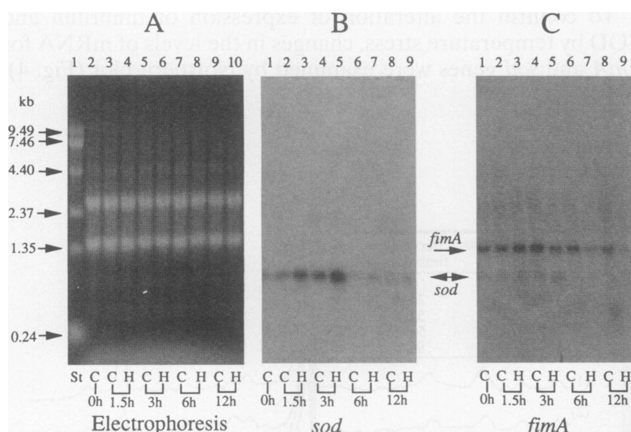

FIG. 4. Northern blot analysis of mRNAs for the *fimA* and *sod* genes from cells temperature stressed for various time periods (0, 1.5, 3, 6, 12 h). C, control cells; H, temperature-stressed cells. The positions of mRNAs for *sod* and *fimA* genes are indicated with arrows. (A) Electrophoresis of isolated RNAs. The same amount of RNA (30 μg) from cells at various growth conditions was analyzed. (B) Northern blot analysis of mRNA for the *sod* gene. (C) Northern blot analysis of mRNA for the *fimA* gene.

These observations suggest that *P. gingivalis* can alter the expression of fimbriae, SOD, and other proteins such as 55- and 75-kDa proteins under the conditions observed in inflamed periodontal pockets. The biological significance of decreased fimbria expression and higher SOD activity at elevated temperatures is not clear. Nevertheless, fimbriae have been shown to be a major target for the host response in patients with advanced periodontal disease. Specific antibodies against *P. gingivalis* fimbriae have been detected in patients with markedly elevated antibodies of the immunoglobulin G and A subclasses (8, 23). *P. gingivalis* fimbriae can also activate mouse peritoneal macrophages and subsequently induce gene expression and production of interleukin-1 (12), as well as induce gene expression of neutrophil chemotactic factor KC via protein kinase C (13). A decrease in fimbriae may allow this organism to evade the host immune response in inflamed periodontal pockets. Fimbriae play an important role in the colonization of the periodontal sulcus. However, subsequent to colonization, heavily fimbriated cells may not be required to evade the host immune defenses while this organism invades and destroys gingival tissues. Heat shock treatment of murine macrophages results in an enhanced capacity to release superoxide anion (25). Therefore, elevated SOD activity would be necessary to help invading bacteria survive the increased superoxide levels produced from neutrophils in inflamed periodontal pockets. In order to understand the alterations which *P. gingivalis* undergoes in the environment of the periodontal pocket, further examination to characterize stress-regulated proteins such as the 55-kDa protein is in progress in our laboratory.

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