

## Lipopolysaccharide Preparation Extracted from *Porphyromonas gingivalis* Lipoprotein-Deficient Mutant Shows a Marked Decrease in Toll-Like Receptor 2-Mediated Signaling

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We recently demonstrated that a new PG1828-encoded lipoprotein (PG1828LP) was able to be separated from a *Porphyromonas gingivalis* lipopolysaccharide (LPS) preparation, and we found that it exhibited strong cell activation, similar to that of *Escherichia coli* LPS, through a Toll-like receptor 2 (TLR2)-dependent pathway. In order to determine the virulence of PG1828LP toward cell activation, we generated a PG1828-deficient mutant of *P. gingivalis* strain 381 by allelic exchange mutagenesis using an *ermF-ermAM* antibiotic resistance cassette. A highly purified preparation of LPS from a PG1828-deficient mutant ( $\Delta$ PG1828-LPS) showed nearly the same ladder-like patterns in silver-stained gels as a preparation of LPS from a wild-type strain (WT-LPS), as well as *Limulus* amoebocyte lysate activities that were similar to those of the WT-LPS preparation. However, the ability of the  $\Delta$ PG1828-LPS preparation to activate NF- $\kappa$ B in TLR2-expressing cells was markedly attenuated. Cytokine production by human gingival fibroblasts was also decreased in response to the  $\Delta$ PG1828-LPS preparation in comparison with the WT-LPS preparation, and the activity was comparable to the stimulation of highly purified lipid A of *P. gingivalis* by TLR4. Further, lethal toxicity was rarely observed following intraperitoneal injection of the PG1828-deficient mutant into mice compared to that with the wild-type strain, while the  $\Delta$ PG1828-LPS preparation showed no lethal toxicity. Taken together, these results clearly indicate that PG1828LP plays an essential role in inflammatory responses and may be a major virulence factor of *P. gingivalis*.

*Porphyromonas gingivalis* has been implicated as a major etiological agent in the development and progression of chronic periodontitis, which is a destructive inflammatory disease of the supporting tissues of the teeth (38). This bacterium is a gram-negative, obligate anaerobic, oral black-pigmented rod that possesses a large number of potential virulence factors, such as fimbriae, hemagglutinin, lipopolysaccharide (LPS), and various proteases (15).

Among these virulence factors, LPS is well known as a major component of the outer membranes of gram-negative bacteria, and it exhibits powerful immunostimulatory and inflammatory activities (32). However, *P. gingivalis* LPS has a lower level of endotoxic potency than other types of enterobacterial LPSs (21, 27), while it and its active center, lipid A, have been shown to have other properties, such as an ability to activate cells from LPS-hyporesponsive C3H/HeJ mice as well as those from LPS-responsive C3H/HeN mice (18, 42).

Toll-like receptor 4 (TLR4) and its accessory protein MD-2 are known to function as signaling receptors for various LPSs (44), and C3H/HeJ mice have been demonstrated to be hypo-

responders, due to a natural point mutation of TLR4 (30). Further, TLR2 has been shown to be an essential signal-transducing molecule for *P. gingivalis* LPS preparations (4, 13), although *P. gingivalis* LPS is thought to be associated with quite different lipid A phosphorylation and acylation patterns (25). More recently, Darveau et al. (6) indicated that *P. gingivalis* LPS activates cells through both TLR2 and TLR4, because it possesses multiple lipid A species. In contrast, it was also reported that *P. gingivalis* LPS exerted antagonistic effects toward TLR4-dependent cell activation by *Escherichia coli* LPS (5, 45).

We previously demonstrated that highly purified lipid A from *P. gingivalis* and its synthetic counterpart activated cells via a TLR4/MD-2-dependent pathway but not via TLR2, which was in contrast to the cell activation activities of a *P. gingivalis* LPS preparation, which were shown to occur via TLR2 (28). In addition, we recently showed that a PG1828-encoded lipoprotein (PG1828LP) was able to be separated from a *P. gingivalis* LPS preparation by using a detergent-modified phenol-water extraction method, and we found that it exhibited TLR2-dependent cell activation and possessed strong cell-activating capacities in comparison with *E. coli* LPS (11). In the present study, we generated a PG1828-deficient mutant of *P. gingivalis* and showed that its LPS preparation significantly reduced cell activation via TLR2.

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## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and plasmid.** *P. gingivalis* strain 381 was grown anaerobically at 37°C in brain heart infusion (BHI) broth (Difco, Detroit, Mich.) containing 0.5% yeast extract (Difco), 5 µg of hemin per ml, and 1 µg of vitamin K<sub>3</sub> per ml. *E. coli* strain DH5α was used for cloning and was grown on Luria-Bertani agar base (Difco) or in LB broth (Difco). Plasmid pVA2198, which carries the *ermF-ermAM* gene and confers erythromycin resistance, was also used (9). For selection or maintenance of the plasmid-containing strains, antibiotics (1 µg of clindamycin per ml for *P. gingivalis* and 100 µg of ampicillin per ml for *E. coli*) were added to the media.

**DNA sequencing.** A DNA fragment from PG1825 to PG1829 of *P. gingivalis* 381 was obtained by PCR, using synthesized primers designed according to the DNA sequence of *P. gingivalis* W83 (24). The sequence of the product was determined with the ABI PRISM 3100-Avant automated DNA sequencer (Applied Biosystems, Foster City, Calif.), using the BigDye Terminator version 3.1 cycle sequencing kit.

**Construction of the PG1828-deficient mutant.** To generate a mutant deficient in the PG1828-encoded lipoprotein, plasmid DNA constructs were made by replacing the PG1828 gene with an *ermF-ermAM* cassette (Fig. 1A). The BamHI/EcoRI fragment of pVA2198 containing the *ermF-ermAM* gene was ligated with BamHI/EcoRI-digested pBluescript II SK(+) (Stratagene, La Jolla, Calif.). The upstream flanking regions, including the entire PG1825, PG1826, and PG1827 genes, and the downstream flanking regions, including the entire PG1829 gene, were amplified from *P. gingivalis* 381 chromosomal DNA by using the following primers: 1825-dw (5'-CGCCGGTCTAGACTAAGGATTGATTAAC-3' [the XbaI site is underlined]), 1827-dw (5'-TTTATTACTAGTGTGTGTGCTGAGCAGGC-3' [the SpeI site is underlined]), 1829-dw (5'-GAGTGTGATATCCG CATGGCTATTCCTTT-3' [the EcoRV site is underlined]), and 1829-up (5'-AAGTATCTCGAGCTGCTCGCTTTTGATT-3' [the XhoI site is underlined]). Fragments digested with enzymes were cloned into pBluescript II SK(+) carrying the *ermF-ermAM* gene and transformed into *E. coli* DH5α by standard techniques (34). The BssHIII fragment of the resulting construct containing the *ermF-ermAM* gene (Fig. 1A) was transformed into *P. gingivalis* 381 by electroporation as previously described, with some modifications (22, 33). Briefly, *P. gingivalis* cells were anaerobically grown at 37°C in 50 ml of BHI broth containing 0.5% yeast extract (Difco), 5 µg of hemin per ml, and 1 µg of vitamin K<sub>3</sub> per ml and then were washed with cold distilled water twice and resuspended in 200 µl of 1 mM MgCl<sub>2</sub> containing 10% glycerol. Linearized DNA samples (10 µg) were added to an 80-µl cell suspension and electroporated in a 0.1-cm-diameter cuvette at 200 Ω, 1.8 kV, and 25 µF (Gene Pulser II system; Bio-Rad, Hercules, Calif.). The cells were then immediately mixed with 10 ml of prewarmed BHI broth containing 0.5% yeast extract, 5 µg of hemin per ml, and 1 µg of vitamin K<sub>3</sub> per ml and incubated anaerobically at 37°C for 16 h, after which they were plated onto BHI agar plates containing 0.5% yeast extract, 5 µg of hemin per ml, 1 µg of vitamin K<sub>3</sub> per ml, and 1 µg of clindamycin per ml. The plates were incubated for 5 to 7 days anaerobically at 37°C to develop colonies.

**PCR analysis.** Chromosomal DNA samples from the wild-type strain and PG1828-deficient mutant were amplified by using primers 1 (5'-CGATGAGCTTGCGGTCAATA-3'), 2 (5'-CCATGTACGTGAGCGGATAT-3'), 3 (5'-GAACTGTCTAGTAGTATACC-3'), 4 (5'-GTTGCTTTTGCAGCT-3'), and 5 (5'-CCATGACGAAGAGTTTCGAGA-3'), as shown in Fig. 1B, with the primer pairs indicated in Fig. 1C.

**DNA probes and Southern blot analysis.** An *ermF-ermAM* probe was prepared as a 2.1-kb fragment and amplified from pVA2198 by using the primers *erm-up* (5'-CCGCTATGCTTTTTTGCTC-3') and *erm-dw* (identical to primer 3). The DNA probe was labeled with alkaline phosphatase (ALP) by using the AlkPhos direct labeling module (Amersham Biosciences, Piscataway, N.J.). BamHI-digested chromosomal DNA samples from the wild-type strain and the PG1828-deficient mutant were electrophoresed on a 1% agarose gel and then transferred to a HyBond-N+ nylon membrane (Amersham Biosciences) by using an alkaline blotting protocol. The membrane was prehybridized at 60°C for 15 min in a hybridization buffer and then hybridized with an ALP-labeled probe at 60°C for 2 h. At the end of the procedure, the membrane was washed under highly stringent conditions, and then a chemiluminescence detection assay was carried out with the CDP-Star detection reagent (Amersham Biosciences). The membrane was exposed to X-ray film for 30 min, and signals were developed for each strain.

**RT-PCR analysis.** Total RNA samples were isolated from the wild-type strain and PG1828-deficient mutant by using TRIzol reagent (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's instructions and then were treated with RNase-free DNase (TaKaRa, Shiga, Japan) based on a method described previously (7). Reverse transcription (RT) was conducted with avian myeloblas-

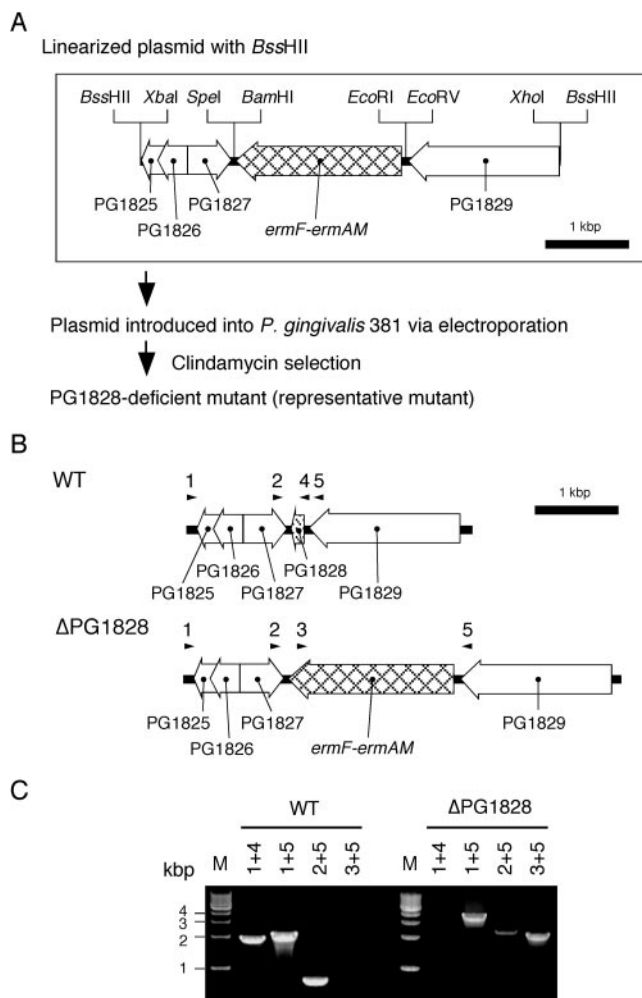


FIG. 1. Inactivation of PG1828 in *P. gingivalis*. (A) Construction of a PG1828-deficient mutant of *P. gingivalis* strain 381 by allelic exchange mutagenesis with an *ermF-ermAM* antibiotic resistance cassette (as described in Materials and Methods). (B) Predicted maps of the genomes of the wild-type and the PG1828-deficient mutant. Arrowheads indicate the numbers and positions of oligonucleotide primers for PCR analysis (as described in Materials and Methods). (C) PCR analysis of WT and ΔPG1828. Numbers above the lanes indicate the primer pairs (B) used for the PCR analysis. Lane M, DNA marker; left margin, molecular sizes.

tosis virus reverse transcriptase (TaKaRa) with random hexamers, and the cDNA was subjected to PCR to amplify the PG1828 gene by using the primers 1828-up (identical to primer 4) and 1828-dw (5'-TTGAGCGGCTTGTTTCAG-3'). For a negative control, a non-RT sample was also amplified by PCR.

**LPS preparation and bacterial component.** Extraction of LPS by a phenol-water method was performed as described previously (11). The preparation was repurified by a detergent-modified phenol-water extraction method, as described by Manthey and Vogel (20), and visualized by Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 15% polyacrylamide gel and periodic acid-silver (Ag) staining (43). *P. gingivalis* lipid A was isolated by aqueous acetic acid hydrolysis of an LPS preparation from the wild-type strain, followed by successive separation by thin-layer chromatography, as previously described (28). *E. coli* O55:B5 LPS was obtained from Sigma Chemical Co. (St. Louis, Mo.).

**LAL assay.** Various doses of the repurified LPS preparations from the wild-type strain (WT-LPS) and PG1828-deficient mutant (ΔPG1828-LPS) were mixed separately with *Limulus* amoebocyte lysate (LAL) reagent and then incubated at

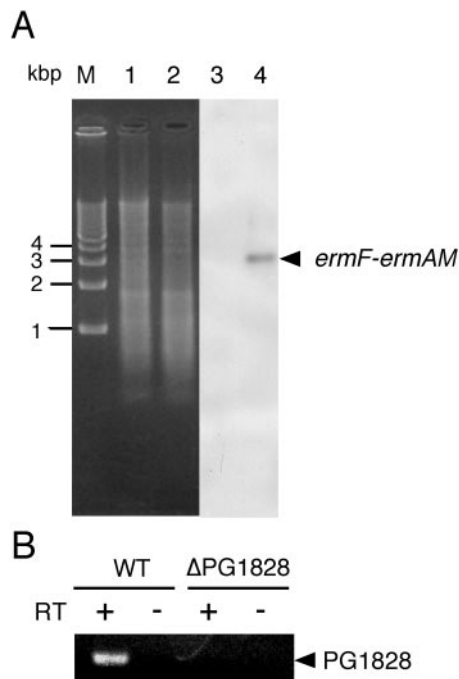


FIG. 2. (A) Detection of *ermF-ermAM* by Southern blot analyses of BamHI-digested chromosomal DNA samples from the wild-type and PG1828-deficient mutant strains. BamHI-digested chromosomal DNA was electrophoresed on a 1% agarose gel (lane M, DNA marker; lane 1, WT; lane 2, ΔPG1828) and transferred to a nylon membrane. The membrane was hybridized with an ALP-labeled *ermF-ermAM* probe, and a chemiluminescence detection assay was carried out (lane 3, WT; lane 4, ΔPG1828). Left margin, molecular sizes. (B) RT-PCR analysis of PG1828 mRNA expression in *P. gingivalis*. The presence (+) or absence (-) of RT in each RT-PCR mixture is indicated above the lanes.

37°C for 30 min, after which the activities were determined with a quantitative chromogenic assay (Seikagaku Kogyo, Tokyo, Japan).

**Visualization of PG1828LP.** The LPS preparation was subfractionated by hydrophobic interaction chromatography according to our previously reported method (11), with a slight modification. Briefly, the LPS preparation was subjected to chromatography on an octyl-Sepharose 4FF column (Amersham Biosciences) and eluted with 0.1 M ammonium acetate with a linear gradient of 1-propanol (15 to 60%). PG1828LP in the LPS preparation was separated by Tris-glycine SDS-PAGE with a 15% polyacrylamide gel and visualized by Coomassie brilliant blue (CBB) staining with Simply Blue SafeStain (Invitrogen Corp., Carlsbad, Calif.).

**Luciferase assay.** Interleukin-3 (IL-3)-dependent murine Ba/F3 pro-B cells stably expressing p55I $\kappa$ B-Luc and an NF- $\kappa$ B/DNA binding activity-dependent luciferase reporter construct (Ba/ $\kappa$ B), murine TLR2 and a p55I $\kappa$ B-Luc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and a p55I $\kappa$ B-Luc reporter construct (Ba/mTLR4/MD-2), were used to detect NF- $\kappa$ B-dependent luciferase activity, as described previously (12, 28). Briefly, the cells were inoculated onto 96-well plates at  $10^5$  cells/100  $\mu$ l of RPMI 1640 supplemented with 10% fetal bovine serum and were stimulated separately with the indicated doses of the WT-LPS or ΔPG1828-LPS preparations. After 4 h at 37°C, 100  $\mu$ l of Bright-Glo luciferase assay reagent (Promega, Madison, Wis.) was added to each well, and luminescence was quantified with a luminometer (Turner Designs luminometer model TD-20/20; Promega). *E. coli* LPS was used as a positive control for TLR4/MD-2 ligand.

Human embryonic kidney 293 cells were transiently cotransfected with 1  $\mu$ g of a human TLR2 expression vector or its empty vector, 50 ng of pNF- $\kappa$ B Luc reporter plasmid (Stratagene), and 50 ng of pRL-TK (Promega) by using Lipofectamine 2000 (Invitrogen Corp.). Twenty-four hours after transfection, the cells were stimulated with the indicated doses of the WT-LPS or ΔPG1828-LPS preparations for 12 h, after which luciferase activity was measured by using a dual-luciferase reporter assay system (Promega).

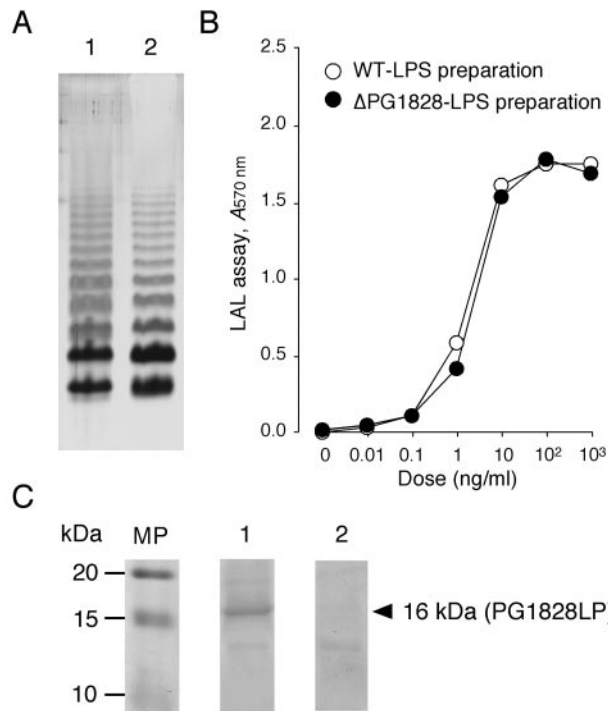


FIG. 3. Characterization of the ΔPG1828-LPS preparation. (A) Tris-glycine SDS-PAGE was performed with a 15% polyacrylamide gel, and proteins were visualized by Ag staining. Lanes: 1, WT-LPS; 2, ΔPG1828-LPS. (B) The indicated doses of the LPS preparations were mixed with LAL reagent, and LAL activity was determined with a quantitative chromogenic assay. Representative results from three independent experiments are shown. (C) The LPS preparation was subjected to chromatography on an octyl-Sepharose 4FF column and eluted with 0.1 M ammonium acetate with a linear gradient of 1-propanol (15 to 60%). The components in the specific fraction were separated by Tris-glycine SDS-PAGE with a 15% polyacrylamide gel, and PG1828LP was visualized by CBB staining. Lanes: MP, protein marker; 1, WT-LPS preparation; 2, ΔPG1828-LPS preparation. Left margin, molecular sizes.

**Mice.** BALB/c mice (male, 8 weeks old) were obtained from Japan SLC, Inc., Shizuoka, Japan. The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan.

**Cytokine assay.** Human gingival fibroblasts were prepared from clinically normal gingival tissues according to a method similar to that described previously (11). After written informed consent under a protocol approved by the Institutional Review Board of Asahi University was received, tissue samples were collected. The cells were cultured in  $\alpha$ -MEM (Sigma Chemical Co.) containing 10% fetal bovine serum, 50  $\mu$ g of gentamicin per ml, and 50 ng of amphotericin B per ml at 37°C in a 5% (vol/vol) CO<sub>2</sub> atmosphere and then were used for the assays at the 5th and 13th passages. The cells were stimulated with the indicated doses of the test specimens for 24 h at 37°C. Following incubation, the culture supernatants were collected and analyzed for secreted IL-8 by using an enzyme-linked immunosorbent assay (ELISA) kit (GT, Minneapolis, Minn.). In some experiments, mice were intraperitoneally injected with 100  $\mu$ g of WT-LPS or ΔPG1828-LPS preparation along with 18 mg of D-galactosamine (D-GalN). Serum specimens from the two groups (each containing five mice) were obtained 1 h after the injection of LPS preparations. These specimens were analyzed for tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-6 concentrations by using an ELISA kit (eBioscience, San Diego, Calif.).

**Virulence model.** It was previously demonstrated that *P. gingivalis* LPS exhibited weak lethal toxicity (40). To sensitize mice to the lethal effects of LPS preparations, we used D-GalN (10). Briefly, a model for lethal toxicity in mice was established with an intraperitoneal injection of the indicated doses of the LPS preparations along with 18 mg of D-GalN diluted in 500  $\mu$ l of saline. For some of the experiments, mice were injected peritoneally with the indicated

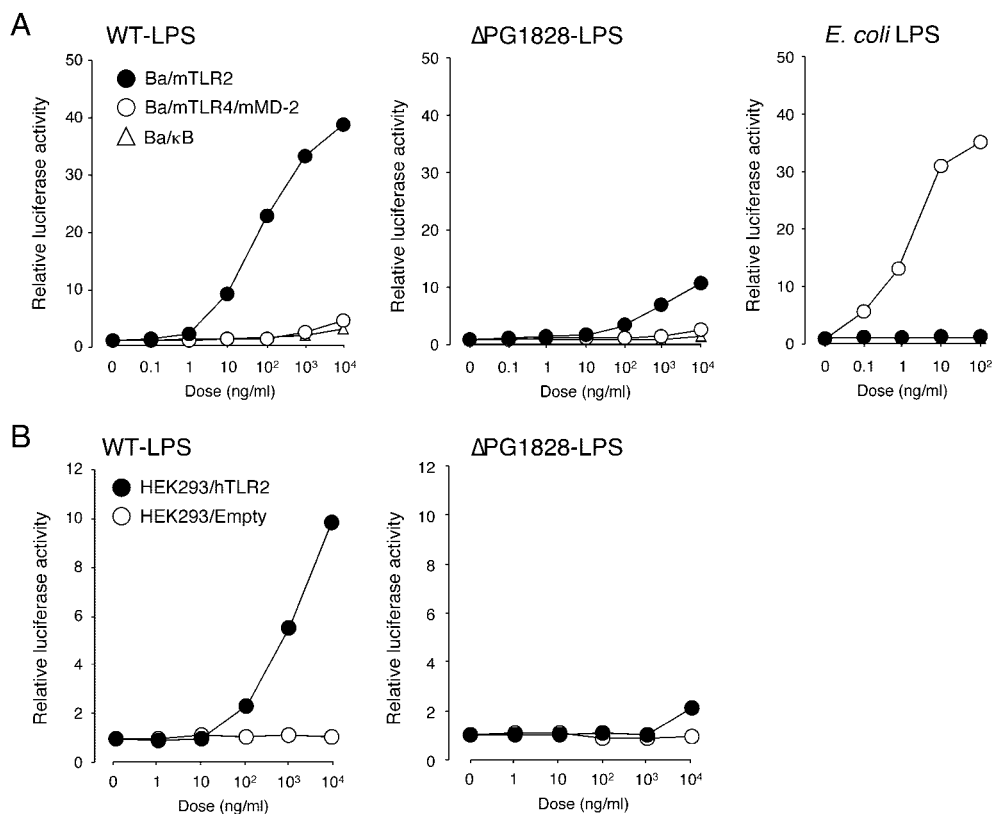


FIG. 4. TLR-dependent activation of the  $\Delta$ PG1828-LPS preparation. (A) Ba/ $\kappa$ B, Ba/mTLR2, and Ba/mTLR4/mMD-2 cells were stimulated with the indicated doses of WT-LPS or  $\Delta$ PG1828-LPS preparations for 4 h. *E. coli* LPS was used as a positive control for TLR4/MD-2 ligand. (B) HEK293/hTLR2 and HEK293/Empty were stimulated with the indicated doses of the WT-LPS or  $\Delta$ PG1828-LPS preparations for 12 h. NF- $\kappa$ B activation was measured with a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to nonstimulated activity.

doses of living *P. gingivalis* cells. Survival was monitored for up to 7 days, and the 50% lethal dose (LD<sub>50</sub>) was calculated as previously described (16).

**Analysis of enzymatic activity.** The amounts of cell-associated enzymes in the wild-type strain and the PG1828-deficient mutant were determined by a method similar to that described previously (3). Briefly, the cells were centrifuged and suspended in phosphate-buffered saline for enzyme analysis. An API ZYM chromogenic assay system (Bio Mérieux, Marcy-l'Étoile, France) was used for estimation of enzyme levels, and the intensity of the color reaction was graded semiquantitatively in comparison to a standard API ZYM color reaction chart. The results were determined from three independent experiments.

**Statistical analysis.** IL-8 production induced by the various doses of  $\Delta$ PG1828-LPS was assessed with a one-way analysis of variance, using the Bonferroni or Dunn method, and the results are presented as the mean  $\pm$  standard error of the mean. Serum cytokine concentrations in different experimental groups were analyzed for statistical significance by using Welch's *t* test.

**Nucleotide sequence accession number.** The DNA sequence obtained in this study has been entered into the DDBJ database under accession number AB189170.

## RESULTS

**Construction of PG1828-deficient mutant of *P. gingivalis*.** To evaluate the biological properties of PG1828LP isolated from the *P. gingivalis* LPS preparation, we constructed a PG1828-deficient mutant of *P. gingivalis* strain 381. The method used for this inactivation is shown in Fig. 1A. Erythromycin resistance was used as the selective marker for homologous recombination between the knockout cassette and chromosomal DNA. The PG1828 knockout cassette was formed by replacing

PG1828 with the erythromycin resistance genes, *ermF-ermAM*. Following the electrotransformation of *P. gingivalis* 381 cells, multiple clindamycin-resistant colonies appeared and a representative colony was selected. The resulting transformants with erythromycin resistance were verified by PCR to have the desired *ermF-ermAM* gene insertion (Fig. 1B and C). Southern blot hybridization with an *ermF-ermAM* gene probe exhibited a single band for the BamHI-digested genomic DNA of the PG1828-deficient mutant (Fig. 2A), and the mutant expressed no PG1828 mRNA in an RT-PCR analysis (Fig. 2B). Further, the DNA sequences of PG1825, PG1826, PG1827, *ermF-ermAM*, and PG1829 of the mutant were checked with *P. gingivalis* 381 genomic DNA (accession no. AB189170) and that of the *ermF-ermAM* gene (accession no. AF219231).

**Characterization of LPS preparation from the PG1828-deficient mutant.** We extracted an LPS preparation from the PG1828-deficient mutant and evaluated its characteristics. The  $\Delta$ PG1828-LPS preparation showed nearly the same ladder-like pattern as the WT-LPS preparation on Ag-stained gels (Fig. 3A). Further, the clotting activities of the  $\Delta$ PG1828-LPS preparation in an LAL assay were the same as those of the WT-LPS preparation (Fig. 3B). These results indicated that the  $\Delta$ PG1828-LPS preparation was equivalent to the LPS molecule of the WT-LPS preparation. We previously showed that PG1828LP could be visualized as a 16-kDa band by SDS-

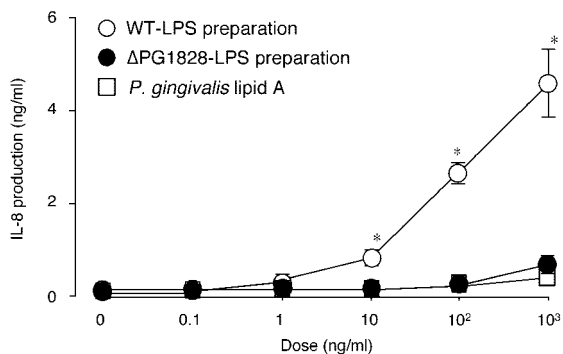


FIG. 5. Cytokine production by human gingival fibroblasts stimulated with the ΔPG1828-LPS preparation. The cells were stimulated with the indicated doses of the WT-LPS preparation, the ΔPG1828-LPS preparation, or *P. gingivalis* lipid A for 24 h. IL-8 production was analyzed by ELISA. Data are shown as the means ± standard error of the means from three independent experiments. *P. gingivalis* lipid A was used for TLR4/MD-2 ligand in the *P. gingivalis* LPS preparation, and the data were described previously (11). \*, significantly different from the mean value of each specimen dose against the ΔPG1828-LPS preparation ( $P < 0.01$ ).

PAGE with CBB staining (11); therefore, the absence of PG1828LP in the ΔPG1828-LPS preparation was confirmed by protein visualization. These preparations were subfractionated by using hydrophobic interaction chromatography to enrich PG1828LP as described previously (11). PG1828LP was then concentrated into specific fractions, and a major CBB-positive band was found at 16 kDa in the WT-LPS preparation; however, no CBB-positive 16-kDa band was found in the ΔPG1828-LPS preparation (Fig. 3C).

**Immunostimulatory activities of the LPS preparation from the PG1828-deficient mutant.** We examined TLR-dependent cell activation by the LPS preparations (Fig. 4). The WT-LPS preparation from *P. gingivalis* 381 exhibited a much higher murine TLR2-dependent NF-κB activation than the ΔPG1828-LPS preparation from the PG1828LP-deficient mutant, whereas only marginal murine TLR4/MD-2-dependent NF-κB activation compared to that with *E. coli* LPS was observed in both LPS preparations. The same results were obtained for the human TLR2-expressing cell line HEK293/hTLR2.

To clarify the cell-activating capacities, we next examined the IL-8-producing activity of the ΔPG1828-LPS preparation by using human gingival fibroblasts, which constitutively express TLR2 and TLR4 (39). ΔPG1828-LPS showed a lower level of IL-8 production than the WT-LPS preparation, which was nearly the same as that of highly purified *P. gingivalis* lipid A (Fig. 5). These results demonstrated that PG1828LP is a

TABLE 1. Lethal toxicity of *P. gingivalis* cells in BALB/c mice

<i>P. gingivalis</i> cells	No. of dead mice/total no. tested with the following dose (10 <sup>8</sup> cells/mouse):					LD <sub>50</sub> <sup>a</sup> (10 <sup>8</sup> cells)
	1	3	10	30	100	
Wild type	0/5	1/5	2/5	5/5	5/5	13.7
PG1828-deficient mutant	0/5	0/5	0/5	0/5	1/5	>93.0

<sup>a</sup> Calculated by the method of Kärber (16).

TABLE 2. Lethal toxicity of *P. gingivalis* LPS preparations in D-GalN-sensitized BALB/c mice

Test specimen	No. of dead mice/total no. tested with the following dose (μg/mouse):			LD <sub>50</sub> <sup>a</sup> (μg)
	1	10	100	
WT-LPS preparation	0/5	2/5	5/5	37.8
ΔPG1828-LPS preparation	0/5	0/5	0/5	>100

<sup>a</sup> Calculated by the method of Kärber (16).

major cell-activating component in the *P. gingivalis* LPS preparation.

**Virulence of the PG1828-deficient mutant in vivo.** To investigate whether PG1828LP is associated with virulence of the bacterium, murine lethal shock was examined. The PG1828-deficient mutant of *P. gingivalis* 381 exhibited a lower level of toxicity than the wild-type strain (Table 1). In addition, an intraperitoneal injection of the WT-LPS preparation resulted in lethal toxicity against D-GalN-sensitized mice, with an LD<sub>50</sub> of 37.8 μg/mouse (Table 2). On the other hand, the ΔPG1828-LPS preparation induced no lethal activities at up to 100 μg/mouse. Further, the mice injected with the ΔPG1828-LPS preparation showed a significant decrease in TNF-α and IL-6 concentrations in serum compared to those injected with the WT-LPS preparation (Fig. 6).

It was previously demonstrated that various proteinases contribute to the virulence of *P. gingivalis* organisms (17, 26, 31). Both wild-type and PG1828-deficient mutant strains exhibit significant levels of alkaline phosphatase, trypsin, acid phosphatase, and *N*-acetyl-β-glucosaminidase, as well as weak butyrate esterase (C4), leucine arylamidase, cysteine arylamidase,

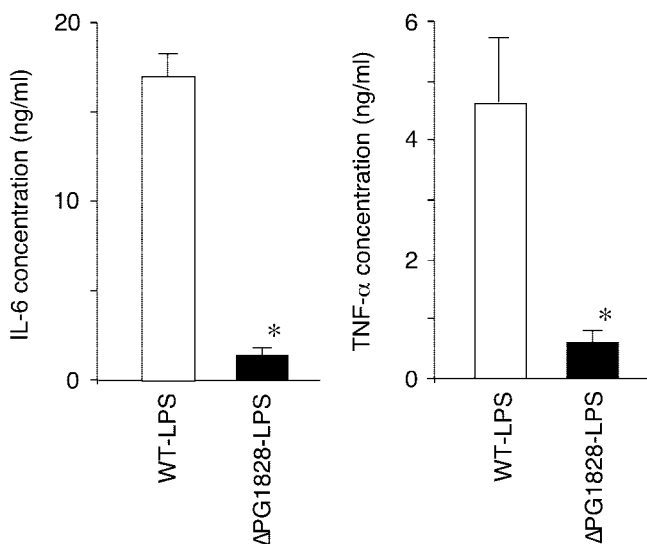


FIG. 6. Cytokine concentrations in sera of mice treated with the ΔPG1828-LPS preparation. Mice were intraperitoneally injected with 100 μg of WT-LPS or ΔPG1828-LPS preparations along with 18 mg of D-GalN, and then serum specimens were collected 1 h after injection of the LPS preparations. TNF-α and IL-6 concentrations were analyzed by ELISA. Error bars indicate standard errors of the means. \*, significantly different from the mean value for the WT-LPS preparation ( $P < 0.01$ ).

and phosphoamidase activities (data not shown). The enzymatic characterizations of these organisms in the present study were consistent with those in a previous report (37). Together, these results show that the reduced lethal activity of the present PG1828-deficient mutant was not associated with its enzymatic properties.

## DISCUSSION

TLR2 is a main signal-transducing receptor that confers sensitivity to a variety of microbial pathogens, such as peptidoglycan, lipoarabinomannan, and bacterial lipoproteins, as well as others (41). Among those pathogens, lipoprotein has been reported to exhibit many biological activities associated with LPS (23, 46). Further, commercially available LPS preparations have been shown to slightly induce signals via TLR2 and TLR4, although the activities, caused by lipoproteins in those preparations, were shown to be removable by phenol reextraction (14, 19).

In the present study, we used a genetic approach to further examine the participation of lipoprotein in a *P. gingivalis* LPS preparation. The PG1828-deficient mutant failed to express PG1828 mRNA (Fig. 2), resulting in the disappearance of the 16-kDa CBB-positive stained band in the  $\Delta$ PG1828-LPS preparation assay results (Fig. 3). These findings indicate that the desired lipoprotein, PG1828LP, was successfully deleted from the deficient mutant. Further, the  $\Delta$ PG1828-LPS and WT-LPS preparations each showed a ladder-like pattern in Ag-stained gels, indicating the presence of a glycoconjugate with repeating units, which is a characteristic of LPS molecules, and there were no differences in LAL clotting activity between the two preparations (Fig. 3). On the other hand, the  $\Delta$ PG1828-LPS preparation exhibited a marked reduction of activation of TLR2-expressing cells compared to the WT-LPS preparation, while it slightly activated human gingival fibroblasts in a manner comparable to the stimulation of highly purified *P. gingivalis* lipid A by TLR4/MD-2 (Fig. 4 and 5). These results indicate that the cell activation induced by the  $\Delta$ PG1828-LPS preparation may have been due to *P. gingivalis* LPS molecules.

Several bacterial lipoproteins have been identified and are thought to play important roles in bacterial pathogenesis. It was previously shown that a 47-kDa lipoprotein (GNA47) of *Neisseria meningitidis* was associated with peptidoglycan metabolism, cell separation, and cell membrane architecture and that it also caused bacteremia and mortality in infant rats (1). In addition, the spirochetal lipoprotein of OspC-deficient *Borrelia burgdorferi* was demonstrated to be unable to invade tick salivary glands, which is a critical step in transmission from the arthropod vector to mammalian host (29). Recently, a murein lipoprotein-deficient mutant of *Salmonella enterica* serovar Typhimurium was demonstrated to be nonvirulent in a murine lethal model (36). In the present study, the PG1828-deficient mutant produced a significantly reduced mortality rate in mice in comparison to the wild-type strain (Table 1), and an intraperitoneal injection of the  $\Delta$ PG1828-LPS preparation was not toxic in D-GalN-sensitized mice, even at the highest dose tested (100  $\mu$ g/mouse) (Table 2). In another study, the lipoprotein obtained from *E. coli* cells was found to induce lethal shock against D-GalN-sensitized mice, and a heat-killed preparation

from a lipoprotein-deficient *E. coli* mutant was less efficient than that from wild-type *E. coli* (46).

It was reported that various proteases were secreted by *P. gingivalis* organisms (17, 26, 31). We found here that both the wild-type and the PG1828-deficient mutant exhibited trypsin-like protease activity (data not shown). The intrinsic protease may digest PG1828LP released from the organisms. The protease, however, seems not to inhibit the virulence of PG1828LP (Table 1). Since mature PG1828LP contains two lysine at the 6th and 42nd amino acid residues from N-terminally lipidated cysteine, tryptic digestion of the lipoprotein gives three peptide fragments, i.e., residues 1 to 6, 7 to 42, and 43 to 52. Our previous study showed that the TLR2-mediated immunostimulating activity of isolated PG1828LP was reduced only about 10-fold by trypsin digestion and that the lipopeptide fragment from residue 1 to 6 still retained the activity (11). It has also been reported that proteinase K or trypsin digestion fails to inhibit the activities of lipoproteins from other bacterial species (8, 35). Further, synthetic lipopeptides with short peptides, such as Pam<sub>3</sub>CSK<sub>4</sub>, show strong TLR2-mediated activities (2). Thus, released PG1828LP, which exhibits immunostimulating activity in vivo and is possibly accessible to intrinsic proteases, acts as a virulence factor of *P. gingivalis*, even if it is digested.

In conclusion, we showed that PG1828LP, a PG1828-encoded lipoprotein from *P. gingivalis*, is a principal component for cell activation and virulence of the organism. The reduced activity of the PG1828-deficient mutant and its LPS preparation in proinflammatory cytokine production and lethal toxicity suggests that PG1828LP is a virulence factor associated with periodontal diseases.

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