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Proteome analysis of *Porphyromonas gingivalis* cells placed in a subcutaneous chamber of mice

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Introduction: *Porphyromonas gingivalis*, an oral anaerobic bacterium, is considered a major pathogen for chronic periodontitis. Pathogenic bacteria usually upregulate or downregulate gene expression to combat the protective responses of their hosts.
Methods: To determine what protein is regulated when *P. gingivalis* cells invade host tissues, we analyzed the proteome of *P. gingivalis* cells that were placed in a mouse subcutaneous chamber by two-dimensional gel electrophoresis and mass spectrometry.
Results: Fourteen proteins were upregulated, while four proteins were downregulated. We focused on three upregulated proteins, PG1089 (DNA-binding response regulator RprY), PG1385 (TPR domain protein), and PG2102 (immunoreactive 61-kDa antigen), and constructed mutant strains that were defective in these proteins. Mouse abscess model experiments revealed that the mutant strain defective in PG1385 was clearly less virulent than the wild-type parent strain.
Conclusion: These results indicate that the PG1385 protein is involved in *P. gingivalis* virulence and that the method used here is useful when investigating the *P. gingivalis* proteins responsible for virulence.

Key words: *Porphyromonas gingivalis*; proteomics; subcutaneous chamber; virulence

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Periodontal diseases are the most prevalent of the oral inflammatory diseases characterized by the destruction of alveolar bone and the supporting connective tissue surrounding teeth (1, 23, 24). These destructive diseases are the outcome of a complex network of molecular and cellular interactions. At the boundary of the microbe and host compartments, many microbial virulence factors are produced to counter the actions of innate and acquired immunity by the host. The molecular activities of these microbes in the confines of a host remain to be determined so understanding the host–microbe interplay would be important to finding potential new targets to inhibit the activities or growth of the organisms responsible for periodontal diseases.

While a large number of different species have been recognized as members of the periodontal environment, *Porphyromonas gingivalis*, a gram-negative anaerobe, has long been considered to play an important role in initiation and progression of periodontal disease (25). The presence of this organism, acting either alone or as a mixed infection with other bacteria, and possibly in concert with certain immunological deficiencies in the host, appears to be essential for disease activity (7, 23). *P. gingivalis* is found to express 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 (8, 11). Expression of these virulent factors is thought to be tightly regulated in response to environmental cues. Although a number of studies describe gene expression of *P. gingivalis* being regulated by environmental stresses (3, 9, 14, 16, 19, 21, 26), gene expression of *P. gingivalis* cells in *in vivo* lesions is not completely understood.

In 1991, Genco et al. (5) reported the development of a mouse subcutaneous chamber model that allows the assessment of host–parasite interactions in localized infections. Burns et al. (2) have shown using the subcutaneous chamber model that the cytokine response to live *P. gingivalis* is impaired in mice that are deficient for Toll-like receptor 2 (TLR-2), but not in TLR-4-deficient mice, and that *P. gingivalis* survival in chamber exudates and blood decreased in TLR-2-deficient mice more than in the wild-type and TLR-4-deficient mice, suggesting that induction of cytokines including tumour necrosis factor-α, interleukin-1β, interferon-γ, and interleukin-10 may not contribute to *P. gingivalis* clearance and rather interferes with it in the subcutaneous chamber model. They also found that the degree of alveolar bone loss in the TLR-2-deficient mice is less than that in the wild-type mice in *P. gingivalis* oral infection experiments.

Using this chamber model, one can easily sample bacteria grown *in vivo* and assess the modulation of potential virulence factors by this specific growth environment. In this study, we describe the identification of genes whose products are upregulated or downregulated in the host using two-dimensional (2D) gel electrophoresis; this can be used to identify changes in gene expression caused by stress treatments and growth conditions. Further, we constructed *P. gingivalis* mutants that were defective in the genes encoding those proteins that were upregulated in the mouse subcutaneous chamber. We also compared the virulence of these mutants using the mouse subcutaneous abscess model.

Materials and methods

Bacterial strain and growth conditions

Porphyromonas gingivalis strain W83 was grown anaerobically (10% CO_2, 10% H_2, 80% N_2) in enriched brain–heart infusion (BHI) broth as described previously (12). Bacterial cells were grown at 37°C until an optical density at 550 nm (OD_{550}) of 1.0 was reached. Cultures were then concentrated by centrifugation at 10,000 ***g*** for 10 min and cells were either collected (control sample), or resuspended in 1/30 of the original volume in fresh, enriched BHI broth.

Experimental animals

Female BALB/c mice approximately 8 weeks old were used in these studies. Coil-shaped subcutaneous chambers were prepared and surgically implanted as previously described by Genco et al. (5). One week after implantation, the chambers were inoculated with 0.4 ml of a concentrated suspension of *P. gingivalis* in enriched BHI broth. Six hours after inoculation, chamber fluid containing bacterial cells (*in vivo* sample) was aseptically removed from each implanted chamber using a hypodermic needle (25-gauge) and syringe. Usually, chamber fluid harvested from three mice was mixed and subjected to 2D gel electrophoresis.

2D gel electrophoresis

P. gingivalis cells were dissolved in 10% trichloroacetic acid. The resulting precipitates were washed several times with acetone and dried. The cells were then suspended in a lyzing buffer [7 M urea, 2 M thiourea, 4% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (CHAPS), 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM tributylphosphin, and 40 mM Tris base]. The lysates were sonicated and centrifuged at 15,000 ***g*** for 10 min. After centrifugation, the supernatants were subjected to 2D gel analysis. Proteins (200 μg) were separated in the first dimension by isoelectric focusing using the Multiphor II system (GE Healthcare Biosciences, Waukesha, WI) according to the manufacturer's instructions. Each protein of the same concentration was loaded on 18-cm rehydrated isoelectric focusing strips with an immobilized pH gradient between pH 4 and 7 (GE Healthcare Biosciences). Proteins were then electrofocused at 20°C in four stages. Focused gel strips were equilibrated for 10 min in equilibration solution A [50 mM Tris–HCl (pH 8.8), 30% glycerol, 6 M urea, 2% sodium dodecyl sulfate (SDS), and 40 mM dithiothreitol) and for further 10 min in equilibration solution B [50 mM Tris–HCl (pH 8.8), 30% glycerol, 6 M urea, 2% SDS, and 135 mM iodoacetamide). SDS–polyacrylamide gel electrophoresis was then performed using 12% separating gels, according to the manufacturer's instructions. After electrophoresis, proteins in the gels were stained with Coomassie brilliant blue R-250.

Peptide mass fingerprinting

Protein spots that differed in their expression profiles were semi-automatically extracted, digested with trypsin, and purified using a ProteomIQ Xcise In-Gel Digest kit (Proteome Systems Inc., Sydney, Australia) and a high-throughput gel-excise processor (Xcise; Shimadzu Co., Kyoto, Japan). Peptide mass analyses were performed using an AXIMA CFR (Shimadzu Co.) matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer in reflectron mode.

Protein identification

Amino acid sequences of the *P. gingivalis* W83 proteins were obtained from The Institute for Genomic Research website (http://www.tigr.org). Proteins were identified from peptide mass data using the database search engine (MASCOT; Matrix Science Ltd, London, UK). The MASCOT score is the probability score for the peptide match calculated from the amino acid sequences database based on the Mowse scoring algorithm (15).

Construction of *P. gingivalis* mutants

P. gingivalis W83 genome sequence data were obtained from The Institute for Genomic Research website (http://www.tigr.org). *P. gingivalis rprY* mutants were constructed as follows. A DNA fragment corresponding to a 0.73-kilobase (kb) region within the *rprY* gene upper region was generated by polymerase chain reaction (PCR) using *P. gingivalis* W83 chromosomal DNA as the template with a forward primer, 5′-GGGTACCCAAAGCATTGATTCCGTTTGC, containing a *Kpn*I site (underlined) and a backward primer, 5′-GGAATTCCCCCTCTTCTCCATCACCAGA, containing an *Eco*RI site (underlined). The resulting fragment was cloned into the pGEM-T Easy Vector (Promega, Madison, WI), to yield pKD714. A DNA fragment corresponding to a region (0.50-kb) located in the *rprY* gene lower region was obtained by PCR with a forward primer, 5′-GGGATCCCATGATGCCACGTAAGGATGG, containing a *Bam*HI site (underlined) and a backward primer, 5′-GGCGGCCGCCGCCCTTTCCGTGGATATTCT, containing a *Not*I site (underlined). The resulting fragment was cloned into the pGEM-T Easy Vector to yield pKD715. The *Kpn*I–*Eco*RI region of pKD714 containing the 0.73-kb fragment was inserted into the same sites of pBluescript SK-, resulting in pKD716.

The *Sac*II–*Sac*I fragment (unique sites of the pGEM-T Easy Vector) of pKD715 containing the *rprY* lower region was inserted with the same sites of pKD716, yielding pKD717. To generate a non-polar mutant, the *erm*F DNA block (1.0-kb), which contains the putative promoter and ribosome binding site but not the terminator region, was generated by PCR using pKD399 (22) as the template with a forward primer, 5′-GGGATCCCCCGATAGCTTCCGCTAT, containing a *Bam*HI site (underlined) and a backward primer, GGATCCCCTACGAAGGATGAAATTT, containing a *Bam*HI site (underlined). The resulting fragment was cloned into the pGEM-T Easy Vector to yield pKD718. To generate an insertion mutant or a non-polar mutant, the *erm*F–*erm*AM DNA block (2.1-kb) from pKD399 or the *erm*F DNA block (1.0-kb) from pKD718 was inserted into the *Bam*HI site of pKD717 that was located at the junction of the 0.73-kb *rprY*-upstream fragment and the 0.60-kb *rprY*-downstream fragment to yield pKD719 or pKD720. The orientation of the *erm*F–*erm*AM DNA block of pKD719 was opposite to that of the *rprY* gene, whereas the orientation of the *erm*F DNA block of pKD720 was the same as that of the *rprY* gene. The pKD719 or pKD720 plasmid DNA was linearized by *Fsp*I digestion and introduced into cells of *P. gingivalis* W83 by electroporation. The cells were spread on tryptic soy (TS) agar containing 10 μg/ml erythromycin (Em) and incubated anaerobically for 7 days. Em-resistant transformants (KDP156 and KDP157) were obtained after the above procedure with pKD719 and pKD720, respectively. Proper DNA replacement of these transformants was verified by Southern blot analysis.

The *P. gingivalis* PG2102 insertion mutant was constructed as follows. A DNA fragment corresponding to a region (0.62-kb) within the PG2102 gene upper region was generated by PCR using *P. gingivalis* W83 chromosomal DNA as the template with a forward primer, 5′-GGGTACCCCGGCAATAGCAATGTGAAGA, containing a *Kpn*I site (underlined) and a backward primer, 5′-GGAATTCCCCACTGAAATTCGGGATCAT, containing an *Eco*RI site (underlined). The resulting fragment was cloned into the pGEM-T Easy Vector (Promega), to yield pKD721. A DNA fragment corresponding to a region (0.64-kb) located in the PG2102 gene lower region was obtained by PCR with a forward primer, 5′-GGGATCCCACGATCAATGGGGAGAGTTG, containing a *Bam*HI site (underlined) and a backward primer, GGCGGCCGCCTGCACGTTCAGCCTGTATTC, containing a *Not*I site (underlined). The resulting fragment was cloned into the pGEM-T Easy Vector to yield pKD722. The *Kpn*I–*Eco*RI region of pKD721 containing the 0.62-kb fragment was inserted into the same sites of pBluescript SK-, resulting in pKD723. The *Bam*HI–*Not*I fragment of pKD722 containing the PG2102 lower region was inserted with the same sites of pKD723, yielding pKD724. The *erm*F–*erm*AM DNA block (2.1-kb) from pKD399 was inserted in the *Eco*RI–*Bam*HI site of pKD717, which was located at the junction of the 0.62-kb PG2102-upstream fragment and the 0.64-kb PG2102-downstream fragment to yield pKD725. The pKD725 plasmid DNA was linearized by *Not*I digestion and introduced into cells of *P. gingivalis* W83 by electroporation. Proper DNA replacement of the resulting Em-resistant transformant (KDP158) was verified by Southern blot analysis.

The *P. gingivalis* PG1385 insertion mutant was constructed as follows. A DNA fragment corresponding to a region (1.17-kb) within the PG1385 gene was generated by PCR using *P. gingivalis* W83 chromosomal DNA as the template with a forward primer, 5′-CCTGTTTGCAGCAGTTATGC and a backward primer, 5′-TTTCCTTCGTGACGCTCTGT. The resulting fragment was cloned into the pGEM-T Easy Vector (Promega), to yield pKD726. The *erm*F–*erm*AM DNA block (2.1-kb) from pKD399 was inserted in the *Bgl*II site of pKD726 to yield pKD727. The pKD727 plasmid DNA was linearized by *Fsp*I digestion and introduced into cells of *P. gingivalis* W83 by electroporation. Proper DNA replacement of the resulting Em-resistant transformant (KDP159) was verified by Southern blot analysis.

Mouse subcutaneous abscess model assay

Virulence of *P. gingivalis* strains W83, KDP157, KDP158 and KDP159 was determined as described by Neiders et al. (13). Each bacterial strain was grown in enriched BHI broth to an OD_{550} of 1.0. The cells were harvested, resuspended, and adjusted to a concentration of 1×10^{11} colony-forming units/ml in the same medium. Female BALB/c mice (8–10 weeks old) were challenged with subcutaneous injections of 0.1 ml bacterial suspension at two sites on the depilated dorsal surface (0.2 ml per mouse). Injected mice were examined daily for the presence and locations of secondary lesions and the health status of each mouse was recorded.

Statistical analysis

Statistical analysis was by two-way repeated measures analysis of variance (ANOVA) for comparison of the survival and one-way ANOVA for comparison of the virulence between wild-type and mutant strains. Tukey's honestly significant differences (HSD) test was used as a *post hoc* test. A difference with $P < 0.05$ was considered significant. All statistical analyses were performed using the STATISTICAL ANALYSIS SYSTEM (Version 14.0, SPSS Japan Inc., Tokyo, Japan). Student's *t*-test was used to evaluate the difference in the mean value of abscess formation rate of three experiments.

Results

Proteome analysis

We attempted to compare the protein expression in the *in vivo* sample with that in the control sample. Two representative 2D separations of cellular proteins obtained from the control sample and *in vivo* sample are presented in Fig. 1A and Fig. 1B, respectively. Fourteen protein spots of the *in vivo* sample were markedly increased, and four spots were decreased compared to those of the control sample (Fig. 1). Next, the peptide mass analysis of these proteins and database analysis with the *P. gingivalis* genome sequence were performed (Table 1). Of the 14 upregulated proteins examined, 10 proteins were identified as *P. gingivalis* proteins. Four proteins were not determined or were derived from mouse. We found that the upregulated-proteins were related to protein synthesis, detoxification, energy metabolism, and degradation of proteins. Interestingly, heat-shock protein DnaK was found to be downregulated in the *in vivo* sample.

Virulence of *P. gingivalis* mutants in the mouse subcutaneous abscess model

From the protein list given in Table 1, three upregulated proteins, PG1089 (RprY), PG2102 (immunoreactive 61-kDa antigen), and PG1385 [tetratricopeptide repeat (TPR) domain protein], were selected, and chromosomal mutants defective in the proteins were constructed to analyze the involvement of these proteins in *P. gingivalis* virulence. BALB/c mice were challenged with subcutaneous injections of bacterial suspensions of strain W83 and its mutant strains, KDP157, KDP158, and KDP159 (2×10^{10} colony-forming units per animal). The wild-type

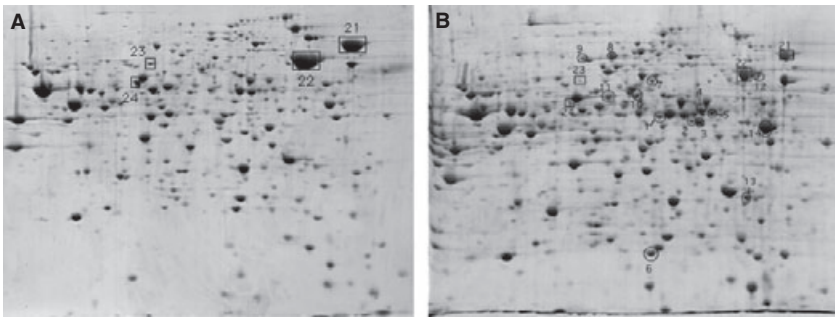

Fig. 1. Representative two-dimensional electrophoresis gels of the *Porphyromonas gingivalis* cell preparation. (A) *P. gingivalis* cells grown in enriched brain–heart infusion broth. (B) *P. gingivalis* cells placed in a mouse subcutaneous chamber for 6 h. Experiments were performed more than three times. The averaged gels were examined using image matching software (PDQUEST Version 7.2; Bio-Rad, Hercules, CA) to detect spots. Spots were analyzed with MALDI-TOF mass spectrometry. The identified proteins are listed in Table 1. The upregulated protein spots are numbered from 1 to 14 and the downregulated protein spots are numbered from 21 to 24.

Table 1. Identification of protein spots with MALDI-TOF mass analysis

Spot no.[1]	TIGR locus	Definition	MASCOT score[2]
Upregulated			
1	PG0387	Translation elongation factor Tu	122
2	–	Not determined	
3	–	Actin from the host	
4	PG1055	Thiol protease	156
5	PG1360	Phosphoribosylamine-glycine ligase	58
6	PG0618	Alkyl hydroperoxide reductase, C subunit	105
7	PG1424	Peptidylarginine deiminase	110
8	–	A serum protein from the host	
9	PG0249	Oxaloacetate decarboxylase, putative	65
10	PG1844	Lysine-specific cysteine proteinase Kgp	70
11	–	Not determined	
12	PG2102	Immunoreactive 61-kDa antigen PG91	145
13	PG1089	DNA-binding response regulator RprY	167
14	PG1385	TPR domain protein	204
Downregulated			
21	PG1208	DnaK protein	58
22	PG0520	Chaperonin, 60-kDa	92
23	PG0962	Prolyl-tRNA synthetase	107
24	PG0690	4-Hydroxybutyrate coenzyme A-transferase	48

[1]The protein spots indicated in Fig. 1 were analyzed using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.
[2]Proteins were identified using MASCOT with a custom *P. gingivalis* W83 open reading frame database obtained from The Institute for Genomic Research (TIGR).
TPR, tetratricopeptide repeat.

strain W83 induced spreading, ulcerative lesions on the abdomens of all mice tested by 24 h and the mice showed ruffled hair, whereas the mice injected with KDP159 displayed fewer lesions with regard to the number of mice with lesions, the size of the abscess, and the whole body state (Fig. 2). The average abscess sizes for the mice injected with KDP157 and KDP158 were smaller than that for the mice injected with W83, and the whole body state of the mice injected with KDP157 was better than that of the mice injected with W83; however, there were no statistical differences in the number of mice with lesions and the size of abscess.

Discussion

In the present study, we used 2D electrophoresis technology to study host–microbe interactions. 2D electrophoresis is a powerful tool for resolving protein mixtures into their component polypeptides and can be used to compare different test samples. It is reported that 2D electrophoresis of proteins extracted from *P. gingivalis* often gave poor resolution and/or the disappearance of certain protein spots as a result of the presence of abundant proteases within the samples. However, degradation of proteins could be prevented by pretreatment of cells with trichloroacetic acid as previously described by Pridmore et al. (17).

P. gingivalis strain W83 was selected as the virulent strain because many previous reports have described its virulence-associated activities and disease-promoting characteristics *in vitro* and *in vivo* (6, 10). When the strain was directly inoculated into the subcutaneous tissues of mice, this inoculation resulted in rapidly spreading purulent infection. We, therefore, tried to use the subcutaneous chamber model to obtain sufficient samples of *in vivo*-grown bacterial cells.

Ten proteins, the amounts of which were increased *in vivo*, included proteins with various functions such as oxidative stress protection and protein degradation. In this study, we focused on three proteins [immunoreactive 61-kDa antigen (PG2102), DNA-binding response regulator RprY (PG1089), and TPR domain protein (PG1385)] and constructed mutant strains that were defective in these proteins. These mutant strains formed pigmented colonies on the blood agar plates and produced gingipains as well as the wild-type parent strain (data not shown). Virulence analysis with subcutaneous injection in mice revealed that the PG1385-defective mutant was less virulent than the wild-type parent strain, whereas we could not find statistical differences in the number of mice with lesions and the size of abscess among the PG2102-defective mutant, the PG1089-defective mutant, and the wild-type parent strain. However, the whole body state of the mice injected with the PG1089-defective mutant was obviously better than that of the mice injected with the wild-type parent strain.

PG1385 is one of the TPR-containing proteins. TPR is a structural motif present in a wide range of proteins. It mediates a variety of different protein–protein interactions. TPR-containing proteins are found in a number of different organisms including prokaryotes and eukaryotes. Okano et al. (14) reported that 19 proteins of *P. gingivalis* W83 are upregulated by aeration and that the 19 proteins include HtpG (PG0045), chaperonin 60-kDa (PG0520), DnaK (PG1208), TPR domain protein (PG1385), trigger factor (PG0762), RagA (PG0185), and AhpC (PG0618). In the subcutaneous chamber in the present study, TPR domain protein (PG1385) and AhpC (PG0618) were increased, whereas chaperonin 60-kDa (PG0520) and DnaK (PG1208) were decreased, indicating that oxidative stress is among the stresses suffered by *P. gingivalis* cells in the chamber. The TPR domain protein

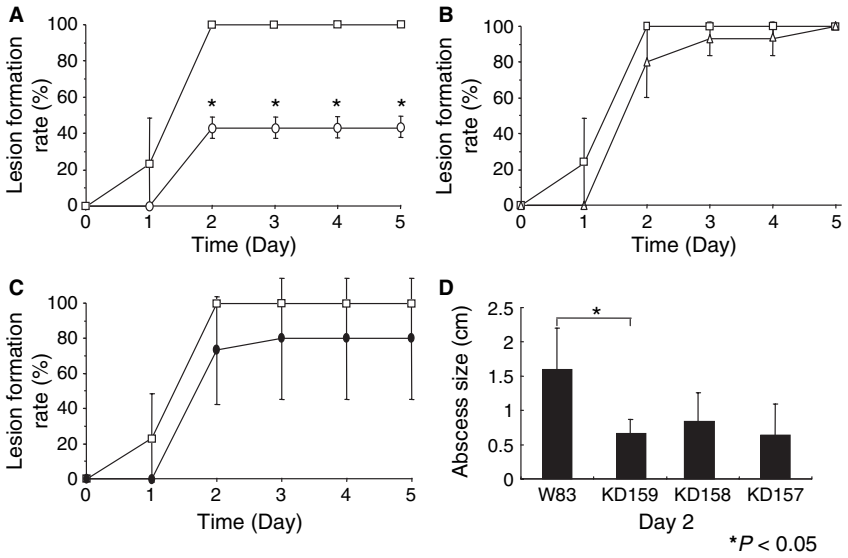

Fig. 2. Mouse subcutaneous abscess model assay. *Porphyromonas gingivalis* cells were subcutaneously injected at two sites on the depilated dorsal surface of female BALB/c mice. The animal experiments used six mice for each bacterial strain and were performed three times. (A–C) Lesion formation rates: (A) open rectangle, W83; open triangle, KDP159 (PG1385 mutant); (B) open rectangle, W83; open circle, KDP158 (PG2102 mutant); (C) open rectangle, W83; closed circle, KDP157 (PG1089 mutant). (D) Average diameter of abscesses on the abdomens 2 days after inoculation.

(PG1385) is a major protein located in the periplasm (20), implying that PG1385 binds to other proteins in the periplasm or protruding from the inner or outer membrane.

PG1089 is a feature of the DNA-binding response regulator of the two-component system and its homologue, RprY, is found in *Bacteroides fragilis*. The *B. fragilis rprY* gene is located downstream of *rprX*, which encodes one of the histidine protein kinase components of the two-component regulatory system (18). However, there is no such a gene encoding the histidine protein kinase around PG1089 in the *P. gingivalis* genome. Expression of *B. fragilis rprX* and *rprY* genes in *Escherichia coli* downregulates the outer membrane porin proteins OmpF and OmpC, suggesting that the two-component system regulates the expression of outer membrane proteins (18). It was reported that the gene encoding PG1089 is induced during contact of *P. gingivalis* cells with Hep-2 epithelial cells (9). Very recently, Duran-Pinedo et al. (4) have found that RprY directly binds and upregulates the *ahpC* gene as well as the NADH : ubiquinone oxidoreductase operon.

In conclusion, we found 10 up-regulated genes and 4 down-regulated genes by proteomics analysis of *P. gingivalis* cells placed in a mouse subcutaneous chamber. Mutant analysis of the up-regulated genes suggested that the PG1385-encoding gene was clearly responsible for virulence of *P. gingivalis*.

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