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A member of the peptidase M48 superfamily of *Porphyromonas gingivalis* is associated with virulence *in vitro* and *in vivo*

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Background: *In vivo*-induced antigen technology was previously used to identify 115 genes induced in *Porphyromonas gingivalis* W83 during human infection. One of these, PG2197, a conserved hypothetical protein which has homology to a Zn-dependent protease, was examined with respect to a role in disease.
Design: The expression of PG2197 in human periodontitis patients was investigated, but as there is increasing evidence of a direct relationship between *P. gingivalis* and cardiovascular disease, a mutation was constructed in this gene to also determine its role in adherence, invasion, and persistence within human coronary artery endothelial cells (HCAEC) and neutrophil killing susceptibility.
Results: Plaque samples from 20 periodontitis patients were analyzed by real-time PCR, revealing that PG2197 was expressed in 60.0% of diseased sites compared to 15.8% of healthy sites, even though *P. gingivalis* was detected in equal numbers from both sites. The expression of this gene was also found to be up-regulated in microarrays at 5 and 30 min of invasion of HCAEC. Interestingly, a PG2197 mutant displayed increased adherence, invasion, and persistence within HCAEC when compared to the wild-type strain.
Conclusion: This gene appears to be important for the virulence of *P. gingivalis*, both *in vivo* and *in vitro*.

Keywords: *Porphyromonas gingivalis*; *IVIAT*; *periodontitis*; *cardiovascular disease*

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An estimated 80% of American adults currently have some form of periodontal disease. In 2000, the cost of therapy in the USA for the treatment of periodontal disease was estimated at $5–6 billion (1). Periodontitis is an inflammation of the periodontium that results in destruction of the bone and connective tissue attachment of the teeth. Periodontal diseases can range in severity from the mildest form, gingivitis, to the more common, chronic periodontitis, to the most severe, necrotizing periodontal disease (2). The major characteristics of the disease are microbial plaque formation, periodontal inflammation, loss of attachment and alveolar bone, and consequent periodontal pocket formation (3). *Porphyromonas gingivalis* is recognized as an important bacterial species in the pathogenesis of periodontitis and is most often isolated from subgingival sites (4–7). Many elements of periodontitis can be attributed to virulence factors from *P. gingivalis*, such as proteolysis, edema, neutrophil accumulation, and bleeding on probing (8). This bacterial species is also capable of invading several epithelial and endothelial cell lines including human coronary artery endothelial cells (HCAEC) (9, 10). Cellular invasion is a mechanism by which bacteria can evade host immunologic defenses. Previous studies on the invasion of HCAEC by *P. gingivalis* were performed in relation to an epidemiological and experimental link observed between cardiovascular disease and the presence of periodontal pathogens such as *P. gingivalis* (9–13). Gene expression during invasion of HCAEC was also determined and microarray analyses demonstrated that 62 genes were differentially regulated (14).

Virulence gene expression is modulated by bacteria in response to their environment (15). Previously, *in vivo*-induced antigen technology (IVIAT) (16) identified 115 *P. gingivalis* genes that were induced *in vivo* in humans during periodontal disease (17). One of the genes identified, PG2197 (conserved hypothetical protein, J. Craig Venter Institute; http://cmr.jcvi.org/tigr-scripts/CMR/shared/GenePage.cgi?locus=PG_2197), has domain homology to a Zn-dependent protease with chaperone function and may be an HtpX homologue. HtpX is a stress-controlled protease identified in *Escherichia coli* (18) that is under control of the two-component stress response system Cpx (19, 20). In this study, RT-PCR and real-time PCR were used to probe patient plaque samples to confirm *in vivo* gene expression of PG2197. In addition, microarrays were used to examine differential expression of PG2197 during co-cultivation with HCAEC. Additionally, a mutation was constructed in PG2197 and the mutant, as well as the parent strain were tested for (1) the ability to adhere, invade, and persist within HCAEC and (2) the ability to survive phagocytosis by mouse neutrophils.

Materials and methods

Bacterial and cell culture conditions

P. gingivalis W83 and W83Δ2197 were grown as described elsewhere (21). HCAEC (Lonza, Walkersville, MD) were maintained at 37°C with 5% CO_2 in endothelial cell basal medium-2 (EBM-2) supplemented with EGM-2-MV singlequots (Lonza) according to the manufacturer's protocol. HCAEC were seeded at 1×10^5 cells/well into 24-well tissue culture plates and incubated overnight in antibiotic-free EBM-2.

RNA sample collection

P. gingivalis W83 cultures were grown in supplemented brain heart infusion (BHI) broth and subcultured. RNA was extracted as described below. Using criteria described elsewhere (22) and after Institutional Review Board approval, subgingival plaque samples were collected from two healthy and two diseased sites from 20 patients using a Gracey curette. Plaque was removed from the curette by sterile paper point and placed into 1 mL of Trizol (Invitrogen). Samples were vortexed and stored at –80°C for subsequent RNA extraction.

RNA isolation and reverse transcription (RT)

RNA isolations were performed by using Trizol and the RNeasy kit (Qiagen) with DNase treatment as described elsewhere (12). Reverse transcription (RT) was performed by using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (Qiagen) following the manufacturer's protocol, reactions were incubated at 48°C for 16 hours. cDNA samples were purified by using the PCR purification kit (Qiagen) following the manufacturer's protocol.

Real-time RT-PCR

Primers (Table 1) were designed by using Beacon Designer software (Premier Biosoft International). *P. gingivalis*-specific 16S rRNA primers used were those previously reported (22). All other primers used were blasted against nucleotide databases to ensure specificity. Real-time RT-PCR was performed by using an iCycler Thermal Cycler and iQ SYBR green supermix according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). DNA fragments of each gene were used as internal controls and in the generation of

Table 1. Gene-specific primers used for real-time PCR, mutant construction, and mutant confirmation

Application/Gene	Primer name	Sequence (5′ → 3′)	Product size (bp)
Real-time RT-PCR			
P. gingivalis 16S rRNA	Pg 16s F	CATAGATATCACGAGGAACTCCGATT	436
	Pg 16s R	AAACTGTTAGCAACTACCGATGTGG	
PG2197	PG2197 realtime F	ACATCCACGGCACAGGTG	81
	PG2197 realtime R	TCCCAATCCGTTTTGCTTCAG	
Mutant construction			
PG2197	2197 5′ F	GGTATGCCATGGAGGAAT	423
	2197 5′ R	AAAAGGTACCGCAAGGCACTGGAGGACA	
	2197 3′ F	TTTTTCTAGAATCGGATAGCTGCCATTC	449
	2197 3′ R	AGCTTCTACCTCTGCCTCTATT	
Mutant confirmation			
W83Δ2197	2197 confirm F	GATTTCGGGGCGCAGTCAGA	2,236/3,837[a]
	2197 confirm R	AGCGGATCGGAAAATTGGAGTCG	

[a]Product sizes are listed for both the wild-type and the mutant strains.

standard curves. All real-time PCR reactions were run with an annealing temperature of 55°C. Data normalization and analysis were performed by using iCycler and Microsoft Excel software. Gene expression was normalized to the *P. gingivalis* 16S rRNA expression.

Mutant construction

W83Δ2197 was constructed by allelic replacement as described elsewhere (21). Briefly, upstream and downstream regions of PG2197 were amplified by PCR using gene-specific primers (Table 1) and cloned into the suicide vector pPR-UF1. This vector has been described previously (11). The mutation was confirmed by PCR and growth curves were performed to confirm that growth rate was not altered (data not shown). The mutation was not expected to cause any polar effects since PG2197 does not appear to exist in an operon.

Adhesion assays

Adhesion assays were performed as described elsewhere (23) with minor modifications. *P. gingivalis* W83 or W83Δ2197 from overnight cultures were added to antibiotic-free EBM-2 and bacteria were allowed to adhere to HCAEC at a multiplicity of infection (MOI) of 100 for 30 min at 4°C. Non-adherent bacteria were washed away with cell-culture medium. Cells and adherent bacteria were fixed with 5% formalin for 15 min at 37°C, washed, and then blocked (5% BSA, 1% goat serum, and 0.1% Tween20 in PBS) for 1 hour at room temperature. Adherent bacteria were detected with polyclonal rabbit anti-*P. gingivalis* serum followed by a horseradish peroxidase (HRP)-labeled mouse anti-rabbit antibody (Cappel, Solon, OH). An ELISA was performed to confirm that the polyclonal antibody bound similarly to both W83 and W83Δ2197. Colorimetric substrate 3,3′,5,5′-tetramethylbenzidine (Sigma, St. Louis, MO) was added to each well and incubated with the cells for 5 min at room temperature. The reaction was stopped with the addition of an equal volume of 1 N HCl and the OD_{450} was measured with an ELISA plate reader (Bio-Rad). Wells containing cells alone or cells and bacteria with no primary antibody were used as controls.

Invasion and persistence assays

Invasion and persistence assays were performed as described previously (11). A total of 10^5 HCAEC/well were seeded into a 24-well tissue culture plate and incubated at 37°C overnight. *P. gingivalis* W83 or W83Δ2197 was added at a concentration of 10^7 bacterial cells/ml per well (MOI of 100) and bacteria were allowed to invade the cells for 1.5 hours. After washing, 300 μg/mL gentamycin and 200 μg/mL metronidazole were added to kill extracellular bacteria. After an additional 1-hour incubation, the cells were washed and either lysed with 1 mL of water for 20 min and plated for enumeration on blood agar plates or antibiotic-free medium was added to the cells before reincubating. In the later case, at 6, 12, and 24 hours post-inoculation, the supernatant was collected and centrifuged at $600 \times g$ for 5 min. The pelleted bacteria were resuspended in PBS and plated on blood agar to enumerate the extracellular bacteria. Subsequently, HCAEC were lysed with water and intracellular bacteria were enumerated by plating on blood agar. All individual cell culture experiments were performed by using triplicate wells and each experiment was performed three times.

Microarray hybridizations and data analysis

P. gingivalis W83 microarray glass slides (version 2) were obtained from J. Craig Venter Institute (http://www.jcvi.org/). HCAEC were infected as described above with *P. gingivalis* W83. After 5 and 30 min, total RNA was isolated from internalized bacteria, RT and microarray reactions were performed as described elsewhere (14). Control reactions by using broth cultures and data analysis were done as described previously (14).

Neutrophil killing

The susceptibility of *P. gingivalis* to neutrophil killing was measured as described previously with minor modifications (24). Briefly, overnight cultures of *E. coli* S17-1 (control) and *P. gingivalis* W83 and W83Δ2197 were pelleted and resuspended to 1×10^8 CFU/mL in PBS. Each bacterial suspension (70 μL) was opsonized with 50 μL of polyclonal rabbit anti-*P. gingivalis* serum. After opsonization, 0.5 mL of the neutrophil suspension (5×10^5 polymorphonuclear leukocytes) was added to the bacteria/serum mixture and incubated at 37°C at 300 rpm in a thermomixer. After 4 hours, a 30 μL sample was collected, diluted, and plated. Experiments were performed in triplicate.

Statistical analysis

Comparisons between groups were done by using a Student *t*-test. Data that were not normally distributed were compared by using the Mann–Whitney Rank sum test. Differences in proportions were compared by using the Fisher's exact test plus Yate's correction. A value of $P < 0.05$ was considered statistically significant.

Results

Confirmation of in vivo-induced antigen technology (IVIAT) gene expression in patient plaque samples

Real-time PCR was used to assay *in vivo* expression of gene PG2197 in plaque samples from human periodontitis patients. No statistically significant differences were observed in *P. gingivalis* detection ($P > 0.05$) between the plaque sites; 73.5% of samples taken from the diseased sites were positive for *P. gingivalis* as measured by previously reported *P. gingivalis*-specific 16S primers

(22) while 54.3% of healthy plaque samples tested positive for *P. gingivalis*. However, when comparing the expression pattern in diseased versus healthy plaque sites, PG2197 was expressed significantly more in diseased sites (60.0%) than in healthy sites (15.8%, $P < 0.01$).

Microarray experiments

Analysis of microarrays showed that a total of 160 *P. gingivalis* genes were differentially regulated at 5 min and a total of 67 genes were differentially regulated at 30 min of co-culture with HCAEC (Fig. 1). Of these, 18 genes were differentially regulated at both time points. At 5 min of contact, 56 and 104 genes were up and down-regulated, respectively (data not shown). At 30 min, 13 and 54 genes were up and down-regulated, respectively (data not shown). Gene PG2197 was, among those, found to be up-regulated at both the time points, 3.0 and 4.5-fold at 5 and 30 min, respectively. These data were confirmed by Real-Time PCR by using *P. gingivalis*-specific primers showing a 4.3 and 5.4-fold up-regulation when compared to the control.

Adherence, invasion, and persistence

Mutant W83Δ2197 demonstrated significantly enhanced adherence to HCAEC at 30 min when compared to W83 (2.6-fold, $P < 0.001$, Fig. 2**A**) and the ability to invade HCAEC was also increased by 2.3-fold for W83Δ2197 at 2.5 hours ($P < 0.01$, Fig. 2**B**). However, when invasion efficiency was normalized to the adherence data, W83Δ2197 showed no increase in invasion. The persistence of W83 and W83Δ2197 within HCAEC was also examined. The results are expressed as the percentage of the wild-type that was recovered at 2.5 hours post-inoculation (Fig. 3). After 6 hours, more W83Δ2197 were recovered from the medium (3.6-fold, $P = 0.06$, Fig. 3**A**) than W83, although not statistically significant. After 12 hours, a similar percentage of W83Δ2197 was recovered from within the cells when compared to W83 (Fig. 3**B**). However, at 6 and 24 hours greater numbers of W83Δ2197 (1.6-fold, $P < 0.05$; 3.1-fold, $P < 0.05$) were recovered when compared to W83 (Fig. 3**B**).

Neutrophil killing

There was no statistical difference in the susceptibility to killing by neutrophils when *P. gingivalis* W83Δ2197 was compared to W83 (Fig. 4).

Discussion

Previously, IVIAT was used to identify immunogenic bacterial genes of *P. gingivalis* expressed specifically during infection but not in regular culture conditions (11). One of the genes identified by this technique, PG2197, is described as a conserved hypothetical protein and likely localizes to the inner or outer membrane (PSORT). PG2197 domain homology is recognized as a Zn-dependent protease with chaperone function. This class of metalloproteases belongs to the MEROPS peptidase family M48 and often is described as probable HtpX homologues (25). As IVIAT has previously identified *in vivo*-expressed genes that are likely related to virulence, characterization of this newly discovered gene in *P. gingivalis* was pursued to determine its relation to disease.

This study reports that the PG2197 gene is significantly expressed in periodontitis disease sites (60%), but it is not frequently expressed in healthy sites (16%) even though *P. gingivalis* was detected in equal amounts from both sites. Thus, PG2197 shows a correlation to disease. Previously, we have reported that another IVIAT identified gene of *P. gingivalis* W83 (PG1334; a band 7/MEC-2 family gene) also had a higher expression in diseased sites versus healthy sites and again *P. gingivalis* was present in similar amount in both sites (11). Detection of *P. gingivalis* in similar frequency from periodontally healthy and diseased sites is not uncommon and has been previously observed (26, 27). Therefore, bacteria could be present but not necessarily expressing virulence genes until a triggering event happens and initiation of disease occurs.

Since invasion of HCAEC has been used a model of host/pathogen interactions in relation to cardiovascular disease (9–13), microarray analysis was used to identify those genes of *P. gingivalis* W83 that are up-regulated in response to co-culture with HCAEC. This analysis detected differential expression of 160 and 67 genes during infection of HCAEC at 5 and 30 min, respectively. Most pertinent to this study, PG2197, was one of the genes identified as being up-regulated after only 5 and 30 min of contact with host cells, suggesting its importance in early colonization events. To further characterize the phenotypic trait of this gene, a mutation in the gene was constructed using allelic replacement. The mutant was tested for its ability to resist killing by mouse neutrophils and loss of this protein does not impact its susceptibility to killing by neutrophils. Quantitative invasion assays of the mutant and wild-type were then performed by using HCAEC, a cell model previously used to study virulence factors. Surprisingly, the adherence of W83Δ2197 was increased when compared to adherence of the wild-type strain. As adherence is an initial event in invasion, it is possible that the enhanced invasion and persistence seen with the PG2197 mutant were only due to its increased adherence. This was confirmed when the invasion data were normalized to the adherence data. Cellular invasion is an important survival mechanism by which bacteria can evade host immunologic defenses. *P. gingivalis* has been previously shown to use this mechanism for survival (10, 28). Furthermore, extracellular bacteria were also recovered in the medium at all time points for both the wild-type and the mutant strains. This could be due to cell lysis and subsequent release of bacteria or by a mechanism used by *P. gingivalis* to ensure transmission from one cell

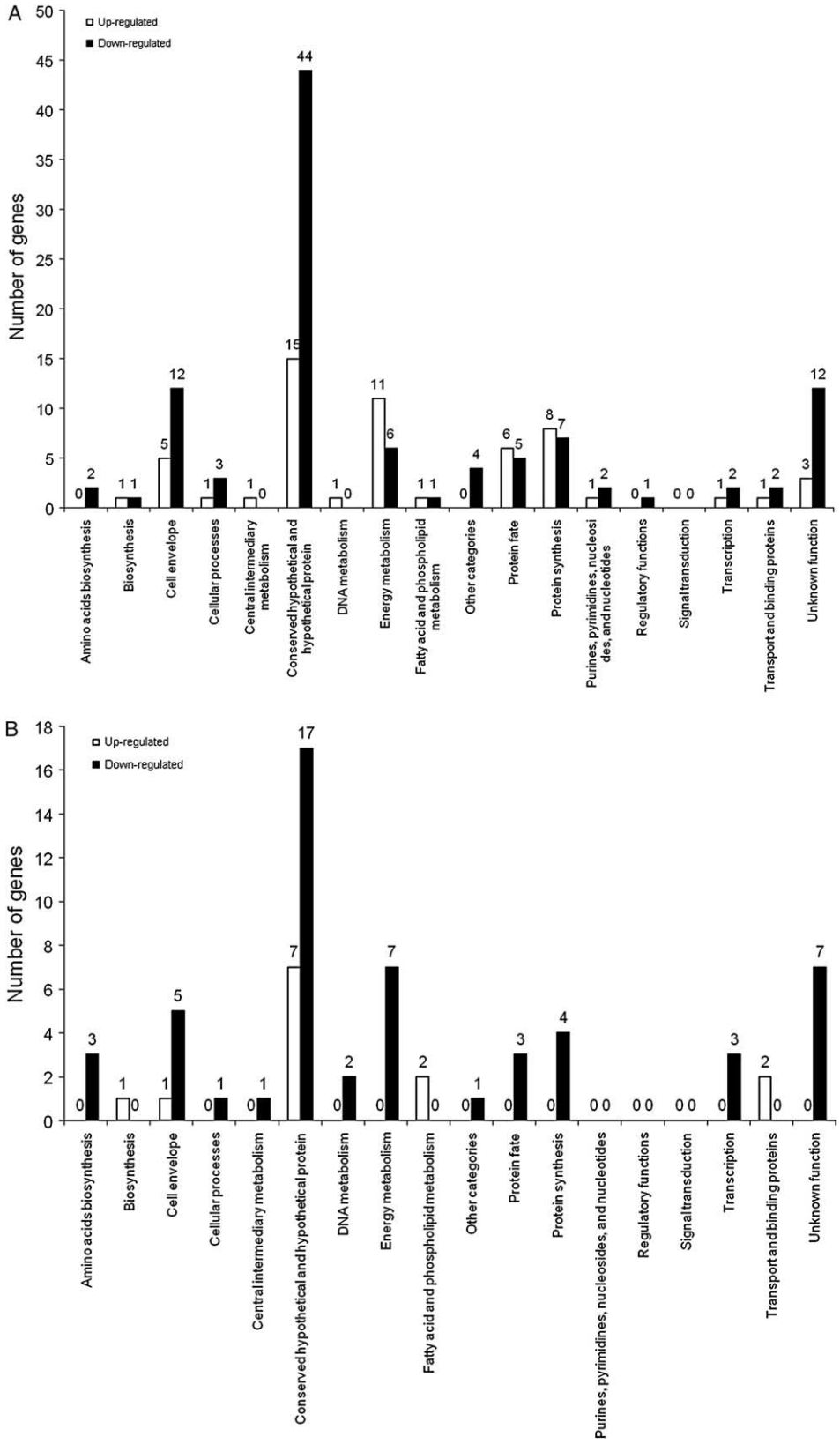

Fig. 1. Distribution of differentially expressed genes grouped by functional classification according to the TIGR *P. gingivalis* genome database. Numbers above the bars indicate the number of genes differentially expressed in each functional group after (A) 5 min or (B) 30 min adherence to HCAEC.

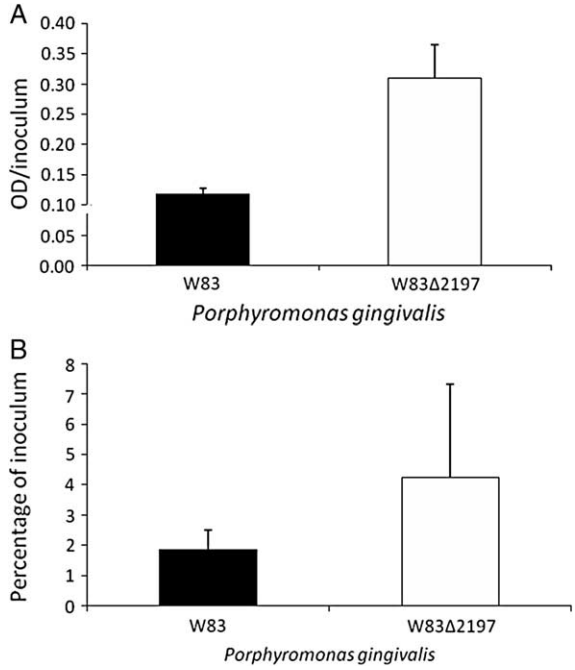

Fig. 2. Infection of HCAEC by *P. gingivalis* wild-type (black bar) and mutant strain (white bar). (A) Adherence to HCAEC at 4°C. The *P. gingivalis* strains were allowed to adhere to HCAEC for 30 min at 4°C (to prevent internalization of bacteria) before non-adherent bacteria were washed away. Host cells were then fixed with 5% formalin and probed with polyclonal rabbit anti-*P. gingivalis* serum followed by an HRP-labeled mouse anti-rabbit secondary antibody. The mutant strain W83Δ2197 showed enhanced adherence to HCAEC (2.6-fold, $P < 0.001$). (B) Invasion of HCAEC (2.5 hours) expressed as the percentage of inoculum. *P. gingivalis* W83 or W83Δ2197 were allowed to invade HCAEC for 1.5 hours. Cells were washed and antibiotics were added to kill extracellular bacteria. After a 1-hour incubation, the cells were washed and lysed. The intracellular bacteria were diluted in PBS and plated for enumeration on blood agar. The PG2197 mutant demonstrated increased invasive ability at 2.5 hours compared to the wild-type (2.3-fold, $P < 0.01$).

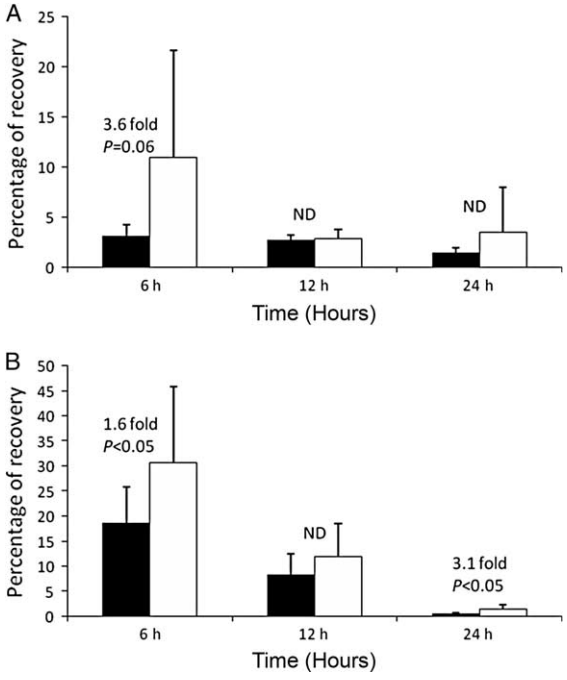

Fig. 3. *P. gingivalis* W83 (black bar) and mutant W83Δ2197 (white bar) detected extracellularly (A) or intracellularly (B) at 6, 12, and 24 hours after inoculation of HCAEC, expressed as the percentage of that strain recovered at 2.5 hours post-inoculation. *P. gingivalis* W83 or W83Δ2197 were initially allowed to invade the HCAEC for 1.5 hours. The cells were then washed and antibiotics were added to kill extracellular bacteria. After an additional hour, the cells were again washed and antibiotic-free medium was added to the wells. At 6, 12, and 24 hours post-inoculation, the supernatants were collected, centrifuged the pelleted bacteria were resuspended in PBS and plated on blood agar to enumerate the extracellular bacteria (A). When compared to W83, more W83Δ2197 were recovered from the medium at 6 hours (3.6-fold, $P = 0.06$) although the difference was not statistically significant. In addition for (B), the infected HCAEC were washed and lysed with water to enumerate intracellular bacteria in a similar manner. More W83Δ2197 (1.6-fold, $P < 0.05$; 3.1-fold, $P < 0.05$) were recovered at 6 and 24 hours when compared to the wild-type strain, W83. ND: No difference.

to another. For example, a recent study demonstrated that *P. gingivalis* can exit infected cells and reinfect new host cells (29). Therefore, intracellular transmission without host cell lysis could be used as an important survival mechanism by this periodontal pathogen.

The mechanism by which a mutation in the PG2197 gene affects adhesion to cells is at this point unknown. Based on homology, PG2197 or HtpX is a membrane-bound, stress-controlled protease identified in *E. coli* whose proteolytic active site is on the cytosolic side of the inner membrane (19, 20). This protease is under the control of the Cpx stress response, a two-component phosphorelay system capable of both autoamplification and feedback inhibition mechanisms (19, 20). The Cpx stress response may also become activated by the loss of HtpX function (20). In addition, it has been suggested that this system is important in the regulation of virulence factors. For example, Cpx regulates VirF in *Shigella*, which in turn activates the transcription of genes necessary for host-cell invasion (30). Furthermore, Cpx-regulated DsbA and DegP are essential for pili assembly on the surface of uropathogeneic *E. coli* (31, 32). Homologues for the two components of the Cpx system appear to exist in *P. gingivalis*: PG1797 (CpxA) and PG1089 (CpxR). If PG2197 is related to HtpX, then it is conceivable that this gene is regulated by a similar two component-response

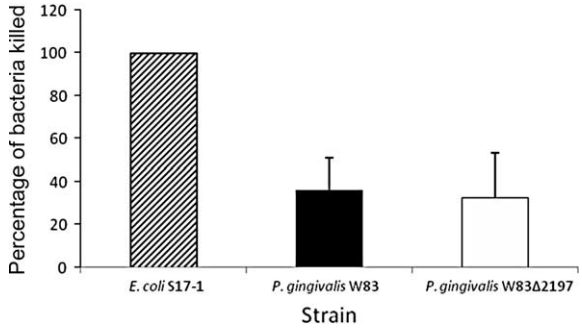

Fig. 4. Killing of *P. gingivalis* after four hours of incubation with mouse neutrophils. Overnight cultures of *P. gingivalis* W83, W83Δ2197 and *E. coli* S17-1 were pelleted and resuspended to 1×10^8 CFU/mL in PBS. Seventy five microliters of each bacterial suspension was opsonized with 50 μL of polyclonal rabbit anti-*P. gingivalis* serum. After opsonization, 0.5 mL of the neutrophil suspension (5×10^5 PMN) was added to the bacteria/serum mixture. The mixtures were incubated at 37°C at 300 rpm in a thermomixer, and a 30 μL sample was diluted and plated after 4 hours. There was no statistical difference in the susceptibility to killing by neutrophils of *P. gingivalis* W83Δ2197 when compared to the wild-type. This experiment was performed in triplicate.

system. It is plausible that, similar to the loss of HtpX activating the Cpx system, the loss of PG2197 could activate its homologous response system and lead to up regulation of other virulence factors. This may explain the enhanced adhesion, invasion and persistence seen with the PG2197 mutant. Future experiments are needed to clarify the role of this gene including characterization of the proteolytic activity of PG2197 and determination if this gene is a component of the Cpx system.

In conclusion, PG2197 appears to be important for the virulence of *P. gingivalis*, both *in vivo* and *in vitro*.

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Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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