Porphyromonas gingivalis htrA is involved in cellular invasion and in vivo survival

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HtrA is a heat-stress protein that functions both as a chaperone and as a serine protease. HtrA has been shown in several organisms to be involved in responses to stressful environmental conditions and involvement of HtrA in virulence has been reported in pathogenic species. A Porphyromonas gingivalis htrA mutant demonstrated no significant difference to the W83 parent strain when subjected to high temperature and pH values from 3 to 11. However, the htrA mutant showed increased sensitivity to H2O2. Cell invasion assays indicated that the total interaction (adherence) with KB cells, human coronary artery endothelial cells and gingival epithelial cells (GEC) was the same for both the wild-type and the htrA mutant. However, the htrA mutant showed increased invasion in KB cells and GEC. Microarray experiments indicated that a total of 253 genes were differentially regulated in the htrA mutant, including a group of stress-related genes, which might be responsible for the observed decreased resistance to H2O2. In animal experiments, a competition assay showed that the htrA mutant did not survive as well as the wild-type. In another in vivo assay, fewer mice infected with the htrA mutant died than mice infected with W83, suggesting that the htrA gene is virulence-related. These data indicate that the htrA gene in P. gingivalis does not relate to stress conditions such as high temperature and pH, but rather to H2O2 stress. The htrA gene also appears to be important for virulence and survival in in vivo animal models.

INTRODUCTION

HtrA (high temperature requirement protein A) is conserved among many genera, including Salmonella, Brucella and Yersinia (Pallen & Wren, 1997). HtrA has a dual function: one function is as a chaperone at lower temperatures, while its proteolytic activity is activated at elevated temperatures (Spiess et al., 1999). When bacteria confront stressful environments, including elevated temperatures or reactive oxygen intermediates, damaged proteins tend to form in the cell. Thus, elimination of the damaged proteins becomes essential for the bacteria to survive. In Escherichia coli, the periplasmic HtrA has been found to act as a stress response protease (Davies & Lin, 1988; Laskowska et al., 1996; Lipinska et al., 1990; Strauch & Beckwith, 1988). In other bacteria, such as Yersinia enterocolitica and Brucella abortus (Elzer et al., 1994; Li et al., 1996), HtrA has been shown to play a role in the response to high temperature and oxidative stress conditions.

HtrA has also been found to be related to virulence in some species. For example, in Y. enterocolitica, Salmonella typhimurium and B. abortus, htrA mutants show decreased virulence compared to the wild-type, indicating the involvement of HtrA in pathogenicity (Chatfield et al., 1992; Elzer et al., 1996; Li et al., 1996). The essential role of HtrA in virulence prompted the use of a Salmonella htrA mutant as a live vaccine (Tacket et al., 2000).

Porphyromonas gingivalis, a Gram-negative, anaerobic, black-pigmented bacterium, is considered to be a primary aetiological agent of certain types of periodontal diseases (Socransky & Haffajee, 1992). P. gingivalis is found in subgingival plaque in periodontal pockets that have temperatures higher than normal body temperature and varying levels of oxygen (Fedi & Killoy, 1992; Kung et al., 1990; Lindskog et al., 1994; Mettraux et al., 1984; Meyerov...
et al., 1991; Tanaka et al., 1998). This is an environment in which the bacteria are frequently stressed and one in which they must compete in order to survive.

_P. gingivalis_ can attach to and invade oral epithelial cells (Dorn et al., 2000; Lamont et al., 1995; Sandros et al., 1994). Furthermore, it has been reported to be present in atherosclerotic plaque (Kozarov et al., 2005) and is able to invade human coronary artery endothelial cells (HCAEC) (Dorn et al., 2001). In these environments, _P. gingivalis_ may be subjected to multiple conditions of stress; thus, the expression of heat-shock proteins in _P. gingivalis_ should be highly significant for _P. gingivalis_ survival.

A conserved homologue of the _htrA_ gene in _P. gingivalis_ has been identified (http://cmr.tigr.org/tigr-scripts/CMR/shared/AnnotationSearch.cgi, locus number PG0593). In a previous study, the _htrA_ gene was determined to be upregulated in a luxS mutant of _P. gingivalis_ compared to wild-type strain W83 (Yuan et al., 2005). Here we report the construction of an _htrA_ mutant, W83A0593, which has been used to characterize the functions of _htrA_ in relation to stress, cell invasion and virulence in animal models.

**METHODS**

**Bacterial and cell culture conditions.** _P. gingivalis_ wild-type W83 was grown on blood agar plates (BAPs) consisting of 4 % (w/v) trypticase soy agar, 0.5 % (w/v) yeast extract, 5 % (v/v) sheep blood, 5 μg haemin ml⁻¹ and 1 μg vitamin K₁ ml⁻¹. _P. gingivalis_ was also cultured in trypticase soy broth (TSB) supplemented with 5 μg haemin and 1 μg vitamin K₁ ml⁻¹ (Belanger et al., 2007). Cells were grown and maintained at 37 °C in an anaerobic chamber containing an atmosphere of 85 % (v/v) N₂, 10 % (v/v) H₂ and 5 % (v/v) CO₂. The _P. gingivalis_ W83 _htrA_ mutant, strain W83A0593, was maintained as described for the wild-type strain, except that 5 μg clindamycin ml⁻¹ was added to the medium to maintain the antibiotic selective pressure.

**Mutant construction.** The technique to create mutants has been described elsewhere (Belanger et al., 2007). Briefly, a 573 bp internal fragment of the _htrA_ gene (positions 301–874 of the gene) was generated by PCR using the primer PG0593F (MT), 5'-AAAAAGG-ATCCTCGGAGGGCAATCCAGAAAC-3' (starting at position 301), and PG0593B (MT), 5'-AAACCTGACAATGGTAGTGGATGC-ACCAC-3' (ending at position 874). Underlined sequences indicate BamHI and PstI sites, respectively. The DNA fragment was then cloned into the _P. gingivalis_ suicide vector pVA3000 (Lee et al., 1996) at the BamHI and PstI sites. _E. coli_ strain S17-1 was used to deliver the vector containing the internal fragment into _P. gingivalis_ W83 by conjugation on blood agar, then the transconjugants were selected on plates containing clindamycin (5 μg ml⁻¹). Homologous recombination of the vector with the chromosome resulted in two copies of the gene, truncated at either the 5' or the 3' end. Southern blot analysis was done to confirm the construction of the _htrA_ mutant, W83A0593, using an ECL detection kit according to the manufacturer’s protocol (Amersham). Growth curves were performed to verify if the insertion of the vector into the _P. gingivalis_ genome would affect the growth of the _htrA_ mutant.

**Stress experiments**

For all stress-related experiments, strains W83 and W83A0593 were cultured in supplemented TSB without antibiotics until they reached the mid-exponential phase of growth.

(i) **Temperature stress experiments.** One millilitre each of W83 and W83A0593 cultures was centrifuged at 15 600 g for 2 min, then the cell pellets were washed using 1 ml 0.1 M glycine buffer (pH 7). Cell pellets were then resuspended in 1 ml of the same buffer and incubated at 50 °C. Aliquots were removed at 0 and 8 min.

(ii) **Hydrogen peroxide (H₂O₂)-induced stress.** Ten millilitres each of W83 and W83A0593 cultures was centrifuged at 4000 g for 10 min at 4 °C, then washed with 10 ml 0.1 M glycine buffer (pH 7). The bacterial pellets were resuspended in 0.1 M glycine buffer containing 0.35 mM H₂O₂. Aliquots were taken at 0, 15 and 30 min.

(iii) **pH stress experiments.** Bacterial cells were treated as described above and the pellets were resuspended in 0.1 M glycine buffer (pH range from 3 to 10 in 1 unit increments). Aliquots were taken at 0, 30 and 45 min and 1 and 2 h.

Decimal dilutions from the above experiments were made and plated onto BAPs. Plates were incubated in an anaerobic chamber for 7–10 days and c.f.u. were enumerated. The percentage survival of the bacteria was calculated using the number of c.f.u. at time 0.

**Cell cultures.** KB cells were once thought to be derived from an oral cancer, but in fact they were derived from a glandular cancer of the cervix (Masters, 2002). Nevertheless, KB cells have been used extensively as a model to study host cell interactions with _P. gingivalis_. HCAEC, a primary cell line (Cambrex), were maintained in EBM-2 medium supplemented with EGM-2-MV single-use aliquots (Cambrex). Primary gingival epithelial cells (GEC) (kindly provided by Dr Richard Lamont) were cultured in KMB medium supplemented with insulin, triiodothyronine, transferrin, recombinant human epidermal growth factor, epinephrine, bovine pituitary extracts and hydrocortisone (Cambrex), as described by Lamont et al. (1995). Cells were cultured in 75 cm² flasks at 37 °C in a humidified atmosphere with 5 % (v/v) CO₂. Confluent monolayers were split by treatment with trypsin-verseae. KB cells (ATCC CCL-17) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum and 100 mg penicillin/streptomycin ml⁻¹.

**Cell invasion assay.** Invasion experiments were done in 24-well plates containing 10⁴ cells per well. _P. gingivalis_ was then added at an m.o.i. of 100:1 and a standard antibiotic protection assay was performed as described previously (Lamont et al., 1995; Yuan et al., 2007). The experiments were performed in triplicate, each time using three wells for each strain.

**Transmission electron microscopy.** KB and HCAEC were infected for 2.5 h with wild-type _P. gingivalis_ or the _htrA_ mutant as described above. The infected cells were then fixed with 2 % glutaraldehyde/2 % paraformaldehyde/2 mM CaCl₂/0.1 M sodium cacodylate, pH 7.4, and acid phosphatase activity was localized using cytidine 5’-monophosphate and cerium chloride as described by Robinson (1985). Specimens were post-fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate, dehydrated and embedded in Epon 812 resin. Thin sections (60–80 nm) were cut and examined on a JEOL 100CX transmission electron microscope.

**RNA preparations.** _P. gingivalis_ wild-type W83 and mutant strain W83A0593 were grown in 40 ml TSB to an OD₅₀₀ of 2.0. The bacterial cells were then collected by centrifugation at 4 °C and processed immediately for RNA extraction. Total RNA was isolated from independent quadruplicate broth cultures with 10 ml Trizol reagent (Invitrogen). Bacterial RNA was subsequently isolated following the protocol described by the manufacturer. All RNA samples were treated with RNase-free DNase I (Ambion) and purified using the RNeasy kit (Qiagen), according to the manufacturer’s instructions for the optional DNase in-column treatment. Reference
RNA (refRNA) was isolated from W83 cells grown in TSB to an OD$_{600}$ of 0.5, 1.0, 1.5 and 2.0. This refRNA was used in the microarray hybridizations. refRNA was purified as above, aliquoted and stored at −80 °C until further use. All RNA preparations were tested to confirm the absence of DNA using conventional PCR with primers for the *P. gingivalis* gene *ftn* (ferritin, locus number PG1286; http://cmr.tigr.org/tigr-scripts/CMR/shared/GenePage.cgi?locus=PG_1286).

### cDNA synthesis and preparation of probes
Reverse transcription reactions were performed according to the protocol provided by The Institute of Genomic Research (TIGR) at http://pfgrc.tigr.org/protocols.shtml with the following minor modifications: Superscript III (Invitrogen) was used as the reverse transcriptase, the amount of RNA used was increased to 5.0 μg and the molar ratio of amino acid-dUTP/dTTP used was 2:1. Purified W83 and LY2003 cDNAs were then coupled with indocarbocyanine (Cy3)-dUTP, while reference cDNAs were coupled with indocarbocyanine (Cy5)-dUTP (Amersham).

#### Microarray hybridizations
*P. gingivalis* W83 microarray glass slides (version 2) were kindly provided by TIGR. The microarrays consisted of 1907 70-mer oligonucleotides representing 1990 ORFs. The full 70-mer complement is printed four times on the surface of each microarray slide. Additional details regarding the arrays are available at http://pfgrc.tigr.org/slide_html/array_descriptions/P_gingivalis_2.shtml. Four individual Cy3-labelled cDNA samples originating from four different cultures of W83 or W83Δ0593 grown in broth without antibiotics were hybridized to the arrays along with Cy5-labelled reference cDNA samples, for a total of eight slides. With this approach, dye swap is not necessary. Hybridizations were carried out in a MAUI 4-bay hybridization system (BioMicro Systems) for 16 h at 42 °C. The slides were then washed according to TIGR protocols and scanned using a GenePix scanner (Axon) at 532 (Cy3 channel) and 635 nm (Cy5 channel) with a Cy3/Cy5 ratio of 1:1.

#### Microarray data analysis
16-bit TIFF single-channel images were stored and later loaded into TIGR Spotfinder software (http://www.tm4.org/spotfinder.html). The intensity values of each spot were measured and then normalized using LOWESS (LocFit normalization; mode, global) and InterLog Mean (Interactive Log Mean Centering Normalization) using the default settings with the following modification: one bad channel tolerance, generous; followed by in-slide replicate analysis using TIGR MIDAS software (www.tm4.org/midas.html). Spots that were flagged as faulty, due to either low intensity values or signal saturation, were automatically discarded. Final statistical analysis was carried out using IBB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). A total of 1902 genes passed hybridization reactions were performed according to the protocol provided by The Institute of Genomic Research (TIGR) at http://pfgrc.tigr.org/protocols.shtml with the following minor modifications: Superscript III (Invitrogen) was used as the reverse transcriptase, the amount of RNA used was increased to 5.0 μg and the molar ratio of amino acid-dUTP/dTTP used was 2:1. Purified W83 and LY2003 cDNAs were then coupled with indocarbocyanine (Cy3)-dUTP, while reference cDNAs were coupled with indocarbocyanine (Cy5)-dUTP (Amersham).

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## RESULTS
In preparation for the experiments, the growth rates in broth culture of W83 and W83Δ0593 were compared, but no difference was observed (data not shown). This indicated that integration of the vector into the *P. gingivalis* chromosome did not adversely affect the growth rate of strain W83Δ0593 under the growth conditions tested.

### Heat stress experiments
W83 and W83Δ0593 were treated at 50 °C for 8 min. The resulting percentage survival was then calculated based on comparison of the surviving number of bacteria to the inoculum number. After 8 min at 50 °C, although the percentage survival of W83Δ0593 (27.3%) was lower than that of W83 (32.9%), the difference was not great enough to reach a statistically significant level (*P*>0.05).

### Hydrogen peroxide stress experiments
W83 and W83Δ0593 were stressed using 0.35 mM hydrogen peroxide in 0.1 M glycine buffer for 15 and 30 min; then the percentage survival was compared between the wild-type and the mutant. The *htrA* mutant W83Δ0593 survival rate decreased when compared to that of W83 at both time points, 20.8 versus 6.2% at 15 min and 2.1 versus 0.2% at 30 min, respectively. A statistically significant difference was observed between the strains at both time points (*P*=0.04 and 0.03, respectively). These data indicate that, in *P. gingivalis*, *htrA* is involved in resistance to hydrogen peroxide stress.

### pH stress experiments
The *htrA* mutant W83Δ0593 showed no difference in terms of survival compared to W83 at any of the tested pH values (pH 3–11 in 1 unit increments) at any of the time points tested (data not shown).

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**htrA in stress response, invasion and virulence**

[1](http://mic.sgmjournals.org)
**Total interaction and invasion of cell lines**

KB cells, primary HCAEC and primary human GEC were used in this study. The *E. coli* strain MC1061 was used as a non-invasive control (Dorn et al., 1999).

**(i) KB cells.** Total interaction for this study is defined as the number of adherent plus the number of intracellular (invaded) bacterial cells at the time the host cells were lysed. In terms of total interaction, *P. gingivalis* W83Δ0593 was not found to have any statistically significant difference compared with W83 in KB cells (*P>* 0.05) (Fig. 1a). However, the htrA mutant was found to invade KB cells better than the wild-type strain since the number of recovered W83Δ0593 cells was approximately six times that of W83 (*P* < 0.001) (Fig. 1b). This difference was statistically significant, indicating that in KB cells W83Δ0593 is more invasive than W83.

**(ii) GEC.** The invasion of GEC showed a trend similar to KB cells, with no difference found for total interaction (*P* > 0.05) (Fig. 1c), while the htrA mutant W83Δ0593 was found to be more invasive than W83 (*P* < 0.001) (Fig. 1d), since the number of intracellular W83Δ0593 cells was four times that of W83 in GEC.

**(iii) HCAEC.** The total interaction of W83Δ0593 with HCAEC was significantly lower than W83 (*P* = 0.001) (Fig. 1e), but no difference in the numbers of internalized bacteria (invasion) (*P* > 0.05) (Fig. 1f) was observed.

**Microscopy**

After 2.5 h of infection, both *P. gingivalis* W83 (Fig. 2a) and the htrA mutant (Fig. 2b) were found within vacuoles in HCAEC. The presence of autophagic vacuoles and autolysosomes in the infected HCAEC suggested that both bacterial strains were capable of promoting autophagy. Both W83 and the htrA mutant were localized predominantly in vacuoles which contained multiple bacteria but lacked the lysosomal enzyme acid phosphatase.

**Microarray experiments**

The microarray experiments indicated that a total of 253 genes (ORFs) were differentially regulated in the htrA mutant at a significance level of *P* < 0.005. One hundred and forty genes were upregulated and 113 genes were downregulated. Thus the absence of htrA significantly altered the expression of a large number of genes in the *P. gingivalis* genome. A group of stress-related genes, such as htpG, groEL/groES, clpB, dnaK, grpE and a gene encoding the universal stress protein (PG0245), were all differentially downregulated in W83Δ0593 (Table 1) as was gingipain Kgp/HagD (PG1844), a well known *P. gingivalis* virulence factor. In addition, 83 hypothetical proteins, 24 cell-envelope genes, 5 regulatory genes and 11 transport and binding proteins genes were differentially regulated in the htrA mutant. Among these are cell-envelope genes related to various cellular processes, including adhesion to host cells and degradation of surface proteins, and thus these genes

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**Fig. 1.** Total interaction with and invasion of various cell types as determined using a standard antibiotic protection assay. Values represent the means ± SD for triplicate samples of lysates from the infection of 10⁵ cells by 10⁷ bacteria from independent experiments. W83, wild-type; W83Δ0593, htrA mutant; MC1061, *E. coli* strain used as a non-invasive control. (a) Total interaction with KB cells (*P* > 0.05); (b) invasion of KB cells (*P* < 0.001); (c) total interaction with GEC (*P* > 0.05); (d) invasion of GEC (*P* = 0.001); (e) total interaction with HCAEC (*P* = 0.001); (f) invasion of HCAEC (*P* > 0.05).
may relate to bacterial virulence. It is noteworthy that there were no polar effects observed as a result of the htrA gene inactivation, as no alteration in the expression of flanking genes could be found after analysis of the microarray data.

**Competition assay**

The mutant was assayed in a mouse abscess model for alterations in virulence relative to strain W83. A group of 18 mice were injected subcutaneously with a 1 : 1 mixture of W83 and W83Δ0593. For 3 days after injection, six mice were sacrificed each day and the subcutaneous abscess contents were analysed. During the first 2 days of sampling, the percentage of recovered W83Δ0593 was higher than the number of recovered W83 (58.5 versus 41.5% for the first day and 57.3 versus 42.7% for the second day) (Fig. 3). However, the percentage of recovered W83Δ0593 dropped to 28.4% on the third day, and it was significantly lower compared to day 1 (P=0.0008) and day 2 (P=0.001), indicating a decreased survival ability of W83Δ0593 over a longer time period compared to W83.

**Virulence study**

In a second set of experiments using a mouse abscess model, two groups of eight mice were challenged with

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**Table 1.** Examples of differentially regulated genes in the *P. gingivalis* htrA mutant as determined by microarray analysis

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Gene*</th>
<th>Common name*</th>
<th>Cellular role*</th>
<th>Expression (-fold)†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG1759</td>
<td><em>Adhesion protein, putative</em></td>
<td>Cell envelope</td>
<td>+9.1</td>
<td>0.000317</td>
<td></td>
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<tr>
<td>PG0186</td>
<td><em>ragB</em></td>
<td>Lipoprotein RagB</td>
<td>Cell envelope</td>
<td>−8.0</td>
<td>0.001155</td>
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<tr>
<td>PG0245</td>
<td><em>Universal stress protein family</em></td>
<td>Cellular processes</td>
<td>−4.9</td>
<td>0.000372</td>
<td></td>
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<tr>
<td>PG1844</td>
<td><em>kgp (hagD)</em></td>
<td>Gingipain Kgp – haemagglutinin protein</td>
<td>Cellular processes</td>
<td>−6.3</td>
<td>0.002625</td>
</tr>
<tr>
<td>PG0419</td>
<td><em>Hypothetical protein</em></td>
<td>Hypothetical protein</td>
<td>−7.5</td>
<td>0.001175</td>
<td></td>
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<tr>
<td>PG0045</td>
<td><em>hrtG</em></td>
<td>Heat-shock protein</td>
<td>Protein fate</td>
<td>−34.6</td>
<td>0.000416</td>
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<tr>
<td>PG0520</td>
<td><em>groEL</em></td>
<td>Chaperonin, 60 kDa</td>
<td>Protein fate</td>
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<td>0.000256</td>
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<tr>
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<td><em>groES</em></td>
<td>Chaperonin, 10 kDa</td>
<td>Protein fate</td>
<td>−16.3</td>
<td>0.001839</td>
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<tr>
<td>PG0553</td>
<td><em>Extracellular protease, putative</em></td>
<td>Protein fate</td>
<td>−36.9</td>
<td>0.00001</td>
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<tr>
<td>PG1118</td>
<td><em>clpB</em></td>
<td>ClpB</td>
<td>Protein fate</td>
<td>−80.6</td>
<td>0.00001</td>
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<tr>
<td>PG1208</td>
<td><em>dnaK</em></td>
<td>DnaK</td>
<td>Protein fate</td>
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<td>0.000315</td>
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<tr>
<td>PG1775</td>
<td><em>grpE</em></td>
<td>GrpE</td>
<td>Protein fate</td>
<td>−144.9</td>
<td>0.000024</td>
</tr>
<tr>
<td>PG1007</td>
<td><em>Transcriptional regulator, GntR family</em></td>
<td>Regulatory functions</td>
<td>+14.6</td>
<td>0.000645</td>
<td></td>
</tr>
<tr>
<td>PG1040</td>
<td><em>Transcriptional regulator, putative</em></td>
<td>Regulatory functions</td>
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<td>0.003347</td>
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<tr>
<td>PG0173</td>
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<tr>
<td>PG1431</td>
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<td>Regulatory functions</td>
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<tr>
<td>PG2167</td>
<td><em>Immune-reactive 53 kDa antigen PG123</em></td>
<td>Unknown function</td>
<td>−12.9</td>
<td>0.002296</td>
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</tr>
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</table>

†Negative values indicate downregulation; positive values indicate upregulation.
either W83 or W83Δ0593 at a dose of 7.5 × 10⁹ c.f.u. per mouse. The general health status of the mice treated with W83 and W83Δ0593 was the same for days 1 and 2 as all showed diffused lesions on their bodies and some were unable to walk normally. On day 3, 6/8 mice died in the W83 group while 3/8 in the W83Δ0593 group died. However, this difference was not statistically significant (P=0.143).

**DISCUSSION**

HtrA is a periplasmic protein that functions as both a chaperone and a serine protease, which degrades damaged proteins formed under stress conditions. Thus, HtrA protease activity is important for the ability of bacteria to respond to various stress situations. In the periodontal pocket, *P. gingivalis* survives various environmental stresses during the progression of periodontal disease (Fedi & Killoy, 1992; Kung et al., 1990; Lindskog et al., 1994; Meyerov et al., 1991). The role of HtrA under different stress conditions has been shown for several bacterial species, although the relevance is not universal among all the species tested.

Environmental pH plays an important role in the survival of human pathogens. For *P. gingivalis*, resistance to various pH conditions is necessary for the bacteria to survive in oral cavities and periodontal pockets where the pH is known to vary considerably (Bickel & Cimasoni, 1985; McDermid et al., 1988). In this study, the *P. gingivalis* htrA mutant was not affected by changes in pH. The unchanged survival rate between the wild-type and the htrA mutant under different pH values indicates that other gene products are probably responsible for resistance to pH stress in *P. gingivalis.*

The importance of htrA for survival and/or growth at elevated temperatures varies according to the bacterial species (Biswa & Biswas, 2005; Brondsted et al., 2005; Lipinska et al., 1989; Stack et al., 2005). Even though its role is critical for several species, it is not important for *Salmonella typhimurium* and *Staphylococcus aureus* (Johnson et al., 1991; Rigoulay et al., 2005). Our data indicate that the *P. gingivalis* htrA also is not involved in the response to heat shock, at least at 50 °C. However, in another study, a *P. gingivalis* htrA mutant was found to grow more slowly than the wild-type at 42 °C over a period of 24 h (Roy et al., 2006). Thus, *P. gingivalis* may have different mechanisms for dealing with heat shock (50 °C) versus heat adaptation (42 °C) and these data indicate that htrA is likely to play a role in long-term adaptation to elevated temperature, but not in short-term adaptation.

In response to H₂O₂, the htrA mutant was more sensitive than the wild-type when treated with H₂O₂ for 15 and 30 min. Roy et al. (2006) demonstrated that the growth of a *P. gingivalis* htrA mutant was adversely affected by H₂O₂ over a period of 28 h. Therefore, htrA in *P. gingivalis* appears to play a role in the oxidative stress response. Previous reports have indicated that several genes, such as sod, ahpC–F, oxyR and the gene encoding ruberythrin are involved in the response of *P. gingivalis* to oxidative stress (Diaz et al., 2004, 2006; Lynch & Kuramitsu, 1999; Sztukowska et al., 2002). Our microarray data did not show any change in the expression of these genes. However, a separate microarray study of the *P. gingivalis* htrA response to H₂O₂ showed that stress-response-related genes grpE, dnaJ, htpG and groEL were upregulated under these conditions (McKenzie & Fletcher, 2007). Our data demonstrate that all these genes, as well as clpB, were downregulated in the htrA mutant, suggesting that the lower oxidative stress tolerance observed for the htrA mutant may be due to downregulation of the chaperone machinery. In a previous study, we report that the mutation of clpB in *P. gingivalis* also downregulated several genes involved in the stress response, but not htrA. The clpB mutant was sensitive to heat, but not to oxidative and pH stress (Yuan et al., 2007). Thus, several proteins appear to be involved in the molecular mechanism of the oxidative stress response in *P. gingivalis*, but the details of their action remains to be elucidated.

The relevance of HtrA to cell invasion by *P. gingivalis* seems to be cell-type dependent, as the invasion rate of the htrA mutant with epithelial cells (KB and GEC) was increased when compared to the parent strain while no difference was observed with endothelial cells (HCAEC). This might be related to the fact that *P. gingivalis* does not utilize the same trafficking mechanisms within different cell types. For example, in epithelial cells, internalized *P. gingivalis* cells are found freely in the cytoplasm or within single-membrane-bound vacuoles (Belton et al., 1999; Duncan et al., 1993; Houalet-Jeanne et al., 2001; Lamont et al., 1995; Njoroge et al., 1997), while in endothelial cells, *P. gingivalis* is internalized in phagosomes and later...
sequestered by the autophagy machinery (Dorn et al., 2001). In this study, the htrA mutant induced an autophagic response within the host HCAEC as shown previously for P. gingivalis (Dorn et al., 2001). Both wild-type and mutant cells were found with similar cell morphology in autophagic vacuoles. These results are consistent with the data obtained in the invasion assays in which similar numbers of internalized bacteria were recovered for both wild-type and mutant. These different invasion strategies suggest that P. gingivalis may have a different genetic profile when invading epithelial and endothelial cells, and may gain entry into the different cell types by diverse mechanisms.

In a previous study with HEp-2 cells, which P. gingivalis does not invade, htrA, htpG (PG0045), groEL (PG0520), dnaK (PG1208), grpE (PG1775), PG2167 and PG0419 were all shown to be upregulated when P. gingivalis was in contact with the HEp-2 cells (Hosogi & Duncan, 2005). Our microarray data demonstrated that these genes are downregulated in the htrA mutant, suggesting that they may not be relevant to internalization and survival of internalized P. gingivalis in epithelial cells. Ultimately, the higher recovery of mutant cells compared to wild-type cells may be due to changes in the expression of other genes affected by the mutation in htrA. Since there are 140 genes upregulated in the htrA mutant, particularly genes involved in cell envelope composition (15), transport and binding of proteins (9), genes of unknown function (25) and hypothetical proteins (35), it is certainly possible that other unknown invasion/virulence-related genes contributed to the increased invasion observed in epithelial cells. In addition, no difference in the expression of fimA, another relevant P. gingivalis adhesin, was observed, as confirmed by the microarray data and negative staining of the htrA mutant (data not shown).

A regulatory role of htrA in virulence gene expression has been previously identified in P. gingivalis. Roy et al. (2006) demonstrated that an htrA-deficient mutant had decreased Arg-gingipain activity after heat-shock treatment. In Staphylococcus aureus, the expression of α, β and γ haemolysins, in addition to several other virulence factors, were affected by an htrA mutation (Rigoulay et al., 2005). Lyon & Caparon (2004) demonstrated that a mutation of htrA in Streptococcus pyogenes affected the production of the virulence factors SpeB, cysteine protease and the streptolysin S haemolysin. Mutation of htrA has also been shown to affect biofilm formation in Listeria monocytogenes (Wilson et al., 2006) and Streptococcus mutans (Biswas & Biswas, 2005).

In vivo, an L. monocytogenes htrA mutant has been shown to have attenuated virulence in mice compared to the wild-type (Stack et al., 2005; Wilson et al., 2006; Wonderling et al., 2004). Similar results have been observed with htrA mutations in several other bacterial species (Brondsted et al., 2005; Farn & Roberts, 2004; Ibrahim et al., 2004). This probably reflects a requirement for adaptation to an unfavourable and/or changing environment, such as that encountered during disease progression in a host. In the mouse competition assay, the htrA mutant did not survive as well in the mice compared to the wild-type. In addition, when the wild-type and the htrA mutant were injected separately into the mouse abscess model, half as many mice infected with the htrA mutant died as those infected with the wild-type strain. Collectively, these animal data suggest that htrA plays a role in P. gingivalis survival and virulence in vivo.

Gene profiling data demonstrated that there are a total of 253 differentially regulated genes in the htrA mutant, indicating that knocking down the expression of htrA directly or indirectly affects the expression of many other genes. A total of 24 cell-envelope-related genes were differentially regulated in the htrA mutant. Cell-envelope genes are an important class that are likely to be involved in bacterial pathogenesis. They generally function as a dynamic interface between the bacteria and the environment, maintaining the cell structure, protein transport, cellular adhesion and binding to other substrates. It has been shown that the knockout of htrA affects the expression of surface proteins in other bacterial species. For example, Biswas & Biswas (2005) demonstrated that the expression of glucosyltransferases, fructosyltransferases and other surface proteins was affected by an htrA mutation in Streptococcus mutans. Stack et al. (2005) demonstrated that htrA expression in L. monocytogenes is dependent upon the LisRK sensor-kinase, a system known to respond to changes in the integrity of the cell envelope. In addition, capsule formation has been shown to be affected by an htrA mutation in Klebsiella pneumoniae (Cortes et al., 2002). Of significance, our data showed that the htrA mutation altered the expression of transcription regulators PG0173, PG1007 and PG1040, and also PG1431, a regulator of the LuxR family, suggesting that HtrA ultimately may participate in the regulation of several genes in P. gingivalis. Finally, the microarray data were obtained from in vitro cultures of W83 and the htrA mutant, and may not therefore precisely reflect the situation that occurs during invasion and survival in vivo. A more direct analysis using RNA obtained from invading/infecting P. gingivalis may provide additional information as to why the htrA mutant invades epithelial cells better than the wild-type strain and help to explain its low virulence in vivo.

In conclusion, we have demonstrated that htrA in P. gingivalis is responsible for resistance to hydrogen peroxide stress, but not to other stress conditions tested (high temperature and unfavourable pH). The disruption of htrA in P. gingivalis altered the expression of multiple genes, affecting several different cellular functions. Significantly, the downregulation of the chaperone machinery in the htrA mutant may indicate that HtrA in P. gingivalis may play a central role in the oxidative stress response. The htrA mutant did not show a difference in terms of total interaction with two epithelial cell types, and invaded the latter cell types better than the wild-type. In addition, the
animal experiments performed demonstrated that the htrA gene is probably involved in the virulence of this pathogen in vivo.

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htrA in stress response, invasion and virulence


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