HtrA in *Porphyromonas gingivalis* can regulate growth and gingipain activity under stressful environmental conditions

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In several micro-organisms, HtrA, a serine periplasmic protease, is considered an important virulence factor that plays a regulatory role in oxidative and temperature stress. The authors have previously shown that the *vimA* gene product is an important virulence regulator in *Porphyromonas gingivalis*. Further, purified recombinant VimA physically interacted with the major gingipains and the HtrA from *P. gingivalis*. To further evaluate a role for HtrA in the pathogenicity of this organism, a 1.5 kb fragment containing the *htrA* gene was PCR-amplified from the chromosomal DNA of *P. gingivalis* W83. This gene was insertionally inactivated using the *ermF-ermAM* antibiotic-resistance cassette and used to create an *htrA*-deficient mutant by allelic exchange. In one randomly chosen isogenic mutant designated *P. gingivalis* FLL203, there was increased sensitivity to hydrogen peroxide. Growth of this mutant at an elevated temperature was more inhibited compared to the wild-type. Further, in contrast to the wild-type, there was a significant decrease in Arg-gingipain activity after heat shock in FLL203. However, the gingipain activity in the mutant returned to normal levels after a further 30 min incubation at room temperature. Collectively, these data suggest that HtrA may play a similar role in oxidative and temperature stress in *P. gingivalis* as observed in other organisms.

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INTRODUCTION

Periodontitis is a destructive inflammatory disease of the supporting tissues of the teeth. An important aetiological agent implicated in chronic adult periodontitis is the Gram-negative, anaerobic, black-pigmented bacterium *Porphyromonas gingivalis* (reviewed by Genco *et al.*, 1998, 1999; Griffen *et al.*, 1999; Travis *et al.*, 2000). This organism is also associated with other systemic diseases, including atherosclerosis (reviewed by Okuda & Ebihara, 1998; Teng *et al.*, 2002; Kinane & Marshall, 2001). The major virulence factors of *P. gingivalis*, the gingipains, possess high levels of proteolytic activity and have been the focus of much attention (Genco *et al.*, 1999; Imamura, 2003; Nakagawa *et al.*, 2003). The major gingipains are extracellular and/or cell-associated. The Arg-specific gingipains, RgpA and RgpB, are encoded by the genes *rgpA* and *rgpB* respectively, whereas the Lys-specific protease (Kgp) is encoded by one gene, *kgp* (Nakayama, 2003). There is a gap in our understanding of the regulation and processing of the gingipains. We have reported the post-translational regulation of the gingipains in the *vimA*-defective mutant *P. gingivalis* FLL92 (Abaibou *et al.*, 2001). The *vimA* gene is a part of the *bcp-recA-vimA* operon. Further,

protein–protein interaction studies using the purified rVimA showed that this protein interacts with the gingipains and the HtrA homologue in *P. gingivalis* (Vanterpool *et al.*, 2006). The role, if any of HtrA in gingipain regulation and virulence in *P. gingivalis* is unclear.

Protein quality control, which is essential for bacterial survival, is regulated by chaperones and proteases (Lu & McBride, 1994; Goulhen *et al.*, 2003). HtrA (high temperature requirement A) is a periplasmic heat-shock serine protease that functions as a molecular chaperone at low temperatures and has proteolytic activity at elevated temperatures (Kim & Kim, 2002). In several organisms, including bacteria, yeast, plant and humans, HtrA is considered an important factor involved in protein folding and maturation as well as in the degradation of misfolded proteins (Poquet *et al.*, 2000). Inactivation of the *htrA* gene has been shown to affect the sensitivity of many organisms to thermal and oxidative stress (Pallen & Wren, 1997; Lipinska *et al.*, 1990). Here we report that HtrA physically interacts with the gingipains RgpA, RgpB and Kgp in *P. gingivalis*. HtrA is also involved in resistance to thermal and oxidative stress in *P. gingivalis*.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *P. gingivalis* strains were

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Table 1. Plasmids and bacterial strains

Plasmid or strain		Phenotype/description	Source
Plasmids			
pCR2.1-TOPO	Ap ^r Km ^r		Invitrogen
pUC19	Ap ^r		Invitrogen
pFLL200	pCR 2.1-TOPO : <i>htrA</i>		This study
pFLL201	pUC19 : <i>htrA</i>		This study
pFLL202	pUC19 : <i>htrA</i> :: <i>ermF-ermAM</i>		This study
pVA2198	Sp ^r , <i>ermF-ermAM</i>		Fletcher <i>et al.</i> (1995)
<i>P. gingivalis</i>			
W83	Wild-type		This study
FLL203	<i>htrA</i> -defective mutant		This study
<i>E. coli</i>			
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1</i> <i>hsdR17</i> (<i>r_k⁻</i> , <i>m_k⁻</i>) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>		Invitrogen
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80lacZ Δ M15 Δ <i>lacX74 recA1</i> <i>ara139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>		Invitrogen

grown in brain heart infusion (BHI) broth (Difco) supplemented with haemin (5 μ g ml⁻¹), vitamin K (0.5 μ g ml⁻¹) and cysteine (0.1%). Experiments with hydrogen peroxide were performed in BHI without cysteine. *Escherichia coli* strains were grown in Luria-Bertani broth. Unless otherwise stated, all cultures were incubated at 37 °C. *P. gingivalis* strains were maintained in an anaerobic chamber (Coy Manufacturing) in 10% H₂/10% CO₂/80% N₂. Growth rates for *P. gingivalis* and *E. coli* strains were determined by measuring OD₆₀₀. Antibiotics were used at the following concentrations: clindamycin, 0.5 μ g ml⁻¹; erythromycin, 300 μ g ml⁻¹; carbenicillin, 100 μ g ml⁻¹.

DNA isolation and analysis. *P. gingivalis* chromosomal DNA was isolated as previously reported (Vanterpool *et al.*, 2004). For plasmid DNA analysis, DNA extraction was performed by the alkaline lysis procedure as previously described (Johnson *et al.*, 2004). For large-scale preparation, plasmids were purified using the Qiagen plasmid maxi kit.

Generation of the *htrA*-defective mutant *P. gingivalis* strain. A 1.5 kb fragment carrying the intact *htrA* ORF (gene number PG0535, <http://www.oralgen.lanl.gov>) was amplified by PCR using

primers P1 and P2 (Table 2). This fragment was cloned into the pCR2.1-TOPO plasmid vector (Invitrogen) and designated pFLL200. The 1.5 kb fragment was then isolated from pFLL200, digested with *Bam*HI and *Xba*I and ligated to pUC19 linearized with the same enzymes. The new recombinant plasmid was designated FLL201. Orientation was confirmed by restriction analysis. The *ermF-ermAM* cassette, which confers erythromycin/clindamycin resistance in *E. coli* and *P. gingivalis*, was PCR-amplified from pVA2198 using *Pfu* turbo (Stratagene) with primers P5 and P6 (Table 2). The amplified fragment was inserted into the *Msc*I restriction site of the *htrA* gene. The resultant recombinant plasmid, pFLL202, was used as a donor in electroporation of *P. gingivalis* W83 as previously reported (Vanterpool *et al.*, 2004).

Preparation of *P. gingivalis* extracellular fractions and protease assays. One-litre cultures of *P. gingivalis* strains FLL203 and W83 were grown to stationary phase (OD₆₀₀ 1.5) from actively growing cells. Cells were harvested by centrifugation at 10 000 *g* for 30 min. The cell-free culture fluid was precipitated with cold (-20 °C) 37.5% or 60% acetone, and the protein pellet was resuspended in 7 ml 100 mM Tris/HCl buffer (pH 7.4), dialysed for 24 h against the same buffer and then stored on ice at 0 °C. The presence of Arg-X- and Lys-X-specific cysteine protease activities was determined with a microplate reader (Bio-Rad) as previously reported (Potempa *et al.*, 1998).

Protein concentration determination. Protein concentration was calculated spectrophotometrically using an Eppendorf Biophotometer according to the manufacturer's instructions (Brinkman).

SDS-PAGE and immunoblot analysis. SDS-PAGE and transfer to nitrocellulose membranes were as described by Vanterpool *et al.* (2006). The blots were probed with antibodies against specific protease domains (Olango *et al.*, 2003) or HagA (generously donated by Ann Progluske-Fox, University of Florida). The secondary antibody was immunoglobulin G (heavy plus light chains)-horseradish peroxidase conjugate (Zymed Laboratories). Immunoreactive proteins were detected by using the Western Lighting Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences).

Analysis of *P. gingivalis* *htrA*-defective mutant genes by RT-PCR. Total RNA was extracted from *P. gingivalis* strains grown to

Table 2. Primers

Primer	Sequence (5'-3')
P1 <i>htrA</i> forward	ATGAACAAAGTGTGGAAATAC
P2 <i>htrA</i> reverse	TAGTTCTACTTCGAAGGTCCG
P3 <i>pro-kgp</i> forward	ATGAGGAAATTATTATGCTGATCG
P4 <i>pro-kgp</i> reverse	TTGAAGAGCTGTTTATAAGCTGTTT
P5 <i>erythromycin</i> forward	TATTAGGCCTATAGCTTCGCTATT
P6 <i>erythromycin</i> reverse	AATAGGCCTTAGTAACGTGTAACTTT
P7 <i>sigA</i> forward	ATGAGGCAACTTAAAATTTC
F8 <i>sigA</i> reverse	TTAGCCGAGATAACCTTTTCAG

early stationary phase (OD₆₀₀ 1.2) using the RiboPure kit (Ambion). The primers used for RT-PCR analysis were specific for the *kgp* (primers P3 and P4), *htrA* (P1 and P2) and *sigA* genes (P7 and P8) (Table 2). The RT-PCR reaction (50 µl) contained 1 µg template RNA in the Superscript One-step RT-PCR reaction mix (Invitrogen). Negative controls were RT-PCR in the absence of reverse transcriptase.

Cell fraction preparation. One-litre cultures of *P. gingivalis* strains FLL203 and W83 were grown to stationary phase (OD₆₀₀ 1.5) from actively growing cells. Preparations of whole-cell culture, cell-free medium, cell suspension, vesicles and vesicle-free medium were made as previously reported (Olango *et al.*, 2003). The whole-cell culture (WC) fraction is a sample of the culture after the bacterium has been grown to a specific growth phase. This sample has the bacterial cells suspended in the growth medium, and the enzyme activity includes the gingipains that are attached to the bacterial cell surface plus those secreted into the culture medium. After centrifugation, the cell pellet was resuspended, and the enzyme activity in this sample (the cell suspension fraction, CS) represents the gingipains that are attached to the bacterial cell surface. The enzyme activity in the supernatant (the cell-free medium fraction, CF) includes the gingipains that are secreted into the culture medium. Secreted gingipains can either be associated with vesicles or soluble in the culture medium; thus, ultracentrifugation of the cell-free fractions will yield a vesicle pellet (V) and a supernatant of soluble gingipains (VF).

Effect of heat shock on gingipain activity. Cultures of *P. gingivalis* strains FLL203 and W83 were grown for 16 h to an OD₆₀₀ of 1.5; 2 ml samples from these cultures were incubated for 10 min at 55 °C and proteolytic activities were analysed immediately and 30 min after heat shock treatment.

Growth at elevated temperatures and under oxidative stress conditions. Cultures (100 ml) of *P. gingivalis* strains FLL203 and W83 were grown at 37 °C for 16 h to an OD₆₀₀ of 1.5. For growth at elevated temperatures, the cultures were then incubated at 42 °C under anaerobic conditions for 28 h. Growth was determined at intervals of 0, 4, 8, 24 and 28 h as described above. For growth under oxidative stress conditions, cultures were grown in BHI without cysteine in the presence of 0.25 mM hydrogen peroxide. Controls were grown (with cysteine) in the absence of hydrogen peroxide.

Cloning and expression of rHtrA. Oligonucleotide primers (P1 and P2) specific for the ORF of the *htrA* gene were synthesized and used for PCR amplification. The amplified fragment carrying the *htrA* ORF was cloned into pTrcHis2-TOPO expression plasmid (Invitrogen) carrying coding for a C-terminal His-tag. The recombinant plasmid (designated pFLL137) was then transformed into competent *E. coli* TOP10F' cells (Invitrogen). Orientation of the *htrA* gene was determined by restriction endonuclease digestion. The nucleotide sequence of the insert was determined to rule out the occurrence of mutations. *E. coli* carrying the *htrA* ORF was grown in Luria-Bertani broth to exponential phase (OD₆₀₀ 0.7) in the presence of 50 µg carbenicillin ml⁻¹. IPTG was added at exponential phase and the cells were incubated at 37 °C with shaking for an additional 6 h. Cells were harvested by centrifugation and lysed by sonication. Cell membrane and debris were then harvested by centrifugation. The supernatant and pellet were analysed for the presence of the rHtrA protein. The presence of the poly-histidine tag was confirmed using the GelCode 6 × His Protein Tag kit according to the manufacturer's instructions (Pierce).

Protein-protein interactions. The *E. coli* cells carrying the expressed rHtrA were lysed and the rHtrA was purified using Ni-NTA magnetic beads. The purified rHtrA protein was then incubated for 30 min with magnetic beads coated with Ni-NTA. Beads

were washed four times with wash/interaction buffers (50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole and 0.005 % Tween 20). Cell lysates from *P. gingivalis* W83 grown to stationary phase were then incubated for 30 min with the with rHtrA-attached Ni-NTA magnetic beads. As a negative control, the lysates were incubated with the magnetic beads without the recombinant protein. After incubation with cell lysates, unbound proteins were eliminated by extensive washing in wash buffer. Proteins were eluted off the beads under denaturing conditions (1 × lithium dodecyl sulfate sample buffer, heated to 90 °C for 5 min). Eluates were then analysed by SDS-PAGE and immunoblot analysis as described above.

RESULTS

Inactivation of the *htrA* gene in *P. gingivalis* W83 by allelic-exchange mutagenesis

Isogenic mutants of *P. gingivalis* W83 defective in the *htrA* gene were constructed by allelic-exchange mutagenesis. The recombinant plasmid pFLL202, which carries the *ermF-ermAM* cassette in the unique *MscI* restriction site of the *htrA* gene, was used as a donor in electroporation of *P. gingivalis* W83. We predicted that two double-crossover events between the regions flanking the *erm* marker and the wild-type gene on the chromosome would result in replacement of a segment of the wild-type gene with a fragment conferring erythromycin resistance. Following electroporation and plating on selective medium (BHI containing 10 µg erythromycin ml⁻¹), we detected erythromycin-resistant colonies after a 7 day incubation period. To compare their phenotypic properties with those of wild-type strain W83, all mutants were plated on *Brucella* blood agar plates. Similar to the wild-type strain, all mutants displayed a black-pigmented, β-haemolytic phenotype.

Confirmation of inactivation of *htrA* by PCR analysis

Chromosomal DNA from six randomly chosen clindamycin/erythromycin-resistant colonies and the wild-type W83 strain was analysed by PCR to confirm the inactivation of the *htrA* gene. If the *htrA* gene was interrupted by the *ermF-ermAM* cassette, a 3.5 kb fragment was expected to be amplified using *htrA* primers P1 and P2. In addition, a 2.1 kb fragment should be amplified from the mutant using the *ermF-ermAM* primers. The expected 3.5 kb (using *htrA* primers) and 2.1 kb (using erythromycin primers) fragments were observed only in the erythromycin-resistant strains, not in the wild-type (Fig. 1a). To further confirm the absence of the *htrA* transcript in the erythromycin-resistant mutant, DNase-treated RNA from *P. gingivalis* FLL203 and the wild-type was subjected to RT-PCR. As shown in Fig. 1(b), no *htrA* transcript was detected from the mutant, in contrast to the wild-type W83. Using *kgp*-specific primers (Table 2) as a control, the expected 0.8 kb fragment was amplified from all the *P. gingivalis* strains. No amplified fragment was observed from similar reactions in the absence of reverse transcriptase. These results indicated that the insertional inactivation of the chromosomal *htrA* gene with the 2.1 kb *ermF-ermAM* antibiotic-resistance cassette was

Fig. 1. Confirmation of inactivation by PCR and RT-PCR analysis. (a) Primers specific for the *htrA* gene (P1 and P2; Table 2) were used to amplify it from total cellular DNA from *P. gingivalis*: lane 1, *P. gingivalis* FLL203.1 (*htrA::ermF-ermAM*); lane 2, *P. gingivalis* FLL203.2 (*htrA::ermF-ermAM*); lane 3, *P. gingivalis* FLL203.3 (*htrA::ermF-ermAM*); lane 4, *P. gingivalis* W83 (wild-type). Primers specific for the *ermF-ermAM* cassette (P5 and P6) were used to amplify the 2.1 cassette: lane 4, *P. gingivalis* FLL203.1 (*htrA::ermF-ermAM*); lane 5, *P. gingivalis* FLL203.2 (*htrA::ermF-ermAM*); lane 6, *P. gingivalis* FLL203.3 (*htrA::ermF-ermAM*). (b) Total RNA isolated from *P. gingivalis* W83 and the *htrA*-defective mutant in the stationary phase (OD_{600} 1.4–1.5) was subjected to RT-PCR using primers for *htrA* and *pro-kgp*. No transcription of *htrA* was detected in *P. gingivalis* FLL203 when reactions were performed in the absence of reverse transcriptase or in the negative controls. (c) DNase-treated RNA from *P. gingivalis* FLL203 and W83 grown to stationary phase was subjected to RT-PCR for the transcription of the *sigA* gene. For the analysis of *htrA*, primers P1 and P2 were used. For the analysis of the *sigA* gene, primers P7 and P8 were used (Table 2).

successful. One mutant designated *P. gingivalis* FLL203 was randomly chosen for further study. The wild-type and the *htrA*-defective mutant FLL203 showed a similar generation time of 3 h (data not shown). To rule out any polar effect on the gene downstream of *htrA*, DNase-treated RNA from FLL203 was further analysed by RT-PCR. Using specific primers (P7 and P8; Table 2) for the downstream *sigA* gene, a fragment of the predicted size was amplified from both the *htrA*-defective mutant and the wild-type (Fig. 1c).

***P. gingivalis* FLL203 is more sensitive to oxidative stress than the wild-type**

P. gingivalis W83 and the *htrA*-defective mutant FLL203 were evaluated for oxidative stress resistance by their ability to grow in the presence of hydrogen peroxide. Compared to the parent strain, FLL203 demonstrated increased sensitivity to hydrogen peroxide at a final concentration of 0.25 mM (Fig. 2a). The greatest sensitivity was observed during the stationary growth phase (Fig. 2b).

Growth and Rgp activity at elevated temperatures are affected in *P. gingivalis* FLL203

P. gingivalis FLL203, the *htrA*-defective mutant, grew at a similar rate to that of the wild-type at 37 °C (data not shown). However, in contrast to the parent strain, the mutant grew more slowly at 42 °C, and in addition did not grow over an OD_{600} of 0.9 (Fig. 3a). In cells grown at 42 °C for 8 h or 28 h, Rgp activity was increased by approximately 20% and 10% respectively in the mutant compared to the wild-type strain (Fig. 3b). In contrast to the parent strain at a similar cell density, there was greater Rgp activity in the *HtrA*-defective mutant grown at 42 °C (Fig. 3c). The ability of *P. gingivalis* FLL203 to recover from heat shock was also evaluated. After a 10 min incubation at 55 °C, both the wild-type and the mutant strains were further incubated for 24 h at 37 °C. A slightly longer lag phase was observed in the *htrA*-defective mutant in comparison to the wild-type (data not shown). To determine if the gingipains are affected by brief

Fig. 2. The *htrA*-defective mutant is more sensitive to hydrogen peroxide than the wild-type. Actively growing *P. gingivalis* W83 and FLL203 were incubated with 0.25 mM hydrogen peroxide in the absence of cysteine. Sensitivities to hydrogen peroxide were determined by measuring OD₆₀₀ over time. (a) ▲, FLL203 control; ■, FLL203 treated; ◆, W83 control; ▼, W83 treated. (b) Difference in OD₆₀₀ between treated and untreated cultures at exponential (E) and stationary (S) phase. The data in both panels are means $\pm$ SE of three independent experiments.

incubation at elevated temperatures in the *htrA*-defective mutant, *P. gingivalis* W83 and FLL203 were incubated at 55 °C for 10 min. At 37 °C, there was no significant difference in Rgp activity between the mutant and the wild-type (data not shown). However, immediately after heat treatment, Rgp activity in the wild-type was elevated by 7 % in comparison to the untreated control. Interestingly, the Rgp activity in the *htrA*-defective mutant was decreased by 13 % in comparison to the untreated control. Rgp activities determined 30 min after heat treatment were similar for the wild-type and FLL203 (data not shown).

Gingipain profiles and distribution are altered under temperature stress

The distribution of gingipains in the wild-type (Fig. 4a) and the *htrA*-defective mutant (Fig. 4b) was very similar under normal growth conditions. However, the distribution of the Rgps appeared to be slightly altered by incubations at 42 °C. There appeared to be approximately 30 % less cell-associated Rgp activity in the *htrA*-defective mutant (Fig. 4d) in comparison to the wild-type (Fig. 4c). Very little alteration in Kgp activity was observed (data not

Fig. 3. Growth and Rgp activity are affected in *P. gingivalis* FLL203 at elevated temperatures. Cultures of *P. gingivalis* W83 and FLL203 were grown at 42 °C for 28 h under anaerobic conditions. (a) Growth at 42 °C: ■, FLL203; ◆, W83. (b) Rgp activity at exponential (E) and stationary (S) growth phase. (c) Rgp activity per OD₆₀₀ unit. The error bars represent SE.

shown). Immunoblot analysis of extracellular fractions demonstrated the absence of RgpA and RgpB immunoreactive bands in the *htrA*-defective mutant (data not shown).

DISCUSSION

The inflammatory microenvironment of the periodontal pocket (Chapple, 1997; Katsuragi *et al.*, 2003; Wolff *et al.*,

Fig. 4. Distribution of Rgp activity is altered in the *htrA*-defective mutant. *P. gingivalis* FLL203 and W83 were grown at either 37 °C or 42 °C. Activities against *N*^ε-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were tested in whole cell culture (WC), cell suspension (CS), cell free medium (CF), vesicles (V) and vesicle-free (VF) fractions of the *P. gingivalis* strains (see Methods for details). (a) W83 grown at 37 °C, (b) FLL203 grown at 37 °C, (c) W83 grown at 42 °C, (d) FLL203 grown at 42 °C.

1997) would suggest that factors that are important in oxidative and temperature stress would be critical for the survival of *P. gingivalis*. In many organisms, HtrA is known to play a regulatory role in response to environmental signals and is protective against oxidative and temperature stress (Cortes *et al.*, 2002; Foucaud-Scheunemann & Poquet, 2003; Lipinska *et al.*, 1990; Lyon & Caparon, 2004; Noone *et al.*, 2000; Pallen & Wren, 1997; Poquet *et al.*, 2000). HtrA is also involved in the biogenesis of bacterial virulence factors. Thus its inactivation results in attenuated virulence (Cortes *et al.*, 2002; az-Torres & Russell, 2001; Lyon & Caparon, 2004). In our study, we further evaluated the role of HtrA in *P. gingivalis*. We have demonstrated that the *htrA*-defective mutant is more sensitive to elevated temperature than the wild-type. In another study, L. Yuan & A. Progulsk-Fox (unpublished) demonstrated that the survival of the *htrA*-defective mutant is not significantly affected by a 50 °C heat shock. Collectively, these data may suggest that HtrA may play a role in long-term adaptation to prolonged elevated temperature, as demonstrated in this study. This could have implications for growth in the periodontal pocket, where there is usually increased temperature due to inflammation.

In addition to the altered growth of the *htrA*-defective mutant at elevated temperature, there was an increase in gingipain activity in the mutant in comparison to the wild-type. The decreased cell density of the *htrA*-defective mutant at elevated temperature compared to the wild-type may indicate that the level of gingipain activity is significant. Our results indeed indicated that at a similar cell density, there was significantly more gingipain activity in the mutant than in the wild-type. Collectively, these results could suggest an involvement of HtrA in gingipain regulation at elevated temperatures. We are currently investigating the molecular changes that occur with the gingipains under prolonged growth at 42 °C. Prolonged growth of *P. gingivalis* at temperatures above 37 °C has previously been observed to result in a significant decrease in gingipain activity (Amano *et al.*, 1994; Forng *et al.*, 2000; Lynch & Kuramitsu, 1999; Murakami *et al.*, 2004a, b; Percival *et al.*, 1999). Under these environmental conditions, it is likely that the decrease in proteolytic activity could be correlated with a down-regulation in the expression of the gingipain genes (Percival *et al.*, 1999). HtrA and other members of this family, including DegS and DegP, have a stress sensor function and are associated with the periplasmic side of the

bacterial membrane (Day & Hinds, 2002; Krojer *et al.*, 2002; Pallen & Wren, 1997). The stress sensor function is modulated via the PDZ domain and initiates a cascade of events that results in the induced expression of several genes critical to the stress response (Day & Hinds, 2002; Krojer *et al.*, 2002; Pallen & Wren, 1997). A comparison of the *P. gingivalis* HtrA homologue with those of other known organisms reveals a PDZ domain and an N-terminal hydrophobic region, indicating membrane association (<http://www.tigr.org>) (van & Hendriks, 2003; Pallen & Wren, 1997). The mechanism for the regulatory role of HtrA in the expression of the gingipain genes in *P. gingivalis* is unknown. Other virulence genes in *P. gingivalis* are known to be downregulated at elevated temperature. However, it is unclear if a common mechanism is involved in their regulation. This is the subject of further investigation in our laboratory.

In this study, the gingipain activity in response to heat shock appeared to be different from the response of the organism grown at elevated temperature for an extended period. Decreased gingipain activity was observed in the *htrA*-defective mutant, in contrast to the wild-type, which showed an increase in activity. It is possible that during heat shock, denaturation of the gingipains could lead to decreased gingipain activity. In the wild-type, it is likely that the presence of the HtrA protein may stabilize the gingipains. HtrA is also known to have a chaperone function, and could play a role in the protection of the gingipains under these conditions, although we cannot rule out the possibility of other heat-shock proteins being involved. Preliminary results from our laboratory have demonstrated that in protein-protein interaction experiments with rHtrA, this protein can interact with the gingipains as well as HagA (data not shown). These findings are currently being confirmed. In addition, HtrA can interact with the VimA protein, which can bind to the gingipains and regulate their activity (Vanterpool *et al.*, 2006). Collectively, these data could further support a direct involvement of HtrA in the regulation of gingipain activity. It is likely that a complex of proteins stabilize the gingipains at elevated temperature, although HtrA is not essential for the maturation/activation of the gingipains under normal conditions. We cannot rule out the possibility that HtrA may regulate the expression of other heat-shock proteins that would be missing in the *htrA*-defective mutant. Several heat-shock proteins in *P. gingivalis*, including GroES, GroEL, DnaK and HtpG, have been documented (Yamazaki *et al.*, 2004; Yoshida *et al.*, 1999; Goulhen *et al.*, 2003; Maeda *et al.*, 1994).

In addition to having elevated temperatures, the periodontal pocket is an oxidative environment due to the presence of reactive oxygen species (Chapple, 1997; Katsuragi *et al.*, 2003; Sculley & Langley-Evans, 2002). Consistent with reports of other organisms (Ibrahim *et al.*, 2004; Mutunga *et al.*, 2004; Wonderling *et al.*, 2004), our data also suggest that the *htrA*-defective mutant is more susceptible to hydrogen peroxide than the wild-type. This was also

observed in a similar study by L. Yuan & A. Progulsk-Fox (unpublished). Given the multiple mechanisms of oxidative stress resistance in *P. gingivalis*, the relative significance of HtrA in this process is unclear. The virulence potential of the *htrA*-defective mutant was altered compared to that of the wild-type (L. Yuan & A. Progulsk-Fox, unpublished).

In conclusion, we can envision a scenario in *P. gingivalis* where the HtrA protein may be important for regulation of gingipain activity in the inflammatory microenvironment of the periodontal pocket. A specific mechanism for this interaction will be the subject of further study.

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