regT can modulate gingipain activity and response to oxidative stress in Porphyromonas gingivalis

E. Vanterpool,¹ A. Wilson Aruni,² F. Roy² and H. M. Fletcher²

¹Department of Biological Sciences, Oakwood University, Huntsville, AL 35896, USA
²Department of Basic Sciences, Division of Microbiology and Molecular Genetics, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA

Recombinant VimA protein can interact with the gingipains and several other proteins that may play a role in its biogenesis in Porphyromonas gingivalis. In silico analysis of PG2096, a hypothetical protein that was shown to interact with VimA, suggests that it may have environmental stress resistance properties. To further evaluate the role(s) of PG2096, the predicted open reading frame was PCR amplified from P. gingivalis W83 and insertionally inactivated using the ermF-ermAM antibiotic-resistance cassette. One randomly chosen PG2096-defective mutant created by allelic exchange and designated FLL205 was further characterized. Under normal growth conditions at 37 °C, Arg-X and Lys-X gingipain activities in FLL205 were reduced by approximately 35 % and 21 %, respectively, compared to the wild-type strain. However, during prolonged growth at an elevated temperature of 42 °C, Arg-X activity was increased by more than 40 % in FLL205 in comparison to the wild-type strain. In addition, the PG2096-defective mutant was more resistant to oxidative stress when treated with 0.25 mM hydrogen peroxide. Taken together these results suggest that the PG2096 gene, designated regT (regulator of gingipain activity at elevated temperatures), may be involved in regulating gingipain activity at elevated temperatures and be important in oxidative stress resistance in P. gingivalis.

INTRODUCTION

The capacity of micro-organisms to rapidly and specifically adapt to environmental conditions is an essential characteristic for their pathogenic potential (Bronner et al., 2004; Dorman, 2009). While adaptation is mostly regulated by complex networks that may act at the transcriptional level, post-translational control of several virulence factors is important in the host–microbe interaction. Porphyromonas gingivalis, a Gram-negative, anaerobic bacterium, has been shown to be associated with periodontal disease and other systemic diseases, including cardiovascular disease (Lamont & Jenkinson, 1998; Seymour et al., 2007; Amano, 2003). The significance and environmental regulation of several of the major virulence factors of P. gingivalis, including the gingipains, are well documented (Abaibou et al., 2001; Hasegawa et al., 2003; Mikolajczyk et al., 2003; Potempa et al., 2003; Vanterpool et al., 2004; Fitzpatrick et al., 2009; Curtis et al., 2005). The 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 still a gap in our understanding of post-translational control of these factors and their potential impact on the pathogenicity of this organism.

In P. gingivalis there is emerging evidence of a unique regulatory mechanism(s) for gingipain biogenesis. Several genes, including vimA, vimF, gppX, porR, sovPG27 and porT, have been shown to play regulatory roles in gingipain activation and/or maturation (Abaibou et al., 2001; Hasegawa et al., 2003; Ishiguro et al., 2009; Saiki & Konishi, 2010; Vanterpool et al., 2004, 2005, 2006). In P. gingivalis FLL92, a vimA-defective mutant, we previously reported a late onset of proteolytic activity during the late exponential phase and altered gingipain distribution even during the stationary phase. In addition, a 64 kDa partially processed RgpB that was altered in its glycosylation was secreted from the vimA-defective mutant (Olango et al., 2003; Vanterpool et al., 2006). Protein–protein interaction studies using the purified rVimA showed that this protein interacts with the gingipains, HtrA, PG2096 [Oralgen database gene ID: PG1833 (http://www.oralgen.lanl.gov/)] and other proteins in P. gingivalis (Roy et al., 2006; Vanterpool et al., 2004). PG2096 appears to have homology with other G proteins; however, its role in gingipain regulation and virulence in P. gingivalis is unclear.

The involvement of G proteins as regulatory molecular switches in the signal transduction pathways that may modulate virulence and environmental stress has been
demonstrated in several bacteria (Baev et al., 1999; Mauriello et al., 2010; Scott & Haldenwang, 1999). The annotated genomic database for Porphyromonas gingivalis has identified several putative genes (PG1241, PG2142, PG2143, PG0346, PG0048 and PG0711) that may have G-protein-like properties and could modulate various stress functions such as protein intake and transport of iron and iron–copper cluster regulation (http://www.oralgen.lanl.gov/). Here we report that PG2096, designated regT (regulator of gingipain activity at elevated temperatures), plays a role in regulating the gingipains at elevated temperatures. RegT is also involved in resistance to oxidative stress in Porphyromonas gingivalis.

METHODS

In silico analysis. Nucleotide sequences for Porphyromonas gingivalis and all the other strains described below were obtained from the Oralgen database (http://www.oralgen.lanl.gov/) and NCBI web server (http://www.ncbi.nlm.nih.gov/), respectively. The sequences were analysed using CLUSTAL W (Larkin et al., 2007) and Lasergene Version 8 (Burland, 2000).

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Porphyromonas gingivalis strains were grown in Brain Heart Infusion (BHI) broth (Difco) supplemented with haematin (5 μg ml⁻¹), vitamin K (0.5 μg 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. Porphyromonas gingivalis strains were maintained in an anaerobic chamber (Coy Manufacturing) in 10 % H2/10 % CO2/80 % N2. Growth rates for all the other strains described below were obtained from the Oralgen database (http://www.oralgen.lanl.gov/) and all the other strains described below were obtained from the Oralgen database (http://www.oralgen.lanl.gov/) and all the other strains described below were obtained from the Oralgen database (http://www.oralgen.lanl.gov/).

DNA isolation and analysis. Porphyromonas gingivalis chromosomal DNA was prepared as described by Marmur (1961). For plasmid DNA analysis, DNA extraction was performed by the alkaline lysis procedure as previously reported (Vanterpool et al., 2004). For large-scale preparation, plasmids were purified using the Qiagen plasmid maxi kit.

Generation of a PG2096-defective mutant Porphyromonas gingivalis strain. A 4.7 kb fragment carrying the intact regT loci was amplified by PCR using the oligonucleotide primers P1 and P2 (Table 2). This fragment was cloned into the pCR2.1-TOPO plasmid vector (Invitrogen) and designated pFLL99. The recombinant plasmid was digested with HinClI, to remove an internal 3.1 kb fragment of the gene. The ermF-ermAM cassette which confers erythromycin/cefotiamycin resistance in E. coli and Porphyromonas gingivalis was PCR amplified from pVA2198 using Pfu turbo (Stratagene) and inserted into the HinClI site using standard methods (Sambrook & Russell, 2001). Orientation of the erythromycin cassette was determined by restriction endonuclease analysis. The resultant recombinant plasmid, pFLL204, was used as a donor in electroporation of Porphyromonas gingivalis W83 as previously reported (Fletcher et al., 1995; Vanterpool et al., 2004). Confirmation of the regT-defective mutant was carried out by performing PCR using regT-specific primers, which amplified a 3.7 kb amplicon from the mutant in contrast to a 4.7 kb amplicon from the wild-type strain. ermF-specific primers (Table 2) were used to confirm the amplification of a 2.1 kb amplicon specific for the erythromycin cassette.

Growth analysis at elevated temperatures and under oxidative stress conditions. Actively growing cultures of Porphyromonas gingivalis wild-type W83 and the PG2096-defective mutant FLL205 were incubated at 42 °C under anaerobic conditions for 28 h. Growth was determined by OD600 readings at 0, 4, 8, 24 and 28 h. For adaptation to oxidative stress conditions, the strains were grown in BHI without cysteine in the presence of 0.25 mM H2O2. Controls were grown in the absence of H2O2. Growth was determined by OD600 readings at 0, 2, 4, 6, 24 and 28 h.

Protease activity under environmental stress conditions. Protease activity was determined as previously reported (Sheets et al., 2006) for cells grown to exponential phase (OD600 0.8) and stationary phase (OD600 1.2). For analysis of gingipain activity of Porphyromonas gingivalis strains at elevated temperatures, cells were incubated at 42 °C under anaerobic conditions for 28 h.

SDS-PAGE and immunoblot analysis. SDS-PAGE was performed with a 4–12 % Bistris separating gel in MOPS-SDS running buffer according to the manufacturer’s instructions (NuPAGE Novex gels; Invitrogen). Samples were prepared (65 % sample, 25 % 4 × NuPAGE LDS sample buffer, 10 % NuPAGE reducing agent), heated at 72 °C for 10 min, and then electrophoresed at 200 V for 65 min in the XCell SureLock Mini-Cell System (Invitrogen). The protein bands were visualized by staining with Simply Blue Safe stain (Invitrogen). The separated proteins were then transferred to BioTrace nitrocellulose membranes (Pall Corporation) and processed at 15 V for 25 min with a Semi-Dry Trans-Blot apparatus (Bio-Rad). The blots were probed with gingipain-specific antibodies (Potempa et al., 1998).

Table 1. Plasmids and bacterial strains

| Strain or plasmid | Phenotype/description | Source |
|-------------------|-----------------------|--------|
| **Plasmids**      |                       |        |
| pCR2.1-TOPO       | Ap, Km, Tet             | Invitrogen |
| pFLL99            | pCR2.1-TOPO : PG2096   | This study |
| pFLL204           | pCR2.1-TOPO : PG2096 : ermF-ermAM | This study |
| pVA2198           | Sp, ermF-ermAM         | Fletcher et al. (1995) |
| **Porphyromonas gingivalis** |                   |        |
| W83               | Wild-type              | This study |
| FLL205            | regT-defective mutant  | This study |
| E. coli           |                       |        |
| DH5z              | F<sup>+</sup> lacZAM15  
Δ(lacZYA-argF)U169 recA1 endA1  
hsdR17<sup>c</sup> (<sub>M15</sub> supE44 )  
phoA supE44 i. <sub>thi-1</sub> gyrA96 relA1 | Invitrogen |
Immunoreactive proteins were detected by the procedure described in the Western Lightning Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences). The secondary antibody was goat anti-rabbit or anti-chicken IgG alkaline (heavy plus light chains)–horseradish peroxidase conjugate (Zymed Laboratories).

Analysis of sialidase activity. Strains were assessed for their sialidase activities using the Amplex Red Sialidase Activity kit according to the manufacturer’s protocol (Invitrogen).

RESULTS

In silico analysis of PG2096

PG2096 (regT) shows 62 % homology with the gene for the Sgp G protein from Streptococcus mutans (Fig. 1). These genes also clustered with PG0411, which encodes a possible outer-membrane protein in P. gingivalis. The six annotated genes that encode G proteins in P. gingivalis formed a separate cluster (see Supplementary Fig. S1, available with the online version of this paper). The Sgp G protein from S. mutans shares structural similarity with G proteins from Pseudomonas aeruginosa and E. coli (Baev et al., 2000). CLUSTAL W analysis using amino acid sequences showed similarity between RegT and other G proteins from P. gingivalis, P. aeruginosa, E. coli and S. mutans (Supplementary Fig. S2). RegT was more closely related to the P. aeruginosa lineage. In the PG2096 protein there are eight conserved G-protein-like regions between positions 418–429, 460, 515–518, 588, 629–631, 645, 689–690 and 717 (Fig. 1, Supplementary Fig. S3). There is also conserved domain architecture that is similar to that of other hypothetical proteins found in Bacteroides fragilis (gi 81443196, gi 81313587), Treponema denticola (gi 81411469) and Prevotella spp (gi 281300483) (Geer et al., 2002). It is noteworthy that while PG0411 is highly homologous to RegT, it lacks the conserved G-protein-like domains, but shows domain similarity with the TonB, PorT, Kgp and RgpB proteins (Aravind & Koonin, 1999). These domains are missing in the RegT protein. No DNA-binding domains were identified in RegT; however, several RNA-binding sites were predicted (data not shown).

Inactivation of the regT gene in P. gingivalis W83 by allelic-exchange mutagenesis and confirmation by RT-PCR

Isogenic mutants of P. gingivalis W83 defective in the PG2096 gene (designated regT) were constructed by allelic-exchange mutagenesis. The circular recombinant plasmid pFLL204, which carries the ermF-ermAM cassette in the Hincl restriction site (0.8 kb of the open reading frame) of the regT gene, was used as a donor in electroporation of P. gingivalis W83. Following electroporation and plating on selective medium (BHI containing 10 μg erythromycin ml−1), we detected approximately 70 erythromycin-resistant colonies after a 6 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 had a black-pigmented and b-haemolytic phenotype. Chromosomal DNA from two randomly chosen

| Table 2. Primers | Primer  | Sequence (5′–3′) |
|------------------|---------|------------------|
| **P1** PG2096 forward | ATGGTGCAATACTAGCAACGT |
| **P2** PG2096 reverse | TCCGGTGTCCGAAATGATAT |
| **P3** erm forward | TATTAGGCGCATTAGCTTCCCGATAT |
| **P4** erm reverse | AATAGGCCTTAGAAGCTGAAC |

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Fig. 1. Multiple sequence alignment of the amino acid sequences showing conserved G-protein-like domains in relation to the G protein sequences from P. gingivalis and other bacteria.
erythromycin-resistant colonies and the wild-type were analysed using PCR to confirm the inactivation in the regT gene. If the regT gene was interrupted by the ermF-ermAM cassette, a 3.7 kb fragment was expected to be amplified using primers P1 and P2 (Table 2). The expected 3.7 kb and 4.7 kb fragments were observed in the two erythromycin-resistant strains and the wild-type W83, respectively. Using erm primers should amplify the 2.1 kb ermF-ermAM cassette from the regT-defective mutants. PCR analysis using erm cassette primers yielded an amplified 2.1 kb fragment as predicted. RT-PCR analysis of the defective mutant showed the absence of the regT fragment in the regT-isogenic defective mutant in contrast to its presence in the wild-type (data not shown). Taken together, these results indicated the insertional inactivation of the regT gene with the 2.1 kb ermF-ermAM antibiotic cassette. One mutant, designated P. gingivalis FLL205, was randomly chosen for further study.

**Growth analysis under thermal and oxidative stress**

To determine if regT played a role in growth of P. gingivalis at elevated temperatures, strains W83 and FLL205 were incubated at 42°C under anaerobic conditions for 28 h. There was no significant difference (P > 0.1) in the growth in the regT-defective mutant and the wild-type when grown at 42°C up to 24 h (Fig. 2). However, autolysis of FLL205 was somewhat increased when compared to the parent strain at 28 h (Fig. 2). To determine if regT played a role in regulating P. gingivalis growth under oxidative stress, the regT-defective mutant and wild-type were grown in the presence of 0.25 mM H₂O₂. As shown in Fig. 3, the regT-defective mutant appeared to be more resistant to 0.25 mM H₂O₂ in comparison to the wild-type, although the differences between treated and untreated cells were not significant (P > 0.1) at most time points.

**Protease activity under normal and elevated temperature conditions**

To determine if regT can affect proteolytic activity, P. gingivalis FLL205 and the wild-type were evaluated for gingipain activity at different growth temperatures. There was a 35% and 21% decrease in Rgp and Kgp activities, respectively, in P. gingivalis FLL205 compared to the wild-type strain grown to stationary phase at 37°C. A time-course of gingipain activities at 42°C was further evaluated. In FLL205 after 4 h of incubation, there was a 25% and 17% increase in Rgp and Kgp activities, respectively in comparison to the wild-type grown under similar conditions (Fig. 4). After 8 h of incubation, there was a 48% increase in Rgp activity (Fig. 4a) and 45% increase in Kgp activity (Fig. 4b) in FLL205 in comparison

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**Fig. 2.** Growth of P. gingivalis FLL205 is not affected at elevated temperature. Cultures of strains W83 (wild-type; ◆) and FLL205 (regT-defective; □) were grown at 42°C for 28 h under anaerobic conditions. Means ± SEM (n=3) are plotted.

**Fig. 3.** The regT-defective mutant is more resistant to hydrogen peroxide than the wild-type. Actively growing P. gingivalis W83 (a) and FLL205 (b) were incubated with 0.25 mM hydrogen peroxide in the absence of cysteine and growth was measured over 28 h. ■, Hydrogen peroxide-treated; ◆, untreated controls. Means ± SEM (n=3) are plotted.

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to the wild-type. After 28 h, the Rgp activity in FLL205 returned to a level similar to that seen at the 4 h time point (Fig. 4a). The Kgp activity, however, increased by 69% in FLL205 in comparison to the wild-type (Fig. 4b).

Immunoblot analysis using specific antibodies to the extracellular gingipains showed that at 37 °C the haemagglutinin domains for Rgp and Kgp were missing in FLL205 (Fig. 5a, arrow). Immunoblot analysis of the secreted RgpA and RgpB from cells grown at 42 °C showed that the catalytic domain (a 48 kDa immunoreactive band) was more stable in the regT-defective isogenic mutant compared to the wild-type strain (Fig. 5b, arrowed). In addition, the RgpB and Kgp membrane protein profiles of P. gingivalis W83 and regT-defective mutant (FLL205) were altered (Fig. 5c). There was no change in the expression of the gingipain genes in the wild-type compared to the regT-defective mutant (Supplementary Fig. S4).

**Sialidase activities of the regT-defective mutant**

Because VimA can interact with several other proteins, including a putative sialidase, it is likely that a common protein complex may have pleiotropic regulatory functions (Vanterpool et al., 2006). P. gingivalis W83 and FLL205 were assessed for their sialidase activities. Fetuin was used as the substrate for the sialidase activity assessment. Fetuin contains both the α2,3 and α2,6 linkages of sialic acid residues. A Pg0234 (sialidase)-defective mutant was also generated and used as a comparison for sialidase activity (unpublished data). The sialidase activities from the membrane fractions were not significantly different from that of the wild-type; however, there was a 45% decrease in secreted sialidase activity in the regT-defective mutant.

**DISCUSSION**

Factors that are important in adaptation to environmental stress in the inflammatory microenvironment of the periodontal pocket would be critical for the survival of P. gingivalis. In previous reports we showed that the vimA gene can modulate several virulence factors in P. gingivalis but not all (Abaibou et al., 2001; Olango et al., 2003; Vanterpool et al., 2006). Furthermore, several other proteins were observed to interact with rVimA (Abaibou et al., 2001; Vanterpool et al., 2006). These proteins in other systems are known to be involved in post-translational regulation. HtrA, one such protein, was shown to play a similar role in oxidative and temperature stress in P. gingivalis as observed in other organisms (Roy et al., 2006).

In this study we have further characterized the hypothetical RegT protein, which was also shown to interact with rVimA (Vanterpool et al., 2006). Bioinformatic analysis of RegT has demonstrated G-protein-like properties. This protein was homologous to the other annotated G proteins in P. gingivalis and other species including E. coli, P. aeruginosa and S. mutans (http://www.oralgen.lanl.gov; http://www.ncbi.nlm.nih.gov). Amino acid sequence analysis of RegT revealed eight G protein conserved domains. Furthermore, there is conserved domain architecture with other hypothetical proteins from other anaerobes, including Bacteriodes fragilis, Treponema denticola and Prevotella spp. The functions of these proteins are still unknown.

G proteins are known to play an important role in regulating stress-related functions in many organisms (Scott & Haldenwang, 1999; Jiang et al., 2007). They can act as secondary messengers that change the inactive state of GDP to GTP, which thus triggers downstream cellular processes including the secretion of stress proteins such as heat-shock proteins (Kedzierska et al., 1999). The regulatory role of G proteins can occur at both the transcriptional and post-transcriptional level (Jiang et al., 2007). At the post-transcriptional level these proteins may modulate mRNA stability, and the assembly of ribosomes and other structural proteins (Sayed et al., 1999). Our study suggests that RegT could be a post-transcriptional regulator. This would be consistent with the absence of any predicted DNA-binding motif. However, the presence of predicted
RNA-binding sites could raise questions about its role in mRNA stability and ribosome assembly in *P. gingivalis*. This will be further evaluated in the laboratory.

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). In this study, growth of the *regT*-defective mutant was not altered at elevated temperatures in comparison to the wild-type. This could suggest that *regT* does not play a role in survival of the organism under temperature stress. Interestingly, however, the *regT*-defective mutant was found to be more resistant to oxidative stress compared to the parent strain. These findings are similar to what was observed in the *vimA*-defective mutant FLL92 (Johnson et al., 2004). Because RegT and VimA can interact, we cannot rule out the possibility that in the *vimA*-defective mutant, the function of the *regT* gene product could be altered, which would result in the increased resistance to oxidative stress observed in *P. gingivalis* FLL92 (Johnson et al., 2004). On the other hand, loss of GTPase activity can promote oxidative stress resistance. This would be similar to observations in *Saccharomyces cerevisiae*, where guanine nucleotide exchange promotes resistance to oxidative stress (Olarewaju et al., 2004). Loss of the ability to regenerate active GTP-bound elongation factor favours the cells’ ability to respond to oxidative stress. Nucleotide exchange is a critical regulator of most G-proteins. It is unclear if this mechanism is functional in *P. gingivalis*. It is also likely that other genes that may play a role in oxidative stress resistance are upregulated in the *regT*-defective mutant; this will be the subject of further investigation.

We have also provided evidence that the absence of RegT altered regulation of the gingipains at normal or elevated temperatures. At normal temperature the presence of RegT will facilitate the normal maturation/processing of the gingipains. The increase in gingipain activity at elevated temperatures in FLL205 may result from a more stable catalytic domain which may occur in the absence of RegT. The fact that there was no observable change in the expression of the gingipain genes in the mutant compared to the wild-type suggests that RegT modulation is post-transcriptional. Normally, *P. gingivalis* can modulate its virulence factors to ensure adaptation to its changing host environment (Amano et al., 1994, 2001; Forng et al., 2000; Kesavalu et al., 2003; Murakami et al., 2004; Percival et al., 1999). Because temperature is one of the factors that is known to change as a consequence of an increased inflammatory response in the periodontal pocket (Fedi & Killoy, 1992), it is likely that RegT is important in regulating gingipain activity in *vivo*. At increased temperature, a possible downregulation in gingipain activity could influence a decrease in the inflammatory response. Other reports have documented a downregulation of gingipain

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**Fig. 5.** (a, b) Western immunoblot analysis of the extracellular proteins from *P. gingivalis* using specific anti-RGP and anti-KGP antibodies as probes. All lanes contained 20 μg of acetone (60%)-precipitated proteins from the supernatant fractions of cultures grown in BHI medium at 37 °C (a) or 42 °C (b). The blots were reacted with antiserum raised in rabbits or chicken against the Arg-X- and Lys-X-specific protease from *P. gingivalis*. Secondary antibody was goat anti-rabbit or anti-chicken IgG alkaline (heavy plus light chains)–horseradish peroxidase conjugate. Lanes 1 and 3, W83; lanes 2 and 4, FLL205. In (a), lanes 1 and 2 were reacted with chicken anti-Kgp; lanes 3 and 4 were reacted with rabbit anti-RgpA. In (b), lanes 1 and 2 were reacted with rabbit anti-RgpB; lanes 3 and 4 were reacted with rabbit anti-RgpA. (c) Immunoblot analysis of membrane fractions. Lanes 1 and 3, W83; lanes 2 and 4, FLL205. Lanes 1 and 2 were reacted with rabbit anti-RgpB; lanes 3 and 4 were reacted with rabbit anti-Kgp.
activity at elevated temperatures (Percival et al., 1999). The coordinate downregulation of gingipain activity in response to an environmental cue linked to the intensity of the host inflammatory response would be consistent with the clinically observed cyclical nature of disease progression in periodontal diseases.

In conclusion, we can envision a scenario in *P. gingivalis* where RegT may be important for regulation of gingipain activity and oxidative stress resistance 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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