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***Filifactor alocis* collagenase can modulate apoptosis of normal oral keratinocytes**

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SUMMARY

To successfully colonize host cells, pathogenic bacteria must circumvent the host's structural barrier such as the collagen-rich extracellular matrix (ECM), as a preliminary step to invasion and colonization of the periodontal tissue. *Filifactor alocis* possesses a putative Peptidase U32 family protein (HMPREF0389_00504) with collagenase activity that may play a significant role in colonization of host tissue during periodontitis by breaking down collagen into peptides and disruption of the host cell. Domain architecture the HMPREF0389_00504 protein predicted the presence of a characteristic PrtC-like collagenase domain, and a peptidase domain. Our study demonstrated that the recombinant *F. alocis* peptidase U32 protein (designated PrtFAC) can interact with, and degrade type I collagen, heat denatured collagen and gelatin in a calcium dependent manner. PrtFAC decreased viability and induced apoptosis of normal oral keratinocytes (NOKs) in a time and dose dependent manner. Transcriptome analysis of NOK cells treated with PrtFAC showed an upregulation of the genes encoding human pro-apoptotic proteins: Apoptotic Peptidase Activating Factor 1 (Apaf1) Cytochrome C, as well as Caspase 3 and 9, suggesting the involvement of the mitochondrial apoptotic pathway. There was a significant increase in caspase 3/7 activity in NOK cells treated with PrtFAC. Taken together, these findings suggest that *F. alocis* PrtFAC protein may play a role in the virulence and pathogenesis of *F. alocis*.

Keywords

Periodontal disease; Collagen; Collagenase; proteases; Virulence; Normal Oral Keratinocytes; Apoptosis

INTRODUCTION

Polymicrobial infection-induced periodontal disease is characterized by an inflammatory condition that can result in a significant reduction in collagen fiber density in the extracellular matrix (ECM) of the gingival tissues (Lorenzini *et al.*, 2009), and alveolar bone loss (Cochran, 2008). The initial infection is mediated by interaction of periodontopathogenic bacteria with the host ECM. To successfully colonize host cells, these

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bacteria must bypass this structural barrier as a preliminary step to invade and colonize the periodontal tissue. Collagen, the main constituent of the ECM, accounts for a total of 30% of the body protein composition (Ricard-Blum, 2011). It is the major component of the gingival connective tissue, connecting the cementum and alveolar bone (Nanci and Bosshardt, 2006). Periodontal tissues such as dentine, cementum and pulp are made up of mostly type 1 collagen (Scully *et al.*, 2005). Therefore an investigation into the mechanisms employed by periodontopathogenic bacteria to compromise the physical barrier of the host's collagen-rich gingival ECM is relevant to understand its virulence and pathogenicity. Numerous human bacterial pathogens such as *Helicobacter pylori* (Kavermann *et al.*, 2003), *Salmonella typhimurium* (Wu *et al.*, 2002), *Proteus miralis* (Zhao *et al.*, 1999), *Aeromonas veronii* (Han *et al.*, 2008), and *Porphyromonas gingivalis* (Kato *et al.*, 1992) have been reported to produce peptidase U32 family proteins; which function as collagenases and contribute to the pathogenicity of the bacteria by collagen breakdown. *P. gingivalis* a keystone periodontal pathogen produces a peptidase U32 protein; PrtC, which plays a role in tissue destruction and progression of periodontitis (Kato *et al.*, 1992). The PrtC protein can degrade soluble type 1 collagen, fibrillar collagen, as well as gelatin (Kato *et al.*, 1992); therefore, bacterial collagenases can likely play a role in periodontitis pathogenesis.

Filifactor alocis, a Gram-positive, asaccharolytic, obligate anaerobic rod is now considered one of the marker organisms associated with periodontal disease (Kumar *et al.*, 2006). Further, in comparison to the other traditional periodontal pathogens, the high incidence of *F. alocis* in the periodontal pocket compared to its absence in healthy or periodontitis-resistant patients supports its importance in the infectious state of the disease (Kumar *et al.*, 2003, Kumar *et al.*, 2006). Studies from our laboratory (Aruni *et al.*, 2015, Aruni *et al.*, 2011, Aruni *et al.*, 2012, Aruni *et al.*, 2014a, Aruni *et al.*, 2014b) and those reported elsewhere (Moffatt *et al.*, 2011, Wang *et al.*, 2014, Wang *et al.*, 2013) suggest that *F. alocis* has an assortment of potential virulence attributes, which are necessary for host invasion, survival, persistence and pathogenesis. Since collagen is an important component of the periodontium and collagenase activity can play a crucial role in tissue destruction and the progression of periodontitis (Kato *et al.*, 1992), it is unclear if *F. alocis* has this specific property.

In addition to several collagen-binding proteins that might be modulated during *F. alocis* infection of host cells (Aruni *et al.*, 2012, Aruni *et al.*, 2014a), interrogation of its genome has identified 2 putative Peptidase U32 family protein (HMPREF0389_00122, HMPREF0389_00504) predicted to have collagenase activity (http://www.ncbi.nlm.nih.gov/nuccore/NC_016630.1). Preliminary observations from our laboratory indicated that inactivation of the HMPREF0389_00122 gene in *F. alocis* did not appear to alter its collagenase activity (unpublished). This study is focused on evaluating the role of the HMPREF0389_00504 protein in collagen destruction and a potential factor in the pathogenesis of *F. alocis*. The observations presented in the report suggest a role for the HMPREF0389_00504 protein (designated PrtFAC) in collagen destruction and may play a role in the virulence and pathogenesis of *F. alocis* by inducing death of host cells.

METHODS

Bioinformatics Analysis

Bioinformatics analysis was carried out to predict the domains present in *F. alocis* Peptidase U32 family protein (HMPREF0389_00504). Amino acid sequence, and information on the conserved domains were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/nuccore/NC_016630.1). Sequence analysis and alignment was carried out using the ClustalW version 2.0 (<http://www.ebi.ac.uk/>) software. Phylogenetic analysis was calculated by neighbor-joining method using kimura parameter. Amino acid sequence for human apoptotic genes were derived from NCBI RefSeq Gene record (NG 005905).

Bacterial Strain Culture Conditions

All strains and plasmids used in this study are all listed in Table 1. The *F. alocis* ATCC 35896 wildtype strain was grown in Brain Heart Infusion (BHI) broth supplemented with vitamin K (0.5 µg/ml), hemin (5 µg/ml), cysteine (0.1%), and arginine (100 µg/ml). Blood agar plates were prepared by adding 5% of sheep blood (Hemostat Laboratories, Dixon CA), and 2% agar to the supplemented BHI broth. *F. alocis* cultures were grown anaerobically at 37°C, and maintained in an anaerobic chamber (Coy Manufacturing) with 10% H₂, 10% CO₂, and 80% N₂.

Epithelial Cell Culture Conditions

Normal Oral Keratinocytes (NOKs) were obtained as a gift from Dr. Penelope Duerksen-Hughes Laboratory (Division of Biochemistry, Loma Linda University School of Medicine) and grown as previously described (Piboonniyom *et al.*, 2003). Briefly, NOKs were grown in keratinocyte serum-free medium (Keratinocyte-SFM; Life Technologies, Inc.) supplemented with epidermal growth factor and bovine pituitary extract at 37°C and 5% CO₂. Confluent mono cultures were trypsinized, and adjusted to approximately 5×10^3 cells/ml, followed by seeding (1 ml per well) into 12-well plates (3.5 cm² area) (Nunc, Rochester, NY), and further incubation for 48 h to reach semi confluence (10^5 cells per well).

Purification of PrtFAC Protein in *E. coli*

The open reading frame (ORF) of the *F. alocis* peptidase U32 family protein (HMPREF0389_00504) was PCR-amplified with primers P1 and P2 (Table 2), purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA), then cloned into the pET 102-TOPO® vector (Invitrogen, USA). The recombinant plasmid was used to transform *E. coli* Top 10 F' chemically competent cells (Life Technologies, Carlsbad CA). A recombinant plasmid designated pFLL215 (Table 1) was randomly isolated from ampicillin resistant *E. coli* Top10F' cells and selected for expression after the presence, orientation and nucleotide sequence of the insert was confirmed by PCR and DNA sequencing. The pFLL215 plasmid was then transformed into the *E. coli* One shot® BL21 (DE3) strain (Life Technologies, Carlsbad CA), and purification of the recombinant protein (PrtFAC) was carried out using Ni-NTA agarose beads (Qiagen, Valencia, CA) as per the manufacturer's protocol. Briefly, four liters of room temperature Luria-Bertani (LB) media containing ampicillin (100 µg/ml)

was inoculated with 200 ml of overnight culture of the One shot® BL21 (DE3) *E. coli* cells carrying the pFLL215 plasmid. The bacterial culture was grown to exponential phase ($O.D_{600} \sim 0.6$), and induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for an additional 6 h. Using centrifugation (19,000 g for 45 min at 4°C) the culture, post induction, was washed in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 pH 7.4), and pelleted. The bacterial pellet was re-suspended in equilibration buffer (EB) containing 150 mM NaCl, 50 mM Tris at pH 7.5, and subsequently lysed with a French press. Following lysis, the lysate was centrifuged (19,000 g for 45 min at 4°C), and the resultant supernatant containing the induced protein was passed through a column containing the Ni-NTA agarose beads for protein purification. The column was washed twice with EB (containing 10% glycerol and 15 mM imidazole), and the purified protein of 60.72 KDa was eluted with EB containing 500 mM imidazole. Four elution fractions of 1 ml each were then analyzed by SDS-PAGE. The purest fraction of the protein was dialyzed in buffer containing 10mM Tris-HCl (pH 7.4) using 10,000MW cutoff membrane (Amicon Inc., Beverly, MA) to remove the imidazole. The dialyzed protein was then concentrated, analyzed by SDS-PAGE gel and immunoblotting, and aliquoted and stored at -20° C. The purified protein was evaluated for lipopolysaccharide (LPS) contamination using the Limulus amoebolysate (LAL) assay kit (Lonza, MD).

SDS-PAGE and Immunoblotting

SDS-PAGE was used to separate and analyze protein samples as previously reported (Aruni *et al.*, 2012). Proteins from the SDS-PAGE were transferred to a nitrocellulose membrane (Schleicher & Schuell, FL) using a Semi-Dry Trans-blot apparatus (Bio-Rad, CA) at 15 V for 30 min. The blots were probed with primary antibody against the His-tag (mouse; 1 : 1000 dilution), and the secondary antibody (goat anti-mouse; 1 : 1000 dilution) was horseradish-peroxidase-conjugated (Zymed Laboratories, San Francisco, CA). Immunoreactive bands were detected using the Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer's recommendations.

Binding of PrtFAC Protein to Collagen

To determine if PrtFAC binds to collagen, a solid phase direct enzyme-linked immunosorbent assay (ELISA) was employed as previously described (Mishra *et al.*, 2011). Briefly, 96-well Greiner bio-one, medium binding plates (Sigma, MO) were coated with 100 μ l of 10 μ g/ml collagen type 1 (Sigma Aldrich, MO), and incubated overnight at 4°C. The plates were blocked with 5% milk at room temperature for 1 h, and washed 3X with phosphate buffered saline (PBS-T). PrtFAC was added in varying dilutions to a final concentration of 1 μ g, 4 μ g, and 16 μ g/well respectively. The plates were incubated for 1 h at room temperature, followed by washing and hybridization with HisProbe™ - HRP (Thermo Scientific, IL). After a 1 h incubation time, and 3 washes with PBS-T, the cells were incubated with 3, 3', 5, 5'-tetramethylbenzidine substrate for 30 min. Reactions were stopped with 2 M sulphuric acid and read at OD_{450nm} . Bovine serum albumin (BSA) was used as the negative control.

Collagenase Assay

The collagenase activity of PrtFAC was analyzed using the EnzChek® Gelatinase/Collagenase Assay Kit (Molecular Probes Inc. Eugene OR), as per the manufacturer's protocol. Briefly, protein samples were incubated with DQ-gelatin or DQ-Collagen I (Molecular Probes Inc. Eugene OR), respectively in the dark at 25°C. Active enzymes cleaved the fluorescent-labeled gelatin or collagen and the change in fluorescence was measured (Excitation/Emission 485/528 nm). Collagenase from *Clostridium histolyticum* (0.25 U/ml) was used as the control and the negative control was without the PrtFAC protein. A solution of 5 mM CaCl₂ was used to assay for the activation of the PrtFAC protein. 1 mM EDTA and 1 mM 1, 10 phenanthroline were used as inhibitors. To determine if breaking the collagen into smaller peptides enhanced collagenase activity, substrates were boiled for 20 min at 100°C.

Induction of Cell Death

NOKs were grown to semi confluence (10⁵ cells per well), and treated with PrtFAC in 10, 50 and 100 µM concentrations for 48 h. As a caspase control, cells were pre-incubated with Z-VAD-FMK (20 µM) for 1 hour before treatment with the protein. TRAIL (20 µM) a known apoptotic protein (Hymowitz *et al.*, 1999) was used as a positive control. Cell death was visualized by Olympus I x 70 microscope, equipped with Hoffmann Modulation Contrast. Images were acquired using a digital Spot Imaging System.

Caspase 3 and 7-activity assay

NOK cells were treated with 50 µM PrtFAC for 48 h, after which cells were incubated with Caspase-Glo assay substrates at room temperature in the dark for 1 h to measure caspase-3/-7 activity using the Caspase-Glo® 3/7 Assay Kit (Promega, WI USA), according to the manufacturer's instructions. Luminosity was measured using FLx800™ Multi-Detection Microplate Reader (Biotek Instruments INC, Vermont, USA).

RNA Isolation and Real Time Quantitative PCR Analysis

RNA was extracted from the *F. alocis* wild-type strain co-cultured with NOK, or NOK cells alone using the TRI Reagent® Soln. (Ambion, CA). Briefly all cultures were centrifuged at 13,000 rpm for 5 min, and washed in 1X phosphate buffer saline (PBS). Cell lysis was done in 1 ml of TRI Reagent® Soln. and aqueous layer containing the RNA was collected by addition of chloroform and centrifugation at 13,000 g for 15 min at 4°C. RNA was washed with isopropyl alcohol and RNA wash buffer (SV total RNA isolation system Promega, CA). Reverse transcription was carried out using the SV total RNA isolation system (Promega, CA) according to the manufacturer's recommendations. All gene primers used in this study are listed in Table 2. SYBR green kit (Qiagen, Valencia, CA) was used for the real time PCR amplification, and the Cepheid Smart Cyclyer Real Time PCR equipment detected the real-time fluorescence signal. Each reading was performed in triplicate for each gene. 28S rRNA (RNA28S1) was used as control to normalize variations due to differences in reverse transcription. The relative expression ratio of the genes selected and critical threshold cycle (Ct) were calculated (Pfaffl *et al.*, 2002).

Flow Cytometric Apoptosis Analysis

Flow cytometry analysis of NOK cells was used to assay for cell viability and mechanism of cell death. NOKs were grown to semi confluence (1.5×10^4 cells per well) in 96-well plates (0.33 cm^2 area) (Nunc, Rochester, NY), and treated with PrtFAC (10, 50, and $100 \mu\text{M}$) for 48 h. Post treatment, adherent cells were trypsinized and washed with 1X PBS and centrifuged (at 500 g for 5 min) in sterile FACS 5 ml centrifuge tubes. The supernatant was discarded and the cells were stained for flow cytometry analysis using pacific blue™ Annexin-V and 7-Aminoactinomycin D (7-AAD) [Biolegend, San Diego, CA], according to the manufacturer's instructions. Test volumes were decreased for staining in a 96 well plate. Briefly, cells were resuspended in $10 \mu\text{l}$ of Biolegend Annexin V binding buffer then stained with $0.5 \mu\text{l}$ of pacific blue™ Annexin V and $1 \mu\text{l}$ of 7-AAD for 15 min at room temperature in the dark. Cells were washed with $200 \mu\text{l}$ of Annexin-V binding buffer then resuspended in 1% paraformaldehyde. TRAIL protein ($20 \mu\text{M}$) was used as a positive control, while Z-VAD-FMK ($20 \mu\text{M}$), a pan-caspase inhibitor, was used as a caspase control. Data acquisition and analysis of apoptotic cells were performed using the MACSQuant 10 Analyzer (MiltenyiBiotec, Inc, Auburn CA) and FlowJo vX Data Analysis Software (FlowJo, Ashland, OR).

Statistical Analysis

All experiments were performed in triplicate for each condition, and repeated at least three times unless otherwise stated. Error bars represent the standard deviation from the mean, and comparisons for statistical significance were made by paired t-test.

RESULTS

F. alocis HMPREF0389_00504 encodes a peptidase U32 protein with putative collagenase activity

Sequence comparison of the peptidase U32 family protein from several pathogenic organisms such as *H. pylori*, *P. gingivalis*, *Bacillus subtilis*, and *S. typhimurium*, showed a close relationship with the *F. alocis* Peptidase U32 family protein (Figure 1A). *F. alocis* genome contains a peptidase U32 gene (HMPREF0389_00504) which is 1241 nucleotides in length (413 amino acids long) that encodes a protein annotated as a Peptidase U32 family protein. This protein is homologous to the Peptidase U32 of the Clostridia class. The domain architecture of the protein (Figure 1B) showed the presence of overlapping domains: a PrtC-like collagenase domain (amino acid 1-354), as well as a putative peptidase domain (PRK15452) (amino acid 1-403). The PrtC-like domain is similar to the PrtC collagenase protein present in *P. gingivalis*, while the protease domain (PRK15452) is predicted to have peptidase function. Protein modeling showed a probable transmembrane segment (Figure 1C). The *F. alocis* Peptidase U32 shows a 38% identity to PrtC, whereas it shares 70% identity with a peptidase of *Proteocatella sphenisci* also belonging to the U32 family. Based on the ProtoMap database (Yona *et al.*, 2000) the *F. alocis* PrtFAC protein (HMPREF0389_00504), can be classified as members of the cluster- 1872. This cluster contains 24 members, with 13 containing the Peptidase family U32 signature. Bioinformatics analysis of PrtFAC revealed characteristic signature motif of peptidase U32 family members- E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S (Figure 1C). The

F. alocis Peptidase U32 family protein (HMPREF0389_00504) designated PrtFAC was further characterized.

PrtFAC can interact with collagen type I

A surface model of the *F. alocis* PrtFAC protein showed a potential catalytic interacting site consisting of a tetrad of cysteine and arginine residues forming an interactive groove (Figure 1C), which could likely play a role in collagen interaction. The *F. alocis* *prtFAC* gene was cloned and expressed in the pET 102-TOPO® vector as described above. To evaluate the ability of the 60.73 kDa recombinant PrtFAC protein (Figure 2A) to interact with collagen, we use a solid-phase collagen binding ELISA assay. As shown in Figure 2B, the PrtFAC recombinant protein adhered strongly to the collagen-coated plates in a concentration dependent manner. As a control, BSA did not bind.

PrtFAC exhibits Ca²⁺ dependent collagenase activity

In bacteria, peptidase U32 family proteins have been shown to degrade collagen, hence they function as collagenases (Kato *et al.*, 1992, Kavermann *et al.*, 2003, Matsushita and Okabe, 2001). The collagenase activity of the PrtFAC protein was analyzed using fluorescein-labeled collagen substrates. In the presence of the PrtFAC protein, there was a significant increase in the release of the fluorescein label from the type I collagen, and gelatin substrates (Figure 2B, 2C). This activity was Ca²⁺ dependent. Decreased enzyme activity was observed in the presence of EDTA and 1, 10 phenanthroline. There was no enzyme activity observed when the PrtFAC protein was denatured by boiling at 100 °C for 15 min. Fluorescein release was increased when the substrates were boiled prior to incubation with the purified PrtFAC protein (Figure 2D).

PrtFAC treatment of NOK cells leads to a decrease in cell viability via apoptosis

Apoptosis is one of the hallmarks of periodontal disease (Gamonal *et al.*, 2001, Jarnbring *et al.*, 2002, Stathopoulou *et al.*, 2009), and collagenases have been documented to cause apoptosis in a time and dose dependent manner (Lo and Kim, 2004, Zhao *et al.*, 2000). To evaluate the effects of PrtFAC protein on host cells, NOK cells were incubated for 24 or 48 h with varying concentrations (10, 50, and 100 µM) of the purified protein. The cells were visualized using an Olympus I x 70 Microscope equipped with Hoffmann Modulation Contrast or were co-stained with the appropriate apoptotic marker and evaluated by flow cytometry. Cell death observed after 24 h was less than that observed after 48 h (Figure 3A and 3B). As shown in the arrows in Figure 3A, PrtFAC protein treatment of NOK cells growing in monolayers induced shrinkage, loss of adhesion properties and apoptotic cell death, in contrast to the untreated controls. The number of early and late apoptotic populations increased with increasing concentrations of the purified PrtFAC protein (Figure 4A). Inversely, compared to the controls, there was decreased cell viability to approximately 50, 20, and 7% post treatment with PrtFAC at concentrations of 10, 50, and 100 µM respectively (Figure 4B). Cell viability was increased from 20% to 40% when NOK cells were pre-treated with Z-VAD-FMK (20 µM) prior to incubation with the purified PrtFAC protein (Figure 4B). Because the purified PrtFAC protein was determined to contain approximately 0.001 µg/ml of LPS, NOK cells were treated with a PrtFAC protein sample that was denatured by boiling at 100 °C for 15 min. Compared to the control, there was no

observed cell death of NOK cells even with a concentration of up to 100 μ M of the denatured PrtFAC protein (data not shown).

PrtFAC protein-induced apoptosis is regulated by the activation of caspases

Caspases are cysteine proteases that regulate the apoptotic process (Chang and Yang, 2000). To ascertain the direct involvement of caspase activation in PrtFAC-NOK induced apoptosis, and the mechanism by which PrtFAC induced cell death by apoptosis of NOK cells, we evaluated the relative expression of several genes involved in the apoptotic pathways. There was upregulation of genes coding for caspases and pro-apoptotic proteins including apoptosis peptidase activating factor 1 (APAF1), caspase 3, caspase 9, and Cytochrome C all key players in the intrinsic mitochondrial apoptotic pathway (Figure 4C). Additionally we detected significant elevated levels of caspase 3/7-activity 48 h post treatment of NOK cells with PrtFAC (Figure 4D). Caspase 8 was not detected in the NOK cells treated with PrtFAC (Figure 4D).

DISCUSSION

Virulence of bacterial pathogens is dependent on their invasiveness and cytotoxic abilities. Invasiveness reflects the ability of the bacteria to adhere to host cells and penetrate through the structural barriers including the epithelium and extracellular matrix. Periodontal tissue is primarily composed of type I collagen, which is made up of three parallel polypeptide chains of the sequence Gly-X-Y, with X representing proline and Y representing hydroxyproline (Scully *et al.*, 2005). These amino acids give collagen stability and restrict the rotation of the polypeptide backbone (Dung and Liu, 1999). Collagen is extremely resistant to degradation, and can only be cleaved by collagenases (Harrington, 1996). Bacterial collagenases are capable of hydrolyzing denatured as well as native collagen with broad specificity unlike vertebrate collagenases that are more specific in their cleavage sites (Matsushita *et al.*, 1994). Hydrolysis of collagen in general helps in spreading of bacterial infection; hence collagenase is a common invasive enzyme that can play an important role in the invasiveness of bacteria (Han *et al.*, 2008).

Many oral bacteria that produce collagenolytic enzymes are asaccharolytic (e.g., *F. meningosepticum*, *Peptostreptococcus asaccharolyticus*, and *Porphyromonas spp.*), and their metabolism is dependent on the uptake of small peptides and amino acids (Leduc *et al.*, 1996, Takahashi and Sato, 2002). Hence the collagenolytic enzymes by these organisms may thus be essential for survival and growth and may give both saccharolytic and asaccharolytic organisms capable of producing collagenases a greater nutritional diversity compared with non-collagenolytic strains. This may confer a selective advantage for periodontal pathogens. Moreover the degradation of collagen in dental tissues leads to the development of a region that has a lower redox potential level compared to the normal surrounding tissue (Harrington, 1996). This environment promotes the colonization of anaerobic organisms, which can lead to periodontal disease. Collagenases that lead to tissue destruction and subsequent invasion may allow bacteria gain access to more anaerobic sites deep in host tissues, and evade the host's immune response.

This study has provided insights into the role of a *F. alocis* collagenase in collagen degradation and induction of cell death of host cells. We have shown that the PrtFAC protein is able to bind to and degrade type I collagen. In addition, its ability to degrade the denatured collagen could indicate a broad specificity consistent with other bacterial collagenases (reviewed in Zhang *et al.*, 2015). The sensitivity of the PrtFAC protein activity to EDTA and phenanthroline could support its metalloprotease classification and hence it could belong to a subfamily of the U32 family that includes other proteins from *P. gingivalis* and *H. pylori* (Kato *et al.*, 1992, Kavermann *et al.*, 2003). The first crystal structure analysis of a U32 catalytic domain from *Methanopyrus kandleri* did not reveal any discernible typical proteolytic motifs in its three-dimensional structure (Schacherl *et al.*, 2015). Moreover biochemical assays failed to demonstrate proteolytic activity. This is in contrast to our study that has shown proteolytic characteristics for the *F. alocis* PrtFAC protein. Further comprehensive analysis of the function and mechanism of action of this enzyme is under further investigation in the laboratory.

Previous studies have shown the ability of *F. alocis* to adhere to and invade host cells (Aruni *et al.*, 2011, Aruni *et al.*, 2012, Aruni *et al.*, 2014a), thus it's likely that PrtFAC may play a role in this process. While there are other *F. alocis* surface molecules (Aruni *et al.*, 2015, Aruni *et al.*, 2011) that can mediate interaction with host cells and would be a critical virulence attribute, the putative PrtFAC collagenase could facilitate a selective advantage for the survival of *F. alocis* in the periodontal pocket. This would be consistent with the high abundance of *F. alocis* in the periodontal pocket (Kumar *et al.*, 2003, Kumar *et al.*, 2006). We cannot rule out the possibility that the PrtFAC protein can degrade collagen in addition to fulfilling the asaccharolytic nutrition requirement via its peptidase activity. This is under further investigation in the laboratory.

Cell death by apoptosis can be demonstrated in periodontal lesions and could be the direct result of specific bacterial factors or the indirect result of pro-inflammatory cytokines induced by the infection (Moffatt *et al.*, 2011). Previous studies have documented the ability of *F. alocis* to induce apoptosis in gingival epithelial cells (Moffatt *et al.*, 2011), keratinocytes and neutrophils (Wang *et al.*, 2014). While the apoptotic effects of specific bacterial factors were not clarified in these studies, the data from this report suggest cytotoxic activity for the PrtFAC protein. We observed a significant concentration dependent increase in apoptosis in NOK cells treated with PrtFAC. This activity was abrogated in the presence of the denatured PrtFAC protein. Moreover, because LPS is insensitive to boiling at a temperature of 100 °C, it is unlikely that it may have any cytotoxic effect on the NOK cells under our experimental conditions. This would be consistent with previous observations which demonstrated that LPS levels of up to 10 µg/ml were not cytotoxic to NOK cells (Nakata *et al.*, 2013). Apoptosis was associated with the activation of caspase 3/7 and other pro-apoptotic markers that represent key players in the intrinsic mitochondrial apoptotic pathway. It is noteworthy that activation of caspase 8 was not detected. Moreover the inability of Z-VAD-FMK to completely inhibit apoptosis in the presence of the PrtFAC protein may indicate a caspase-independent apoptotic mechanism. Collectively, our data suggest that the purified *F. alocis* PrtFAC protein may induce both a caspase-dependent and caspase-independent apoptosis of NOK cells. *F. alocis* caspase-dependent apoptosis of keratinocytes has been reported earlier (Moffatt *et al.*, 2011), however a mechanism was not

fully described. It is likely that the caspase-dependent mechanism may involve anoikis, a type of caspase-dependent apoptosis that can be triggered by cellular detachment that is associated with loss of integrin survival signaling or inappropriate cell matrix contacts (Frisch & Francis, 1994). The ECM signals have been earlier shown to prevent cells from actively undergoing apoptosis (Meredith *et al.*, 1993). It is unclear if this is similar to *P. gingivalis* gingipain-induced cell death of endothelial cells (Sheets *et al.*, 2005). Anoikis has been described in numerous cell types, including epithelial cells, endothelial cells, keratinocytes, thyroid cells, fibroblasts, and osteoblasts (Aoudjit & Vuori, 2001, Bretland *et al.*, 2001, Gniadecki *et al.*, 1998, Jensen *et al.*, 2011, Sakai *et al.*, 2000). Anoikis can also be regulated in a caspase-independent manner by Bit1, a key regulator (Tan *et al.*, 2013). This mitochondrial protein which contains a cell death domain can translocate to the cytoplasm when integrin–ECM interactions are disrupted (Ekert & Vaux, 2005). In the cytoplasm the cell death domain is believed to be activated to promote cell death signaling of the ECM-detached cells (Biliran *et al.*, 2008). The modulation of Bit1 in our studies is unknown however, Bid another anoikis caspase-independent regulator (Valentijn & Gilmore, 2004) was observed to be up-modulated in the PrtFAC treated NOK cells. Because Bid can regulate apoptosis via the intrinsic pathway (Valentijn & Gilmore, 2004), it is likely the cytotoxic effects of PrtFAC on NOK cells may occur via a similar mechanism. This is under further investigation in the laboratory. It should be noted that serine proteases can also play a role in caspase-independent apoptosis. Several classes of proteases, such as calpains, cathepsins, and the granzyme serine proteases, have also been found to induce caspase-independent apoptosis (Borner & Monney, 1999). It is unclear if the PrtFAC protein which might be able to alter the ECM, can traverse the cell membrane and modify any cell death signaling substrate(s) via its peptidase activity. Current efforts in the laboratory are aimed at elucidating the pathways involved in PrtFAC-induced caspase-dependent and caspase-independent apoptosis in host cells.

In conclusion, our study has confirmed a role for the *F. alocis* collagenase in collagen destruction and its ability in the virulence and pathogenesis of *F. alocis* by inducing cell death of host cells.

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Figure 1.
: Bioinformatics analysis of *F. alocis* Peptidase U32 family protein (HMPREF0389_00504) as described in methods (A) Phylogenetic analysis of Peptidase U32 collagenases among various bacterial pathogens (B) Domain architecture showing the PrtC-like domain (amino acid 1–354), peptidase domain (amino acid 1–403), and peptidase U32 family signature motif. (C) *In silico* protein modeling highlighting the carbon and nitrogen terminals, transmembrane segments (highlighted with arrow), and tetrad of cysteine and arginine residues that compose the potential catalytic interacting site.

Figure 2.

(a) SDS PAGE analysis of purified PrtFAC after purification under native conditions. Lane 1: Protein ladder, lane 2–6: PrtFAC 0.5, 1, 1.5, 2, 2.5, 3 µl, and Western Blot confirmation of PrtFAC showing immunoreactive bands to anti His-tag antibody. (B) Increasing concentrations of PrtFAC (1, 4, 16 µg/well) showed increased binding to collagen substrate, unlike the BSA negative control. (C) Collagenase assay using PrtFAC protein and Collagen type I, and heat treated collagen substrates showed Ca²⁺-dependent collagenase activity, while the use of EDTA (1 mM) and 1, 10 phenanthroline (1 mM) inhibited collagenase activity. To activate collagenase activity 5 mM CaCl₂ was used. (D) Gelatinase assay using PrtFAC protein and gelatin, and heat treated gelatin as substrates. Negative control was without the PrtFAC protein and values were subtracted from the all the test values. Experiment was carried out in three repeats. Error bars represent the standard deviation from the mean. *** P < 0.001

Figure 3. PrtFAC induces Apoptosis in Normal Oral Keratinocytes

NOKs were grown to semi confluence in a 96-well plate (1.5×10^4 cells per well), and treated with PrtFAC in 10, 50 and 100 μ M concentrations for 24 and 48 h respectively. Hoffman Modulation phase contrast I x 70 microscopy was used to visualize cell death. Images were acquired using a digital Spot Imaging System. Trail protein (20 μ M) and ZVAD-FMK (20 μ M) caspase inhibitor were used as controls. There is a time dependent increase in cell shrinkage and cell death in the treated PrtFAC cells compared to the untreated controls.

Figure 4. Stimulation of Normal Oral Keratinocytes (NOK) with PrtFAC Protein

NOKs were grown to semi confluence in a 96-well plate (1.15×10^4 cells per well), and treated with PrtFAC in 10, 50 and 100 μ M concentrations for 48 h. As a caspase control, cells were pre-incubated with ZVAD-FMK (20 μ M) for 1 h prior to treatment with the recombinant protein. TRAIL (20 μ M) was used as a positive control. (A) Apoptosis assay 48 h post treatment with PrtFAC showed an increase in apoptotic populations (B) NOK cell viability post treatment PrtFAC showed dose-dependent significant decrease in cell viability. (C) Transcriptome analysis on regulation of apoptotic genes in NOK cells post treatment with PrtFAC showed the upregulation of pro-apoptotic genes and caspases (D) There was a significant increase in caspase 3/7 activity compared to the untreated. Comparisons were made by paired t-test; n = 6. *** P < 0.001

Table 1**Bacterial Strains and Plasmids Used In This Study**

Bacterial Strains	Description	Source
<i>Filifactor alocis</i> ATCC 35896	Wild-type strain	Aruni <i>et al.</i> , (2011)
<i>Escherichia coli</i> Top 10	Genotype: <i>F</i> - <i>mcrA</i> (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) Φ 80 <i>lacZ</i> <i>M15</i> <i>lacX74</i> <i>recA1</i> <i>araD139</i> (<i>ara</i> - <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (<i>StrR</i>) <i>endA1</i> <i>nupG</i>	Life technologies
<i>E. coli</i> BL21 StarTM (DE3)	Genotype: <i>F</i> - <i>ompT</i> <i>hsdSB</i> (<i>rB</i> - <i>mB</i> -) <i>gal</i> <i>dcm</i> <i>rne131</i> (DE3)	Life technologies
pFLL215	PrtFAC (HMPREF0389_00504) in Top 10 <i>E.coli</i>	This Study

Table 2

Primers Used In This Study

Primer	Description	Nucleotide Sequence	Use
P1	FA 504 PET-Forward	5'-CAC CAT GAA AAA AAT TGA GTT ACT TTC T-3'	cloning
P2	FA 504 PET- Reverse	5'-TTC GAT AAT CGC ATT TGC TGC TTT C-3'	cloning
P3	Apaf1F	5'-GTGGAAAACCTCCTGTGCCG-3'	Real-time PCR
P4	Apaf1R	5'-CCTTTGCGCCTAGGTCTTAGT-3'	Real-time PCR
P5	FaddF	5'-CTCAAGTTCCTATGCCTCGGG -3'	Real-time PCR
P6	FaddR	5'-CAGCATGGAGAAGAGGTCTAGG-3'	Real-time PCR
P7	Casp8F	5'-GCCTGGACTACATTCCGCA -3'	Real-time PCR
P8	Casp8R	5'-CCTTCAGGAAGGACAGATTGCT -3'	Real-time PCR
P9	Bcl2F	5'-GATTGATGGGATCGTTGCCT-3'	Real-time PCR
P10	Bcl2R	5'-ACAGCATGATCCTCTGTCAAGTT-3'	Real-time PCR
P11	CyscF	5'-GAGCGAGTTTGGTTGCACTT-3'	Real-time PCR
P12	CyscR	5'-CGGACGTCCCCACTCTCTAA-3'	Real-time PCR
P13	Casp9F	5'-AAAGTTGTCTGAAGCCAACCC-3'	Real-time PCR
P14	Casp9R	5'-CTGAGAACCTCTGGTTTGCGA -3'	Real-time PCR
P15	BidF	5'-GCTCCGTGATGTCTTTCACAC-3'	Real-time PCR
P16	BidR	5'-TCTGGCTAAGCTCCTCACGTA-3'	Real-time PCR
P17	XiapF	5'-TACGAATGGGGTTCAGTTTCAAG -3'	Real-time PCR
P18	XiapR	5'-GTCTGACTTGACTCATCTTGCAT-3'	Real-time PCR
P19	Casp3F	5'-CAGTTTGTGTTGTGTGCTTCTGAG-3'	Real-time PCR
P20	Casp3R	5'-AACGATCCCTCTGAAAAAGTT-3'	Real-time PCR
P21	28S-F	5'-AAC GAG ATT CCC ACT GTC CC -3'	Real-time PCR
P22	28S-R	5'-CTT CAC CGT GCC AGA CTA GAG-3'	Real-time