The Role of Collagen Interacting Proteins in the Pathogenesis of Filifactor alocis

Ozioma Salomey Chioma
The Role of Collagen Interacting Proteins in the Pathogenesis of *Filifactor alocis*

by

Ozioma Salomey Chioma

A Dissertation Submitted in Partial Satisfaction of the Requirements for the Degree Doctor of Philosophy in Microbiology and Molecular Genetics

June 2016
Each person whose signature appears below certifies that this dissertation in his opinion is adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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<th>Description</th>
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<tr>
<td>AAA</td>
<td>Acute apical abscesses</td>
</tr>
<tr>
<td>AAGPR</td>
<td>Asaccharolytic anaerobic Gram-positive rods</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>AP</td>
<td>Adult periodontitis</td>
</tr>
<tr>
<td>Apaf1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2 antagonist of cell death</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>B-cell lymphoma protein long isoform</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma protein 2</td>
</tr>
<tr>
<td>BCL-10</td>
<td>B-cell lymphoma protein 10</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting-domain death agonist</td>
</tr>
<tr>
<td>BIK</td>
<td>BCL2 interacting killer</td>
</tr>
<tr>
<td>BIM</td>
<td>BCL2 interacting protein BIM</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase-activated DNase</td>
</tr>
<tr>
<td>CASP 3</td>
<td>Cysteinyl aspartic acid-protease-3</td>
</tr>
<tr>
<td>CASP 8</td>
<td>Cysteinyl aspartic acid-protease-8</td>
</tr>
<tr>
<td>CASP 9</td>
<td>Cysteinyl aspartic acid-protease-9</td>
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<tr>
<td>CBD</td>
<td>Collagen binding domain</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>FLICE-inhibitory protein</td>
</tr>
<tr>
<td>CP</td>
<td>Chronic periodontitis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CYSC C</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharides</td>
</tr>
<tr>
<td>FA</td>
<td><em>Filifactor alocis</em></td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthetase</td>
</tr>
<tr>
<td>FADD</td>
<td>FAS-associated death domain protein</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FasL</td>
<td>Fatty acid synthetase ligand</td>
</tr>
<tr>
<td>FasR</td>
<td>Fatty acid synthetase receptor</td>
</tr>
<tr>
<td>GAgP</td>
<td>Generalized aggressive chronic periodontitis</td>
</tr>
<tr>
<td>GChP</td>
<td>Generalized chronic periodontitis</td>
</tr>
<tr>
<td>HtrA2/Omi</td>
<td>High temperature requirement</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>NOKs</td>
<td>Normal oral keratinocytes</td>
</tr>
<tr>
<td>LAP</td>
<td>Localized aggressive chronic periodontitis</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LPXTG</td>
<td>(Sorting signal in Gram-positive bacteria)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MMPs</td>
<td>Metalloproteinases</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial Surface Components Recognizing Adhesive Matrix Molecules</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>Noxa</td>
<td>Phorbol-12-myristate-13-acetate-induced protein 1</td>
</tr>
<tr>
<td>O.D</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Periodontal disease</td>
</tr>
<tr>
<td>PG</td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic kidney disease-like</td>
</tr>
<tr>
<td>PLBW</td>
<td>Pre-mature labor low- birth-weight</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonucleocytes</td>
</tr>
<tr>
<td>Puma</td>
<td>BCL2 binding component 3</td>
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<tr>
<td>PrtC</td>
<td><em>P. gingivalis</em> collagenase protein for P1542</td>
</tr>
<tr>
<td>PrtFAC</td>
<td><em>F. alocis</em> peptidase U32 protein (collagenase)</td>
</tr>
<tr>
<td>PPD</td>
<td>Probing pocket depth</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>Smac/ DIABLO</td>
<td>Second mitochondrial activator of caspases/Direct IAP binding protein with low PI</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor-associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
<tr>
<td>zVAD-FMK</td>
<td>Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
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**FILIFACTOR ALOCIS STRAINS AND MUTANTS USED IN THIS STUDY**

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<td>Wild-type 35896 ATCC strain</td>
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<td>FLL211</td>
<td><em>F. alocis</em> protease mutant</td>
</tr>
<tr>
<td>FLL212</td>
<td><em>F. alocis</em> collagen-adhesin mutant</td>
</tr>
<tr>
<td>pFLL215</td>
<td>Plasmid with <em>F. alocis</em> peptidase (HMPREF0389_00504) gene</td>
</tr>
<tr>
<td><em>P. gingivalis</em> W83</td>
<td><em>P. gingivalis</em> wild-type strain</td>
</tr>
<tr>
<td><em>P. gingivalis</em> 33277</td>
<td><em>P. gingivalis</em> wild-type ATCC strain</td>
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ABSTRACT OF THE DISSERTATION

The Role of Collagen Interacting Proteins in the Pathogenesis of Filifactor alocis.

By

Ozioma Salomey Chioma

Doctor of Philosophy, Graduate Program in Microbiology and Microbial Genetics
Loma Linda University California USA June 2016
Dr. Hansel M. Fletcher, Chairman

*Filifactor alocis* is a Gram-positive, asaccharolytic; obligate anaerobe that has emerged as a potential periodontal pathogen due to its presence in high numbers in patients who have periodontal disease and least detection in healthy individuals (Kumar *et al.*, 2003, Kumar *et al.*, 2006, Wade, 2011). There is compelling evidence in the literature that demonstrates that *F. alocis* has virulence properties that may play an important role in infection-induced periodontitis (Abusleme *et al.*, 2013, Griffen *et al.*, 2012, Hutter *et al.*, 2003, Khader *et al.*, 2006, Siqueira and Rocas, 2003, Siqueira *et al.*, 2009). However, there is a gap in our knowledge about *F. alocis* mediated host-pathogen interaction and virulence. There is therefore a critical need to study the specific virulence attributes that manifest its abundance only during infectious state as a gateway to proffering potential therapeutic interventions to prevent *F. alocis*-induced periodontal disease in the host. This introduction is geared towards providing a concise picture into all that is currently known about *F. alocis*, particularly the role of collagen related proteins of *F. alocis* in the pathogenicity periodontal disease. The goal is to investigate the specific role of *F. alocis* proteases in the virulence and pathogenicity of periodontal disease. The overall objective of this study is to evaluate the specific role of *F. alocis* collagen-degrading proteases in virulence modulation.
CHAPTER ONE

INTRODUCTION

Periodontal Disease

The periodontium is composed of gingiva and tissues surrounding the teeth (periodontal ligament, cementum and alveolar bone) (Fig 1.1) it impacts structural integrity to the tooth, and is often the site of chronic infections (Hassell, 1993). Periodontal disease is a chronic infectious inflammatory disease condition that affects the periodontium, which if left untreated could lead to destruction of alveolar bone, and tooth attachment loss (Kinane, 2000, Xu et al., 2002). This disease is highly prevalent amongst the adult population, and well over 64.7 million Americans have some form of periodontal disease (Eke et al., 2015, Oliver et al., 1991). The National Health and Nutrition Examination Survey (NHANES), conducted in 2012 concluded that 44.7% of adults ≥30 years old in the USA had periodontitis (Eke et al., 2015).

Furthermore, certain studies have shown an association between periodontal disease and several systemic diseases such as; diabetes (Casanova et al., 2014, Chavarry et al., 2009, Khader et al., 2006, Roeder and Dennison, 1998, Salvi et al., 2008), atherosclerosis (Bartova et al., 2014), stroke (Dennison, 1998b, Dorfer et al., 2004, Grau et al., 2004, Sfyroeras et al., 2012), pre-term low birth weight babies (Huck et al., 2011, Saini et al., 2010a, Zachariasen and Dennison, 1998), cardiovascular disease (Beck et al., 1996, Dennison, 1998a), bacterial endocarditis (Lieberman, 1992, Lockhart et al., 2009), rheumatoid arthritis (Bingham and Moni, 2013, Koziel et al., 2014), osteoporosis (Esfahanian et al., 2012), and respiratory diseases (Saini et al., 2010c, Zeng et al., 2012).
Therefore, treatment therapies for periodontal disease could prevent other systemic diseases.

The two main forms of periodontal disease are gingivitis and periodontitis (Highfield, 2009). Gingivitis is inflammation of the gum characterized by redness, swelling, bleeding and halitosis (bad breathe). However, regular brushing, flossing and routine cleaning to remove plaque and calculus by a dental hygienist can reverse gingivitis (Kamath and Umesh Nayak, 2014, Kugel and Boghosian, 2002, Sambunjak et al., 2011). If gingivitis is left untreated, it can progress to periodontitis. Periodontitis is inflammation of the tissues around the tooth, and is characterized by gradual separation of the gum from the teeth and sulcus leading to the formation of pockets, which could get infected (Pihlstrom et al., 2005). Periodontitis is characterized by loose teeth, redness, swelling and pocket depths >3mm. Left untreated, periodontitis can progress and eventually lead to loss of the affected tooth (Oliver et al., 1998).
Figure 1.1. The Periodontium. Figure reproduced from http://www.slideshare.net/marisolvirola/periodontium-11831194.
Periodontal disease can be classified into two major categories based on loss of supporting tissues surrounding the affected tooth namely; chronic periodontitis and aggressive periodontitis (Armitage, 1999). Chronic periodontitis, also called adult periodontitis, is the most frequently occurring form of periodontitis (Armitage, 1999), and is characterized by chronic inflammation, gingival recession, formation of periodontal pockets and loosening of the tooth. In contrast, aggressive periodontitis is not as commonly occurring as chronic periodontitis, and is characterized by rapid attachment loss and bone destruction, and occurs in otherwise healthy individuals.

Periodontal disease can be further characterized based on the severity of the disease (slight, moderate, or advanced), and the extent of bone loss (localized or generalized). The different stages of periodontal disease are highlighted in Figure 1.2.
Figure 1.2. Stages of Periodontal Disease. A= Healthy, B= Gingivitis, C= Mild Periodontitis, D= Moderate Periodontitis, E= Severe Periodontitis. [Link](http://www.newtondentalassociates.com/news/recent-news/boston-area-dentist-discusses-diabetes-and-oral-health). Healthy gums with no inflammation do not bleed, and they have a gingival sulcus probing depth of approximately 2 mm. Generally, in the case of gingivitis, there is buildup of plaque. Bacteria present in the plaque release toxic by-products that elicit an immune response from the host. As a result, inflammation of the gums occurs, characterized by redness and swelling, easy bleeding upon probing and a probing depth of approximately 3 mm, which also leads to halitosis. Mild periodontitis is characterized by the gradual detachment of the gums from the tooth (attachment loss), and deeper pockets. Moderate periodontitis, involves more bone and tissue destruction, and formation of calculus. Finally severe periodontitis is characterized by extensive bone loss, tissue destruction, as well as significant damage to the periodontal ligament.
The role of bacteria in periodontal disease is well established (Graves et al., 2000). The oral cavity is home to over 700 bacterial species, among them, only 300 can be readily cultivated (Belstrom et al., 2014b, Preza et al., 2009). Early colonizers such as *Actinomyces spp.*, *Capnocytophaga spp.*, *Eikenella spp.*, *Haemophilus spp.*, *Prevotella spp.*, *Propionibacterium spp.*, and *Veillonella spp.*, can coaggregate with other early colonizers, and bind to complementary salivary receptors via adhesins, in the acquired pellicle coating the tooth surface (Demuth et al., 1996, Rogers et al., 2001, Takahashi et al., 2002), thereby establishing the early dental plaque. Late colonizers which include *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Treponema denticola* sequentially bind to previously bound bacteria, and form new surfaces for the next coaggregating bacteria (Kolenbrander et al., 2002). The major bacteria demonstrated to be key players in periodontal disease are the late colonizers, which are comprised of the Gram-negative “red complex” bacteria; *Tannerella forsythia*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, *P. gingivalis* and *T. denticola* (Novak, 2002). Recently, novel culture independent techniques such as 16S RNA sequencing have identified newer bacteria implicated in periodontal disease (Dewhirst et al., 2010, Gross et al., 2010, Paster et al., 2001). These findings have resulted in a paradigm shift in periodontal infection due to incidence of both Gram-positive and Gram-negative bacteria, suggesting the presence of a heterogeneous microbial population. Among the many bacteria identified in cases of periodontitis, *Filifactor alocis* has emerged as one of the bacteria redefining the pathogenic scenario of the disease (Kumar et al., 2003, Kumar et al., 2006). Although periodontal pathogens are capable of directly causing destruction of the periodontal tissues (Amano, 2007, Saito et al., 2008), by expressing virulence factors
which enable them to invade and replicate within gingival epithelial cells and underlying connective tissue, the tissue destruction that occurs during periodontal disease is as a result of both bacterial virulence factors, and other tissue-degradative substances produced by the host cell during invasion by periodontal pathogens (Bartold and Van Dyke, 2013). Invasion of host tissue by periodontal pathogens stimulates production of cytokines and prostaglandins that induce tissue loss (Graves et al., 2000). Cytokines such as IL-1, IL-6, and IL-8 are likely to be important in the destructive process by stimulating bone resorption and degradation of the extracellular matrix (ECM) (Birkedal-Hansen, 1993). Therefore, the severity of periodontal disease is a result of the combined effect of the host immune response, as well as the action of the red complex bacteria.

**Risk Factors for Periodontal Disease**

Invasion of the host periodontal tissue by periodontal pathogens, and host response to invasion are not solely responsible for periodontal disease. Age (Eke et al., 2015, Grossi et al., 1995), genetic factors (Kinane and Hart, 2003, Michalowicz, 1994, Michalowicz et al., 2000), osteoporosis (Geurs et al., 2003, Payne et al., 1999), hormones (Juluri et al., 2015, Koreeda et al., 2005, Wu et al., 2015), disease (diabetes) (Emrich et al., 1991, Kinane and Marshall, 2001), medications (Bharti and Bansal, 2013, Hasan and Ciancio, 2004, Saini et al., 2010b), and lifestyle choices (substance abuse, diet, stress, and smoking) (Bergstrom et al., 1991, Pistorius et al., 2002, Souza et al., 2009) may also predispose an individual to periodontal disease.
Gender and Age

It has been extensively documented that men of all ages, races, ethnic groups and geographical locations have a significantly higher rate of periodontal disease compared to women (Eke et al., 2015, Grossi et al., 1995). A recently published follow-up analysis of the NHANES 2009–2012 data collected by the gold standard, full mouth periodontal probing at six sites per tooth, showed that within the socio-demographic groups studied, severe periodontitis was higher among males, and more prevalent with increasing age (≥50 years).

Genetics and Hormones

Genetic factors may play a role in occurrence of periodontal disease. Studies on identical twins show evidence of a substantial genetic basis for risk of adult periodontitis. Adult periodontitis (AP) was estimated to have approximately 50% heritability (Kinane and Hart, 2003, Michalowicz, 1994, Michalowicz et al., 2000). Female hormonal changes during ovulation, pregnancy and menopause, can adversely affect periodontal health. Hormone related gingivitis may arise in menstruating women during ovulation, when progesterone levels are high, causing blood vessel dilation and inflammation (Koreeda et al., 2005). During menopause, estrogen levels drop, and bone mineral density decreases which can lead to bone loss and osteoporosis (1989, Aspalli et al., 2014, Juluri et al., 2015). Hormonal changes in pregnancy cause aggravation of existing gingivitis, however this condition usually gets reverted post-delivery, with adequate attention from a dental hygienist (Wu et al., 2015). Gingivitis can also be as a side effect of birth control pills (Saini et al., 2010b). All these hormonal changes can lead to dry mouth resulting in
changes in the oral microbiota, which facilitate growth of pathogenic bacteria, thereby increasing the risks of getting some form of periodontal disease.

**Medications**

Saliva has a protective effect on the mouth; therefore drugs such as oral contraceptives, anti-depressants, and certain heart medicines, that limit the flow of saliva and promote dry mouth (xerostomia), can leave the mouth vulnerable to accumulation of plaque causing bacteria, and ultimately some form of periodontal disease (Saini et al., 2010b). Furthermore, medications that cause abnormal growth of the gum tissue (i.e. epilepsy anticonvulsant medication-phenytoin and antihypertensive calcium antagonists medication-nifedipine) can result in drug-induced overgrowth of gingival tissue, trap plaque and make the gum difficult to clean (Bharti and Bansal, 2013). This could result in accumulation of bacterial plaque and trigger inflammation, leading to gingivitis. If left untreated, this can progress to periodontitis. Also, patients taking amphetamines have an increased risk of gingival enlargement and abnormal growth of gum tissue which can promote periodontal disease (Hasan and Ciancio, 2004). Therefore side effects of some medication can adversely affect oral health.

**Lifestyle Choices**

Lifestyle choices such as smoking, substance abuse, diet and excessive alcohol consumption play a contributory role to the incidence of periodontal disease. Smoking is considered an important behavioral risk factor in poor periodontal health (Gautam et al., 2011, Mullally, 2004). It encourages chronic inflammation, bone
resorption, and bacterial growth by antibody suppression (Matthews et al., 2011).

Various well-documented epidemiological and clinical studies have shown that smoking can increase periodontal pocket depth, and lead to alveolar bone loss (Bergstrom et al., 1991). Smoking cessation is associated with improved periodontal health in heavy smokers (Albandar et al., 2000, Stoltenberg et al., 1993).

Stress can also adversely affect periodontal health. There is evidence of a link between psychological stress and progression of periodontal disease as evidenced by attachment loss and alveolar bone loss (Hugoson et al., 2002, Pistorius et al., 2002). This could be due to the release of inflammatory hormones such as corticotropin-releasing hormone (CRH) released under stress conditions. CRH stimulates the release of pro-inflammatory molecules such as neuropeptides and cytokines, which may contribute to periodontal pathology (Papathanasiou et al., 2013). The correlation between stress and periodontal disease could also be due to the secretion of pro-inflammatory cytokines i.e. IL-6 released during stress conditions which could exacerbate periodontal destruction (Giannopoulou et al., 2003), or as a result of decline in oral hygiene during stressful situations/conditions (Croucher et al., 1997).

Long-term abuse of alcohol and some illegal drugs (i.e. amphetamines) can damage gums and teeth. Studies show that regular alcohol users required more periodontal treatment due to a positive correlation between frequency of alcohol consumption and periodontitis (Novacek et al., 1995, Rehm et al., 2010, Souza et al., 2009). Alcohol can also aggravate existing cases of periodontal disease (Lages et al., 2015, Shepherd, 2011) and is also associated with increase in clinical attachment loss in a dose dependent manner (Tezal et al., 2004). One study showed long-term alcohol abuse
increases periodontitis, and attachment loss through recession of gingival margin (von Mengden and Brodda, 1976). Others showed that alcohol consumption may moderately increase severity of periodontal disease independent of one’s dental hygiene status (Tezal et al., 2001). An explanation for this could be that alcohol impairs neutrophil, macrophage, and T-cell functions, thereby increasing the likelihood of infections and thus could be a risk factor for periodontal disease (Pitiphat et al., 2003).

**Diabetes**

Periodontitis has been considered a major complication of diabetes (Loe, 1993). Several studies have shown diabetes to be an important risk factor of periodontal disease (Chavarry et al., 2009, Khader et al., 2006, Salvi et al., 2008). However, the relationship between diabetes and periodontal disease is a two-way relationship (Dennison, 1998b, Kinane and Marshall, 2001, Nishimura et al., 2000). Diabetic patients are three times more likely to have periodontitis than non-diabetics (2000, Emrich et al., 1991), and glycemic control seems to play a major role in this (Papapanou, 1996, Taylor et al., 1996). In like manner, periodontitis is also a risk factor for diabetes. This pattern is observed in both type I, and type II diabetes (Lalla et al., 2007, Nelson et al., 1990, Taylor et al., 1998). A study on 350 diabetic children (6-18 years) with type I diabetes and 350 non-diabetic children showed attachment loss and increased gingival inflammation in the diabetic children compared to the non-diabetic control (Lalla et al., 2007). Some of the mechanisms postulated for this observation are a) impairment in neutrophil function in diabetics, which affects neutrophil phagocytosis and clearing of periodontal pathogens in periodontal pockets (Manouchehr-Pour et al., 1981, McMullen
et al., 1981, Mealey, 1999) and b) increase in the production of pro-inflammatory cytokines in diabetics (Nishimura et al., 2000). Therefore it is highly important that diabetics, as well as persons predisposed to diabetes engage in excellent oral care practices.

**Diagnosis and Treatment of Periodontal Disease**

A qualified periodontist is required to properly diagnose periodontal disease (Carranza, 1986, Choi, 2011). Most diagnosis is based on examination of the patient’s gum for signs of inflammation (bleeding, swelling, redness, and pain), the presence of receding gum line, halitosis, and looseness of teeth. Next, probing is done to identify presence of periodontal pockets, and an x-ray can also be used to ascertain the extent of bone loss (McLeod, 2000). Treatment options for periodontal disease depend on the severity of the disease, and are geared towards controlling the inflammation. This can be done by scaling and root planing to remove tartar above and below the gum line (Cobb, 2008), the use of medications such as prescription antimicrobial mouthwashes, antibiotic gels, and oral antibiotics to reduce pocket size and bacteria load (Garrett et al., 1999, Paquette et al., 2008), and surgical procedures such as flap surgery to remove tartar deposited in deep pockets (Nyman et al., 1982). In addition, bone and tissue grafts can be put in place to aid regeneration of lost tissue (Ramseier et al., 2012). To maintain good oral health, individuals must practice good oral hygienic practices (regular brushing and flossing of teeth), and as much as possible avoid the risk factors of periodontal disease (e.g. smoking). During periodontitis there is evidence of low serum/plasma micronutrient levels, as well as evidence of positive outcomes from nutritional interventions; therefore
a daily intake of antioxidants, vitamin D, and calcium could be beneficial for the prevention and treatment of periodontitis (Van der Velden et al., 2011).

**Systemic Diseases Associated with Periodontal Disease**

In recent times, research has shown an increasing relationship between periodontal disease and systemic diseases such as cardiovascular disease (Beck et al., 1996, Dennison, 1998a), pre-term low birth weight babies (Huck et al., 2011, Saini et al., 2010a, Zachariasen and Dennison, 1998), osteoporosis (Esfahanian et al., 2012), respiratory diseases (Saini et al., 2010c, Zeng et al., 2012), stroke (Dennison, 1998b, Dorfer et al., 2004, Grau et al., 2004, Sfyroeras et al., 2012), bacterial endocarditis (Lieberman, 1992, Lockhart et al., 2009), diabetes (Casanova et al., 2014, Chavarry et al., 2009, Khader et al., 2006, Roeder and Dennison, 1998, Salvi et al., 2008), Atherosclerosis (Bartova et al., 2014), rheumatoid arthritis (Bingham and Moni, 2013, Koziel et al., 2014) and Alzheimer’s disease (Martande et al., 2014, Shaik et al., 2014, Singhrao et al., 2015, Sparks Stein et al., 2012).

**Cardiovascular Disease (CVD)**

CVD is a term used to describe heart and blood vessel diseases (Sfyroeras et al., 2012). The etiology of CVD involves a process known as atherosclerosis. Atherosclerosis develops when there is plaque buildup in the arteries, making it impossible for normal blood flow. This could eventually lead to blood clots, causing a heart attack, and sometimes stroke due to blockage of blood vessels to the brain (Kebschull et al., 2010, Sfyroeras et al., 2012). Epidemiological, interventional and functional studies show a
significant association between periodontitis and CVD. Periodontal bacteria and their products could be involved in all stages of atherogenesis. Furthermore, in vitro, animal, and clinical studies showed that periodontal pathogens positively influence all major pro-atherogenic mechanisms and hence periodontal infection could play a contributory role in atherosclerotic pathogenesis. Periodontal disease has been suggested to be involved with cardiovascular (CV) risk factors (Desvarieux et al., 2013, Vedin et al., 2015). Some of the postulated mechanisms for this relationship include elevation of cytokines, and inflammatory biomarkers (Joshipura et al., 2004), while good oral hygiene practices can lead to reduction in inflammatory biomarkers and risk factors for CVD (Lam et al., 2011). Studies have also shown that the keystone periodontal pathogen *P. gingivalis* increases the risk of atherosclerosis by increasing accumulation of lipids in the aorta (Jain et al., 2003, Reyes et al., 2013).

**Preterm and Low-birth-weight (PLBW) Babies**

Preterm birth is defined as any delivery that occurs after 23 gestational weeks and less than 37 weeks (Goldenberg et al., 2000, Honest et al., 2009). Pregnant women display an increase in gingival bleeding on probing due to gingival swelling (Gursoy et al., 2008), and the severity of the periodontal disease increases if they had periodontal disease prior to pregnancy (Offenbacher et al., 2006). It is widely accepted that infection is one of the major causes of pre-mature labor and low-birth-weight (PLBW) babies, and that periodontal disease is an infection of the oral cavity (Saini et al., 2009). Research has shown that Gram-negative bacteria (such as periodontal pathogens) can target the fetus and potentially cause low birth weight (birth weight < 2,500g) by the release of
endotoxins, which stimulate the production of cytokines, and prostaglandins (IL-1β, IL-6, and TNF-α), which can stimulate labor (Jeffcoat et al., 2001). These proinflammatory mediators can also cross the placenta barrier and rupture the uterine membranes and result in pre-term delivery (Yeo et al., 2005).

**Osteoporosis**

Osteoporosis is defined as low bone mineral density (BMD) (Guiglia et al., 2013). The relationship between osteoporosis and periodontal disease has been discussed since the 1960’s (Groen et al., 1968). Osteoporosis and periodontal disease have similar signs of bone resorption, risk factors and etiological factors (Darcey et al., 2013). A recent study by Huang et al., 2016, which divided the population into different levels of oral hygiene maintenance, and the severity of periodontitis and osteoporosis, showed a positive correlation between osteoporosis and periodontal disease, and concluded that oral hygiene maintenance plays a significant role in the association between osteoporosis and periodontal disease (Huang et al., 2016). They found that sudden periodontitis in individuals with good oral hygiene practices could be a symptom of osteoporosis. Another study showed a positive correlation between low skeleton BMD, and alveolar bone loss in a large population of postmenopausal women (Mohammad et al., 1996). Others also showed that postmenopausal women with osteoporosis had an increased risk of periodontitis than women without osteoporosis (Geurs et al., 2003, Payne et al., 1999). While some studies suggest a positive correlation between osteoporosis and periodontitis (Lin et al., 2015, Nicopoulou-Karayianni et al., 2009, Sultan and Rao, 2011, Tezal et al., 2011).
2000), others have argued that there is no correlation between the two (Elders et al., 1992, Kribbs, 1990).

**Respiratory Diseases**

Researchers have found that there is an association between periodontal disease and respiratory diseases such as pneumonia (Scannapieco and Mylotte, 1996, Scannapieco et al., 1998), and that periodontitis a risk factor of respiratory diseases (Saini et al., 2010c). Some of the proposed mechanisms to explain the role of oral bacteria in respiratory disease could be by aspiration of oral pathogens (such as *P. gingivalis*) into the lung to cause infection, or cytokines originating from periodontal tissues may alter respiratory epithelium to promote infection by respiratory pathogens (Scannapieco, 1999).

**Filifactor alocis**

*Filifactor alocis* is a Gram-positive, asaccharolytic, obligate anaerobic rod microorganism that has the potential to be a key player in periodontal disease because it is present in high numbers in periodontitis and in low numbers in healthy individuals (Kumar et al., 2003, Kumar et al., 2006, Wade, 2011). *F. alocis* was first isolated in 1985, from the subgingival areas of gingivitis and periodontitis patients, and was classified as *F. nucleatum* (Cato et al., 1985); however, in 1999, it was reclassified as *F. alocis* based on the 16S rRNA sequencing (Jalava and Eerola, 1999). *F. alocis* has unique attributes that make it a successful periodontal pathogen. One such attribute is its resistance to oxidative stress (Aruni et al., 2011), which is an important mechanism for
overcoming the highly reactive oxygen species (ROS) environment in the periodontal pocket. It also has an abundance of proteases, and proteases have been documented to be important virulence factors in oral pathogens (Lamont and Jenkinson, 1998).

**Role of F. alocis in Periodontal Disease**

*F. alocis* is fast gaining importance as an organism of interest in periodontitis (Abusleme et al., 2013, Griffen et al., 2012, Hutter et al., 2003, Khader et al., 2006, Siqueira and Rocas, 2003, Siqueira et al., 2009) due to novel culture-independent molecular techniques (Dewhirst et al., 2010, Paster et al., 2001), which not only make detection of this fastidious organism much easier, but also provide evidence that links *F. alocis* to periodontitis.

Studies from our lab show that *F. alocis* is able to interact with other periodontal pathogens such as *P. gingivalis*, and possesses virulence properties that enable it to survive and persist in the periodontal pocket (Aruni et al., 2011). Others have shown a mutualistic relationship between *F. alocis* and both *A. actinomycetemcomitans*, and *S. parasanguinis*; - an association indicative of future bone loss in localized aggressive periodontitis (LAP) (Fine et al., 2013). This observation is consistent with the report that periodontal disease is of polymicrobial origin. So far *F. alocis* has been implicated in cases of localized aggressive periodontitis (Fine et al., 2013, Shaddox et al., 2012), refractory periodontitis (Colombo et al., 2012, Colombo et al., 2009), and chronic periodontitis (Belstrom et al., 2014a). A study employed to evaluate the subset of pathogens in subgingival biofilm samples from patients with generalized chronic periodontitis (GChP), generalized aggressive periodontitis (GAgP), and healthy patients
(PH) showed that *F. alocis* is found in significantly higher levels in patients with GChP and GAgP, than PH (Oliveira *et al.*, 2016). Studies showed that periodontitis is associated with a characteristic salivary bacterial profile, and *F. alocis* occurs more frequently and at higher levels in saliva samples from periodontitis patients, than in samples from the healthy control cohort (Belstrom *et al.*, 2014b). This observation is independent of an individual’s smoking status (Belstrom *et al.*, 2014a). Another study employing two-step redundancy analysis of saliva, supragingival plaque, and subgingival plaque samples from healthy and periodontitis patients, using pocket depth and attachment loss as diagnostic parameters revealed that *F. alocis* was significantly associated with periodontal disease (Chen *et al.*, 2015).

*F. alocis* has been identified as one of the microbiota that persists in high prevalence in patients with refractory periodontitis (Colombo *et al.*, 2012, Colombo *et al.*, 2009). This is consistent with earlier findings that *F. alocis* is found in higher numbers in patients with periodontitis and least detected in healthy patients (Kumar *et al.*, 2003). The link between *F. alocis* and periodontitis is strengthened by a comparison of subgingival bacterial communities from chronic periodontitis (CP) patients and healthy patients using 454 sequencing of 16S rRNA which identifies *F. alocis* as one of the organisms associated with cases of CP (Griffen *et al.*, 2012). Inflammation of the periodontal tissue is one of the key indicators of periodontitis (Wang *et al.*, 2013). *F. alocis* is able to illicit an inflammatory response in cases of periodontitis (Moffatt *et al.*, 2011, Wang *et al.*, 2013). An in vivo study using a mouse subcutaneous chamber model demonstrated that *F. alocis* was able to establish a pro-inflammatory, pro-apoptotic local infection, which was quickly resolved by the action of host neutrophil influx (Wang *et
In addition *F. alocis* can recruit neutrophils and spread systemically and colonize remote tissues such as lung, spleen, kidney and liver (Wang *et al.*, 2013). This observation is crucial to understanding the role of *F. alocis* in the etiology of periodontal disease (Bahekar *et al.*, 2007, Jared *et al.*, 2009, Negrato *et al.*, 2013, Scannapieco and Mylotte, 1996, Scannapieco *et al.*, 1998, von Wowern *et al.*, 1994). Taken together, the available evidence indicates that *F. alocis* may be a major player in periodontitis, and its involvement in periodontal disease needs to be thoroughly investigated.

Dental implants provide a surgical restorative tool for supporting dental prosthesis such as dentures, crowns, bridge and facial prosthesis. This method of dental treatment has recorded great success (Albrektsson *et al.*, 2012, Ferrigno *et al.*, 2002, Fiorellini *et al.*, 1998); however it has its complications (Renvert *et al.*, 2009). One of such complications is peri-implantitis; late onset inflammatory infection affecting the tissues surrounding an osseointegrated dental implant, which is often characterized by loss of bone support and probing pocket depth (PPD) ≥5 mm combined with bleeding on probing (BOP) or suppuration (Bragger *et al.*, 2005). *F. alocis* has also emerged as one of the species implicated with peri-implantitis (da Silva *et al.*, 2014, Tamura *et al.*, 2013). A study identified *F. alocis* as one of the most prominent bacteria present in cases of peri-implantitis by employing culture techniques and 16S rDNA to characterize the predominant bacterial flora associated with peri-implantitis (Tamura *et al.*, 2013). This was carried out by comparing patients with healthy implants against patients with peri-implantitis, and revealed that the surrounding environment in the sulcus of patients with peri-implantitis is well suited for the growth of both asaccharolytic anaerobic Gram-negative bacteria such as *Parascardovia denticolens, P. intermedia, F. nucleatum, P.*
gingivalis, Centipeda periodontii, and Parvimonas micra, and other asaccharolytic anaerobic Gram-positive rods (AAGPRs) such as Eubacterium nodatum, E. brachy, E. saphenum, F. alocis, and Slackia exigua (Tamura et al., 2013). This suggests that asaccharolytic anaerobic Gram-positive rods (AAGPRs) such as F. alocis may play a contributory role to the etiology of peri-implantitis, and the already known Gram-negative pathogens implicated in periodontal disease may not be the only players in peri-implantitis. Another comparison between microbial diversity in the biofilms associated with peri-implantitis and patients with healthy implants by Sanger sequencing also identified F. alocis as a member of the subgingival microbial population in diseased implants (da Silva et al., 2014). These studies indicate a link between F. alocis and peri-implantitis, suggesting that F. alocis may play a vital role in the etiology of peri-implantitis.

In recent times, there has been an expansion in the microbial profiles associated with the development of endodontic infections (Jacinto et al., 2007, Siqueira and Rocas, 2003). These microbial species implicated in cases of endodontic infections may vary significantly depending to the geographic location in which samples are taken (Rocas et al., 2006, Siqueira et al., 2005), and between symptomatic and asymptomatic endodontic infections (Sakamoto et al., 2006). F. alocis has been discovered in the canals of root-filled teeth with periapical lesions, and is associated with signs and symptoms of endodontic infections (pain, wet canals and swelling) (Gomes et al., 2006). It is also identified as one of the prevalent phylotypes present in cases of failed endodontic treatment alongside other species such as P. gingivalis, P. micra, S. moorei, D. invisus, E faecalis and F. nucleatum (Zhang et al., 2012). These results indicate a heterogenous
microbial diversity in failed endodontic root-filled teeth, in which *F. alocis* is present, and may play a contributory role. Studies examining the presence of endodontic pathogens in samples from necrotic root canal, and acute apical abscesses (AAA) by polymerase chain reaction (PCR) indicate the presence of anaerobic species including *F. alocis*, *Porphyromonas endotalis*, *Prevotella nigrescens*, and *T. forsythia* which exhibit low clustering behavior (Montagner *et al.*, 2012).

*F. alocis* has also been detected in cases of untreated root canal with necrotic pulp and AAA samples alongside other endodontic pathogens such as *P. endodontalis*, *P. nigrescens*, and *T. forsythia* (Gomes *et al.*, 2006, Siqueira and Rocas, 2003). In cases of failed endodontic treatments, microorganisms usually occur in all root-filled teeth associated with periradicular lesions, and *F. alocis* was detected in 48% of all the samples tested in cases of failed endodontic therapy (Siqueira and Rocas, 2004a). *F. alocis* was detected in 57.1% root canal samples from teeth showing asymptomatic periradicular lesions, 30% in samples taken from root canals associated with acute apical periodontitis, and 46% in samples from endodontic infections, suggesting that *F. alocis* is also involved in the etiology of different periradicular diseases (Siqueira and Rocas, 2003). In summary, the high occurrence of *F. alocis* in endodontic infections (Siqueira and Rocas, 2004a, Siqueira and Rocas, 2004b, Siqueira *et al.*, 2004), as well as in apical portions of the root canals (Siqueira *et al.*, 2004, Siqueira *et al.*, 2005), suggests that this fastidious anaerobic bacteria may play a role in the etiology/pathogenesis of periradicular diseases. Further studies need to be undertaken to identify the key role of as-yet-unculturable bacteria in persistent endodontic infections.
Virulence Attributes of F. alocis

Since F. alocis has been identified in the pathogenic scenario of periodontal disease (Kumar et al., 2003, Kumar et al., 2006), it must possess various attributes that make it a successful pathogen. The periodontal pocket is a stressful environment for pathogens because of the presence of ROS associated with chronic inflammation, changes in temperature, pH, and competition with other periodontal pathogens for nutrient availability. Therefore F. alocis must have an array of virulence factors to ensure its continued existence and survival in the harsh environment of the periodontal pocket.

Proteases

Proteases play an important role in virulence and pathogenesis especially in proteolysis (Frees et al., 2013, Ingmer and Brondsted, 2009). While cytosolic proteases are involved in degradation of bacterial virulence factors and providing tolerance to adverse environments membrane proteases are mainly involved in protein processing, protein turnover and quality control (Dalbey et al., 2012). The extracellular proteases are involved in host cell interactions such as degradation of host matrix components, as well as disruption of host cell signaling pathways to herald invasion of the host cell by the bacterium.

In Gram-positive bacteria, proteolysis plays a critical role in maturation of proteins as well post translation regulation of gene expression (Laskowska et al., 1996). Proteome analysis of F. alocis identified 15 unique proteases, several of which might have functions similar to proteins present in other red complex bacteria (Aruni et al., 2012) (see Table 1.1). It is noteworthy that comparison of a low passaged clinical strain
(D62-D) to the wild-type ATCC (35896) strain showed that proteases vary among *F. alocis* strains (Aruni *et al.*, 2012).
Table 1.1. Proteases of *F. alocis*

<table>
<thead>
<tr>
<th>No</th>
<th>Protease</th>
<th>Gene Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oligoendopeptidase F</td>
<td>Oligoendopeptidase F (HMPREF0389_00926)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oligoendopeptidase F (HMPREF0389_00527)</td>
</tr>
<tr>
<td>2</td>
<td>Carboxy-processing protease</td>
<td>Carboxy-processing protease (HMPREF0389_00522)</td>
</tr>
<tr>
<td>3</td>
<td>ATP-dependent Clp protease</td>
<td>ATP-dependent Clp protease ATP-binding subunit ClpX (HMPREF0389_01648)</td>
</tr>
<tr>
<td>4</td>
<td>Serine protease HtrA</td>
<td>Serine protease HtrA (HMPREF0389_01460)</td>
</tr>
<tr>
<td>5</td>
<td>O-sialoglycoprotein endopeptidase</td>
<td>O-sialoglycoprotein endopeptidase (HMPREF0389_01445)</td>
</tr>
<tr>
<td>6</td>
<td>Xaa pro dipeptidase</td>
<td>Xaa-Pro dipeptidase (HMPREF0389_01538)</td>
</tr>
<tr>
<td>7</td>
<td>Glycoprotease family protein</td>
<td>Glycoprotease family protein (HMPREF0389_01443)</td>
</tr>
<tr>
<td>8</td>
<td>Metalloprotease</td>
<td>Metalloprotease (HMPREF0389_00692)</td>
</tr>
<tr>
<td>9</td>
<td>Caax amino protease</td>
<td>CAAX amino protease (HMPREF0389_00677)</td>
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<td>10</td>
<td>Caax amino protease family protein</td>
<td>CAAX amino protease family protein (HMPREF0389_00590)</td>
</tr>
<tr>
<td>11</td>
<td>ATP-dependent zinc metalloprotease FtsH (neutral zinc metalloprotease family protein)</td>
<td>Neutral zinc metalloprotease family protein (HMPREF0389_01001) [Zn_peptidase_2; Putative neutral zinc metallopeptidase]</td>
</tr>
<tr>
<td>12</td>
<td>Zinc protease</td>
<td>Zinc protease (HMPREF0389_00298)</td>
</tr>
<tr>
<td>13</td>
<td>ATP-dependent protease La</td>
<td>ATP-dependent protease La (HMPREF0389_00279)</td>
</tr>
<tr>
<td>14</td>
<td>Protease</td>
<td>Protease (HMPREF0389_00122) [Collagenase domain]</td>
</tr>
<tr>
<td>15</td>
<td>RIP metalloprotease</td>
<td>RIP metalloprotease RseP (HMPREF0389_00112)</td>
</tr>
</tbody>
</table>

Data collated from published reference (Aruni et al., 2012)
**Biofilm Formation**

Biofilm formation is a process whereby microorganisms attach themselves to a surface (living or inanimate surface), secreting extracellular polymeric substances (EPS) as they grow, which results in a change in the phenotype of the biofilm-associated microorganisms (Donlan, 2001). This change may be reflected in growth rate and gene expression (Donlan, 2000). Bacteria in the oral cavity develop biofilm on the surface of the tooth (Huang et al., 2011, Novak, 2002), and the ability of *F. alocis* to form biofilms has been demonstrated in cases of both aggressive and chronic periodontitis (Schlafer et al., 2010). It is noteworthy that there is significant increase in biofilm formation, when *F. alocis* is co-cultured with *P. gingivalis* ATCC 33277 (Aruni et al., 2011). *F. alocis* has also been demonstrated to be part of the periodontal polymicrobial biofilm communities (Schlafer et al., 2010), and preferentially accumulate at sites rich in *F. nucleatum* (Wang et al., 2013). This is a crucial observation, as dental plaque biofilm is comprised of a complex assortment of microbial communities, and is a direct precursor of periodontal disease (Kuboniwa and Lamont, 2010).

Biofilms pose a serious public health concern because of the decrease in antimicrobial susceptibility due to the biofilm mode of growth (Ceri et al., 1999). This antimicrobial resistance could be as a result of a number of factors. In biofilm communities there is transfer of extra-chromosomal DNA between species, therefore antibiotic resistance can be transferred between species that are resistant to certain antimicrobials such as beta-lactams, erythromycin, and tetracycline to less resistant species (Tenover FC, 1998). Furthermore the slow growth rate of microorganisms in biofilms can minimize the rate of uptake of antimicrobials into the cell (Duguid et al.,
1992), and the EPS secreted by biofilms may also bind and react with antimicrobials to halt or attenuate its effect (Hoyle et al., 1992)

Oxidative Stress Resistance

The periodontal pocket is a highly inflammatory environment due to the action of the red complex bacteria that stimulate the host immune system to produce cytokines and polymorphonucleocytes (PMN) (Dahiya et al., 2013). As the part of the defense response to infection, PMNs produce ROS superoxide via the respiratory burst mechanism, which has deleterious effects on the cells (Masi et al., 2011).

Studies that compare P. gingivalis to F. alocis show that F. alocis is more resistant to oxidative stress than P. gingivalis, and its growth is stimulated under these conditions (Aruni et al., 2011). In other studies, there was a high level of F. alocis in smokers (Mason et al., 2015). This observation could be because the growth of F. alocis is stimulated by oxidative stress. There are various possible explanations for the oxidative stress resistance observed in F. alocis. The genome of F. alocis contains genes that encode proteins that could play a role in the oxidative stress resistance observed in F. alocis (http://www.ncbi.nlm.nih.gov/nuccore/NC_016630.1). These genes include; superoxide reductase, iron-sulfur cluster gene, iron permease, ruberythrin, ferrous hydrogenase, and thioredoxin, and they are upregulated when F. alocis is in co-culture with P. gingivalis (Aruni et al., 2012).

F. alocis also possesses a well-developed system that could facilitate efflux of reactive oxygen species, as well as a superoxide reductase (HMPREF0389_00796) that could aid its growth in the presence of hydrogen peroxide.
Macrophage and Neutrophil Evasion

Evasion of host immune response is a major mechanism of survival of any pathogen (Finlay and McFadden, 2006). In periodontitis, the formation of complex biofilm communities by oral pathogens proffers the advantage of evading the host defense mechanism, which include macrophages and neutrophils (Donlan, 2001). Using a mouse subcutaneous chamber model Wang et al., (2014), showed that F. alocis infection in mouse could elicit a proinflammatory, proapoptotic local infection (Wang et al., 2014). Other Studies show a dysfunction in neutrophil response after infection of a mouse chamber model with F. alocis even though there was a normal neutrophil recruitment due to the infection. Collectively, these observations show that F. alocis may have unique ways of evading neutrophils and macrophages (Wang et al., 2013).

Amino Acid Metabolism

As an asaccharolytic organism, F. alocis cannot metabolize carbohydrates as its source of energy; therefore it must rely on other carbon sources such as amino acids, as an important substrate for its growth. F. alocis has been shown to utilize cysteine, lysine, and arginine for nutrition (Uematsu et al., 2003), and its growth is stimulated in arginine-enriched media (Aruni et al., 2011). In the periodontal pocket, arginine is highly abundant from the breakdown of various protein substrates by host-derived and bacteria proteases (Dewhirst et al., 2010). The F. alocis genome shows the existence of a well-developed amino acid metabolism pathway, necessary for the catabolism of proteins and amino acids (http://www.ncbi.nlm.nih.gov/nuccore/NC_016630.1). The arginine deiminase pathway in F. alocis, is involved in metabolism of arginine to citrulline and
ornithine, without the intermediate step of citrulline production (Uematsu et al., 2003). Breakdown of arginine could be beneficial for nutritional support and survival of other oral pathogens. It could also favor an increase in pH of the periodontal pocket, to balance out the acidic conditions generated from breakdown of carbohydrate by the mixed flora in the oral cavity (Kanapka and Kleinberg, 1983). There is also evidence that the nutritional needs of F. alocis are being met during an infection, due to the abundance of proteins involved in arginine metabolism found in F. alocis. These include arginine deiminase (HMPREF0389_01584), acetyl ornithine transferase (HMPREF0389_01570) (Aruni et al., 2012), as well as the presence of arginine decarboxylase (HMPREF0389_00102), aminotransferases (HMPREF0389_01352) and (HMPREF0389_01353), present in the genome of F. alocis, all of which are needed to metabolize the high amount of arginine present in the periodontal pocket.

**Adhesion and Invasion Related Proteins**

In order to become a successful pathogen, pathogenic bacteria have the ability to adhere-to and invade the host cell as one of the first steps in pathogenesis. Bacterial colonization of host tissue is a critical step in the infection process, through binding specifically to the components of the host’s extracellular matrix (ECM) such as collagen. *F. alocis* possesses membrane adhesion proteins and putative Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs), that mediate microbial attachment to the host, by recognizing and binding to specific components of host ECM such as collagen, fibrinogen, fibronectin, laminin, elastin, heparin, and proteoglycans (Bosman and Stamenkovic, 2003). *F. alocis* has also been shown to
successfully adhere and invade HeLa epithelial cells, and this is enhanced in the presence of *P. gingivalis* (Aruni *et al.*, 2011). Some of these MSCRAMMs have been shown to be upregulated when *F. alocis* was in co-culture with *P. gingivalis* (Aruni *et al.*, 2012).

MSCRAMMs have various functions including evasion of host immune response (Foster *et al.*, 2014), biofilm formation (Hendrickx *et al.*, 2009) adhesion, and invasion of host cells and tissues (Foster and Hook, 1998, Patti *et al.*, 1994a). MSCRAMMs include cell wall anchor proteins such as adhesins, collagenases, and invasins (Patti and Hook, 1994). MSCRAMMs related to collagen substrates have been shown to be important virulence factors in pathogenic bacteria. These collagen-related MSCRAMMS facilitate entry and establishment of pathogenic bacteria in the host by binding and degrading the collagen-rich ECM of the host (Kang *et al.*, 2013). *P. gingivalis*, a keystone periodontal pathogen possesses a collagenase protein that is involved in plasma degradation (Holt *et al.*, 1999, Houle *et al.*, 2003, Lawson and Meyer, 1992). The ECM is a physiologically active tissue that provides structural support, as well as initiates biochemical processes such as cell adhesion (Schlie-Wolter *et al.*, 2013), cell division (Thery *et al.*, 2005), homeostasis (Tyagi, 2000), and cell proliferation (Senoo and Hata, 1994), therefore, a breach in this structure would be considered a mark of a successful pathogen.
<table>
<thead>
<tr>
<th>No</th>
<th>Putative MSCRAMM</th>
<th>Gene Annotation</th>
</tr>
</thead>
</table>
| 1) | Collagen-adhesin proteins | Collagen-adhesin protein (HMPREF0389_01006) [Collagen binding domain]  
Gram positive anchor (HMPREF0389_01336), [Collagen binding domain]  
Hypothetical protein (HMPREF0389_01750) [Collagen binding domain] |
| 2) | Collagenase | Protease (HMPREF0389_00122) [Collagenase domain]  
Peptidase, U32 family (HMPREF0389_00504) [Collagenase domain] |
| 3) | Heparin-binding protein | 50S ribosomal protein L29 (HMPREF0389_00839) [Ribosomal L29 HIP; Ribosomal L29 protein/HP* heparin/heparan sulfate interacting protein (HIP)] |
| 4) | Fibronectin-binding protein | Fibronectin-binding protein (HMPREF0389_00575) [Fibronectin binding domain] |

Data collated from published reference [117]
Collagen

Collagen is a major component of the extracellular matrix (Alberts et al.), making up at least 30% of all proteins in mammalian bodies (Ricard-Blum, 2011). It is an insoluble structural matrix protein, consisting of three helically wound polypeptide fibrils, which must be unwound prior to degradation by collagenases (Chung et al., 2004). Collagen is present in the skin, tissues, bones, oral cavity as well as other body structures. In the mouth, collagen is the major component of the gingival connective tissue. Collagen fibers connect cementum and alveolar bone (Naito and Gibbons, 1988, Nanci and Bosshardt, 2006). Collagen is found in abundance in dental tissues; dentine (type 1 collagen), pulp (type 1 and III collagen), cementum (predominantly type I collagen), basement membrane (type IV collagens) (Scully et al., 2005). Collagen is responsible for regulating cell adhesion, tissue development and tensile strength, and is also involved in chemotaxis in vertebrates. (Rozario and DeSimone, 2010). Collagen fibers found in the ECM provide structure from the outside, while collagen fibrils provide the major biomechanical platform for cell attachment and anchorage of macromolecules, to define the structure and form of tissues (Kadler et al., 1996). Interstitial collagens are right-handed triple helices comprised of α-chains of repeating Gly-Xaa-Yaa triplets. When the molecules in the triplet positions are proline and hydroxylproline, the stability of the triplet helix is increased. The stability is also strengthened and stabilized by the close packing of the triplet helix chains by hydrogen bonding (Ramshaw et al., 1998). Based on assembly of the triple helices, collagens can be classified into fibrillar and non-fibrillar collagen.
Fibrillar collagen makes up about 80-90% of collagen in mammals. The triple-helix structure is 3000Å in length and 15Å in diameter. Types I, II, and III are the fibril forming collagen, and the basic unit of the fibrillar collagen is the triple helix, they are characterized by repeating Gly-Xaa-Yaa triplets to form a right-handed helix (Lodish and Darnell, 2000). Collagen type I is the most abundant fibrillar collagen, making up about 95% of the total collagen in animal tissue (Miller and Gay, 1987).

Non-Fibrillar collagens on the other hand are made up of collagen types IV and VI. They have areas of spontaneous non-helical repeats in addition to the Gly-Xaa-Yaa repeating triplets resulting in non-fibrillar structure (Lodish and Darnell, 2000).

Collagen fibrils are formed by the assembly of the three left-handed helices of collagen molecules in a right-handed triple helix (Brodsky and Persikov, 2005, Charvolin and Sadoc, 2012).

Collagenases are enzymes (endopeptidases) that can digest collagen fibers in their insoluble triple-helical form into peptides (Shingleton et al., 1996). Collagenases are first synthesized as a pre-proenzyme, and secreted as an inactive proenzyme. This proenzyme consists of a propeptide, a catalytic domain, a short proline-rich linker region, and a C-terminal hemopexin (Hpx) domain (Clark and Cawston, 1989). Hung et al., 2014, showed that Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis (Chung et al., 2004), and this observation has been supported by studies from others (Han et al., 2010).

**Role of Bacterial Collagen-Interacting Proteins in Pathogenicity of Disease**

The ECM of eukaryotic cells is made up of several molecules such as fibers,
proteoglycans and polysaccharides that provide structural and biological support to the surrounding cells. Of these molecules, collagen is the most abundant component of the ECM (Ricard-Blum, 2011). Therefore pathogenic bacteria must secrete proteases that can interact with host collagen, (by binding and degrading it), to facilitate colonization of the host tissue.

Various bacterial species have been shown to produce collagen-interacting proteins, which contribute to their pathogenicity (Abranches et al., 2011, Aviles-Reyes et al., 2014, Elasri et al., 2002, Harrington, 1996, Miller et al., 2015, Patti et al., 1994b, Rhem et al., 2000, Rich et al., 1999, Xu et al., 2004). Excessive collagen degradation by collagenases and other matrix metalloproteinase (MMPs), and disruption of normal ECM metabolism occurs in the pathology of periodontal disease (Lorencini et al., 2009). This is usually accompanied with inflammation, a significant reduction in collagen fiber density in the gingival tissues (Golub et al., 1997), as well as loss of alveolar bone (Grossi et al., 1995). The collagen degradation leads to the breakdown of the cytoskeleton, to provide substrate for survival, attachment and colonization by other anaerobic bacteria, and leads to the formation of periodontal pockets.

**Collagen Binding/Adhesin Proteins**

Adhesion to host cells is the first step in colonization by pathogenic bacteria, and is a crucial step in the infectious process. The extracellular matrix (ECM) in most cases is the site of bacterial adhesion, and the ability to adhere to components of ECM i.e. collagens, laminin, fibronectin or proteoglycans, is essential for the virulence of several pathogenic bacteria. Therefore pathogenic bacteria must express surface proteins with

In oral bacteria collagen-binding proteins and adhesins also play an important role in colonization (Han \textit{et al.}, 2008, Miller \textit{et al.}, 2015, Moses \textit{et al.}, 2013). \textit{P. gingivalis}, a keystone periodontal pathogen, produces a collagenase protein, PrtC which plays a role in tissue destruction and progression of periodontitis (Kato \textit{et al.}, 1992), therefore, bacterial collagenases can likely play a role in periodontitis pathogenesis (Adamowicz \textit{et al.}, 2014). Bacteria secrete various proteins such as collagen adhesin/binding proteins, and collagenases that promote bacterial adhesion, and ensure the successful colonization of the host cell. One such family of proteins, the MSCRAMMs interact with components of the host extracellular matrices. In Gram-positive bacteria, the amino-terminal (N-terminal) portion of MSCRAMMs contains the ligand-binding domain in the so called A region, while the carboxy-terminal (C-terminal) half, contains repeated motifs often known as the B domains that appear to extend and project the ligand-binding domain away from the bacterial surface (Foster, 1998). The C-terminus also contains the cell wall sorting motifs, including the LPxTG sequence, which is essential for sortase-dependent anchoring to the cell wall, followed by a stretch of hydrophobic residues and a short segment of positively charged amino acids (An \textit{et al.}, 2000). The cna protein of \textit{Staphylococcus aureus} (Xu \textit{et al.}, 2013), and ace collagen-binding MSCRAMM from \textit{Enterococcus faecalis} (Rich \textit{et al.}, 1999), are prime examples of MSCRAMMS whose virulence potentials have been fully documented. Other studies have also shown that collagen-adhesins play critical roles in bacterial colonization (Leo \textit{et al.}, 2010, Ross \textit{et al.}, 2012).
Peptidase U32 Family of Bacterial Collagenases

The peptidase U32 family is recognized as a group of microbial collagen-degrading enzymes with little or no known information about their catalytic site, and fold of the protein, hence the designation “U”-for unknown catalytic mechanism (Rawlings et al., 2012). They are putative collagenases and contribute to the pathogenicity of pathogenic bacteria (Kato et al., 1992, Kavermann et al., 2003, Xiong et al., 2011). So far, P. gingivalis (Kato et al., 1992) Helicobacter pylori (Kavermann et al., 2003) Salmonella Typhimurium (Wu et al., 2002) Proteus mirabilis (Zhao et al., 1999) and Aeromonas veronii (Han et al., 2008) are examples of pathogenic bacteria whose U32 peptidases have been documented. U32 peptidase classification is based on the presence of a consensus sequence E-x-F-x(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S. The first crystal structure of the peptidase domain in the U32 family protein of Methanopyrus kandleri was described recently (Schacherl et al., 2015). In bacteria, peptidase U32 proteins function as collagenases, and the type member of this family is P. gingivalis, which produces a prtC protein that contributes to its virulence. PrtC is able to degrade soluble and reconstituted type 1 collagen, heat denatured type I collagen, and azocoll (Kato et al., 1992). In order to be a successful pathogen, bacteria must overcome the structural barrier of the host’s ECM that is primarily made up of collagen. Bacteria produce collagenases, which break down collagen. Little is known about bacterial collagenases.

The most well understood bacterial collagenase is the collagenase from Clostridium histolyticum (Bond and Van Wart, 1984a, Bond and Van Wart, 1984b, Bond and Van Wart, 1984c). C. histolyticum secretes two types of collagenases; ColG and
ColH which not only causes extensive tissue destruction in myonecrosis (Matsushita et al., 1998) but is also able to cleave native collagen and denatured collagen (gelatin). Thus bacterial collagenases are recognized as virulence factors. Numerous human bacterial pathogens have been reported to possess collagenases as virulence factors which can hydrolyze collagen and gelatin (denatured collagen) (Eke et al., 2015, Han et al., 2008, Harrington, 1996, Kato et al., 1992, Kavermann et al., 2003, Matsushita et al., 1998, Xiong et al., 2011). Bacterial collagenases belong to the MEROPS peptidase family M9 (INTERPRO: IPR002169; PFAM: PF0172) and assigned to the EC 3.4.24.3 by The Nomenclature Committee of The international Union of Biochemistry and Molecular Biology (Duarte et al., 2016). The MEROPS peptidase family M9 is subdivided into subfamilies M9A and M9B, based on amino acid sequence and catalytic function. M9A subfamily includes subfamily types M09.001 Bacterial collagenase V (Vibrio algainolyticus), M09.004 VMC peptidase (V. mimicus), and UP (subfamily M9A unassigned peptidases). M9B on the other hand includes subfamily types M09.002 Collagenase G/A- class I collagenases (C. histolyticum), M09.003002 Collagenase H - class II collagenases (C. histolyticum), and UP (subfamily M9B unassigned peptidases). The difference between M9A and M9B is at the site of collagen cleavage. In M9A (Vibrio bacteria) collagen cleavage occurs at Xaa+Gly, while in the M9B (Clostridial collagenases) collagen cleavage occurs between the Yaa+Gly bonds. The most well understood bacterial collagenase is the collagenase from C. histolyticum (Bond and Van Wart, 1984a, Bond and Van Wart, 1984b, Bond and Van Wart, 1984c). Clostridial collagenases are multi-domain proteins, which have a signal peptide, a putative pro domain, a collagenase unit (formerly known as the catalytic segment 1 (S1) and up to two
linking domains such as polycystic kidney disease-like (PKD) formerly known as the collagen recruitment segment (S2) domain and up to three collagen-binding domains (CBD) formerly known as segment 3 (S3) (Matsushita and Okabe, 2001). On the basis of homology comparison of each segment, *Clostridia* collagenases were first grouped into two classes. While Class I collagenases (subtypes α, β, and γ) are highly active against collagen and moderately active against FALGPA (a synthetic peptide that cannot be hydrolyzed by proteinases such as elastase or trypsin, but used for kinetic studies of collagenases). Class II collagenases (subtypes δ, ε, and ζ) are highly active towards synthetic peptides but moderately active to collagen (Bond and Van Wart, 1984a, Bond and Van Wart, 1984b, Bond and Van Wart, 1984c). Studies show that *C. histolyticum* collagenases ColG and ColH encode class I and II collagenases respectively (Matsushita *et al.*, 1999, Matsushita *et al.*, 1998, Matsushita *et al.*, 2001), and have different sequences and domain organization (Matsushita *et al.*, 1999). Other bacterial collagenases such as collagenases from *C. tetani* have also been implicated in tissue necrosis, and may be involved in its pathogenesis due to its ability to destroy specific component of the ECM (Bruggemann *et al.*, 2003). Thus bacterial collagenases are recognized as virulence factors. Bacterial collagenases contribute to virulence and pathogenesis of disease by:

i. evasion of the host immune system and degradation of ECM components to facilitate initial entry of bacteria into the host by degrading specific components of the hosts ECM, to facilitate successful colonization (Han *et al.*, 2008).

ii. activation of host proteases and inactivation of host protease inhibitors overcoming host defenses (Maeda and Yamamoto, 1996).
iii. release of nutrient from breakdown of the host ECM components such as collagen to provide nutrition for bacteria,

iv. lowering of redox potential via impairing blood supply and buildup of toxic waste metabolites by tissue destructing proteases (Harrington, 1996), and

v. establishment of the pathogen and entry into anaerobic sites to accelerate the disease process.

**Collagen Related Proteases of *F. alocis***

Proteolytic degradation of extracellular matrix (ECM) components by bacterial cells is an important step in pathogenicity as cells attach, and migrate through the ECM.

*F. alocis* possesses some putative collagen-related MSCRAMMs in its genome (Table 1.2), that could interact with the collagen-rich ECM of the host to facilitate colonization and pathogenesis of periodontal disease. They include the collagen-binding proteins and collagenases listed below.

1) Collagen-adhesin protein (HMPREF0389_01006) [collagen binding domains]
2) Gram-positive anchor protein (HMPREF0389_01336) [collagen binding domains]
3) Hypothetical protein (HMPREF0389_01750) [collagen binding domains]
4) Protease (HMPREF0389_00122) [Collagenase domain], and
5) Peptidase, U32 family (HMPREF0389_00504) [Collagenase domain]

The above proteins are modulated when *F. alocis* is cultured with epithelial cells, and this may play a role in colonization to host tissue (Aruni *et al*., 2012). We hypothesize
that *F. alocis* collagen-related proteins play a role in adherence and invasion of host cell, and can modulate virulence through multiple mechanisms.

**F. alocis: Host-Pathogen Interaction**

Host–pathogen interactions are the interactions that take place between a pathogen (e.g. virus, bacteria) and their host. Pathogen interaction with the host cell could either be harmful to the host, or could cause no harm to the host (Alberts *et al.*, 1999). These interactions could be between the host’s proteins and bacterial proteins, referred to as interactomes (Simonis *et al.*, 2012, Uetz *et al.*, 2006).

Numerous studies have shown the ability of *F. alocis* to impact host cells, allowing it to persist in the periodontal pocket. Virulence is one of a number of possible outcomes of host-microbe interaction and refers to the capacity of a microbe to cause disease (Casadevall and Pirofski, 1999). Since *F. alocis* has mainly been identified in the pathogenic scenario of periodontal disease, it is safe to say that most of the *F. alocis*-host interactions are those that may foster periodontal disease progression. The virulence of any microorganism including *F. alocis* is mainly involved with its ability to invade and colonize the host, acquire nutrients, and evade the host’s immune defenses (Ribet and Cossart, 2015, Wilson *et al.*, 2002). Studies from our lab have shown that *F. alocis* possesses an array of putative virulence factors that could aid its involvement in the pathogenicity of periodontal disease (Aruni *et al.*, 2011). These virulence factors include proteins that would enable it establish itself within the cells of the host, and increase its potential to cause disease.
Adhesion of bacteria to the host tissues is the initial step in disease progression, and the adhesion/attachment of *F. alocis* to eukaryotic cells in the oral cavity requires the participation of adhesin proteins that could foster the attachment to host cells, and even lead to the disruption of host cell membranes. *F. alocis* has been documented to possess certain MSCRAMMs (Table 1.2) that recognize specific components on the host cells, such as collagen, laminin, fibronectin, and heparin. Studies from our lab showed the upregulation of a putative collagenase gene, protease (HMPREF0389_00122), a collagen adhesion (HMPREF0389_01006), as well as a hypothetical protein, with collagen binding domain (HMPREF0389_001750) when *F. alocis* is co-cultured with epithelial cells (Aruni *et al.*, 2012).

*F. alocis* infection can modulate host and induce the secretion of proinflammatory cytokines IL-1β, IL-6, and TNF-α (Moffatt *et al.*, 2011), and using a subcutaneous mouse infection model, *F. alocis* infection not only led to an increase in proinflammatory cytokine levels, but also to a rapid influx of neutrophils (Wang *et al.*, 2014). Proteome analysis of *F. alocis* in co-infection with *P. gingivalis* and epithelial cells showed the modulation of proteins involved in several host signaling pathways such as cell-cell interaction, and apoptosis (Aruni *et al.*, 2014a). Among these proteins, cell wall and cell membrane proteins of *F. alocis* have been shown to play a significant role. Other forms of bacterial interactions with host cells may involve death of the host cell (Ashida *et al.*, 2011). Bacteria are sometimes able to completely hijack the cell death machinery of the host cell, when they interact with the host, and can result in the demise of the cell by various cell death pathways such as apoptosis, necrosis, and autophagy.
Cell death by apoptosis has been 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 neutrophil (Wang et al., 2014). Apoptosis is a highly regulated mechanism of cell death, and is a regulatory process to maintain a balance between cell proliferation and cell death. The two main signaling cascades that lead to apoptosis are the extrinsic pathway and the intrinsic/mitochondrial pathway (Krammer, 2000, Suen et al., 2008), and activation of caspases is one of the hallmarks of apoptosis (Chang and Yang, 2000). Many bacteria including *F. alocis* been shown to mediate apoptosis (Moffatt et al., 2011) thereby leading to general tissue destruction. Also, collagenases have been shown to cause apoptosis in bacteria (Lo and Kim, 2004). As a result of the high number of *F. alocis* during periodontal infection, the exact role and mechanisms of *F. alocis* mediated apoptosis and involvement of specific proteins in this process need to be explored.

**Apoptosis**

Cell death by apoptosis has been 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). The two main types of cell death in living organisms are necrosis and apoptosis (Bright and Khar,
Both can be induced by the same pathophysiological stimuli, however the mechanism of cell death differs.

Necrosis is cell death as a result of factors external to the cell, that cause morphological changes such as cell swelling, condensed swollen and ruptured mitochondria, disrupted organelle membranes, and rupture of plasma membrane, which usually leads to release of the cytoplasmic contents into the surrounding tissue, inflammation, and pre-mature death of cell by autolysis (Narula et al., 1997, Trump et al., 1997).

Apoptosis, also known as programmed cell death (PCD), is a mechanism cell death which is essential for homeostasis and development (Renehan et al., 2001). Apoptosis comes from a Greek word, which means falling of leaves from a tree, and denotes the natural removal of dead cells without damage to the entire organism (Fink and Cookson, 2005). Apoptosis is activated by cysteine proteases called caspases (McIlwain et al., 2013), and is characterized by distinct energy-dependent biochemical mechanisms and morphological changes, such as cell shrinkage, chromatin condensation, and containment of the damaged cells in apoptotic bodies to prevent inflammation (Norbury and Hickson, 2001). The morphological changes can be identified by light and electron microscopy (Hacker, 2000). Apoptosis occurs normally during development to maintain a homeostatic balance between cell death and cell division in tissues, and as a defense mechanism when cells are damaged as a result of disease (Norbury and Hickson, 2001). In this study we have explored the role of *F. alocis* collagen interacting protein in modulating host cell death.
The mode of cell death, whether necrotic or apoptotic, depend on the nature of the cell death signal, the tissue type, and physiological milieu (Fiers et al., 1999). It is noteworthy that although the mechanisms and morphologies of apoptosis and necrosis differ, there is an overlap between these two processes. For instance an apoptotic event can be converted to necrosis by a decrease in availability of caspases, and intracellular ATP (Denecker et al., 2001, Leist et al., 1997). Table 1.3 highlights the differences between apoptosis and necrosis, while Fig 1.3 shows the main pathways involved in apoptosis.
Table 1.3: Differences between apoptosis and necrosis.

<table>
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<tr>
<th>No.</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Physiologically programmed cell death</td>
<td>Caused by cell damage or trauma</td>
</tr>
<tr>
<td>2.</td>
<td>No leakage of cell content, little or no</td>
<td>Significant inflammatory response</td>
</tr>
<tr>
<td></td>
<td>inflammation</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Cell shrinkage but intact organelles</td>
<td>Cell swelling</td>
</tr>
<tr>
<td>4.</td>
<td>Membrane blebbing but integrity maintained</td>
<td>Loss of membrane integrity</td>
</tr>
<tr>
<td>5.</td>
<td>Chromatin condensation (pyknosis), Nuclear</td>
<td>DNA degradation increase</td>
</tr>
<tr>
<td></td>
<td>fragmentation (karyorrhexis) and DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fragmentation</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Tightly regulated</td>
<td>Poorly regulated</td>
</tr>
<tr>
<td>7.</td>
<td>Increased mitochondrial membrane permeability,</td>
<td>Rupture of cell and loss of plasma membrane integrity</td>
</tr>
<tr>
<td></td>
<td>release of pro-apoptotic proteins and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>formation of apoptotic bodies</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Packages in apoptotic bodies ingested by</td>
<td>Lysed cells ingested by macrophages</td>
</tr>
<tr>
<td></td>
<td>macrophages</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Affects individual cells</td>
<td>Affects many cells at once</td>
</tr>
<tr>
<td>10.</td>
<td>Energy required</td>
<td>Requires no energy (passive)</td>
</tr>
</tbody>
</table>

Table collated from information from published references (Elmore, 2007, Kroemer, 1998).
Extrinsic Apoptotic Signaling Pathway

Cell-surface death receptors belonging the tumor necrosis factor (TNF) receptor gene superfamily activate the extrinsic apoptotic pathway (Locksley et al., 2001). Extracellular signal proteins trigger this pathway, by binding to cell surface death receptors. Members of this family possess a cysteine-rich extracellular ligand binding domain, a transmembrane domain, as well as an intracellular cytoplasmic death domain, which transmit cell death signals from the cell surface into the cell (Ashkenazi and Dixit, 1998).

A well understood model of how death receptors trigger the extrinsic apoptotic pathway is the FasL/FasR model (Wajant, 2002). This involves binding of homotrimeric ligand to their corresponding homotrimeric receptor, and the subsequent recruitment of cytoplasmic adaptor proteins, which exhibit death domains which bind to the receptors (i.e. activation of Fas ligand on the surface of a cell and binding to Fas receptor results in the activation of the adapter protein FADD which recruits initiator pro-caspase 8 to form a death inducing signaling complex (DISC) (Hsu et al., 1995, Wajant, 2002). Caspase-8 is activated by autocatalytic activation of pro-caspase 8 (Kischkel et al., 1995). The activation of caspase-8 then triggers the activation of execution caspases; caspase-3, 6, 7, which cleave various substrates and ultimately cause the morphological and biochemical changes observed in apoptotic cell death (Slee et al., 2001).

The extrinsic apoptotic pathway is regulated c-FLIP protein, which binds to FADD and caspase-8 to deactivate them (Scaffidi et al., 1999). Similarly another extrinsic pathway inhibitor protein Toso, can inhibit Fas-induced apoptosis in T cells by blocking caspase-8 processing (Hitoshi et al., 1998).
Figure 1.3: Schematic representations of apoptotic pathways (Elmore, 2007).
Intrinsic Apoptotic Signaling Pathway

Cell can activate the apoptotic pathway from within due to non-receptor-mediated stimuli in response to injury, or other stressors such as DNA damage, free radicals, and deprivation of growth factor and cytokines, which trigger the intrinsic apoptotic signaling pathway, to produce signals that can cause deleterious changes within the cell. These signals lead to the release of pro-apoptotic proteins that usually reside in the mitochondria, into the cytosol, and involves the activation of other pro-apoptotic proteins such as Bcl-2-associated protein X (Bax). BAX undergoes a conformational change, which enables its integration into the outer membrane of the mitochondria, resulting in mitochondrial membrane permeabilization (MOMP) (Liu et al., 2003). The intrinsic pathway of apoptosis depends on the mitochondria. Other pro-apoptotic proteins such as cytochrome C, endonuclease G, and apoptosis inducing factor (AIF) are released into the cytosol (Schimmer, 2004, van Loo et al., 2002). Once in the cytosol, cytochrome C a water-soluble component of the electron transport chain binds to pro-caspase activating factor (Apaf1), leading to the formation of an “apoptosome”, other proteins in the apoptosome recruit the initiator caspase 9, and activate it. The activated caspase 9 can then activate downstream executioner caspases, leading to cell death by apoptosis. The intrinsic pathway is regulated by Bcl-2 family proteins (Cory and Adams, 2002). Bcl-2 family is also regulated by P53 tumor suppressor protein (Schuler and Green, 2001).

Perforin/Granzyme Signaling Pathway

T-cell mediated cytotoxicity is able to exert their cytotoxic effects on tumor cells and virus-infected cells through secretion of the transmembrane pore-forming molecule...
perforin with a subsequent release of cytoplasmic granules Granzyme A and B through
the pore and into the target cell (Trapani and Smyth, 2002).

Perforin, Granzyme A and B are serine proteases that are released by cytotoxic T cells
(CTL), and natural killer (NK) cells. Granzyme A and B enter cells by pores made by
perforin, and induce the activation of Bid which enable MOMP and cytochrome C release
(Mathiasen and Jaattela, 2002). Granzyme A mediates caspase-independent pathway by
cleaving lamins A, B, and C, degrading histones, disrupting nuclear lamina, and allowing
DNases access to the chromatin (Zhang et al., 2001b). Granzyme A could function as a
backup system if Granzyme B is inhibited (Turk et al., 2002).

Granzyme B can mediate the intrinsic apoptotic pathway by cleaving Bid, or can directly
activate caspases, and will cleave proteins at aspartate residues, and can activate
procaspase-10 (Sakahira et al., 2015). However in the presence of caspase inhibitors
granzyme B can also induce necrotic cell death (Mathiasen and Jaattela, 2002). Bel-2 can
completely block the granzyme pathway (Zhang et al., 2001a)

Anoikis

Anoikis has been described in numerous cell types, including epithelial cells,
endothelial cells, keratinocytes, thyroid cells, fibroblasts, and osteoblasts (Aoudjit and
Vuori, 2001, Bretland et al., 2001, Gniadecki et al., 1998, Jensen et al., 2011, Sakai et
al., 2000). In this study we show that the collagenase in F. alocis can induce apoptosis
possibly through an anoikis mechanism (Chioma et al., 2016).

Anoikis is defined a form of cell death that is induced by inadequate or
inappropriate cell–matrix interactions, that results in loss of integrin signaling (Frisch and
Francis, 1994, Zhan et al., 2004). It is activated either through the extrinsic or the intrinsic apoptotic pathway, and results in cell death (Gilmore, 2005). In anoikis, the extrinsic pathway is activated through ligation of extracellular death ligands such as FasL to their membrane receptors i.e. Fas receptors. This ligation can trigger the direct proteolysis of targets dependent on caspase-7 activation, or activate the intrinsic pathway. Upregulation of pro-apototic molecules (such as Bim, Bad, Bik, Puma, Noxa) induce the intrinsic pathway by inactivating the Bcl-2 anti-apoptotic proteins. The Pro-apoptotic protein Bim mainly initiates activation of anoikis through the intrinsic apoptosis pathway, although a role has been proposed also for Bid (Valentijn and Gilmore, 2004). These proteins promote the release of mitochondrial cytochrome c and assembly of the apoptosome, which promoting anoikis by inducing the breakdown of caspase-specific targets (Taddei et al., 2012). Figure 1.4 provides a summary of cell death by anoikis.
Figure 1.4: Activation of the Anoikis pathway (Taddei et al., 2012).
Aims

In order to colonize host tissue, *F. alocis* must circumvent the host’s structural barrier such as the collagen-rich ECM. *F. alocis* possesses collagen-related proteins especially MSCRAMMs, that play an important role in bacterial colonization of host tissue; a critical step in the infection process, through binding specifically to the components of the host’s extracellular matrix (ECM) especially collagen (Patti and Hook, 1994). Collagen degradation by collagenases and other matrix metalloproteinase (MMPs) is an early indicator of periodontal disease (Patti *et al.*, 1994b). This degradation leads to the breakdown of the cytoskeleton, which provides substrate for survival, attachment and colonization by microorganisms.

In this study, we examined the role of three collagen-related proteins protease (HMPREF0389_00122), peptidase U32 family protein (HMPREF0389_00504) and collagen-adhesin protein (HMPREF0389_01006), in pathogen virulence and host modulation.

The aim of this study was to

1) investigate the role of *F. alocis* collagen-related proteins in pathogen adherence, and invasion, and virulence, and

2) investigate the role of collagen-related proteins of *F. alocis* in host protein interactions and apoptosis.

The hypothesis to be tested is that *F. alocis* collagen-related proteins i.e. collagenases and collagen-binding proteins, play a vital role in virulence, colonization and host interaction by adhering-to and degrading host collagen.
References


Bruggemann, H., Baumer, S., Fricke, W. F., et al. (2003) The genome sequence of


65


Lin, T. H., Lung, C. C., Su, H. P., et al. (2015) Association Between Periodontal Disease and Osteoporosis by Gender A Nationwide Population-Based Cohort Study. Medicine, 94.


CHAPTER TWO

_Filifactor alocis_ COLLAGENASE CAN MODULATE APOPTOSIS OF NORMAL ORAL KERATINOCYTES

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Running Title: _Filifactor alocis_ Peptidase U32 protein.

Keywords: Periodontal disease, Collagen, Collagenase, proteases, Virulence, Normal Oral Keratinocytes, Apoptosis.

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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 of 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, including *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*. 
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 (Lorencini 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 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 proteins (HMPREF0389_00122, and 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.

**Materials and 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](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/](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 2.1. The *F. alocis* 35896 ATCC wild-type 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 × 10³ 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 (10⁵ 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 2.2), 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.D₆₀₀~0.6), 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 Na₂HPO₄, 1.8 mM KH₂PO₄ 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 lyzed with 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 diayzed 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 µl of 10 µg/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 µg, 4 µg, and 16 µg/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$_{450\text{nm}}$. 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 CaCl$_2$ 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 ($10^5$ 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 Cycler 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 (ΔΔCt) 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 x 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 μM) 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 μl of Biolegend Annexin V binding buffer then stained with 0.5 μl of pacific blue™ Annexin V and 1 μl of 7-AAD for 15 min at room temperature in the dark. Cells were washed with 200 μl of Annexin-V binding buffer then resuspended in 1% paraformaldehyde. TRAIL protein (20 μM) was used as a positive control, while Z-VAD-FMK (20 μM), 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.
Table 2.1. Bacterial Strains and Plasmids Used In This Study

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Description</th>
<th>Source</th>
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<tbody>
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<td><em>Filifactor alocis</em> ATCC 35896</td>
<td>Wild-type strain</td>
<td>Aruni <em>et al.</em>, (2011)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> Top 10</td>
<td>Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Life technologies</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 StarTM (DE3)</td>
<td>Genotype: F- ompT hsdSB (rB-mB-) gal dcm me131 (DE3)</td>
<td>Life technologies</td>
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<tr>
<td><em>pFLL215</em></td>
<td>PrtFAC (HMPREF0389_00504) in Top 10 <em>E.coli</em></td>
<td>This Study</td>
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Table 2.2. Primers Used In This Study

<table>
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<td>P1</td>
<td>FA 504 PET-Forward</td>
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<tr>
<td>P2</td>
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<tr>
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<td>FaddF</td>
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<td>Real-time PCR</td>
</tr>
<tr>
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<td>FaddR</td>
<td>5’-CAGCATGGAGAAGAGGTCTAGG-3’</td>
<td>Real-time PCR</td>
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<td>Casp8F</td>
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<td>P16</td>
<td>BidR</td>
<td>5’-TCTGGCTAAGCTCCTCAGTA-3’</td>
<td>Real-time PCR</td>
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<td>P17</td>
<td>XiapF</td>
<td>5’-TACGAATGGGGTTCAAGTTTCAAG-3’</td>
<td>Real-time PCR</td>
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<tr>
<td>P18</td>
<td>XiapR</td>
<td>5’-GTCTGACTTGAAGACTCTTGTGCAT-3’</td>
<td>Real-time PCR</td>
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<tr>
<td>P19</td>
<td>Casp3F</td>
<td>5’-CAGTTTTTGTGTTGTGGCTTCTGAG-3’</td>
<td>Real-time PCR</td>
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<tr>
<td>P20</td>
<td>Casp3R</td>
<td>5’-AACGATCCCCTCTGAAAAAGTT-3’</td>
<td>Real-time PCR</td>
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<td>P21</td>
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<td>5’-AACGATTCCACTGTCCTGTC-3’</td>
<td>Real-time PCR</td>
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<tr>
<td>P22</td>
<td>28S- R</td>
<td>5’-TTTACGATCTCCAAGAGTTG-3’</td>
<td>Real-time PCR</td>
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</tbody>
</table>
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 2.1). *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 2.2) 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 2.3). 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-(2)-G-[SA]-[LIVM]-C-x(4)-G-x-C-x-[LIVM]-S (Figure 2.3). The *F. alocis* Peptidase U32 family protein (HMPREF0389_00504) designated PrtFAC was further characterized.
Figure 2.1. Phylogenetic analysis of Peptidase U32 collagenases among bacterial pathogens

- *Clostridium beijerinckii* 0.44689
- *Escherichia coli* (YhbV protein) 0.42863
- *Porphyromonas gingivalis* proteinase C (PrtC) 0.3714
- *Salmonella typhimurium* (clg protein) 0.24975
- *Aeromonas veronii* (acg protein) 0.24124
- *Bacillus subtilis* (yrrN protein) 0.0.36332
- *Helicobacter pylori* (hp0169) 0.32048
- *Filifactor alocis* (PrtFAC) 0.28156

0.5
Figure 2.2. Domain architecture of peptidase U32 family protein (HMPREF0389_00504) showing the PrtC-like domain (amino acid 1-354), peptidase domain (amino acid 1-403), and peptidase U32 family signature motif.
Figure 2.3. In silico protein modeling of peptidase U32 family protein (HMPREF0389_00504), highlighting the carbon and nitrogen terminals, trans-membrane segments (highlighted with arrow), and tetrad of cysteine and arginine residues that compose the potential catalytic interacting site.
Figure 2.4. (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 (B) Western Blot confirmation of PrtFAC showing immunoreactive bands to anti His-tag antibody.
**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 2.3), 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 2.4) to interact with collagen, we use a solid-phase collagen binding ELISA assay. As shown in Fig 2.5, the PrtFAC recombinant protein adhered strongly to the collagen-coated plates in a concentration dependent manner. As a control, BSA did not bind.
Figure 2.5. PrtFAC interaction with type I collagen substrate. Increasing concentrations of PrtFAC (1, 4, 16 μg/well) showed increased binding to collagen substrate, unlike the BSA negative control. Experiment was carried out in three repeats. Error bars represent the standard deviation from the mean.
PrtFAC Exhibits Ca$^{2+}$-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 (Fig 2.6). This activity was Ca$^{2+}$ dependent. Decreased enzyme activity was observed in the presence of EDTA and 1, 10 phenanthroline, and there was no enzyme activity observed when the PrtFAC protein was denatured by boiling at 100 °C for 15 min (Fig 2.6A). PrtFAC also had gelatinolytic activity, however this activity was not Ca$^{2+}$ dependent (Figure 2.6B).
Figure 2.6. PrtFAC collagenase and gelatinase assay. (A) 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. (b) 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
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 (Fig 2.7 and 2.8). As shown in the arrows in Fig 2.7, 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 (Fig 2.9). 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 (Fig 2.10). 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 (Fig 2.10). Because the purified PrtFAC protein was determined to contain approximately 0.001μg/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 μM of the denatured PrtFAC protein (data not shown).
Figure 2.7. PrtFAC induces Apoptosis in Normal Oral Keratinocytes. NOKs were grown to semi confluence in a 96-well plate (1.5 X10^4 cells per well), and treated with PrtFAC in 10, 50 and 100 μM concentrations for 24 h. 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 μM) and ZVAD-FMK (20 μM) 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 2.8. PrtFAC induces Apoptosis in Normal Oral Keratinocytes. NOKs were grown to semi confluence in a 96-well plate (1.5 X10⁴ cells per well), and treated with PrtFAC in 10, 50 and 100 μM concentrations for 48 h. 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 μM) and ZVAD-FMK (20 μM) 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 2.9. PrtFAC induces increase in early and late apoptotic populations. Stimulation of Normal Oral Keratinocytes (NOK) with PrtFAC Protein showed an increase in apoptotic populations.
Figure 2.10. Decrease in viable NOK post stimulation with PrtFAC. Stimulation of Normal Oral Keratinocytes (NOK) with PrtFAC Protein showed dose-dependent significant decrease in cell viability. Comparisons were made by paired t-test; n = 6. *** P <0.001
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 (Fig 2.11). Additionally we detected significant elevated levels of caspase 3/7 activity 48 h post treatment of NOK cells with PrtFAC (Fig 2.12). Caspase 8 was not detected in the NOK cells treated with PrtFAC (Fig 2.11).
Figure 2.11. Expression of pro-apoptotic and caspase genes in NOKs treated with PrtFAC. Stimulation of Normal Oral Keratinocytes (NOK) with PrtFAC Protein resulted in upregulation of pro-apoptotic genes and caspases.
Figure 2.12. Increase in caspase 3/7 activity in NOKs treated with PrtFAC. Stimulation of Normal Oral Keratinocytes (NOK) with PrtFAC Protein resulted in increase in caspase 3/7 activity compared to the untreated. Comparisons were made by paired t-test; n = 6. *** P <0.001.
**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 (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 contract 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 and 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 and 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 and 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 and Gilmore, 2004) was observed to be up-modulated in the PrtFAC treated NOK cells. Because Bid can regulate apoptosis via the intrinsic pathway (Valentijn and 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 and 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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References


crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell, 
4: 563-71.

assessment of apoptotic and proliferative gingival keratinocytes in oral and 
sulcular epithelium in patients with gingivitis and periodontitis. J Clin 

of gap junction transfer sensitizes thyroid cancer cells to anoikis. Endocr Relat 

Kato, T., Takahashi, N. & Kuramitsu, H. K. (1992) Sequence analysis and 
characterization of the Porphyromonas gingivalis prtC gene, which expresses a 

Kavermann, H., Burns, B. P., Angermuller, K., et al. (2003) Identification and 
characterization of Helicobacter pylori genes essential for gastric colonization. J 


are associated with bacterial community shifts as assessed by quantitative 16S 

ingivalis strains in a defined basal medium. Anaerobe, 2: 257-61.


CHAPTER THREE

THE ROLE OF *Filifactor alocis* COLLAGEN RELATED MSCRAMMS IN

VIRULENCE AND PATHOGENESIS

Running Title: *Filifactor alocis* collagen related MSCRAMMs.

Keywords: Periodontal disease, Collagen, Collagenase, MSCRAMMs, Adhesins, Virulence, Pathogenesis.
Summary

The ability of pathogenic bacteria to adhere to and invade the hosts collagen-rich extracellular matrix (ECM) facilitates bacterial colonization, and is an initial step in the successful establishment of bacteria in the host. Filifactor alocis a Gram-positive, asaccharolytic, obligate anaerobic bacteria, now considered to play a key role in periodontitis, has been shown to be able to invade host tissue, however, there is a gap in our understanding of the bacterial proteins that play a role in facilitating adherence and invasion of host tissue. The genome of F. alocis contains several putative microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) capable of interacting with host collagen. F. alocis possesses a protease (HMPREF0389_00122) gene, and a collagen-adhesin gene (HMPREF0389_01006), that may play a significant role in colonization of host tissue during periodontitis by binding and degrading host collagen. Domain architecture of the protease gene (HMPREF0389_00122) revealed the presence of a putative collagenase domain, as well as peptidase domains, while the collagen-adhesin (HMPREF0389_01006) gene possesses a characteristic peptidase M1 domain, and two collagen-binding domains. Allelic exchange mutagenesis was employed to generate isogenic mutants deficient in protease (HMPREF0389_00122) and collagen-adhesin (HMPREF0389_01006) genes. These mutants were designated FLL211 and FLL212 respectively. FLL211 exhibited a significant increase in autoaggregation, while FLL212 mutant exhibited a significant reduction in growth rate, decrease in adherence and invasion of epithelial cells, as well as an upregulation of other collagen-related proteins when in co-culture with normal oral keratinocytes. Taken together, these findings suggest that collagen related proteins of F. alocis play a role in virulence.
**Introduction**

Periodontal disease is a polymicrobial infection of the dental pulp that if left untreated can culminate in loss of alveolar bone and the infected tooth. The key players responsible are the red complex bacteria: *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* (Novak, 2002). However, evidence from novel culture-independent molecular techniques have identified *Filifactor alocis*, a Gram-positive, asaccharolytic, obligate anaerobe, as an important marker organism in periodontal disease, owing to its presence in high numbers in periodontitis patients, and least detection in periodontitis free individuals (Kumar *et al.*, 2003, Kumar *et al.*, 2006, Oliveira *et al.*, 2016).

Studies from our lab have shown that *F. alocis* possesses an assortment of potential virulence attributes which are necessary for host invasion, survival, persistence and pathogenesis (Aruni *et al.*, 2011, Aruni *et al.*, 2012, Aruni *et al.*, 2014a). To successfully colonize host cells, pathogenic bacteria must be able to adhere, and invade tissues of the host. Therefore, bacteria must target proteins to its cell surface for interactions with the host tissue. Many pathogenic Gram-positive bacteria make use of cell wall anchor proteins to adhere and invade the host cell by attaching to certain components of the hosts extracellular matrix (ECM) such as collagen. Our earlier studies have shown that *F. alocis* possesses many cell wall anchor proteins that could interact with the ECM (Aruni *et al.*, 2012) Bacterial proteins that bind to specific components of the hosts ECM are called microbial surface component-recognizing adhesion matrix molecules (MSCRAMM’s). Cell wall anchor proteins, and membrane proteins in bacteria, such as collagen-adhesin proteins play a unique role in these processes. Cell
wall anchor proteins have certain characteristic features; N-terminal signal peptide and a C-terminal 30-40 amino acid sorting signal which is composed of a conserved LPxTG motif, followed by the hydrophobic and positively charged tail (Navarre and Schneewind, 1999). These proteins must transverse the thick layer of the bacterial wall in order to be situated on the surface of the cell to display its functional units to the surrounding environment. *F. alocis* possesses several putative cell wall anchored MSCRAMMs, and membrane proteins, some of which are upregulated when *F. alocis* is in co-culture with epithelial cells that can interact with the collagen-rich ECM of the host (Aruni et al., 2014a).

In this paper we focus on two genes in *F. alocis* that encode proteins capable of interacting with host collagen, in order to investigate their involvement in colonization of periodontal tissue during infection. We hypothesize that the protease (HMPREF0389_00122), and collagen-adhesin protein (HMPREF0389_01006) of *F. alocis*; play a role in adhesion, invasion, and colonization of host tissue. This study is focused on evaluating the role of the two genes in pathogenesis of *F. alocis* in order to gain a clearer understanding of the virulence factors that make *F. alocis* an important player in periodontal disease.

**Materials and Methods**

**Bioinformatics Analysis**

The National Center for Biotechnology Information (NCBI) conserved domain database ([http://www.ncbi.nlm.nih.gov/nuccore/NC_016630.1](http://www.ncbi.nlm.nih.gov/nuccore/NC_016630.1)) was used to retrieve amino acid sequences, and information on the conserved domains present in *F. alocis*
collagen interacting proteins: protease (HMPREF0389_00122), and collagen-adhesin protein (HMPREF0389_01006). Amino acid sequence alignment was done using Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The phylogenetic relationships between other oral pathogens and *F. alocis* was analyzed using MEGA software version 4.0 (Tamura *et al.*, 2007). Kimura 2-parameter model was used to calculate phylogenetic distance, and clustering was analyzed using the neighbor-joining method with bootstrap values based on 1,000 replicates (Saitou and Nei, 1987). ClustalW version 2.0 (http://www.ebi.ac.uk/) was used to analyze the amino acid sequences, while protein subcellular localization was predicted using the PSORT and iPSORT programs (Nakai and Kanehisa, 1991).

**Bacterial Strains, Plasmids and Culture Conditions**

All strains and plasmids used in this study are all listed in Table 1. The *F. alocis* ATCC 35896 wild-type 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). BHI blood agar medium was prepared by adding 5% of sheep blood (Hemostat Laboratories, Dixon CA), and 2% agar. *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. Growth rates were determined spectrophotometrically by measuring optical density at 600 nm [OD₆₀₀] every three hours. Antibiotics were used at the following concentrations: Erythromycin 3 μg/ml and tetracycline 10 μg/ml.
Epithelial Cell Culture Conditions

Normal Oral Keratinocytes (NOKs) were grown in keratinocyte serum-free medium (Keratinocyte-SFM; Life Technologies, Inc.) supplemented with epidermal growth factor and bovine pituitary extract as previously described (Piboonniyom et al., 2003). Briefly, NOK cells were grown in a 37°C incubator at 5% CO₂. Confluent monocultures were trypsinized, adjusted to 5 x 10³ cells/ml, then seeded (1ml per well) into a 12-well plate (3.5cm² area) (Nunc, Rochester, NY), and incubated to semi confluence for 48 h (10⁵ cells per well)

Construction of FLL211 and FLL212 Mutants

Long PCR-based fusion of several fragments was done as described previously (Shevchuk et al., 2004). All Primers used for this study are listed in Table 2. Amino acid sequence from the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/CP002390.1), was used to design specific oligonucleotide primers to replace the open reading frame (ORF) of the protease (HMPREF0389_00122) and the collagen-adhesin (HMPREF0389_01006) genes with the tetQ and the ermF antibiotic cassette respectively. Briefly 500 kilobase flanking fragments both upstream and downstream of the target genes were PCR amplified from F. alocis 35896 ATCC chromosomal DNA of with antibiotic cassettes with oligonucleotide primers that contained overlapping nucleotides for the upstream and downstream fragments. The ermF cassette was amplified from the pVA2198 (Vanterpool et al., 2005) plasmid, while the tetQ plasmid was amplified from the pT-COW plasmid (Gardner et al., 1996). These three fragments were fused by PCR using the forward primer of the upstream fragment and the reverse primer of the
downstream fragment. The PCR-fused fragment was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and electroporated into F. alocis 35896 ATCC wild-type strain as previously described (Abaibou et al., 2001). The cells were plated on BHI agar containing erythromycin 3 μg/ml or tetracycline 10 μg/ml, and incubated at 37 °C for 5-10 days in the anaerobic chamber. Colony PCR, and DNA sequencing were used to confirm the correct gene replacements. The resulting mutants showing the deletion of F. alocis collagen related genes; protease (HMPREF0389_00122), and collagen-adhesin protein (HMPREF0389_01006) were designated FLL211 (ΔFA_00122::tetQ) and FLL212 (ΔFA_01006::ermF) respectively.

**RNA Isolation and Real Time Quantitative PCR Analysis**

RNA was extracted from the F. alocis wild-type 35896 ATCC strain co-cultured with HeLa epithelial cells, and FLL211 or FLL212 strains co-cultured with HeLa epithelial cells, or F. alocis wild-type 35896 ATCC, FLL211 or FLL212 strains 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 transcriptor high fidelity cDNA synthesis kit (Roche, Indianapolis, IN) 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 Cycler Real Time PCR equipment detected the real-time fluorescence signal. Each reading was performed in triplicate for each gene. 16s ribosomal RNA gene (HMPREF0389_RS01660) and 28SrRNA(RNA28S1) were used as controls to normalize variations due to differences in reverse transcription The relative expression ratio of the genes selected and critical threshold cycle (ΔΔCt) were calculated (Pfaffl et al., 2002).

**Static Biofilm Formation Assays**

We compared the biofilm forming ability of the mutants FLL211 and FLL212 to the *F. alocis* ATCC 35896 wild-type strain, using a method previously described (Hinsa and O'Toole, 2006). Briefly, actively growing cultures of all the strains were grown overnight then diluted with sterile BHI media to an optical density of 0.02 at [600 nm]. Next, 200 μl of the BHI-bacteria mix, was transferred into a sterile polystyrene 96-well flat-bottomed microplate, and incubated anaerobically at 37°C for 24 h. Post-incubation, the supernatant was discarded; and the attached cells were washed with 1X phosphate-buffered saline (PBS), and stained for 5 min with 100 μl of 0.1% crystal violet. The crystal violet was washed off with double distilled water (ddH₂O), and de-stained with 100 μl of 95% ethanol for 5 min. The crystal violet and bacteria mix was transferred into another sterile 96-well polyvinyl microplate, and the optical density was read at 570 nm using a spectrophotometer. Wells with media only served as the negative control. Biofilm formation was proportional to the absorbance of the crystal violet.

**Autoaggregation Assay**

Autoaggregation assay was performed as previously described (Houle et al.,
2003). Briefly *FLL211*, *FLL212* and *F. alocis* 35896 ATCC wild-type strains in the log phase of 0.02 [O.D$_{600nm}$] were collected by centrifugation, washed once with 1X PBS, and then re-suspended in 1X PBS to 0.4 [O.D$_{600nm}$]. The decrease in OD$_{600nm}$ of each suspension over a three hour period at 37°C under anaerobic conditions was used to monitor autoaggregation (decrease in absorbance as the cells clumped).

**Adherence and Invasion Assay**

Adherence and invasion of NOK cells by *F. alocis* 35896 ATCC wild-type and *FLL211 FLL212* mutant strains were quantified using the standard antibiotic protection assay previously described (Yilmaz et al., 2003). Briefly, a single bacteria colony of each strain was isolated from a BHI agar plate and grown to exponential phase in BHI broth. Next the bacterial cells were centrifuged, washed twice with 1X PBS, and adjusted to $10^7$ CFU/ml. The NOK cell monolayer was washed twice with PBS, and infected with bacteria at a multiplicity of infection (MOI) of 1:100 ($10^5$ epithelial cells), and then incubated anaerobically at 37°C for 45 and 90 min. Supernatant was recovered and adherent cells were washed in 1X PBS to remove non-adherent cells. *F. alocis* is sensitive to 100 μg/ml of metronidazole, therefore this concentration of metronidazole was used to kill bacteria bound to surface of NOK cells. Sterile distilled water was used to release internalized bacteria by osmotic lysis, and internalized bacteria was serially diluted, plated (in triplicate) on BHI agar, and incubated for 7 days anaerobically. The number of adherent bacteria was obtained by subtracting the number of intracellular bacteria from the total bacteria obtained in the absence of 100 μg/ml metronidazole (Aruni et al., 2012).
**Collagenase Assay**

The collagenase activity of *F. alocis* 35896 wild-type and *FLL211* and *FLL212* were compared with *P. gingivalis* strains W83 and 33277 ATCC strains as previously described (Takahashi *et al.*, 1991). Briefly all the strains were grown to log phase, and the EnzChek® Gelatinase/Collagenase Assay Kit (Molecular Probes Inc. Eugene OR), was used to access the collagenase activity as per the manufacturer’s protocol. All strains were incubated with DQ-gelatin or DQ-Collagen I (Molecular Probes Inc. Eugene OR), respectively in the dark at 25°C for 24 h. Active enzymes cleaved the fluorescent-labeled gelatin or collagen and the change in fluorescence was measured (Excitation/Emission 485/528 nm). Collagenase from *C. histolyticum* (0.25 U/ml) was used as the control and the negative control was without the bacterial strains.
Table 3.1. Bacterial Strains Used In This Study

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. alocis</em> ATCC 35896</td>
<td>Wild-type strain</td>
<td>(Aruni <em>et al.</em>, 2011)</td>
</tr>
<tr>
<td><em>FLL211</em></td>
<td>Protease mutant (HMPREF0389_00122)</td>
<td>This Study</td>
</tr>
<tr>
<td><em>FLL212</em></td>
<td>Collagen-adhesin mutant(HMPREF0389_01006)</td>
<td>This Study</td>
</tr>
<tr>
<td>Primer</td>
<td>Description</td>
<td>Nucleotide Sequence</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>---------------------</td>
</tr>
</tbody>
</table>
| P1    | Tet_Q_F     | TGA CTA ACT AGG AGG AAT AAA  
TGA ATA TTA TAA ATT TAG GAA   | Gene inactivation |
| P2    | Tet_Q_R     | GAT TAT TCC CTC CAG GTA TTA TTT  
TGA TGA CAT TGA TTT TGG A    | Gene inactivation |
| P3    | Erm_F_F     | ATG ACA AAA AAG AAA TTG CCC  
GTT CGT TTT ACG GGT CAG CAC TT | Gene inactivation |
| P4    | Erm_F_R     | GAT TAT TCC CTC CAG GTA CTA  
CGA AGG ATG AAA TTT TTC A    | Gene inactivation |
| P5    | Upstream FA1006 F | TTA AAT CAC AAA GAA GTA CAA  
GAA A                      | FA 1006 inactivation |
| P6    | Upstream FA1006 R | CGG GCA ATT TCT TTT TTG TCA  
TTTG CAT CTC TCC CTT ATC CAT TTAT      | FA 1006 inactivation |
| P7    | Downstream FA1006 F | TCG TAG TAC CTG GAG GGA ATA  
ATCCAA AAT ACT TTA TGA AAG  
ATA ATT A                  | FA 1006 inactivation |
| P8    | Downstream FA1006 R | AAT TTC TAT TTC CCA TAA ACG  
ATA C                      | FA 1006 inactivation |
| P9    | Upstream FA0122 F | TTA GAT GGG CAG CGG CAT TAT  
CGG A                      | FA 122 inactivation |
| P10 | Upstream  | TTC ATT TAT TCC TCC TAG TTA GTC  | FA 122 inactivation |
|     | FA0122 R  | ACCG TTT GAT ACA GCA GCA TAC AAT G |
| P11 | Downstream| AAA ATA ATA CCT GGA GGG AAT AAT CGAT CAA GTT AGG AAT ATC ATT CGG TC |
|     | FA0122 F  | GA122 inactivation |
| P12 | Downstream| GTT TTT CCA CCC GTA TTA GGT CCG G |
|     | FA0122 R  | GA122 inactivation |
| P13 | F1750     | GTA GTT ATG GTT CTC TCC ACC TAA A |
|     | Real time F | GA122 inactivation |
| P14 | F1750     | TAA TGA CCT AAA TGC TCC CGT AGA A |
|     | Real time R | GA122 inactivation |
| P15 | F122 Real time F | GGT GTA ACT TGC TTT GCA TTC AAA C |
|     | Real time R | GA122 inactivation |
| P16 | F122 Real time F | TAT TCT ATT CCG GAT GTA GTG ACT G |
|     | Real time R | GA122 inactivation |
| P17 | F1006     | GCG TTG TTA TTA TCA TCC CAA GTC T |
|     | Real time F | GA122 inactivation |
| P18 | F1006     | ACT GTA GAT GAA GAA GTT GTT CCG A |
|     | Real time R | GA122 inactivation |
| P25 | F16S- F   | AGC GGC GAA ATG CGT AGA TA |
|     | GA122 inactivation |
| P26 | F16S- R   | CAC CTT CCG ATA CGG CTA CC |
Results

Bioinformatics Analysis

Multiple sequence analysis of the protease of 16S rRNA from several oral pathogens revealed that *F. alocis* is closely related to several established Gram-negative red complex bacteria such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans* (Fig 3.1). Similarly, phylogenetic analysis of collagen-related proteins in other bacteria reveal that the collagen-adhesin protein (HMPREF0389_01006) in *F. alocis* is closely related to proteins from other Gram-positive pathogens such as, Ace (adhesin of collagen of *E. faecalis*), and Scm (second collagen adhesin of *E. faecium*) Cna (collagen binding protein of *S. aureus*), *Streptococcus gordonii* (Cbp), *Streptococcus mutans* and *Streptococcus sanguinis*, (Fig 3.1). In addition, it is also closely associated with Gram-negative pathogens like *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* (EmaA). The protease (HMPREF0389_00122) is an orthologue of *P. gingivalis* PrtC (PG-1542 collagenase) (Data not shown), and shows close molecular relatedness to proteins of *A. actinomycetemcomitans*, and domain architecture of this protein showed collagenase domains (Fig 3.2). The collagen adhesin protein (HMPREF0389_01006) contains a LPxTG cell wall anchor domain (CnaB) with two collagen binding motifs, as well as a peptidase domain (Fig 3.3). In silico protein modeling (Fig 3.3) also confirmed the domain architecture of the collagen adhesin protein and a characteristic interactive molecular groove.
Figure 3.1. Phylogenetic analysis of Collagenase among various oral pathogens. (A) Collagen-adhesin protein (HMPREF0389_01006) is closely related to proteins from other Gram-positive pathogens such as *S. mutans*, *S. gordonii* Cbp protein, and associated with proteins from Gram-negative pathogens such as *F. nucleatum*. (B) *F. alocis* is closely related to several established Gram-negative red complex bacteria such *P. intermedia*, and *A. actinomycetemcomitans*. 
Figure 3.2. Domain architecture and protein modeling of Protease (HMPREF0389_00122) in *F. alocis*, showing collagenase domains.
Figure 3.3. Domain architecture and protein modeling of collagen-adhesin protein (HMPREF0389_01006) in *F. alocis*. Collagen-adhesin protein contains two collagen binding domains, and probable collagenase domain.
FLL212 Showed a Significant Decrease in Growth Rate

The growth rate of *F. alocis* 35896 ATCC wild-type strain and the mutants; FLL211 and FLL212, were compared over a 24 h period to evaluate the effect of inactivation of the collagen related MSCRAMMs on the ability of bacteria to actively divide and proliferate. FLL211 showed no significant variation in the phenotype and growth rate under normal conditions (Fig 3.4A), while FLL212 showed a significant decrease in growth rate compared to the wild-type (Fig 3.4B).
Figure 3.4. Growth rates of *FLL211* and *FLL212* under normal conditions. (a) Growth rates of *FLL211*. (B) Growth rates of *FLL211*. All Cultures were incubated at 37°C under anaerobic conditions. Growth rates were determined by measuring the optical density at [OD$_{600nm}$] over 24 h. The data are means ± standard deviations (SD) of three independent experiments.
Co-culture of FLL212 with NOKs Cells Resulted in Upregulation of Other F. alocis Collagen-Related MSCRAMMs

We assessed the expression of other collagen-related MSCRAMMs in FLL212 during invasion of NOK cells. As shown in Fig 3.5, we observed an eight-fold upregulation of the protease gene (HMPREF0389_00122), and a four-fold upregulation of the hypothetical protein (HMPREF0389_01750) gene, which possesses collagen-binding domains. As a control we did not see the upregulation of the collagen-adhesin gene, confirming the deletion of that gene in FLL212.
Figure 3.5. Real-time PCR analysis of collagen related proteins in *FLL212* after invasion of NOK cells, showed the upregulation of other collagen-related MSCRAMM genes such as the protease gene (HMPREF0389_00122) and hypothetical protein (HMPREF0389_01750).
**FLL211 Showed an Increase in Biofilm Formation Compared to Wild-Type**

Bacteria in the oral cavity develop biofilm on the surface of the tooth (Novak, 2002) and the ability of *F. alocis* to form biofilms has been demonstrated in cases of both aggressive and chronic periodontitis (Schlafer *et al.*, 2010) The biofilm forming abilities of *FLL211* and *FLL212* were compared to the *F. alocis* 35896 wild-type strain. *FLL211* showed an increase in biofilm formation, however, there was no significant difference in biofilm formation in *FLL212* compared to the wild-type *F. alocis* 35896 ATCC strain (Fig 3.6).
Figure 3.6. Biofilm formation assay of *F. alocis* ATCC 35896, *FLL211* and *FLL212*. There was an increase in biofilm formation in *FLL211*, however, there was no variation in biofilm formation in *FLL212* mutant compared to the *F. alocis* ATCC 35896 wild-type strain.
**Autoaggregation was Increased in FLL211 Compared to the Wild-Type**

Autoaggregation is defined as the adherence of bacteria belonging to the same strain. In this process, bacteria physically interact with each other and settle to the bottom in a static liquid suspension (Nikitina *et al.*, 2001, Sorroche *et al.*, 2010).

Autoaggregation of *F. alocis* 35896 ATCC, *FLL211* and *FLL212* were compared over a 3h period. *FLL211* displayed a significant increase in autoaggregation, while the level of autoaggregation in *FLL212* was similar to the wild-type strain (Fig 3.7)
Figure 3.7. Autoaggregation of *F. alocis* 35896 ATCC, *FLL211* and *FLL212*. *FLL211* displayed a significant increase in autoaggregation compared to *FLL212*, and the *F. alocis* 35896 ATCC wild-type strain.
**FLL212 Showed Significant Decrease in Adherence and Invasion of Epithelial Cells**

In order to become a successful pathogen, pathogenic bacteria have ability to adhere-to and invade the host cell as one of the first steps in pathogenesis. We tested the ability of the isogenic mutant *FLL212* to adhere, and invade HeLa cells. Our invasion studies show a significant decrease in the ability of *FLL212* to adhere to, and invade NOK epithelial cells (Fig 3.8).
Figure 3.8. Adherence and invasion assay of *F. alocis* 35896 ATCC *FLL211*, and *FLL212* strains using NOK cells. Error bars represent mean ± SD of four independent experiments.
**FLL211 and FLL212 Showed No Significant Changes in Collagenase Activity Compared to Wild-Type**

To demonstrate loss of function in *FLL211*, which is deficient in protease gene (HMPREF0389_00122), annotated to have collagenase domains, we carried out a collagenase activity test using the EnzChek® Gelatinase/Collagenase Assay Kit as per manufacture’s protocol. *P. gingivalis* was used as a control owing to the presence of an active collagenase gene (Kato *et al.*, 1992, Takahashi *et al.*, 1991). There was no significant variation in collagenase activity of *FLL211* and *FLL212* as compared to the wild-type strain (Fig 3.9).
Figure 3.9. Collagenase activity of *F. alocis* 35896 ATCC FLL211, and FLL212 strains. Collagenase activity carried out as explained in the methods. No significant difference between FA ATCC, FLL211 and FLL212. *P. gingivalis* W83 and 33277 were used as controls.
Discussion

The ability of an organism to invade the host tissue facilitates bacterial colonization, and is the first step in the infectious process. Pathogenic bacteria possess structural and biochemical mechanisms to invade host cell and cause disease (Wilson et al., 2002). This could be through the secretion of substances that could harm the host such as toxins (Carbonetti, 2015), or by structural components of the bacterial cell e.g. Lipopolysaccharide (LPS) (Raetz and Whitfield, 2002).

Bacterial adherence and invasion of eukaryotic cells is mediated by a bacterial ligand called an adhesin, which is a macromolecule that binds a bacterium to specific components of the host’s extracellular matrix (ECM) such as collagen. Surface proteins that bind to specific components of the hosts ECM are called microbial surface component-recognizing adhesion matrix molecules (MSCRAMM’s). The name MSCRAMM’s was coined by Höök and colleagues representing cell surface adhesins which mediate microbial attachment to the host, by recognizing and binding to specific components of host extracellular matrix such as collagen, fibrinogen, fibronectin, laminin, elastin, heparin, and proteoglycans (Bosman and Stamenkovic, 2003). MSCRAMMs are found in almost all pathogenic Gram-positive species, and their functions include; evasion of host immune response, biofilm formation, adhesion and invasion of host cells and tissues. MSCRAMMs include cell wall anchor proteins such as adhesins, collagenases, and invasins (Dalbey et al., 2012).

*F. alocis* proteome analysis has shown the expression of cell wall anchor proteins. Hence, in this study, we propose the *F. alocis* mediates virulence and host modulation through adherence to the collagen in the host ECM. *F. alocis* collagen-
adhesin protein and collagenase are involved in such processes mediating initial adherence, invasion and host modulation. These two genes and their gene products facilitate binding and break down of collagen; a major component of the hosts ECM, resulting in tissue breakdown thereby facilitating bacterial entry to host cell and providing nutrition for anaerobic bacteria.

In this study, we have inactivated two *F. alocis* collagen related genes, collagen-adhesin protein (HMPREF0389_01006) and Protease (HMPREF0389_00122) and evaluated their involvement in *F. alocis* virulence and pathogenesis.

The collagen-adhesin deficient mutant designated *FLL212* had a significant decrease in growth rate, compared to *F. alocis* 35896 ATCC wild-type strain. Likewise, *FLL212* invasion in NOK cells showed an increase in the expression of other MSCRAMM genes, such as a four-fold upregulation of another collagen-binding gene: hypothetical protein (HMPREF0389_01750) and an eight-fold upregulation in the protease gene (HMPREF0389_00122). The upregulation of these other MSCRAMMs in *FLL212* could be as a result of a compensatory mechanism been put in place, due to the deletion of the collagen-adhesin gene.

*F. alocis* has been demonstrated to form biofilm (Aruni *et al.*, 2011, Schlafer *et al.*, 2010) and this may play a role in its pathogenesis. In this study, we show a significant increase in autoaggregation and an increase in biofilm formation in *FLL211*, compared to the ATCC wild-type strain. The aggregation of *FLL211* may be modulated by the regulation of exopolysaccharides (EPS) synthesis in this mutant (Rinaudi *et al.*, 2010), and could be as a result of the increase in biofilm forming ability observed in this mutant compared to the wild-type strain. Gibbons and Nygaard proposed that specific adhesion
mechanisms are responsible for the formation of aggregates amongst plaque forming bacteria (Gibbons and Nygaard, 1970). A review by Slavkin and Bowden concluded that biofilms and microbial aggregates are a common mechanism for the survival of bacteria in nature (Bowden and Hamilton, 1998, Slavkin, 1997). Therefore, by inactivating the protease gene in *F. alocis*, we trigger the activation of possible survival mechanisms in this organism.

Previous studies from our laboratory has shown that *F. alocis* is able to adhere, and invade epithelial cells (Aruni *et al.*, 2011, Aruni *et al.*, 2012), however, the proteins that play a role in this phenomena remain elusive. In this study we show that the *F. alocis* collagen-adhesin gene plays a significant role in adhesion and invasion of host cells, which is evident by significant decrease in adherence to NOK cells by the gene deletion mutant *FLL212* (Fig 3.8). The collagen-adhesin gene has been shown to play role in virulence of several other pathogenic bacteria such as Cnm protein in *S. mutans* is a major virulence factor required for adherence and invasion of endothelial cells (Abranches *et al.*, 2011, Aviles-Reyes *et al.*, 2014), the Cna protein in *S. aureus* also contributes to pathogenesis of osteomyelitis (Elasri *et al.*, 2002, Xu *et al.*, 2004). Also, collagen related MSCRAMMs such as *P. gingivalis* (PrtC) protein is involved in plasma degradation (Holt *et al.*, 1999). Similarly, the collagen-adhesin in *F. alocis* may play a role in pathogenesis.

The inactivation of *FLL211*; a collagenase gene in *F. alocis* led to no significant changes in collagenolytic activity of the mutant strain, we now know that this observation could be due to the existence of another collagenase in *F. alocis* (HMPREF0389_00504).
designated PrtFAC (Chioma et al., 2016), which may compensate for the loss of collagenase gene in FLL211.

In conclusion, our study has confirmed a role for the F. alocis collagen related genes namely, collagen-adhesin protein (HMPREF0389_01006) and Protease (HMPREF0389_00122) in adherence, invasion, and virulence of F. alocis, and play a significant role in colonization and host interaction.

Acknowledgments

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References


CHAPTER FOUR

GENERAL DISCUSSION

Chronic periodontal infections are based on effective predisposition of bacterial agents to successfully colonize the host and circumvent host biophysical and structural barriers. In order to execute this process, bacterial agents target the host ECM, which is predominantly made up of collagen. Breakdown of collagen is the first step in bacterial colonization that further leads to invasion of host cells. Many bacterial virulence proteins such as MSCRAMMs are functional in breaking down host barrier and are focused on ECM components especially the collagen. Among them, a set of proteins facilitate bacterial adhesion to host and this is followed by the second group of enzymes which breakdown the collagen-rich ECM.

Collagen disintegrating and disruption of ECM metabolism occurs in periodontal disease (lorencini 2009). This is usually accompanied with inflammation, a significant reduction in collagen fiber density in the gingival tissue (Golub et al., 1997), as well as loss of alveolar bone (Grossi et al., 1995). The collagen degradation leads to the breakdown of the cytoskeleton, to provide substrate for survival, attachment and colonization by other anaerobic bacteria, and leads to the formation of periodontal pockets.

Many pathogenic bacteria have been shown to possess collagen-interacting proteins such as collagen-adhesin proteins and collagenases, which may play a role in colonization of host tissue by binding (collagen-adhesins) and degrading collagenases) host collagen. Furthermore, the breakdown of collagen in dental tissues could lead to a region of lower redox potential level compared to the normal surrounding tissue, and
promote colonization by other anaerobic organisms, which lead to periodontal disease (Harrington, 1996). *F. alocis* is now considered a marker organism for periodontitis, because of its high abundance in patients with periodontitis, and least detection in healthy individuals (Kumar et al., 2003, Kumar et al., 2006). *F. alocis* possesses many virulence factors such as resistance to oxidative stress, a well-developed amino acid metabolism pathway, enhanced growth in conditions of oxidative stress, ability to form biofilms, and causes bone loss in vivo animal studies.

The *F. alocis* genome has been completely sequenced, and has allowed for the identification of putative collagen-interacting genes. This study has focused on three collagen-interacting genes namely; protease (HMPREF0389_00122), peptidase (HMPREF0389_00504), and collagen-adhesin (HMPREF0389_01006), that may play a role in the interaction of *F. alocis* with the collagen-rich ECM of the host. Bioinformatics analysis indicates that the two proteins coded by the genes- protease (HMPREF0389_00122), and peptidase (HMPREF0389_00504), belong to the U32 family of bacterial collagenases, and share homology with other bacterial proteases, such as U32 peptidases and collagenases (Fig 2.1). Bioinformatics analysis of the collagen-adhesin (HMPREF0389_01006) shows that it contains two collagen-binding domains, a probable collagenase domain, as well as an LPxTG motif required for translocation of protein from the cytoplasm to the cell membrane/cell wall compartments.

Inactivation of protease (HMPREF0389_00122), and collagen-adhesin (HMPREF0389_01006) genes in *F. alocis* was carried out by allelic exchange mutagenesis, and the mutants were designated *FLL211* and *FLL212* respectively. *FLL211* mutant shared a lot of similarities with the wild-type *F. alocis* 35896 ATCC strain, in
growth characteristics (Figure 3.4), phenotype and collagenolytic activity (Fig 3.9). The protease (HMPREF0389_00122) gene encodes a putative collagenase, however, inactivation of this gene did not show a marked reduction in collagenase activity. This could be as a result of another putative collagenase gene in *F. alocis* genome; peptidase (HMPREF0389_00504 (Chioma et al., 2016), which may play a compensatory collagenase functional role in the *FLL211* mutant, and hence could explain the lack of reduction in collagenase activity in *FLL211*. *FLL211* was however different from the wild-type because of its significantly higher ability to autoaggregate compared to the wild-type strain.

Autoaggregation refers to the adherence of bacteria belonging to the same strain, and could be modulated by the synthesis of exopolysaccharides in this mutant (Rinaudi et al., 2010). The collagen–adhesin deficient mutant; *FLL212* on the other hand showed unique differences from the *F. alocis* 35896 wild-type strain. *FLL212* had significant decrease in growth rate, and ability to adhere and invade epithelial cells. Invasion of NOK cells by *FLL212* showed that other putative collagen-related MSCRAMMs genes such as protease (HMPREF0389_00122), and hypothetical protein (HMPREF0389_01750) were upmodulated in the absence of the collagen-adhesin gene (Fig 3.5). It is important to note that the protease (HMPREF0389_00122) gene in addition to a collagenase domain also possesses collagen-binding domain, and the hypothetical protein (HMPREF0389_01750) also possesses two collagen-binding domains. Therefore these genes collectively could play a role to compensate for the loss of the adhesin in *FLL212*, however, this upregulation of other collagen-related genes was not enough to restore the adhesive and invasive capacity of the mutant. The collagen-
adhesin gene seems to play unique role in adhesion to host cells, as inactivation of the
gene led to a significant decrease in adhesion and invasion in this mutant (Fig 3.8). The
third collagen-related gene of interest to this study was a peptidase (HMPREF0389_00504), coding for U32 family of bacterial collagenases. Phylogenetic analysis revealed that PrtFAC had a high degree of homology with other bacterial proteins belonging to the U32 family (Fig 2.1). Sequence comparison of the peptidase U32 family protein from several pathogenic bacteria show that PrtFAC is closely related to the peptidase U32 proteins in S. Typhimurium, B. subtilis, P. gingivalis, and H. pylori (Fig 2.1). PrtFAC shares 38% homology with PrtC of P. gingivalis, and 70% homology with Proteocatella sphenisci, also belonging to the U32 peptidase family. PrtFAC is homologous to the U32 proteins in the Clostridia class. Bioinformatics analysis of the protein sequence of PrtFAC revealed the presence of the signature motif for peptidase U32 family proteins E-x-F-x(2)-G-[SA]-LIVM-C-x(4)-G-x-C-x-[LIVM]-S.

Multiple attempts to inactivate this gene employing different inactivation strategies proved abortive. Which imply this gene to be essential for FA survival. However the peptidase (HMPREF0389_00504) gene was successfully cloned in E. coli. Recombinant clones harboring the peptidase (HMPREF0389_00504) gene in the right orientation were confirmed by PCR and by DNA sequencing. The optimal protein expression was achieved by induction with 0.5mM IPTG, and the expression of a 6xHistagged fusion protein was confirmed by western blot analysis and probing with anti-His antibodies (Fig 2.4b). As anticipated, the presence of the His-tag facilitated the purification of the protein. Owing to its ability to degrade type 1 collagen, the recombinant protein was designated PrtFAC (Chioma et al., 2016). We demonstrated the
ability of PrtFAC to bind to collagen type 1 (Fig 2.5), and degrade it in a Ca\(^{2+}\)-dependent manner (Fig 2.6). We also observed an increase in collagenase activity upon boiling of the collagen substrates at 100°C for 15mins, which could suggest a peptidase function for this protein. The collagenolytic activity of PrtFAC was inhibited by EDTA, and 1, 10 phenanthroline and a Ca\(^{2+}\)-independent gelatinase activity of PrtFAC was also observed. Since prtC collagenase protein in *P. gingivalis* was not reported to have any gelatinolytic activity (Takahashi *et al.*, 1991), but still belonged to the U32 family of bacterial collagenases, the observation of gelatinolytic activity in PrtFAC denotes heterogeneity of the peptidase U32 family of peptidases/collagenases. Even though first crystal structure of a U32 peptidase catalytic domain from *M. kandleri* did not reveal the presence of any proteolytic motifs, and the purified protein failed to demonstrate proteolytic activity (Schacherl *et al.*, 2015). This is in contrast to the results of our study, which showed proteolytic activity of PrtFAC.

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). In addition to its collagenolytic activity, PrtFAC was also able to induce cell death of NOKs, through a caspase 9 dependent mechanism. However ZVAD-FMK was unable to completely inhibit apoptosis in the presence of PrtFAC, and this could indicate the presence of a caspase independent apoptotic mechanism. It is important to note that boiling of the PrtFAC protein to inactivate the protein, prior to treatment of NOKs with the inactivated protein,
did not lead to apoptosis of NOKs, affirming that the cytotoxic effects observed are as a result of the PrtFAC.

In conclusion, the work presented herein on collagen-related proteins in *F. alocis* has confirmed a role of *F. alocis* collagenase in collagen destruction and its ability in the virulence and pathogenesis of *F. alocis* by inducing cell death of host cells, and has also confirmed a role of the collagen-adhesin protein in adhesion, invasion, and virulence.

The implication of *F. alocis* collagen related proteins in the pathogenesis of periodontitis is enormous. 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). The growth and metabolism of asaccharolytic anaerobic oral pathogen depends on the uptake of small peptides and amino acids (Leduc *et al.*, 1996, Takahashi and Sato, 2002). Therefore the collagenolytic activity in *F. alocis* could provide nutrition peptides needed by asaccharolytic bacteria through protein breakdown helping in survival and growth of *F. alocis*. Asaccharolytic organisms capable of producing collagenase show a greater nutritional diversity compared with non-collagenolytic strains. This work has added to our understanding on the role of *F. alocis* in periodontitis, and provided a direction for future studies. Work is currently underway in our laboratory, to study the molecular mechanisms involved in the *F. alocis* virulence and host modulation
References


