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LOMA LINDA UNIVERSITY

School of Medicine
in conjunction with the
Faculty of Graduate Studies

The role of the *Filifactor alocis* hypothetical protein FA1654 in oxidative stress
resistance

By

Malissa Makaylia Mangar

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

December 2022

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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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“Psalm 121” has literally been my everything over the last five years (thanks to my grandma, my rock, my prayer warrior and one of my biggest supporters), it has been the verse I turn to for every bad day, and there have been a lot of those. Despite it all I give God the glory and the praise. I thank him for granting me mercy and giving me the opportunity to achieve my dream of being a scientist and even more for using me as a testimony to his goodness. Without God, I am nothing and none of this would have ever been possible.

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List of abbreviations

ADHD	Attention deficit hyperactive disorder
Ahpc/Ahpf	Alkyl hydroperoxide reductase
Asp	Aspartic acid
ATCC	American type culture collection
Bcp	Bacterioferritincomigratory protein
BHI	Brain heart infusion
C3	Complement component 3
CD	Circular dichroism
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CFU	Colony forming unit
CP	Chronic periodontitis
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
DPS	DNA starvation and stationary phase protection protein
DSF	Differential scanning fluorimetry
FA	<i>Filifactor alocis</i>
FACIN	<i>F.alocis</i> complement inhibitor
Fe	Iron
FEOOH	Iron oxide hydroxide
FISH	Fluorescence in situ hybridization

GAP	Generalized aggressive periodontitis
GEC	Gingival epithelial cells
Glu	Glutamic acid
H ₂ O ₂	Hydrogen peroxide
His	Histidine
Ig	Immunoglobulin
Il	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
I-TASSER	Iterative Threading Assembly Refinement
kDA	Kilodalton
LB	Luria-Bertani
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NETs	Neutrophil extracellular traps
NOD	Nucleotide-binding oligomerization domain
NOK	Normal oral keratinocyte
OD	Optical density
PCR	Polymerase chain reaction
PD	Periodontal disease
PNM	Polymorphonuclear neutrophils
PrtFAC	<i>F. alocis</i> peptidase U32 protein

qPCR	Real time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SCIN	Staphylococcal complement inhibitor
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOR	Superoxide reductase
TGIKs	Telomerase Immortalized Gingival Keratinocytes
TLR	Toll-like receptor
TNF- α	Tumor Necrosis Factor α
TYR	Tyrosine

ABSTRACT OF THE DISSERTATION

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By

Malissa Makaylia Mangar

Doctor of Philosophy, Graduate Program in Microbiology and Microbial Genetics

Loma Linda University California USA September 2022

Dr. Hansel M. Fletcher, Chairman

The survival/adaptation of *Filifactor alocis*, a fastidious gram positive asaccharolytic anaerobe in the inflammatory environment of the periodontal pocket, requires an ability to overcome oxidative stress. Moreover, emerging observations suggest its pathogenic characteristics are highlighted by its capacity to survive in the oxidative-stress microenvironment of the periodontal pocket and its likely ability to modulate the microbial community dynamics. There is still a significant gap in our understanding of *F. alocis* mechanism of oxidative stress resistance and its impact on the virulence and pathogenicity of the microbial biofilm. The pathogenicity of *F. alocis* may be impacted by its response to oxidative stress in the subgingival crevice and understanding this stress response mechanism is key to providing insight in the pathogen's role in disease onset. This project traverses the current understanding of *F. alocis*, and primarily focuses on elucidating the role of the FA1654 protein in the oxidative stress machinery of *F. alocis*. The data obtained indicates that the FA1654 protein may function as a DNA starvation and stationary phase protection protein (DPS), that can modulate Fenton reaction- mediated oxidative stress via iron sequestration.

CHAPTER ONE

INTRODUCTION

Periodontal Disease

The periodontium is the term used to collectively describe the tissue supporting the teeth and comprises four components; the cementum, gingiva, periodontal ligaments and alveolar bone [(CHO & Garant, 2000), Figure 1]. Failure to maintain the health of the periodontium results in the onset of periodontal disease. Periodontal disease is a group of chronic infection-induced inflammatory diseases that affect the periodontium (Chaffee & Weston, 2010; G. Hajishengallis & Lamont, 2012; George Hajishengallis & Lamont, 2016; Michaud, Fu, Shi, & Chung, 2017) and can impact systemic health. Periodontal disease affects a large portion of the population in the United States and proves to be a dental and public health issue. A study conducted by Eke and colleagues showed that approximately 65 million adults in the United States, ages 30 and over are affected by the disease (Eke *et al.*, 2015). In addition, many studies have indicated that there is a link between periodontal disease, obesity, diabetes, atherosclerosis, pre-term labor and Alzheimer's disease (Chaffee & Weston, 2010; Garcia, Henshaw, & Krall, 2001; Loos, Craandijk, Hoek, Dillen, & Van Der Velden, 2000; Meurman, Sanz, & Janket, 2004). The link between periodontal and systemic health indicates that treating periodontal disease may lead to an improvement in systemic health.



Figure 1.1 Healthy vs. diseased tooth/gum. Adapted <https://www.periodontistphoenix.com/blog/2021/02/11/4-types-of-periodontal-therapy/>

Periodontal disease is classified into three categories; necrotizing periodontal disease, periodontitis and periodontitis as a manifestation of systemic disease (Caton et al., 2018) . Infection of the gingiva is referred to as gingivitis and is a result of biofilm formation, which initiates a non-specific immune response (Armitage, 1999; Caton et al., 2018; Gayatri et al., 2021; Slots, 1979). Infection is characterized by bleeding of gums on probing (Caton et al., 2018; Michaud et al., 2017; Slots, 1979; Stillman et al., 2005; Williams, 1990). The staging of periodontal disease is based on the severity of its presentation and the complexity of treatment required for disease management (Caton et al., 2018). The variables considered in the diagnosis of the disease include clinical

attachment loss, percentage of alveolar bone loss, probing depth and tooth mobility (Caton et al., 2018). The distinction between gingival health, disease and periodontitis emphasizes the requirement for maintenance oral health, in cases where there was success was made in treating periodontitis. Furthermore, it should be noted that with proper treatment, patients with gingivitis can revert to a state of health, whilst periodontitis patients remain periodontitis patients for life (Caton et al., 2018).

Diagnosis of periodontal disease is done by examining the gingiva of the patient to evaluate the presence of inflammation, the integrity of the underlying structure, and the formation of gingival pockets (Graves, Jiang, & Genco, 2000; Williams, 1990). A pocket depth of 3-4mm or more is indicative of the degradation of the periodontal ligament and the onset of disease (Williams, 1990). It should be noted that there are cases where patients are asymptomatic but are diseased. The treatment of periodontal disease aims to slow down the rate of disease progression, whilst regenerating the periodontium (Williams, 1990). Physical removal of dental plaque by mechanisms such as scaling and polishing have proven to be effective in the restoration of gingival health (Graves et al., 2000; Slots, 1979; Williams, 1990). The use of antibacterial agents has also been shown to be effective in slowing disease progression. In severe cases, oral surgery is required, and operations such as bone grafting can be used to regain the integrity of the periodontium. (Slots, 1979; Williams, 1990).

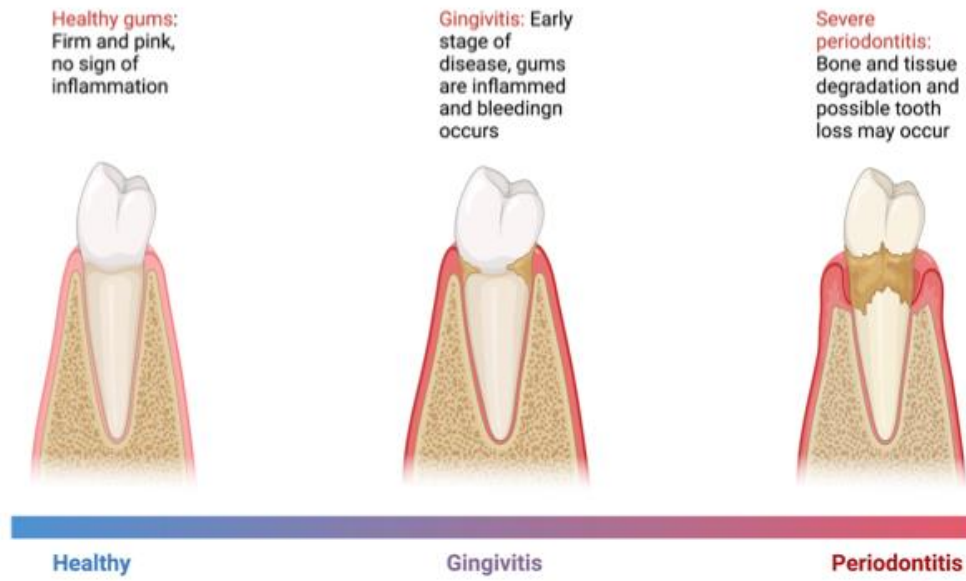


Figure 1.2. Progression of periodontal disease. Adapted from biorender.com

The role of bacteria in the onset of periodontal disease had been thoroughly examined and established (Graves *et al.*, 2000). The oral microbiome comprises over 600 microbes; pathogenic and non-pathogenic, many of which are currently classified as uncultivable (Dewhirst *et al.*, 2010). Microscopic studies have indicated that during the development of disease, the first days of plaque formation are the result of pathogens referred to as early colonizers (Kolenbrander *et al.*, 2002; L oe, 1981). Some examples of these early colonizers are; *Streptococcus gordonii*, *Prevotella denticola* and *Streptococcus oralis*. The second stage of disease and plaque development is a result of *fusobacterium* and *filamentous spp.* (L oe, 1981). The third and final stage of disease development sees the attachment of late colonizers such as *Prevotella. intermedia*, *Porphyromonas. gingivalis*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* (Kolenbrander *et al.*, 2002). The major players in periodontal

disease however are a small group of pathogens known as the ‘Red Complex’ and comprise *P. gingivalis*, *T. forsythia* and *T. denticola* (A. Wilson Aruni, Francis Roy, & H. M. Fletcher, 2011; W. Aruni, Chioma, & Fletcher, 2014; Chen et al., 2015a). Studies utilizing clinical isolates from patients with periodontal disease identified *P. gingivalis* and *A. actinomycetemcomitans* in multiple sites in infected patients (Greenstein & Lamster, 1997). It was further determined that other subgingival organisms such as *F. nucleatum* and *P. intermedia* were present in infected patients (Greenstein & Lamster, 1997). These results thereby strengthened the evidence of the role bacteria plays in periodontal disease. The implementation of new techniques which do not require the involvement of bacterial culture, such as 16S RNA, have identified other pathogens that are involved in disease onset (Dewhirst et al., 2010). One such pathogen is *F. alocis* (W. Aruni et al., 2014; Dewhirst et al., 2010). *F. alocis*, a gram positive, rod-shaped, asaccharolytic obligate anaerobe has been found in high abundance in diseased individuals, indicating that it plays a key role in the onset and persistence of diseased state (W. Aruni et al., 2014).

Periodontal pathogens result in the destruction of tissue during periodontal disease by secreting agents such as proteases that can destroy cells (Jusko et al., 2016b). The immune responses elicited by pathogens are also destructive and may be more so than the pathogen itself. Invading bacteria elicit the release of pro-inflammatory cytokines such as IL-6, IL-8, IL-1beta and TNF-alpha (Armstrong, Klaes, Vashishta, Lamont, & Uriarte, 2018; Dickinson et al., 2011; Q. Wang et al., 2014). Additionally, bacteria have been shown to migrate from the site of infection (Q. Wang et al., 2014), resulting in damage in other tissue types (Dominy et al., 2019; Hishiya et al., 2020).

Therefore, we can conclude that the degree of damage and the progression of periodontal disease is a culmination of the host immune response and the physical action of the invading bacterial species.

Oral Ecology and Dysbiosis in Periodontal Disease

As previously described, the oral microbiome is the habitat of a variety of microorganisms including, bacteria, fungi, viruses and archaea (Curtis, Diaz, & Van Dyke, 2020), of which bacteria have been implicated in the onset and progression of periodontal disease. Recent years have seen a shift in the paradigm of the mechanism of disease, with dysbiosis being implicated as the cause and leading to the development of the “Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation” model. Dysbiosis is characterized as an imbalance in resident microbiota, which is incompatible with health (F. A. Scannapieco & Dongari-Bagtzoglou, 2021). In periodontal disease dysbiosis is not classified as an increase in species diversity, but rather an increase in biomass, resulting from a shift in the balance between pathogenic and non-pathogenic microbes. The shift in equilibrium leads to overall changes in the microbial structure and population of the subgingival biofilm and bacterial load. This is associated with destructive disease (Curtis et al., 2020; F. A. Scannapieco & Dongari-Bagtzoglou, 2021). This shift in equilibrium is attributed to host perturbation such as disease, genetics and lifestyle choices (F. A. Scannapieco & Dongari-Bagtzoglou, 2021). Population changes within the subgingival microbiota initiate inflammatory host responses, which when left “unchecked” become uncontrolled and contribute to the destruction of periodontal tissue (Curtis et al., 2020; T. E. Van Dyke, Bartold, &

Reynolds, 2020). There is a clear association between the onset of disease and dysbiosis in the oral microbiota; however it has not been determined whether dysbiosis is the initiator of disease or is it a consequence of disease onset. Moreover, the nexus between inflammation and dysbiosis is critical to the onset and proliferation of disease (T. E. Van Dyke et al., 2020).

The Oral Microbiome

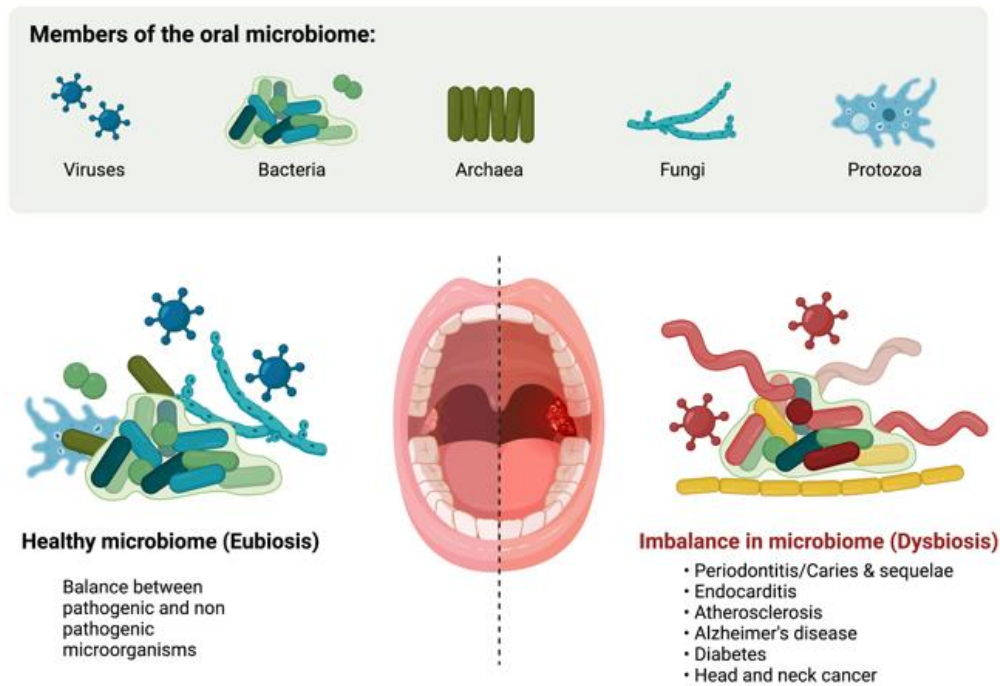


Fig.1.3. Depicting dysbiosis in the oral microbiome. Adapted from Radiac, et al 2021

Risk Factors for Periodontal Disease

The physical invasion of the periodontium by a periodontopathic bacteria is not the sole factor in the onset of periodontal disease (Eke et al., 2015). Studies have

indicated that genetic factors (AlJehani, 2014; Thomas E Van Dyke & Dave, 2005), hormones (Genco, 2000; Genco & Borgnakke, 2013; Koreeda et al., 2005; Wu, Chen, & Jiang, 2015), gender (Genco & Borgnakke, 2013), age (AlJehani, 2014; Genco & Borgnakke, 2013; Thomas E Van Dyke & Dave, 2005), stress and lifestyle choices (Albandar, Streckfus, Adesanya, & Winn, 2000; AlJehani, 2014; Thomas E Van Dyke & Dave, 2005) and the use of prescription drugs (AlJehani, 2014; Bharti & Bansal, 2013; Hasan & Ciancio, 2004) may increase an individual's susceptibility to periodontal disease.

Nutrition

There is constant conversation on the risk factors of periodontal disease and its link to systemic health and disease. However, what is not commonly discussed is the role diet and nutrition plays and the fact that it is at the base of it all. Nutrients can be classed as macronutrients; carbohydrates, proteins, fats and micronutrients; vitamins and minerals (Varela-López, Giampieri, Bullón, Battino, & Quiles, 2016). These nutrients are necessary for growth, energy, metabolism and overall health and wellness (Varela-López et al., 2016). Deficiencies in the serum levels of nutrients, more specifically micronutrients, have been suggested to be involved in the onset of periodontitis and its proliferation. It has been determined that as important as nutrients are to the upkeep of good health, the misuse of nutrients can have detrimental effects, an example of such is the use of carbohydrates. Foods rich in sugar, specifically sucrose and added sugars have been determined to be highly cariogenic (Hujoel & Lingström, 2017; Najeeb, Zafar, Khurshid, Zohaib, & Almas). Sucrose is found to be more cariogenic than its counterparts

glucose and fructose (Najeeb et al.). Fermentation of sugar by bacteria produces acid, which results in the demineralization of teeth (Najeeb et al.). On the other hand the sugar substitute xylitol has been determined to have antibacterial properties against periodontopathogens such as *P.gingivalis* and *A.actinomycescomitans* (Najeeb et al.).

Vitamins A, B complex and C maintain the integrity of epithelial cells, aid in cell repair, partake in the synthesis of collagen and even have antioxidant potential (Najeeb et al.). Vitamin C specifically was found to have anti-ROS activity and its addition to castings and gels and dental implants was shown to increase osteo-integration in implant surgeries and produce better post-surgical healing (Najeeb et al.). Aside from vitamin C, another compound considered to have great antioxidant potential is lycopene. Lycopene is a red pigment found in vegetables such as tomatoes, carrots and watermelon (Najeeb et al.). Studies conducted determined that lycopene can damage to the periodontium by minimizing the generation of free radicals and destroying existing free radicals and their precursors and can even reverse H₂O₂ mediated DNA damage (Belludi et al., 2013). It was also observed that using lycopene in conjunction with mechanical therapy showed an improvement in overall clinical attachment loss and a reduction in gingival index (Belludi et al., 2013). The consumption of food high in antioxidants can be used as adjunct anti-oxidant therapy. Antioxidants may also reduce the clinical attachment loss and bleeding upon probing and are an overall indicator of nutrition playing a role in periodontal health.

Gender and Age

Studies have documented that the occurrence and severity of periodontal disease increases with age. A study conducted by Al Jehani and colleagues showed a mean annual

rate of bone loss of 0.28mm in 70-year-old individuals compared to that of their 25-year-old counterparts where bone loss was observed at a rate of 0.07mm per annum(AlJehani, 2014). This increase in disease prevalence may be due to prolonged plaque exposure and build up over time and an accumulation of damage, rather than an increase in the rate of destruction with age (AlJehani, 2014; Thomas E Van Dyke & Dave, 2005). It is pertinent to note that age is not categorized as an intrinsic risk factor by all the conducted studies, this is since the onset of disease is usually observed approximately at 70-75 years of age and is rare under the age of 40 (AlJehani, 2014; Genco & Borgnakke, 2013). It is yet to be determined if the destruction of the periodontium accompanied by age is due to the reduction in host defense mechanisms or an increase in host destructive mechanisms. It is however, clear that with age comes a higher risk of disease (Genco & Borgnakke, 2013). Along with age, studies have shown that gender may also play in the onset of disease. Disease is more frequently reported in men than women and with varying degrees of severity (Genco & Borgnakke, 2013). Men tend to have poorer oral hygiene and less likely to partake in prophylactic treatments such as dental visits than women (Genco & Borgnakke, 2013; Thomas E Van Dyke & Dave, 2005).

Genetic factors and Hormones

Bacterial infections have been implicated as the etiological agents of periodontal disease (A. W. Aruni, F. Roy, & H. M. Fletcher, 2011; Genco, 2000; Thomas E Van Dyke & Dave, 2005), however studies have determined that host factors can increase an individual's susceptibility to developing periodontal disease in certain demographics (AlJehani, 2014; Thomas E Van Dyke & Dave, 2005). Some demographics have inherited

traits rendering them more susceptible to disease progression. These heritable traits are influenced by factors such as socioeconomic status, environmental and epigenetic factors and lifestyle risks such as smoking and drug use, and can negatively impact oral health (AlJehani, 2014). Along with genetic factors, hormones have been implicated in disease onset and progression. Studies have shown that estrogen may have a role in protecting against periodontal disease (Genco & Borgnakke, 2013), however, a contradictory study indicated that gingival inflammation and disease onset is influenced by hormonal levels (Koreeda et al., 2005). In a clinical case focused on a 35-year-old woman, it was observed that gingival inflammation worsened during menstruation and ovulation when estrogen levels were higher (Koreeda et al., 2005). The use of birth control comprising of progesterone and estradiol increased the loss of attachment (Wu et al., 2015). Hormone fluctuations occurring during pregnancy (estrogen and progesterone) have been suggested to influence the composition of the oral microbiota and the accompanying inflammatory response in gingival tissue (Wu et al., 2015).

Medications

Medications such as anti-hypertensives, sedatives and antihistamines have been determined to reduce the flow of saliva in the oral cavity (AlJehani, 2014; Bharti & Bansal, 2013). Meanwhile, other medications such as vitamins and supplements have a high sugar content, that can alter the pH of the oral cavity and the composition of plaque. Alteration in the composition of plaque can increase its adhesion to teeth (AlJehani, 2014). Anti-convulsants, calcium channel blockers, and immunosuppressant drugs, inhibit the influx of intracellular calcium (Bharti & Bansal, 2013). These drugs have

varying functions and biochemical compositions, and can cause gingival hyperplasia, leading to the hypothesis that the overgrowth of gingival tissue is a result of the mechanism of action of these drug classes and not their biomedical composition (Bharti & Bansal, 2013). The use of amphetamines for the treatment of conditions such as ADHD has also been attributed as a causative agent of gingival hyperplasia (Hasan & Ciancio, 2004). The overgrowth of the gingiva makes plaque removal difficult, increasing the build-up of plaque and inflammation, and the probability of disease (Bharti & Bansal, 2013).

Lifestyle Choices (smoking, stress, and drug use)

Smoking over long periods is detrimental to systemic health and leads to conditions such as cancer and chronic obstructive pulmonary disease (COPD). Studies, have also shown that smoking is detrimental to oral health (Albandar et al., 2000). Smoking results in a reduction of gingival bleeding, tobacco usage and the nicotine from cigarettes results in localized vasoconstriction, diminishing the flow of blood to the gingival tissue and changes in the composition of the oral microbiome (AlJehani, 2014; Gayatri et al., 2021; Thomas E Van Dyke & Dave, 2005). Insufficient blood supply to the periodontium results in the degradation of the alveolar bone, whilst masking indicators used for clinical diagnosis. Delayed diagnosis and treatments allow for exponential diseased progression (Albandar et al., 2000; AlJehani, 2014; Gayatri et al., 2021).

It is also suggested that individuals experiencing psychological stress are at high risk for the development of periodontal disease (Thomas E Van Dyke & Dave, 2005). These individuals are also unable to cope with the stress of everyday life, neglecting

basic hygiene, and leaving room for substandard oral and dental hygiene (AlJehani, 2014; Thomas E Van Dyke & Dave, 2005). A possible mechanism to be considered, is that stress and psychological illness increase the production of hormones such as cortisol along with cytokines, for example IL-6 (Thomas E Van Dyke & Dave, 2005). However, this mechanism has not been fully elucidated. Stressful lifestyles increase the possibility of risky lifestyle choices such as drug abuse, excessive alcohol consumption and poor dieting choices, increasing the occurrence of disease.

Systemic Disease associated with Periodontal Disease

As interest in the etiology of periodontal disease grows, so does the observance of the impact it has on systemic health. It has been demonstrated that there is a link between cardiovascular disease (AlJehani, 2014; Dave & Van Dyke, 2008; Nogueira et al., 2021; Sanz, D'Aiuto, Deanfield, & Fernandez-Avilés, 2010; Frank A Scannapieco, Bush, & Paju, 2003; Sfyroeras, Roussas, Saleptsis, Argyriou, & Giannoukas, 2012), diabetes (AlJehani, 2014; Kuo, Polson, & Kang, 2008; Nogueira et al., 2021), pre-term birth (Graves et al., 2000; Varadan & Ramamurthy, 2015; Zadeh-Modarres, Amooian, Bayat-Movahed, & Mohamadi, 2007), osteoporosis (AlJehani, 2014; BDent, BScDent, & FRACDS, 2010; Kuo et al., 2008; C.-W. J. Wang & McCauley, 2016), respiratory disease (Agado & Bowen, 2012; F. Scannapieco, 1999; Sharma & Shamsuddin, 2011) and periodontal disease.

Cardiovascular Disease (CVD)

Cardiovascular disease is defined as a disease of the heart and blood vessels (Sfyroeras et al., 2012), the main contributor being atherosclerosis (Frank A Scannapieco

et al., 2003). The disease is characterized by arterial constriction due to sub-endothelial deposition of cholesterol, esters and calcium in blood vessels (Frank A Scannapieco et al., 2003). Plaque deposits also comprise a variety of cell types including fibroblast and immune cells (Frank A Scannapieco et al., 2003). CVD is one of the leading causes of death in the world (Sanz et al., 2010), and has been linked to periodontal disease. It is suggested that the link between the two diseases is based on the formation of atherosclerotic and periodontal disease plaques. The proposed mechanisms are as follows: 1) bacterial invasion of the blood stream results in the release of toxins that lead to the degradation of the endothelium (Sfyroeras et al., 2012); 2) the initiation of an anti-inflammatory responses to bacterial invasion that releases cytokines which may damage the endothelium of vascular walls (AlJehani, 2014; Sfyroeras et al., 2012), 3) heat shock proteins released from periodontal pathogens, such as *P.gingivalis* and *P. intermedia*, can degrade vascular walls (Sfyroeras et al., 2012). Studies have also determined that bacteria from periodontal disease may also enter into the bloodstream, imbedding in the plaque buildup (Sfyroeras et al., 2012). This bacteria-filled plaque increases the risk of bacterial endocarditis in patients who undergo heart valve surgery. Periodontal disease also upregulates the production of C-reactive proteins, fibrinogen, IL-6 and other inflammatory factors associated with CVD (AlJehani, 2014; Bansal, Rastogi, & Vineeth, 2013; Sfyroeras et al., 2012). Furthermore, animal studies conducted have established that there is indeed a link between periodontal disease and CVD. A study also showed that the formation of atherosclerotic plaque progressed at an exponential rate as a result of destruction initiated by periodontal disease (Dave & Van Dyke, 2008).

Obesity

The initial link between obesity and periodontal disease was made in 1977 when histopathological changes were observed in the periodontium of Zucker rats with a genetic predisposition to obesity. The association was first made in humans in 1998 (Jagannathachary & Kamaraj, 2010; Martinez-Herrera, Silvestre-Rangil, & Silvestre, 2017). These rats experienced an increase in alveolar resorption and inflammation (Jagannathachary & Kamaraj, 2010). Obese individuals tend to experience 60% macrophage infiltration as opposed to the 5-10% in individuals who are not (Martinez-Herrera et al., 2017; Nogueira et al., 2021). The mechanistic link to obesity and periodontal disease is the expression of adipokines: these cytokines are produced by fat cells or adipocytes and other cell types in adipose tissue (Chaffee & Weston, 2010; Scorzetti et al., 2013; Suresh & Mahendra, 2014). These adipokines bind to receptors on target cells to initiate signaling cascades that can affect the expression and regulation of genes (Martinez-Herrera et al., 2017). Obesity and other obesity-related diseases such as cardiovascular disease and diabetes, increase the presence of macrophages in the host along with the secretion of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-alpha. This increase in pro-inflammatory cytokines exacerbates periodontal disease (Chaffee & Weston, 2010; Jagannathachary & Kamaraj, 2010).

Diabetes Mellitus

Diabetes mellitus is a condition categorized by a deficiency in the secretion or insulin from the pancreas (Bascones-Martinez, Gonzalez-Febles, & Sanz-Esporrin, 2014).

The condition falls into two categories based on its pathophysiology, these sub-types are Type 1 and Type 2 (Mealey, 2006). Type 1 is characterized by insufficient production of insulin due to the auto-immune destruction of β -cells (Mealey, 2006). Type 2 is characterized by the development of insulin resistance as a result of lifestyle choices such as dietary habits (Kuo et al., 2008). Hyperglycemia and hyperinsulinemia are factors indicative of diabetes (Kuo et al., 2008). Studies have indicated that individuals with uncontrolled diabetes are at higher risk for developing chronic periodontitis and rapid disease progression. Individuals with diabetes experience increased gingival bleeding and inflammation (AlJehani, 2014; Kuo et al., 2008; Mealey, 2006). Acute bacterial infections as seen in chronic periodontitis, induces the chronic resistance to insulin triggering hyperglycemia (Kuo et al., 2008). Insulin resistance may then progress to the generation of advanced glycation end products (Kuo et al., 2008). The accumulation of these end products and subsequent interaction with its cell receptor is suggested to upregulated the production of NF- κ B, which can intensify the degradation of periodontal tissue and alveolar bone by periodontal pathogen (Taylor & Borgnakke, 2008). Blocking the receptor of advanced glycation end products reduced the damage to the periodontal bone caused by *P. gingivalis* whilst limiting the detrimental inflammatory response in the surrounding tissue (Kuo et al., 2008; Taylor & Borgnakke, 2008). Bacterial factors such as the LPS from the keystone pathogen *P. gingivalis* induces the production of IL-1 β , TNF- α and PGE₂, factors that indicate insulin resistance and that impair its function (Bansal et al., 2013; Bascones-Martinez et al., 2014; Kuo et al., 2008; Mealey, 2006). Periodontal disease was also shown to inhibit the process of glucose uptake mediated by insulin into skeletal muscles, resulting in whole body insulin resistance (Kuo et al.,

2008). The mechanism of action whereby diabetes is impacted by periodontal disease has recently been elucidated. Bacterial and viral infections such as the common cold increases inflammation, resulting in an increase in insulin resistance causing difficulties for an individual with diabetes to control their blood sugar (Mealey, 2006). This phenomenon is increased in cases of chronic periodontitis (Mealey, 2006). Well controlled diabetes, however can aid in the maintenance of an overall healthy prognosis and allows for a positive response to periodontal therapy (AlJehani, 2014).

Osteoporosis

Osteoporosis is the decrease in bone density, this decrease compromises the strength and integrity of bone, increasing the risk of fractures (Kuo et al., 2008; Thomas E Van Dyke & Dave, 2005). Osteoporosis does not only occur in the major bones of the skeletal system but spreads to the mandible and maxilla (BDent et al., 2010; Kuo et al., 2008). Periodontal disease leads to complications in patients with osteoporosis. Low jaw bone density increases the porosity of the alveolar bone. This increased porosity renders the alveolar bone more susceptible to bone resorption by periodontal pathogens (BDent et al., 2010; Kuo et al., 2008). A study conducted in a cohort of post-menopausal women, indicated that the development of osteoporosis results in a loss of alveolar bone and the onset of periodontal disease (Chen et al., 2015a; C.-W. J. Wang & McCauley, 2016). Furthermore, it was shown that osteopenia of the mandible has a correlation to the diminishing of the height of alveolar bone and an increase in tooth loss (AlJehani, 2014; C.-W. J. Wang & McCauley, 2016). Animal studies using ovariectomized sheep with pre-existing periodontal disease exhibited changes in the mineral density of the alveolar

bone that positively correlate with the reduction of the mineral density of skeletal bones (BDent et al., 2010). These animal models not only provide a basis for an explanation for the link between periodontal disease and osteoporosis, but they also show the effects of estrogen deficiency in the alveolar bone (BDent et al., 2010).

Pre-termed labor

Pre-termed labor is the delivery of a fetus after 23 weeks of gestation but before 37 weeks (Bansal et al., 2013; Varadan & Ramamurthy, 2015). Babies born prematurely are at high risk for neurological impairment, respiratory complications, behavioral disorders and a plethora of comorbidities and even death (Varadan & Ramamurthy, 2015; Zadeh-Modarres et al., 2007). During the second trimester of a pregnancy the oral microbiome of the expectant mother comprises of a larger population of gram-negative anaerobic bacteria (Varadan & Ramamurthy, 2015). These gram-negative species involved in the onset of periodontal disease, have adverse effects on pregnancy (AlJehani, 2014; Santos-Pereira et al., 2006). Women with periodontal disease during gestation are 7.5 times more likely to have complications during pregnancy (Varadan & Ramamurthy, 2015). Periodontal pockets are reservoirs for pathogenic microbes, many of which are LPS-rich. These microbes easily enter the blood stream as a result of gingival bleeding and a lack of gingival epithelium (Santos-Pereira et al., 2006). Periodontopathogens produce endotoxins that trigger the production of prostaglandins and cytokines, such as TNF- α , which can induce labor prematurely (AlJehani, 2014; Genco & Borgnakke, 2013; Zadeh-Modarres et al., 2007). In a case report of a still birth, it was found that fetal death was a result of *F. nucleatum* found in the mother's oral

cavity, which was transmitted to the placenta via the hematological pathway, initializing fetal complications (Varadan & Ramamurthy, 2015). Another study found *F. nucleatum* and *P. gingivalis* in the amniotic fluid of a woman who experienced pre-termed labor (Varadan & Ramamurthy, 2015). These pathogens can also cause chorioamniotic infections (Varadan & Ramamurthy, 2015). Animal studies have also determined that LPS from *P. gingivalis* results in pre-termed labor, birth defects and low birth weight (Bansal et al., 2013; Genco & Borgnakke, 2013). It should be noted that in some cases, the failure of the mother to initiate an IgG response to infections by ‘red complex’ bacteria resulted in a fetal IgM response (Varadan & Ramamurthy, 2015). This fetal IgM response can initiate premature labor (Varadan & Ramamurthy, 2015).

Respiratory Disease

Respiratory diseases like chronic obstructive pulmonary disease (COPD) and pneumonia are a result of the aspiration of pathogenic bacteria from the oropharynx into the lower respiratory tract (Sharma & Shamsuddin, 2011). Periodontal disease has been associated with respiratory infections. Dental plaque acts as a reservoir for oral pathogens, which can ‘dislodge’ and be aspirated into the respiratory tract (Agado & Bowen, 2012; Bansal et al., 2013; F. Scannapieco, 1999). Failure of host defense systems to remove these pathogens from the mucosa allows for bacterial growth (Sharma & Shamsuddin, 2011). Pathogens such as *P. gingivalis* produce proteases and other enzyme, which in conjunction with hydrolytic salivary enzymes, degrade the protective domains of the host secretory components. Degradation components such as mucins diminish host defenses and increase disease risk (F. Scannapieco, 1999; Frank A Scannapieco et al.,

2003; Sharma & Shamsuddin, 2011). The production of cytokines resulting from periodontal disease affects the integrity of the respiratory endothelium, promoting infections by respiratory pathogens (Agado & Bowen, 2012; F. Scannapieco, 1999). Isolation of pleural fluid from a patient with pneumonia yielded a bacterial culture comprising *P. gingivalis*, *F. nucleatum* A. *actinomycetemcomitans* (Agado & Bowen, 2012; F. Scannapieco, 1999).

Immune response in Periodontitis

The onset of periodontal disease initiates both the innate and adaptive immune responses (Muñoz-Carrillo et al., 2019; Silva et al., 2015). Pathogens trigger the innate immune system, initiating pattern recognition receptors that bind to Pathogen Associated Molecular Patterns (PAMPs). PAMPs include toll-like receptors, nucleotide-binding oligomerization domain (NOD) protein, cluster of differentiation (CD) proteins and lectins. Neutrophils are the most common white blood cell and are the first line of defense against bacterial infection (San Miguel, Opperman, Allen, & Svoboda, 2011; Y. Wang, Andrukhov, & Rausch-Fan, 2017). In a healthy oral cavity, polymorphonuclear neutrophils (PMN) are responsible for the maintenance of the commensal biofilm at the interface of the tooth and gingival crevice. Oral inflammation leads to hyperactive oral PMNs that result in elevated phagocytosis and neutrophil degranulation, increasing the generation of ROS and NETs. Hyperactive PMNs also initiate the upregulation of proinflammatory cytokines such as NF- κ B (Fine et al., 2020; Muñoz-Carrillo et al., 2019). An increase in oxidative stress is also linked to a reduction in serum immunoglobulin G (IgG) levels (San Miguel et al., 2011). Aside from the neutrophilic

response, bacterial infection also activates the complement system, facilitating the opsonization of microbial pathogens, marking them for clearance by antibodies and phagocytes (Becerra-Ruiz, Guerrero-Velázquez, Martínez-Esquivias, Martínez-Pérez, & Guzmán-Flores; Nędzi-Góra, Kowalski, & Górska, 2017).

The adaptive immune system comprises B and T lymphocytes. There are a variety of T-cell responses, with CD4⁺ T-helper cells amplifying the immune response. The immune response elicited is determined by the type of cytokines released: Th1 produces IL-2 and IFN-gamma, whilst Th2 produces IL-4, IL-5, IL-6, IL-10 and IL-13 (Becerra-Ruiz et al.). CD8⁺ T-cells are found in the gums of healthy patients and those with induced periodontitis. These cells may be suppressors since they can regulate other immune cells. CD8⁺ regulatory cells also impact alveolar bone homeostasis, protecting it by reducing osteoclast generation and the modulation of local immune response (Becerra-Ruiz et al.).

B-cells are responsible for adaptive humoral immunity and the production of antibodies and cytokines. B-cells are protective during chronic periodontitis and cease the progression of disease by bacterial elimination. They are also the source of inflammatory mediators such as IL-1 and TNF-alpha and act as antigen-presenting cells (Nędzi-Góra et al., 2017)

Oxidative stress and Periodontitis

Oxidative stress is an imbalance between free radicals/reactive oxygen species and antioxidants (Tóthová & Celec, 2017). The ROS are the oxygen free radicals and are

generated by normal cellular metabolism and the activity of cells and tissues (Tomofuji et al., 2009). As periodontal disease is inflammatory in nature, it has been determined that ROS production increases with the progression of the disease (Y. Wang et al., 2017). This is a result of the initiation of the innate immune system, specifically the activation of neutrophils and macrophages during phagocytosis (Y. Wang et al., 2017). ROS causes damage to tissues, lipids, nucleic acids and the oxidation of enzymes (Sczepanik et al., 2020; Tóthová & Celec, 2017).

Oxidative stress induced by other pathogens

Oral diseases including periodontitis, gingivitis and halitosis are not the result of a single microbial species, but rather a dysbiotic microbial population (Džunková et al., 2018). The most noted pathogens involved in the onset of disease is *P. gingivalis* and its counterparts in the ‘red complex’ (G. Hajishengallis & Lamont, 2012), however other pathogens involved include *S. sanguis*, *S. oralis* and *Neisseria spp.*, all of which are considered to be early colonizers. These species form the scaffolding for late colonizers and are less sensitive to the ROS they generate (Marquis, 1995). They also offer protection to late colonizers via biofilm association. *S. mutans* and *Lactobacilli spp.*, which have been identified as prevalent in the formation of dental caries (Loesche, 1996) along with colonization by *Actinomyces* and *Veillonella spp.* in the oral biofilms, initiate the production of ROS, thus facilitating oxidative stress. Biofilms when left unperturbed, shift in favor of anaerobic species, increasing the generation of ROS and the strain of oxidative stress (Loesche, 1996).

***Filifactor alocis* and its role in Periodontal Disease**

Filifactor alocis, a novel oral pathogen, was first discovered in 1985 in the human gingival crevice and was originally classified as *Fusobacterium alocis* (Cato, MOORE, & Moore, 1985). The pathogen was later reclassified in 1999 as *Filifactor alocis* because of developments in phylogenetic analysis (Jalava & Eerola, 1999). *F. alocis* is usually found in conjunction with *P. gingivalis* and *T. forsythia*, two of the three 'red complex' bacteria, ranking the pathogen with the same significance in disease onset as the 'red complex' bacteria (Jusko et al., 2016b).

Cato and colleagues examined the flora of the periodontal pocket of patients with periodontal disease. This study found 170 isolates that exhibited characteristics different from known oral pathogens. The isolates were butyrate-producing, non-spore forming, rod-shaped, obligate anaerobes and were named *Fusobacterium alocis* (Cato et al., 1985). *Fusobacterium alocis* and *Fusobacterium sulci* were names generated from this study. Subsequent 16S rRNA analysis determined that the discovered strain was not a member of the *Fusobacterium* genus, but instead resembled *Filifactor vilosus*, a member of the *filifactor* genus, promptly reclassifying the pathogen as *Filifactor alocis* (Citron, 2002; Jalava & Eerola, 1999). *F. alocis* is found in increasing prevalence in individuals with periodontal disease, more so than previously identified pathogens such as pathogen *P. gingivalis* (A. Wilson Aruni, Roy, Sandberg, & Fletcher, 2012; W. Aruni et al., 2014; Q. Wang, Wright, Dingming, Uriarte, & Lamont, 2013). Recent studies, however have unearthed inconsistencies in the gram-staining status of the pathogen. *F. alocis* cells are non-flagellated, ranging from 0.4-0.7µm wide and 1.5-7.0µm long. On blood agar *F. alocis* is non-hemolytic and grows in small colonies that are 1.0mm in diameter (Cato et

al., 1985). *F. alocis* is a slow growing, arriving at a maximum optical density of ~0.25. Such slow growth makes biochemical and functional analyses difficult to conduct. In our laboratory, *Filifactor alocis* is cultured in brain heart infusion broth fortified with yeast extract (0.5%), 5µg/ml hemin, 0.5µl/ml vitamin K and 100µM L-arginine and cysteine. The bacterium is then grown anaerobically at 37°C in 10% H₂, 10% CO₂ and 80% N₂.

F. alocis has been isolated in individuals with chronic and aggressive periodontitis but is relatively undetected in healthy gums (Gomes et al., 2006; Hutter et al., 2003; P.S. Kumar et al., 2003; Purnima S Kumar, Griffen, Moeschberger, & Leys, 2005; Li, Guan, Sun, & Hou, 2014; Paster et al., 2001; Sakamoto, Rôças, Siqueira Jr, & Benno, 2006; Siqueira Jr & Rôças, 2003; Siqueira Jr & Rôças, 2004). The presence of *F. alocis* in the periodontal pocket of diseased individuals suggests that the pathogen plays a role in the onset of disease and possesses key-stone like characteristics (Cato et al., 1985; Gomes et al., 2006; Goncalves et al., 2016; Paster et al., 2001). In a study conducted by Goncalves and colleagues examining healthy individuals and those with Chronic Periodontitis, *F. alocis* was observed in 8% of the sites studied in healthy participants, a value which upon analysis was considered insignificant. Along with *F. alocis*, *Dialister pneumosintes* was also found in low quantities. *P. endodontalis*, however, was observed in a high percentage in healthy patients but was not considered a cause for concern since it is considered an opportunistic pathogen and its presence is not necessarily an indicator of disease (Goncalves et al., 2016).

Another study employing 100 individuals with periodontal disease, 50% of which received prior treatment, determined that *F. alocis* was present in 46% of individuals with primary endodontic infection, and in 24% with secondary infection. The study also

observed that *T. denticola* and *T. forsythia* were also present in both primary and secondary infections. The presence of *F. alocis* in secondary endodontic infections (after treatment) is indicative of the pathogen playing a role in the failure of endodontic therapies (Gomes et al., 2006). These results corroborated those observations made by R. Spooner and colleagues (Spooner et al., 2016). They observed, a reduction in the abundance of periodontal pathogens such as *P. gingivalis*. However, the abundance of *F. alocis* was not reduced (Spooner et al., 2016).

The efficacy of antibiotics in the treatment of *F. alocis* is observably not the same as it is in the case of other periodontopathogens (Spooner et al., 2016). This indicates that *F. alocis* persists in the diseased state and that non-surgical treatment is not effective against the pathogen (Spooner et al., 2016). As previously mentioned, *F. alocis* has been associated with both generalized and localized aggressive periodontitis. The pathogen was also shown to have a mutualistic relationship with other periodontopathogens such as *T. forsythia*, *A. actinomycetemcomitans*, *T. denticola* and *P. gingivalis* (Ikeda et al., 2020). The pathogen is relatively resistant to oxidative stress and has a plethora of virulence factors including collagenase, proteases and the ability to enhance biofilm formation with other oral microbes, which are yet to be fully examined (Chen et al., 2015a, 2015b; Q. Wang et al., 2013). These factors may aid in the organism's survival in the periodontal pocket (Chen et al., 2015b; Q. Wang et al., 2014). In vitro studies to examine the interactions of *F. alocis* with members of oral microbiome demonstrated that *F. alocis* and *S. gordonii* had an antagonistic relationship, which was contrary to the relationship observed between *S. gordonii* and *P. gingivalis*. It was shown that *S. gordonii* provided a scaffold and metabolic aid for *P. gingivalis* (Chen et al., 2015b). This

behavior has been linked to increased alveolar bone loss in mouse models (Chen et al., 2015b).

F. alocis has been shown to improve the biofilm-forming capacity of other members of the oral microbial community (Chen et al., 2015a; Q. Wang et al., 2013). *In vitro* studies have also determined that *F. alocis* enhances the formation of biofilms in co-culture with *P. gingivalis* (Q. Wang et al., 2013). *F. alocis*' ability to facilitate biofilm formation is not limited to *P. gingivalis*, and has been shown to have a synergistic relationship with other bacterial species such as *F. nucleatum*. The ability of *F. alocis* to interact with other oral microbes, aggregating to form biofilms is congruent with this characterization as a periodontopathogen (Q. Wang et al., 2013). Along with enhanced biofilm formation *F. alocis* can adhere to and invade epithelial cells and shows similar characteristics to pathogens capable of tissue degradation (Y. Li et al., 2014). In co-culture with *P. gingivalis*, increased adhesion to epithelial cells. The initiation of apoptosis and morphological changes in epithelial cells were also observed, however apoptosis was triggered after prolonged infection with the pathogens (A Wilson Aruni, Zhang, Dou, & Fletcher, 2014). This data is indicative of *F. alocis* virulence being enhanced by co-infection with other periodontopathogens (A. W. Aruni et al., 2014). Aside from the ability to enhance biofilm formation and adhesion and invasion, *F. alocis* has been implicated in the degradation of periodontal tissue (Y. Li et al., 2014). *In vitro* studies have also indicated that the pathogen stimulates the production of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α (A. W. Aruni et al., 2014; Chen et al., 2015a).

Virulence attributes of *Filifactor alocis*

Bacterial Migration from the site of infection

In a study conducted by Wang and colleagues (Q. Wang et al., 2014), it was shown that *F. alocis* can migrate from the site of infection. A mouse subcutaneous chamber model was used, whereby a titanium ‘chamber’ was implanted into eight to ten-week-old female nc57BL/6 mice and subsequently infected with *F. alocis* at 10^9 CFU in 100µl PBS. Two hours post-infection, bacterial loads were between 10^5 to 10^6 ; these levels were reduced after 72 hours. FISH analysis revealed easily detectable levels of *F. alocis* in the liver, lung and kidney of the subject at 2, 24 and 72 hours post-infection, leading to the discovery that *F. alocis* colonized in the organs of test subjects. Repeated experiments yielded similar results which were corroborated by the use of PCR. These results indicated that *F. alocis* could exit the chamber and enter surrounding organs indicating the ability of the pathogen to migrate from the site of infection.

Recently, *F. alocis* has been found outside the oral cavity. A sixty-five-year-old male with a history of cerebrovascular disease was admitted to the hospital for acute shortness of breath. Testing showed the patient to have a pleural effusion. Pleural fluid was collected and cultured after which gram staining showed the organism to be *F. alocis* (Gray & Vidwans, 2019). This strengthens the conclusion from the mouse study, and indicates that *F. alocis* can also migrate from the site of infection in humans (Q. Wang et al., 2014).

Biofilm Formation

Bacterial populations are unable to survive in a planktonic state and therefore tend to form biofilms. In periodontal disease, mixed species biofilms are formed and exist in the form of plaque on the tooth surface. Research has indicated that the onset of periodontal disease is the result of the proliferation of biofilm-forming species such as *P. gingivalis*, *P. intermedia*, *T. Forsythia*, *T. denticola* and more recently *F. alocis*. To understand the complex pathogenesis of periodontal disease, it has become increasingly necessary to do more than simply isolate and characterize these bacteria from diseased sites. Organisms involved in periodontitis live as a sessile community attached to the surface of the tooth in a matrix of extracellular polymers (Donlan and Costerton 2002). Exploration of the biofilm architecture is essential in periodontal research, and *F. alocis* has been experimentally linked to the co-aggregation of periodontal biofilms (Schlafer et al. 2010). Using 490 patient samples from patients with Generalized Aggressive Periodontitis (GAP) and Chronic Periodontitis (CP) and Periodontitis Resistant individuals, it was found that *F. alocis* was more prevalent in GAP and CP than *P. intermedia* and *A. actinomycetemcomitans*. *F. alocis* was also prevalent in 77.8% of Generalized Aggressive cases and 76.7% of Chronic cases. These results were significant when compared to periodontitis resistant patients, who showed less than 20% prevalence of the pathogen. The study also highlighted that the prevalence of *F. alocis* increased as the depth of the periodontal pocket increases, indicating that as disease progresses so does the growth of the pathogen. In patients suffering from GAP and CP, *F. alocis* colonizes the area of the gingival pocket close to the soft tissue where an immune response can be easily initiated (Schlafer et al. 2010). Symbiotic relationships

between periodontopathogens may contribute to the fastidious nature of *F. alocis* (Schlafer et al. 2010). It has also been shown that *P. gingivalis* ATCC 33277 in co-culture with *F. alocis* showed a significant increase in biofilm formation, which may indicate a commensal relationship between *F. alocis* and *P. gingivalis*. This can also be observed in the ability of the organism to auto-aggregate and express unique characteristics (A. Wilson Aruni et al., 2011). A study by Wang et al evaluated the community interactions of *F. alocis* with *S. gordonii*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans*, and suggests that *F. alocis* likely interacts with a variety of oral bacterial and participates in community development (Wang et al, 2013)

***F. alocis* can escape the complement system**

The complement system is an immune response system comprising of a variety of plasma proteins and is activated by the onset of pathogenic infections which induces an inflammatory response to facilitate the killing and clearance of the infectious agents. In Schlafer and colleagues 2010 study it was observed that *F. alocis* was attached in areas around soft tissue where it is in contact with host immune factors and can promptly initiate an immune response(Schlafer et al., 2010b). One such immune response is the activation of the complement system.

A 2016 study conducted by Blom et al (Jusko et al., 2016b) uncovered the novel *F. alocis* FACIN protein. FACIN is known to inhibit complement by binding to C3 and suppressing the activity of all complement pathways (Jusko et al., 2016a). In the experiments conducted, FACIN significantly inhibited both the classical and alternative pathways. These results were similar to that obtained by its positive control DAF (Jusko

et al., 2016a). It was further determined that FACIN can counteract the normal bactericidal activity of human serum against invading pathogens. It was also shown that FACIN reduces the activity of the alternative pathway convertase in interactions between C3 and the *F. alocis* surface (Jusko et al., 2016a). An increase in complement evasion was also observed when *F. alocis* is in a biofilm. These findings indicate that despite *F. alocis* being found in soft tissue where it can initiate an immune response, it has developed mechanisms to evade the very host response it activates (Jusko et al., 2016a). The evasion mechanism may not only be beneficial to *F. alocis* but also to other organisms involved in periodontal disease. This can be observed in a biofilm population whereby the presence of *F. alocis* increases the survival of organisms such as *P. gingivalis*. This is indicative of *F. alocis* role in the onset and progression of periodontal disease.

Effects of *F. alocis* on the Immune system

A key component of the immune system is neutrophils, which are a class of white blood cells (granulocytes and phagocytes) and are the first to migrate to a site of infection. This makes neutrophils the first line of defense for the human immune system. Neutrophils fight infection by engulfing invading microorganisms and releasing bactericidal enzymes. It was demonstrated that *F. alocis* possesses neutrophil-activating abilities and can elicit the release of TNF-alpha, IL-8 beta and IL-1a (Vashishta et al., 2019), upon bacterial challenge along with the periodontopathogens *P. gingivalis* and *P. stomatis*, however this response was not of the same magnitude as its counterparts *P. stomatis*. It was further shown that *F. alocis* and *P. stomatis* induced the TLR2/TLR6

heterodimers more than the TLR2/TLR1 heterodimers. These heterodimers are formed when *F. alocis* interacts with neutrophils, resulting in the induction of the MAPK pathway and TLR2-dependent neutrophil granule exocytosis (Vashishta et al., 2019). *F. alocis* promotes neutrophil degranulation and chemotactic activity, can impact the migration and infiltration to the site of infection (Armstrong et al., 2016) and increase random migration of neutrophils, which promotes damage to host cells (Armstrong et al., 2016). Degranulation of neutrophils in a TLR2-dependent manner also activates the ERK and p38 MAPK pathways (Armstrong et al., 2016), and the activation of TLR2 can lead to the induction of neutrophil function such as the activation of neutrophil granule exocytosis (Armstrong et al., 2016). The blocking of this neutrophil granulation was also shown to inhibit *F. alocis*-dependent neutrophil cell migration.

Neutrophil extra-cellular traps (NETS), are extracellular fibers generated by neutrophils and have constituents that neutralize bacteria extracellularly (Armstrong et al., 2018; Brinkmann et al., 2004). Gram-positive oral pathogens such as *P. stomatis* and *S. gordonii* have been shown to successfully induce the release of NETs upon infection along with the generation of ROS and neutrophil granulation (Armstrong et al., 2016). *F. alocis* do not affect pre-formed NETs based on their inability to initiate their degradation and did not inhibit the formation of NETs by other oral pathogens. However, the extracellular vesicles of *F. alocis* produced multiple proteins including lipoproteins, and SCIN, which are believed to aid in its virulence mechanism and to have an immunostimulatory effect on host tissue (Kim, Lim, An, & Choi, 2020). *F. alocis* can also stimulate the production of Matrix Metalloprotease-1 in host cells upon infection and increases the expression of MMP-1 via the TLR-2 pathway (Nokhbehshaim et al., 2019).

The studies conducted present *F. alocis* as a key player in the onset and proliferation of periodontal disease.

***F. alocis* activates cytokine production in GECs**

The gingival crevice is lined with epithelial cells which are a part of the first host cells that the pathogen encounters. These gingival epithelial cells also have effectors of innate immunity such as pro-inflammatory cytokines and can elicit an immune response (Moffatt, Whitmore, Griffen, Leys, & Lamont, 2011; Q. Wang et al., 2014). A study conducted by Moffatt and colleagues showed that upon treatment of GECs with *F. alocis* there was a production of pro-inflammatory cytokines (Moffatt et al., 2011). After 24hrs of treatment, there was an increase in the production of IL-1B which gradually increased over 48hrs. IL-6 was also produced upon infection and 24hrs post-treatment, its production was 4-fold more than observed in the control (Moffatt et al., 2011). A significant increase in the levels of TNF-alpha was observed 24hrs post-infection and continuously increased for up to 48hrs. However, no change in the levels of IL-8 after infection was noted (Moffatt et al., 2011). These results indicated that *F. alocis* may selectively induce a pro-inflammatory response from GECs. *F. alocis* infection may also result in cell death via apoptosis. At 24 hrs post-infection, *F. alocis* induced 50% apoptosis in GECs, and at 48 hrs, the apoptosis rate increased to 88% (Moffatt et al., 2011). The *F. alocis*-induced apoptosis of GECs occurred extrinsically via the caspase-3 pathway (Moffatt et al., 2011). The pro-apoptotic activity also occurs via the inhibition of MEK1/2, which prevents the phosphorylation and subsequent activation of the MAPK pathway, which is required for cell proliferation and differentiation (Moffatt et al., 2011).

These studies indicate that *F. alocis* can activate the immune system by producing pro-inflammatory cytokines. They also suggest that the pathogen can survive within the host cell.

Proteases

Proteases are key virulence factors present in periodontopathogens and are involved in post-translational regulation of genes, gene expression and the processing of proteins. Proteases such as the gingipains of *P. gingivalis* plays a role in virulence (A Wilson Aruni et al., 2015). The *F. alocis* genome contains a multitude of proteases that exhibit characteristics like those of other periodontopathogens. However, in *F. alocis* the role of these proteases are yet to be characterized and they may be implicated in tissue destruction and the inactivation of host immune response (A Wilson Aruni et al., 2015).

Collagenase

Collagen is known to be the most abundant structural protein (Shoulders & Raines, 2009). It is one-third of the protein content of humans and a great portion of the dry weight of skin, nails and hair (Kadler, Holmes, Trotter, & Chapman, 1996; Lee, Singla, & Lee, 2001; Shoulders & Raines, 2009) and is also present in organs and tissues. The strength and stability of collagen and its supreme biocompatibility and weak antigenicity make it excellent for biomedical applications (Lee et al., 2001). Collagens may be classified as Fibril associated collagens (FACITs), Membrane-associated collagens (MACITs) and Multiplexins (Shoulders & Raines, 2009). Collagen has also been implicated in wound healing after injuries and in the body's immune system, aiding

in protection against microbial invasion (Kadler et al., 1996). Periodontal tissue has collagen, which serves as a protective barrier against invading pathogens, however, some pathogenic bacteria possess collagenases that hydrolyze collagens, facilitating bacterial invasion and disease progression (Chioma, Aruni, Milford, & Fletcher, 2017). In vitro studies of PrtFAC, a protein with collagenase activity in *F. alocis*, demonstrates the ability to bind and degrade Type1 collagen (Chioma et al., 2017). This observed collagenase activity is indicative of PrtFAC playing a possible role in the invasion and adhesion ability of *F. alocis* (Chioma et al., 2017). The collagenase-like behavior of the protein is advantageous to the pathogen's survival to an immune response and in the periodontal pocket (Chioma et al., 2017). The study also showed that normal oral keratinocytes treated with PrtFAC experience a surge in apoptotic activity, with the initiated apoptosis being both caspase dependent and independent (Chioma et al., 2017). This study is suggestive of collagenase enhancing the virulence capacity of *F. alocis*.

Oxidative Stress

The protective systems of bacteria against oxidative stress require the synergistic involvement of multiple systems to allow for the timely response to the changing levels of Reactive Oxygen Species in both the intracellular and extracellular environments (W. Aruni et al., 2014; Henry et al., 2014). Studies examining the oxidative stress machinery of *F. alocis* have been conducted by our laboratory, however further investigation is required to fully elucidate the mechanism by which the pathogen combats oxidative stress. Our lab has shown thus far that *F. alocis* is relatively more resistant to oxidative stress than its counterpart *P. gingivalis* (A. Wilson Aruni et al., 2011). It is suggested that

F. alocis may aid in the survival of other periodontopathogens in the oral microbiome in the presence of oxidative stress. It has also been speculated that *F. alocis* may function as an “oxidative sink” aiding in oxidative stress resistance (W. Aruni et al., 2014; Henry et al., 2014). Preliminary data obtained by our lab indicates that in H₂O₂-induced oxidative stress, the survival of *P. gingivalis* is enhanced in the presence of *F. alocis* (W. Aruni et al., 2014). In studies of co-culture between *P. gingivalis* and *F. alocis*, an upregulation of proteins known to be involved in oxidative stress was observed. Among these genes were superoxide reductase, iron-sulfur cluster, thioredoxin and ferrous hydrogenase family, and SOR (Arunima, Ezinne, & Fletcher, 2020; Henry et al., 2014). Sialidase activity reported in *F. alocis* may aid in virulence by scavenging ROS, reducing the oxidative stress in the periodontal pocket (Henry et al., 2014). RNA seq. analysis was used to compare the regulation of genes of *F. alocis* in monoculture as opposed to in co-culture with *P. gingivalis*, under H₂O₂-induced oxidative stress. There was ~21% change in gene regulation, with an upregulation of genes believed to be involved in oxidative stress resistance (Arunima et al., 2020). Recently, another gene, FA796 has been studied and designated as a superoxide reductase (SOR). This is the first published deletion mutant in *F. alocis*. Characterization of this mutant determined that it was sensitive to air exposure, and it was more sensitive to superoxide radicals and H₂O₂ induced oxidative stress than the wild-type strain of the pathogen. The data obtained is indicative of FA796 playing a key-role in the survival of *F. alocis* in oxidative stress conditions (Arunima et al., 2020). Along with FA796, we also examined FA519, which exhibited peroxidase activity and the ability to protect DNA from damage (Aja, Mishra, Dou, & Fletcher, 2021).

Invasion and Adhesion

Pathogenic success in infection relies on its success at invading into and adhering to host cells (A Wilson Aruni et al., 2015). It has been shown that *F. alocis* invades and adheres to epithelial cells such as Normal Oral Keratinocytes (A Wilson Aruni et al., 2015). The invasive and adhesive ability of *F. alocis* was determined to be bolstered by co-infection with its counterpart *P. gingivalis*. Monoculture and co-culture studies using *F. alocis* and *P. gingivalis* were conducted and results obtained indicated that in monoculture, *F. alocis* was 10-15% less adherent to epithelial cells than *P. gingivalis*. However, co-culture adhesion increased by 22% (A. W. Aruni et al., 2011). Invasion studies determined that *F. alocis* possesses a mere 25% of the invasion capacity of *P. gingivalis*, however, the invasion was significantly increased in co-culture with *P. gingivalis* (A. Wilson Aruni et al., 2011). The mechanism of invasion and adhesion is still being ascertained. Studies have indicated that filopodial projections on host cells upon co-infection with *F. alocis* and *P. gingivalis*, along with vessel internalization may play a key role in adhesion and invasion (A Wilson Aruni et al., 2015). Proteomic analysis has also observed an increase in a multitude of Membrane Adhesion Proteins and Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (A Wilson Aruni et al., 2015). The MSCRAMMs may play an integral role in the pathogens invasive and adhesive abilities. Caax amino peptides have also been suggested as facilitators of invasion into epithelial cells (A Wilson Aruni et al., 2015). It is evident that there may be several mechanisms involved in successful pathogenic invasion and adhesion, many of which are yet to be determined.

Bacterial mechanisms against oxidative stress

Microbial-induced host responses result in the generation of reactive oxygen species such as hydrogen peroxide and hydroxyl radical (Sczepanik et al., 2020). Reactive oxygen species can lead to the damage and destruction of nucleic acids, lipids and proteins, making it detrimental to bacterial survival. Thus, bacteria, have evolved overtime, developing mechanisms to scavenge ROS. These mechanisms include catalase, peroxidase bacterioferritin comigratory protein (BCP), ruberythrin, sortase and alkyl hydroperoxidase reductase enzyme system (AhpC/AhpF) (Aja, Mangar, Fletcher, & Mishra, 2021; Arunima et al., 2020; Mishra & Imlay, 2012). Along with ROS scavenging mechanisms, bacteria also employ mechanisms to minimize the damage caused by ROS and contaminating metals (Farr & Kogoma, 1991), such as the interaction between Fe^{2+} and hydrogen peroxide. These include iron-sulfur cluster proteins, ferritins, bacterioferritins, DPR and DPS proteins, which play a role in iron sequestration (Bellapadrona, Ardini, Ceci, Stefanini, & Chiancone, 2010a; Pulliainen, Kauko, Haataja, Papageorgiou, & Finne, 2005).

DNA Starvation and Stationary Phase Protection Protein (DPS)

DNA Starvation and Stationary Phase Protection Protein (DPS) proteins are a member of the Ferritin superfamily which includes DPR proteins, ferritins and bacterioferritins and are found across a variety of prokaryote species (Tseng et al., 2019). DPS proteins were first observed in 1992 when they were isolated from *E. coli* in stationary phase by Roberto Kolter, et al (Chiancone & Ceci, 2010b). DPS proteins are approximately 16-20 KDa in size and have a dodecameric (12-mer) tertiary structure.

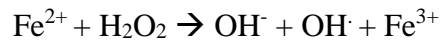
These proteins have an external diameter of 80-90 angstrom and 40-50 angstrom internally (Bellapadrona, Ardini, Ceci, Stefanini, & Chiancone, 2010b). They are formed by 12 identical 4-helix bundle monomers, that assemble into spherical hollow structures endowed with 2:3 symmetry (Bellapadrona et al., 2010b). Across species, DPSs have been shown to have highly conserved folds, however, these proteins possess varying functions beside DNA protection. These functions include virulence protection from multiple stressors such as ionizing radiation, heat and cold shock (Haikarainen & Papageorgiou, 2010). These proteins have also been found to be involved in inflammatory responses upon infection (Haikarainen & Papageorgiou, 2010). Studies have indicated that DPS proteins may have applications in the fields of biotechnology and nanotechnology and their discovery and further study can lead to the advancement in science and technology (Haikarainen & Papageorgiou, 2010). DPS proteins, also referred to as DNA Binding Proteins from starved cells are known to (Tseng et al., 2019) protect DNA under starvation and stress conditions by one of the following mechanisms: 1) binding to DNA forming DPS-DNA complexed (Haikarainen & Papageorgiou, 2010); or 2) binding to and oxidizing iron from its ferrous to ferric form (Haikarainen & Papageorgiou, 2010; Tseng et al., 2019).

Role of DPS in DNA Binding

DNA binding is whereby DPS proteins bind to DNA molecules producing DPS-DNA complexes during stress and starvation. Studies to analyze the phenomena and the mechanism by which it occurs were conducted in the presence of Fe^{2+} and hydrogen

peroxide. This reaction system was utilized since it was shown that the Fenton reaction generates hydroxyl radicals, and it is these radicals that are responsible for DNA damage.

The reaction is as follows:



The formation of DNA-DPS complexes is due to the interactions between the negatively charged phosphate backbone of DNA and the N-terminus of the protein (Bellapadrona et al., 2007). It is believed that it is the positively charged lysine residues of the N-terminus of the protein that aid in the coordination of this interaction. Studies conducted using the *E. coli* DPS, have seemingly confirmed that the three positively charged lysine residues in its N-terminus play a role in DNA binding (Bellapadrona et al., 2007). Site-directed mutants of the *E. coli* DPS decreased DNA binding capabilities (Bellapadrona et al., 2007), indicating that lysine is involved in DNA binding. Lysine's involvement maybe due to its positive charge, an increase in the pH of the protein leads to the deprotonation of lysine, altering its charge and hence decreasing its affinity for the negative phosphate backbone of DNA and allowing the formation of DPS-DNA complexes (Bellapadrona et al., 2010b; Bellapadrona et al., 2007). DNA binding with no condensation occurred when two of the three lysine residues were deleted (Chiancone & Ceci, 2010a). Deletion of the three lysine residues lead to the complete inactivation of the protein's ability to bind to DNA (Chiancone & Ceci, 2010a). The mobility of the N-terminus of DPS proteins have also been implicated in the binding of DNA in stress conditions and may be just as important as the charge gradient of the lysine residues present on the N-terminus (Chiancone & Ceci, 2010b; Zhao et al., 2002). The DPS protein in *A. tumefaciens* also has three lysine residues present in its N-terminus but is unable to interact with DNA due to

the immobilization of its N-terminus (Chiancone & Ceci, 2010a). This immobilization is a result of salt bridge formation and hydrogen bonding on the surface of the dodecameric form of the protein (Chiancone & Ceci, 2010a). The reduction of DPS was also observed in *L. lactis* and may be attributed to a reduction in the mobility in the N-terminus region. Moreover, the N-terminus of DPS proteins have been implicated in DNA binding and condensation (Chiancone & Ceci, 2010a).

However, there is an exception to the phenomenon of N-terminus DNA binding of DPS proteins. In *M. smegmatis*, it was observed that its DPS has a truncated N-terminus and DNA binding is instead coordinated by its C-terminus (Ceci, Ilari, Falvo, Giangiacomo, & Chiancone, 2005).

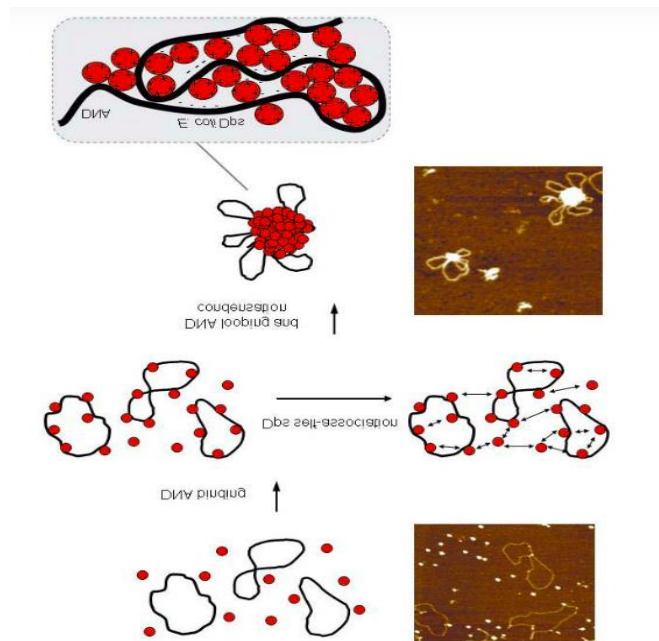


Figure 1.4. Showing the formation of DPS-DNA complexes. Image sourced from (Chiancone & Ceci, 2010b)

Role of DPS in Iron Binding

As previously mentioned, in the presence of iron and oxidants such as oxygen or hydrogen peroxide reactive species are generated, and these ROS can impede the integrity of lipids, proteins, DNA, RNA and other membrane structures (Henry et al., 2014; Ortiz de Oru  Lucana, Wedderhoff, & Groves, 2012). DPS proteins have been shown to bind and oxidize iron, reducing the likelihood of DNA damaging occurring.

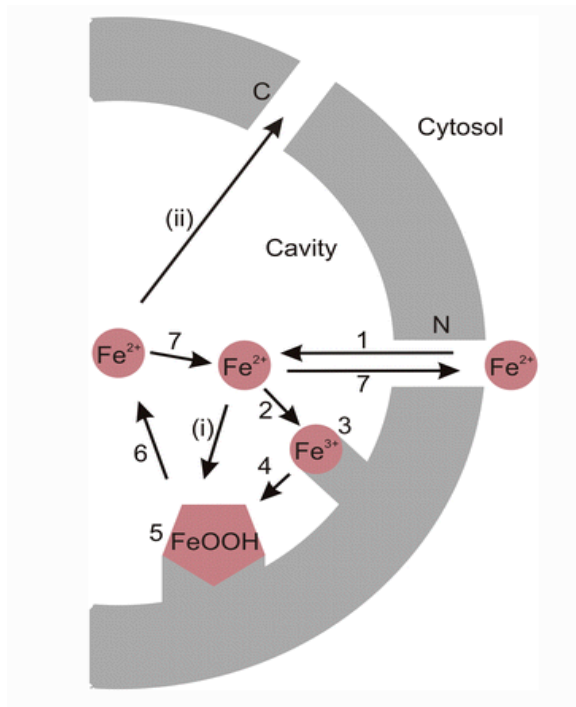


Figure 1.5. Showing the binding of iron to the DPS iron binding site. Image sourced from (Haikarainen & Papageorgiou, 2010)

Iron enters the dodecamer, it then binds to the ferroxidase center of the protein and is oxidized from its 2^+ oxidation state to 3^+ (Haikarainen & Papageorgiou, 2010). The oxidized iron is nucleated forming a FeOOH core, where it is stored and then mineralized. If needed, this oxidized iron can be reduced to its 2^+ oxidation state to be utilized by the cell (Haikarainen & Papageorgiou, 2010). DPS uses H_2O_2 as its oxidant rather oxygen, which is utilized by ferritins. Studies have shown that DPS can oxidize

iron 10^2 - 10^3 fold more, when H_2O_2 is used as the oxidant (Bellapadrona et al., 2010b). The internal cavity of DPS proteins where iron is stored after oxidation has both hydrophilic and hydrophobic channels which connect to the external medium (Bellapadrona et al., 2010b). These channels allow iron to enter the protein's shell to gain access to the ferroxidase center of the protein (Bellapadrona et al., 2010b). The functional domain is the ferroxidase center, which has two binding sites called A and B. Of these sites, A has a higher affinity for iron (Bellapadrona et al., 2010b). In *E. coli*, the affinity of iron to the ferroxidase center is due to a lysine residue, which forms a salt bridge with the aspartic acid residue, facilitating iron coordination (Chiancone & Ceci, 2010a). In *D. radiodurans* the iron-binding ligands in its DPS2 are a His⁷⁰, Glu¹⁰¹ and Asp⁷⁰ (Reon, Nguyen, Bhattacharyya, & Grove, 2012). Try⁷¹ is a conserved residue that aids in preventing the release of the byproducts of radicals from the ferroxidase center by capturing free electrons present (REON et al., 2012). It is evident from the literature that iron binding is coordinated by specific amino acid residues present in the ferroxidase site of the protein (Bellapadrona et al., 2010b; Bellapadrona et al., 2007; REON et al., 2012). . However, despite the presence of conserved residues in the functional domain of these proteins, each DPS has some variation in the amino acids responsible for coordinating iron binding (Bellapadrona et al., 2010b; Bellapadrona et al., 2007; Reon et al., 2012).

Aims

To survive and even thrive in the presence of Reactive Oxygen Species, *F. alocis* must detoxify these toxic elements. *F. alocis* possesses the hypothetical protein FA1654 which has DPS-like activity and can possibly protect DNA in the presence of Fenton reaction-mediated oxidative stress. DPS proteins are known to provide DNA protection by either one or a combination of the following mechanisms binding to DNA forming DPS-DNA complexes, binding to iron, or detoxifying hydrogen peroxide. This protection allows for the pathogen to survive under oxidative stress. In this study, we examine the role of FA1654 in the mechanism of virulence of *F. alocis*. The study aims to characterize the function of FA1654 and determine the role it plays in the oxidative stress resistance. Understanding the role of this gene/protein may provide insight in to the mechanism of action of *F. alocis* in the onset and progression of periodontal disease.

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Characterization of FA1654: a putative DPS protein in *Filifactor alocis*

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Summary

The survival/adaptation of *Fillifactor alocis*, a fastidious Gram-positive asaccharolytic anaerobe, to the inflammatory environment of the periodontal pocket requires an ability to overcome oxidative stress. Moreover, its pathogenic characteristics are highlighted by its capacity to survive in the oxidative-stress microenvironment of the periodontal pocket and a likely ability to modulate the microbial community dynamics. There is still a significant gap in our understanding of its mechanism of oxidative stress resistance and its impact on the virulence and pathogenicity of the microbial biofilm. Coinfection of epithelial cells with *F. alocis* and *P. gingivalis*, resulted in the upregulation of several genes including HMPREF0389_01654(FA1654). Bioinformatics analysis indicates that FA1654 has a “di-iron binding domain”, and could function as a DNA Starvation and Stationary Phase Protection (DPS) protein. We have further characterized the FA1654 protein to determine its role in oxidative stress resistance in *F. alocis*. In the presence of hydrogen peroxide induced oxidative stress there was a ~1.3-fold upregulation of the *FA1654* gene in *F. alocis*. Incubation of the purified FA1654 protein with DNA in the presence of hydrogen peroxide and iron resulted in the protection of the DNA from Fenton-mediated degradation. Circular Dichroism (CD) and Differential Scanning Fluorimetry (DSF) studies have documented the intrinsic ability of rFA1654 protein to bind iron, however the rFA1654 protein is missing the intrinsic ability to reduce hydrogen peroxide. Collectively, the data may suggest that FA1654 in *F. alocis* is involved in oxidative stress resistance via an ability to protect against Fenton-mediated oxidative stress-induced damage.

1 | Introduction

Bacteria in the periodontal pocket are consistently exposed to oxidative stress (Ortiz de Orué Lucana et al., 2012). Oxidative stress can be described as a disruption in the balance of reactive oxygen species (ROS; e.g., superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\bullet$) and nitric oxide (NO)) in the environment, and biological systems must have some mechanism to cope with this dysregulation (Ortiz de Orué Lucana et al., 2012; Sczepanik et al., 2020) (Aja, Mishra, et al., 2021). ROS are generated both as a result of immune responses such as phagocytosis, triggering the activation of NADPH oxidase (DeLeo, Allen, Apicella, & Nauseef, 1999) to fight pathogenic infections, and as a result of aerobic metabolism (Aja, Mishra, et al., 2021; Sczepanik et al., 2020).

Oxidative stress can be detrimental to the survival of organisms and can impede the integrity of lipids, proteins, DNA and RNA and other membrane structures (Henry, McKenzie, Robles, & Fletcher, 2012; Ortiz de Orué Lucana et al., 2012). Bacterial species however have evolved and developed sophisticated regulatory, repair and protective mechanisms to enable the detection of oxidative stress and produce an effective and efficient response to mediate any damage that may ensue. One such mechanism is DNA Starvation and Stationary Phase Protection protein (DPS) and DPS like proteins (Bellapadrona et al., 2010b; Karas, Westerlaken, & Meyer, 2015; Pulliainen et al., 2005; Tseng et al., 2019).

DPS and DPS-like proteins (DPR) are members of the Ferritin super family and protect DNA from oxidative stress damage (Chiancone & Ceci, 2010a; Pulliainen et al., 2005). DPS and DPS-like proteins are dodecamers, with a four-helix structure (Bellapadrona et al., 2010b). Protection against oxidative stress-induced DNA damage by these proteins

occurs via two mechanisms. DNA binding provides physical protection for DNA from damaging agents such as ROS. Chemical protection is provided via the proteins' ferroxidase center, which may lead to the oxidation of iron from its Fe²⁺ to Fe³⁺ state, using hydrogen peroxide as the oxidizing agent.

Fillifactor alocis is a gram-positive, rod-shaped obligate anaerobe. Upon its initial isolation from the gingival sulcus in 1985, *F. alocis* was classified as *Fusobacterium* species, to be later reclassified as *Fillifactor sp.* in 1999 (Aja, Mangar, et al., 2021; Cato et al., 1985; Moffatt et al., 2011). *F. alocis* is present at a higher extent than the “Red complex” species; *Porphyromonas gingivalis*; the keystone pathogen, *Treponema denticola* and *Tannerella forsythia* ((W. Aruni et al., 2014; Chen et al., 2015a; G. Hajishengallis & Lamont, 2012); in patients with periodontal disease, whilst being relatively undetected in healthy patients. This implies it plays a role in the onset of periodontal disease. Periodontal disease is an inflammatory disease affecting the integrity of the supporting structures of the teeth and is characterized by the destruction of periodontal tissue and loss of alveolar bone (Aja, Mangar, et al., 2021; Moffatt et al., 2011). Periodontal disease has been linked to systemic diseases such as diabetes, obesity, pre-termed labor and Alzheimer's (Bascones-Martinez et al., 2014; Kuo et al., 2008). *F. alocis* has been shown to increase the resistance of oral pathogens such as *P. gingivalis* to oxidative stress. A previously conducted study by our lab demonstrated an increased resistance above four-fold in *P. gingivalis* to hydrogen peroxide-induced oxidative stress in co-culture with *F. alocis* (Aja, Mishra, et al., 2021; A. W. Aruni et al., 2011; A. Wilson Aruni et al., 2012), along with increased invasion and adhesion abilities of epithelial cells, TGIKs and NOKs (Aja, Mishra, et al., 2021; A. W. Aruni et al., 2014;

Arunima et al., 2020). Recently a superoxide reductase (SOR) along with a multifunctional protein with peroxidase and disulfide oxo-reductase activity, FA519, have been determined to play a role in shielding *F. alocis* from oxidative stress (Aja, Mishra, et al., 2021; Arunima et al., 2020).

Despite the progress in understanding the virulence mechanism of *F. alocis*, a gap in our understanding of the role of other genes in the oxidative stress resistance mechanism of *F. alocis* still exists. In a co-infection study of epithelial cells with *F. alocis* with *P. gingivalis* several genes were upregulated, one of which was *FA1654*, this gene encodes for the hypothetical protein FA1654 (A. Wilson Aruni et al., 2012). Bioinformatics analysis of FA1654, a hypothetical protein with DPS-like function in *F. alocis*, has shown that the protein has a putative di-iron binding domain and is approximately 16kDa in size. In this study, we characterize FA1654 to determine its role in oxidative stress resistance and the virulence mechanism of the pathogen.

2 | Materials and Methods

2.1 | Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *F. alocis* strain ATCC 35896 was cultured in Brain Heart Infusion (BHI) broth supplemented with 0.5µg/ml vitamin K, 5µg/ml hemin, 0.1% cysteine and 100µm arginine. *F. alocis* was grown anaerobically at 37°C and maintained in an anaerobic chamber (Coy Manufacturing in 10% H₂, 10% CO₂ and 80% N₂). TOP10 and BL21 *E. coli* strains were cultured in Luria-Bertani (LB) broth supplemented with 100µg/ml ampicillin at 37°C.

Growth rates of bacterial cultures were determined by measuring the optical density at 600nm (OD₆₀₀).

2.2 | qPCR analysis

Primers generated for the gene of interest are listed in Table 2. The amplification of the genes of interest was performed with the SYBR green kit (Qiagen) and the signal detection for real-time fluorescence was done via the Cepheid Smart Cycler Real Time PCR device. The cycles are as follows: 95°C for 15min, 40 cycles of 94°C for 15sec, 54°C for 30 secs and 72°C for 30 secs. Measurements were done in triplicate and 16S RNA was utilized as an internal control (Dou et al., 2014).

2.3 | Bioinformatics Analysis

The nucleotide sequence of the *FA1654* (from *F. alocis* ATCC 35896) and BLAST analysis was retrieved from the NCBI database (Arunima et al., 2020). The structural predictions of the protein was done using SWISS-MODEL(<https://swissmodel.expasy.org/>) (Arunima et al., 2020) and the I-tasser database (<https://zhanglab.dcmf.med.umich.edu/I-TASSER/>)

2.4 | Cloning, expression and Purification of r-FA1654

To characterize FA1654, the *FA1654* gene was amplified from *F. alocis* wildtype genomic DNA, using PCR. The amplified gene product was then cloned into pET 102/D-TOPO vector using the TOPO expression kit from Life Technologies. The pET 102/D-TOPO vector with the *FA1654* gene was then transformed into *E. coli* BL-21 cell. BL-21

cells were cultured in LB broth supplemented with 100mg/ml ampicillin and grown overnight in a 37⁰C shaker. At an OD of 0.6, 0.5mM IPTG; was used to induce the expression of the protein, and the culture was grown for an additional four hours. The culture was then centrifuged. The pellet was collected and re-suspended in lysis buffer (150mM NaCl, 50mM Tris buffer, pH 7.5, 10mM imidazole) and mechanically lysed using a French press. The 'lysate' was then transferred to a nickel column to allow for the elution of the protein. The protein was then collected and dialyzed in EQ buffer with 10% glycerol at 4⁰C for 16 hours. Dialysis was repeated once more (Arunima et al., 2020).

2.5 | DNA Protection Assay

Purified FA1654 (2-8 μ m final concentration) was incubated with DNAJ, FA1654 and RNA Polymerase B (DNA template) in Tris buffer (20mM, pH 7.8), FeSO₄ (50 μ M) and deionized water for 5 minutes at room temperature. 88-264mM of H₂O₂, as necessary, was then added to the reaction mix and further incubated for 30 minutes at room temperature. 10X loading dye was then added to the reaction and subsequently resolved via agarose gel electrophoresis using a 1% agarose gel stained with ethidium bromide (Karas et al., 2015).

2.6 | DNA Binding Assay

Purified FA1654 (2-8 μ m final concentration) was incubated with pUC19 plasmid DNA in Tris buffer (20mM, pH 7.8), FeSO₄ (50 μ M) and deionized water for 5 minutes at room temperature. 88-264mM of H₂O₂, as necessary, was then added to the reaction mix and further incubated for 30 minutes at room temperature. 10X loading dye was then added to

the reaction and subsequently resolved via agarose gel electrophoresis using a 1% agarose gel stained with ethidium bromide (Karas et al., 2015).

2.7 | H₂O₂ detection using the 3,5,3'5'-tetramethylbenzidine (TMB) Assay

A typical colorimetric analysis was performed as follows: first, a fresh 10mM stock of H₂O₂ and FeSO₄ were prepared. The FA1654 samples (0.2mM) were then incubated with 2mM FeSO₄, 1mM H₂O₂ and 0.7mM TMB in 0.1M sodium phosphate buffer (pH 6.0) for 15 minutes at room temperature. The reaction was then terminated by the addition of 50ul 0.5M sulphuric acid (Rhee, Chang, Jeong, & Kang, 2010). Finally, the reaction mixture was used for absorption spectroscopy measurement at 450nm via a SpectraMax® i3xPlate reader with the SoftMax® Pro 6 (version6.5.1) software.

2.8 | Circular Dichroism Analysis

CD spectra were recorded in an OLIS CD spectrophotometer (OLIS, Athens, GA). A quartz cuvettes with a path-length of 0.02 cm was used. The protein concentration was 0.8 mg/ml. The CD cell holder was kept at 15⁰C using an external water bath. Two consecutive CD spectra were recorded from 260 to 195 nm for each sample with an integration time of 8 sec per nm. Buffer baselines were recorded at the same temperature and subtracted from the protein spectra. The two protein spectra were averaged and concentration-normalized to obtain molar ellipticity. The secondary structural content was determined using the CDNN software (CDNN: CD Spectra Deconvolution, Version 2.1, Universität Regensburg - Böhm, 1997).

2.9 | Differential Scanning Fluorimetry

The Thermal unfolding temperature (T_m) of the protein with or without iron sulfate was determined using the Prometheus NT.48 NanoDSF instrument (NanoTemper Technologies, LLC, South San Francisco, CA) with 48 capillary chambers. Each sample was excited at 290 nm, and emission was detected simultaneously at 330 and 350 nm. The samples were heated from 15 to 95°C using a constant heating rate of 3°C/min. The first derivative of the fluorescence signal at 350 nm relative to 330 nm (F350/F330) versus temperature produced a bell-shaped thermal unfolding peak. The midpoint of the peak corresponded to the T_m . The T_m of each sample was automatically determined by the built-in analysis software and tabulated in an Excel output file.

3 | Results

3.1 | *F. alocis* HMPREF0389_01654 (FA1654) encodes for a hypothetical protein with an iron binding domain

The genome of *F. alocis* carries the FA01654 gene, which is 437 base pairs in length and encodes for a 145-amino acid hypothetical protein that is approximately 16.7kDa in size. (<https://www.ncbi.nlm.nih.gov/protein/504028302>) (Fig. 1A). Protein modeling via the I-TASSER software indicates that this protein has a predictive iron binding site at amino acid positions 15,48,51,100,133 and 136 (Fig. 1B and 1C). SWISS-MODEL indicates the protein has a dodecameric tertiary structure (Fig.1D). There were no predicted DNA binding domains in FA1654. The amino acid sequence blast showed that FA1654 is annotated as a putative DPS protein that has identity of 62.24%, 58.04%, 47.92% to *Erysipelotrichaceae bacterium*, *Treponema pedis*, *Fusobacterium spp.* respectively. The

E. coli purified recombinant FA1654 *E. coli* was resolved on an SDS-PAGE gel as an ~33.7 kDa Thioredoxin-His tag fusion protein (Fig. 1E).

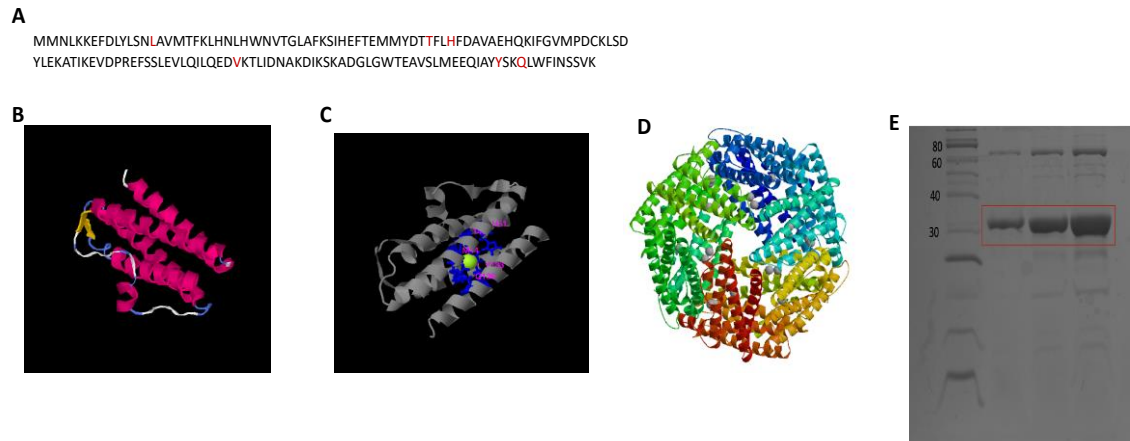


Fig 2.1. In silico analysis of FA1654, a putative DPS protein. (A) Amino acid sequence of FA1654. (B) Structural prediction of FA1654 based on I-tasser modelling. (C) Structural prediction of putative iron binding sites of FA1654. (D) Dodecameric structure of FA1654 based on SWISS-MODEL. (E) SDS-PAGE gel showing rFA1654 with molecular weight ~33.7kDa.

3.2 | FA1654 is upregulated under Hydrogen Peroxide Induced Stress

In the presence of epithelial cells there was an upregulation of the *FA1654* gene in *F. alocis* (A. Wilson Aruni et al., 2012). RT-PCR was used to analyze the expression of the *FA1654* gene in *F. alocis* under both anaerobic and oxidative stress conditions. As shown in Figure 2, the expression of the *FA1654* gene was induced by ~1.3 fold when *F. alocis* was exposed to 0.25 mM of hydrogen peroxide for 30 minutes. However, under coculture conditions with *P. gingivalis*, the *FA1654* gene was downregulated ~1.8 fold. This could likely be the result of a compensatory oxidative stress mechanism that is activated under coculture conditions (Aja, Mishra, et al., 2021).

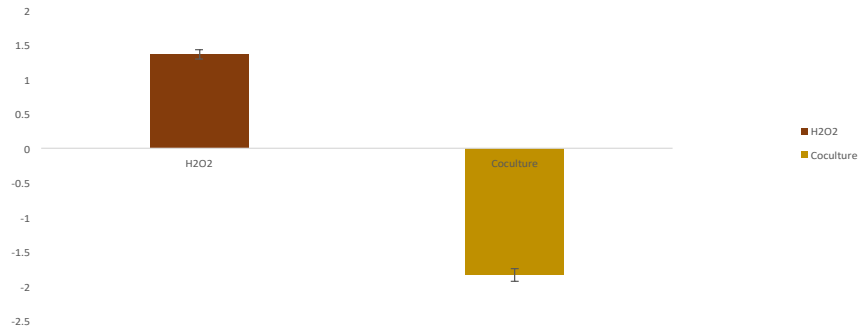


Fig. 2. FA1654 is upregulated when *F. alocis* in the presence of H₂O₂ induced oxidative stress. RT-qPCR was conducted on a monoculture of *F. alocis* incubated with 0.25 mM of H₂O₂ for 15 mins. *F. alocis* in coculture with *P. gingivalis* was incubated with H₂O₂ for 15 mins.

3.3 | FA1654 protects DNA from Fenton reaction- mediated damage

The ability of the FA1654 protein to protect DNA from oxidative stress-induced damage was analyzed using a DNA protection assay in the presence of H₂O₂ and iron (Figure 3). Incubation of DNA in the presence of H₂O₂ showed minimal degradation (lane 3). This is in contrast to the complete degradation of the DNA in the presence of Fe₂SO₄ and H₂O₂ (lane 4). The DNA damage was significantly reduced when it was incubated with FA1654 (lanes 7-9) before the addition of H₂O₂ and Fe₂SO₂ (Figure 3). In the presence of a recombinant collagenase thioredoxin fusion protein (negative control), there was no observed protection against oxidative stress-induced DNA damage (Figure 3, lane 5; Supplemental Figure 1)). Taken together, this may indicate the ability of the FA1654 protein to protect DNA from the Fenton reaction- mediated damage. Moreover, under our experimental conditions, this may indicate that the thioredoxin fusion tag did not protect against the Fenton reaction-mediated observed damage.

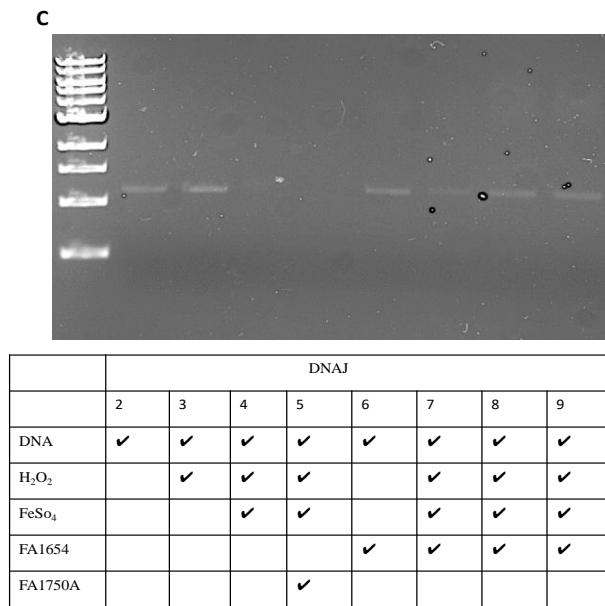
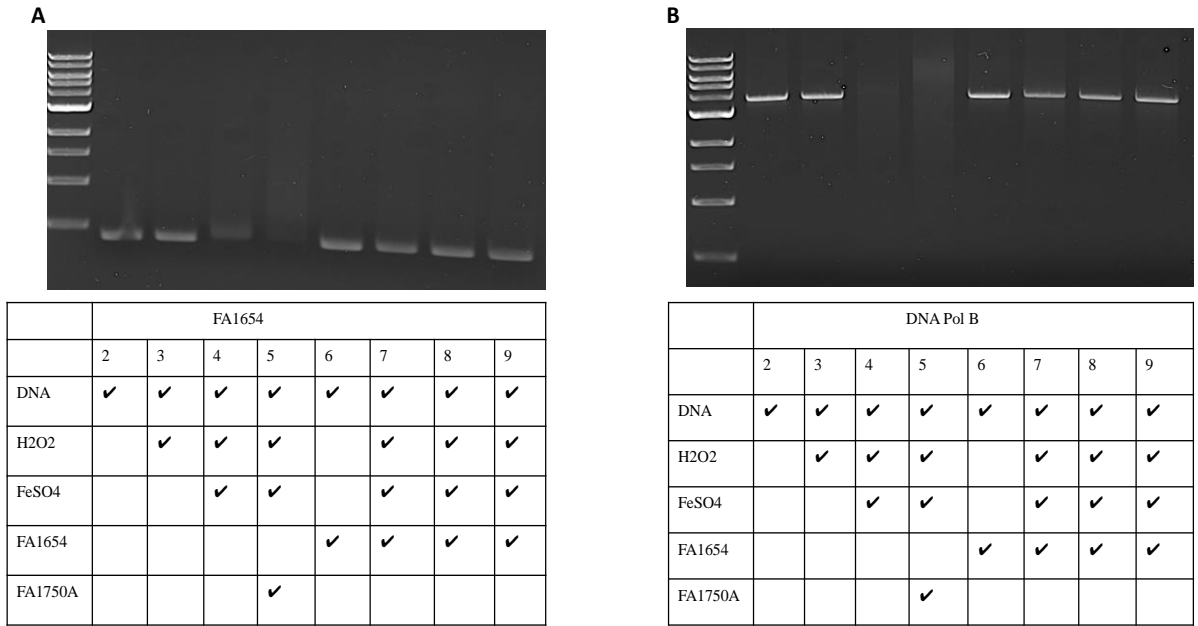


Fig 2.3. FA1654 protects DNA from oxidative damage in vitro. The 1% agarose gels shown represent DNA protection assays. (A) Lane 1, 1 kb ladder; lane 2, DNA alone (positive control); lane 3, DNA + H₂O₂; lane 4, DNA + Fenton reaction (Fe²⁺ and H₂O₂); lane 5, DNA + Fenton reaction + FA1750A (negative control); lane 6, DNA + 2 μM FA1654; lanes 7-9, DNA + Fenton reaction + 2-8 μM FA1654. Lane 7 (A) indicates that as little as 2 μM FA1654 can extinguish the effect of the Fenton reaction, resulting in the protection of DNA.

3.4 | Inability of the rFA1654 protein to form DNA-DPS complexes

While there were no predicted DNA binding domains in FA1654, its ability to bind/interact with DNA was analyzed in the presence of H₂O₂ and iron. Similar, to the control, incubation of the FA1654 protein with DNA resulted in no change in the migration pattern of the DNA through the agarose gel following electrophoresis (Figure 3). There was no observable change in the DNA migration pattern in the presence of iron and H₂O₂.

3.5 | rFA1654 bind iron

The FA1654 protein is predicted to be an iron-binding protein. To determine the ability of FA1654 to bind iron, we performed Circular Dichroism (CD) and Differential Scanning Fluorimetry (DSF) in the absence or presence of iron. The far-UV CD spectra exhibited two negative signals at 222 and 208 nm (Figure 4), which were consistent with significant helical structures in the protein. Deconvolution of the CD signals indicated the presence of 32% of α -helicity in the absence of iron. Upon the addition of 50 μ M Fe, the magnitude of the CD signal at 222nm decreased by approximately 12.5%. This change suggested a small decrease in α -helicity, which may be the result of the binding of Fe to the protein (Fig. 5). To corroborate this data, DSF was used to assess the effect of iron on the thermal stability of the protein. The thermal unfolding of rFA1654 was monitored by measuring the changes in intrinsic fluorescence at 330 and 350 nm (Figure 5). The DSF curve of rFA1654, which contains three Trp residues, displayed one unfolding transition that was centered at 54 °C (this was considered as its thermal unfolding temperature, T_m). The addition of 50 μ M Fe resulted in an increase in Fa1654 T_m by approximately

1.5⁰C, suggesting thermostabilization of the native protein due to specific interactions with iron (Waldron & Murphy, 2003). Taken together, the results from CD and DSF analysis, may provide evidence of the specific binding of iron to the native form of Fa1654 (Figure.6).

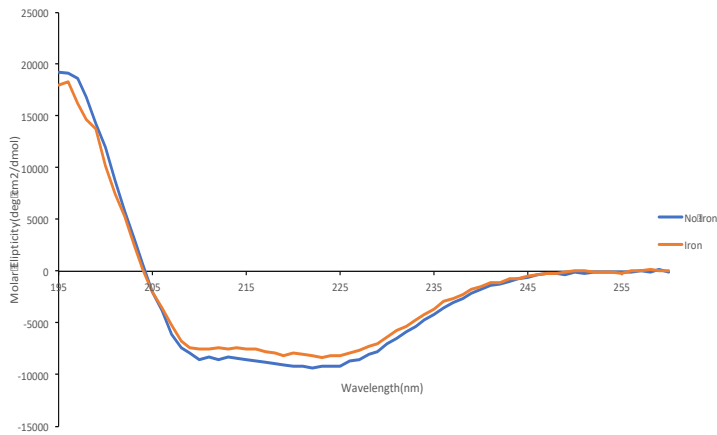


Fig 2.4. CD analysis of FA1654. CD spectra of FA1654 and FA1654 plus 50µM Fe at 222nm. Shift in the molar ellipticity indicates the binding of iron to FA1654.

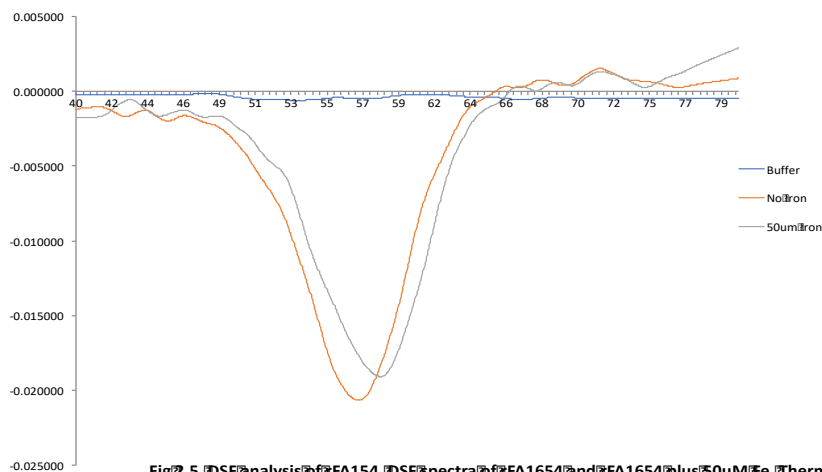


Fig 2.5. DSF analysis of FA1654. DSF spectra of FA1654 and FA1654 plus 50µM Fe. Thermal unfolding increased by 1.5⁰C, indicating the binding of Fe to FA1654.

3.6 | H₂O₂ Detection

A TMB assay was used to determine any intrinsic ability of the FA1654 protein to reduce H₂O₂ (Fig. 6). The assay is based on the colorimetric change of the TMB reagent from yellow to blue when exposed to cation radicals such as OH•. Absorption spectroscopy measurement at 450nm of TMB incubated in the presence of H₂O₂ and the FA1654 protein was 0.015 with no observed colorimetric change. This is in contrast to the control reaction of TMB incubated with Fe and H₂O₂ which resulted in an observed colorimetric change of yellow to blue with an OD_{450nm} of 0.368. This data taken together may indicate that FA1654 does not have the intrinsic ability to reduce H₂O₂.

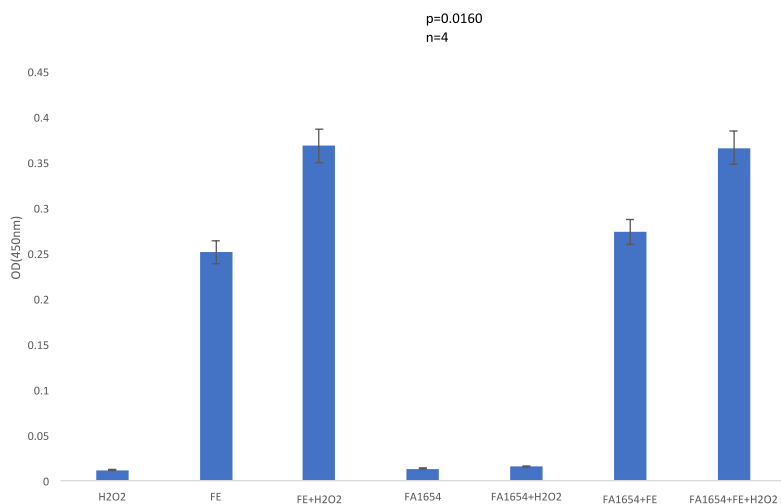


Fig 2.6. The ability of rFA1654 to reduce H₂O₂ was assessed using a TMB assay. TMB when oxidized via cation radicals exhibits a colorimetric change from yellow to blue and can be detected at 450nm. TMB was incubated in the presence of the Fenton reaction (Fe²⁺ and H₂O₂), H₂O₂ alone and TMB plus H₂O₂ plus rFA1654. The data obtained indicates that rFA1654 is unable to reduce H₂O₂.

4 | Discussion

Oxidative stress can play a role in the progression of periodontal disease, which is known to affect more than 65 million adults in the United States (Aja, Mishra, et al., 2021; Eke et al., 2015; Mehrotra & Singh, 2019). The impact of the microbial-induced host response involving the generation of reactive oxygen species such as H₂O₂ and hydroxyl radicals, is important to disease development/progression (Sczepanik et al., 2020). Recently described as part of the Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation” Model, proposes there is a central role for inflammation and its ability to modulate the polymicrobial community along a health to periodontitis continuum (T. E. Van Dyke et al., 2020). As part of this process that can trigger a dysbiotic state to initiate periodontitis, there is inflammation-mediated microbial succession that drives the temporal and spatial emergence of disease-associated species in the periodontal pocket. With disease progression, the transitional dysbiotic microbiota is further altered by an increase in abundance of predominantly disease-associated species particularly at the base of the periodontal pocket resulting in an opportunistic polymicrobial synergistic infection and correlated with an excessive inflammatory response with the accompanying release of ROS, such as H₂O₂ and superoxide radicals from neutrophils and concomitant tissue destruction (Armstrong et al., 2018; Arunima et al., 2020; Sczepanik et al., 2020). There is evidence that *F. alocis* can successfully survive and co-occur with other previously recognized periodontal pathogens in the oxidative stress rich environment of the periodontal pocket.

This study has provided insights into the role of a *F. alocis* hypothetical protein with DPS-like characteristics, involved in oxidative stress resistance via an ability to

protect against Fenton reaction-mediated oxidative stress-induced DNA damage. We have shown that the FA1654 protein shares identity with several DPS proteins including the DPS from *Fusobacterium spp*, *Clostridiales bacterium* and *Treponema pedis*. The FA1654 protein is predicted to have an iron-binding domain and thus is considered to be a putative DPS protein. DPS proteins are associated with the protection of DNA from Fenton reaction-mediated oxidative stress (Antipov et al., 2017; Bellapadrona et al., 2010a) via DNA binding and/or iron binding (Antipov et al., 2017; de Alcântara et al., 2020; Tseng et al., 2019). Our studies have indicated that FA1654 can bind iron *in vitro*. Iron has been demonstrated to be a critical element in bacterial growth, survival and pathogenesis (Andrews, Robinson, & Rodríguez-Quñones, 2003; Frawley & Fang, 2014). However, under conditions such as oxidative stress, iron leads to damage due to its role in the Fenton reaction and the resulting generation of reactive oxygen species. There are three major cellular protective systems against oxidative stress in bacteria. These include DNA repair, chaperone/protease and antioxidant systems (McKenzie et al., 2012). In one of the major antioxidant defenses, iron is carefully sequestered within proteins and is restricted from reacting with ROS (Gutteridge & Halliwell, 2018). Driven by the ability of ROS to bind and oxidize ferrous iron, oxidative stress is a feature of iron metabolism. When H₂O₂ reacts with DNA-bound iron, it generates mutagenic and even lethal DNA lesions. The rFA1654 protein in this study protected DNA from Fenton reaction-mediated damage. The damage protection in the presence of H₂O₂ did not likely occur via the ability of the FA1654 protein to bind DNA. Consistent with the absence of any predicted DNA binding domains in FA1654, an ability to bind DNA was not observed.

The ability of the FA1654 to bind iron, similar to other DPS proteins described in *E. coli*, *L. innocua* and *C. jejuni* (Almiron, Link, Furlong, & Kolter, 1992; Huergo, Rahman, Ibrahimovic, Day, & Korolik, 2013; Ishikawa et al., 2003; X. Yang, Chiancone, Stefanini, Ilari, & Chasteen, 2000), could likely indicate a sequestration function. Thus, a possible mechanism of action of FA1654 is the binding of iron to a putative active site which could render it unavailable to interact with H₂O₂ and possibly prevent the generation of free radicals such as OH•. It is also likely that the protein may utilize H₂O₂ as an oxidant in the multistep oxidation of iron from its 2⁺ to 3⁺ state (de Alcântara et al., 2020) to generate the formation of Iron(III) oxide-hydroxide FeO(OH). This non-reactive form, as the end product of iron oxidation, could be utilized by *F. alocis* proteins to sequester free iron (Haikarainen & Papageorgiou, 2010; Xiaoke Yang, Chen-Barrett, Arosio, & Chasteen, 1998; X. Yang et al., 2000; Xiaoke Yang, Le Brun, Thomson, Moore, & Chasteen, 2000) (Fig.7). Taken together, the sequestering of iron and the formation of FeO(OH), may limit the interaction between iron and H₂O₂, reducing the generation of free radicals. FA1654 was also unable to detoxify H₂O₂, suggesting it has no intrinsic peroxidase activity which could further support its possible role in iron oxidation (Xiaoke Yang et al., 2000). It is unclear if FA1654 may have other functions or can modulate the iron pool within the cell. Because oxidative stress-induced DNA damage is directly proportional to the amount of loose iron in the cell, an ability to scavenge H₂O₂ is important for growth and survival under those environmental conditions. Moreover, oxidants can damage iron enzymes, resulting in the released and several-fold increase in free available iron ((Keyer & Imlay, 1996). FA1654 is likely part of a complex system in *F. alocis* given its relative resistance to oxidative stress (Aja,

Mangar, et al., 2021). While H₂O₂ scavenging enzymes, in most anaerobic organisms, include catalases, peroxidases bacterioferritin comigratory protein (Bcp), ruberythrin and alkyl hydroperoxide reductase enzyme system (AhpC/AhpF) (Aja, Mangar, et al., 2021; Arunima et al., 2020; Mishra & Imlay, 2012) several are missing from the genome of *F. alocis* (Arunima et al., 2020). The *F. alocis* SOR (FA796) protein is a key enzymatic scavenger of superoxide radicals and can protect the bacterium from oxidative stress conditions (Arunima et al., 2020). The multifunctional *F. alocis* FA519 protein which may represent a novel class of thioredoxin family proteins can protect DNA from Fenton reaction-mediated damage with intrinsic peroxidase and disulfide oxidoreductase activities (Aja, Mishra, et al., 2021). It is noteworthy that the genome of *F. alocis* carries the genes for iron-sulphur cluster assembly, ferrous iron transport, FTR1 family and DprA proteins all of which are currently uncharacterized. Additionally, *F. alocis* encodes for glutathione peroxidase, alkyl hydroperoxide reductase subunit AhpC and thioredoxin-disulfide reductase (Aja, Mangar, et al., 2021) genes which may play a role in H₂O₂ scavenging. Together, in *F. alocis* these systems along with FA1654 may be part of a complex network with unknown regulatory components. Although we have made several unsuccessful attempts to inactivate the *FA1654 gene* in *F. alocis*, studies to further validate its role in modulating the response of the bacterium to the oxidative stress environment of the periodontal pocket are ongoing in the laboratory.

Collectively, the data obtained from this study suggests that *F. alocis* FA1654, maybe a member of the ferritin superfamily and a possible DPS. It protects DNA from Fenton reaction-mediated damage and has the intrinsic ability to bind iron. The ability for

FA1654 to modulate important virulence attributes in *F. alocis* is still unclear. What role does FA1654 play in iron homeostasis and how is it regulated in *F. alocis*? Can sequestered iron be released in times of iron deficiency? (de Alcântara et al., 2020) The specific function and relative significance of FA1654 in *F. alocis* are under further investigation in the laboratory.

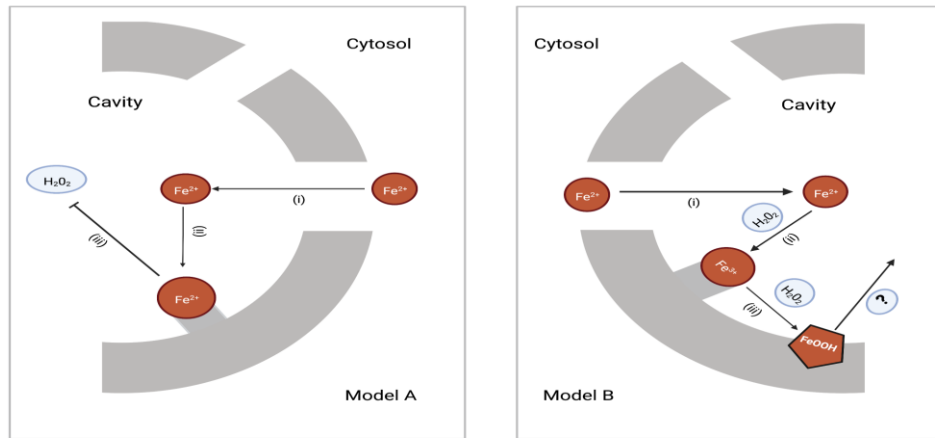


Fig 2.7. Proposed mechanism(s) of action for FA1654. FA1654 binds iron via its 2⁺-iron binding site. (1) This iron may be bound in the 2⁺ state and therefore may be unable to react with H₂O₂, minimizing the generation of Fenton-mediated ROS (Model A). (2) It is also possible that the FA1654 protein may utilize available H₂O₂ as an oxidant, oxidizing iron to produce an iron oxide hydroxide (Model B). The mechanism of iron homeostasis in *F. alocis* is currently not fully understood, it is yet to be determined if bound iron can be recycled and the mechanism by which it occurs. Experiments to determine the amino acid residues involved in iron binding and the kinetics of iron bound to the protein are required to fully elucidate the proposed mechanism.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Malissa Mangar, Arunima Mishra, Zhengrong Yang, Champion Deivanayagam and Hansel M. Fletcher conceived and designed the experiments. Malissa Mangar and Zhengrong Yang conducted experiments, collected and analyzed the data. Malissa Mangar wrote the original draft. Hansel Fletcher validated the data and modified the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement

The data generated or analyzed during this study are included in this article.

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Tables

Strains	Source
<i>F. alocis</i> 35896	ATCC
<i>E. coli</i> TOP10	Life Technology
<i>E. coli</i> BL21	Life Technology
Plasmids	Thermofisher Scientific
pET 102 D-TOPO	

Table 1: Bacterial strains and plasmids used in this study

Use and name of primer	Sequence(5'-3')
Overexpression of FA1654	
FA1654-pET 102 -F1	CACCATGATGAACTTAAAAAAGAATTT
FA1654-pET 102-R1	TTTCACGCTTGAGTTAATGAAC
qPCR analysis	
F1	CAAAGAGGTGGATCCAAGAGAA
R1	CTTCTGTCCAACCCAATCCA

Table 2: Primers used in this study

CHAPTER THREE

GENERAL DISCUSSION

The onset and progression of periodontitis are directly linked to the prolonged exposure of the periodontium to oxidative-induced stress. This inflammatory disease with its hallmark destruction of the tooth-supporting structures involves reactive oxygen species (ROS), such as hydrogen peroxide and superoxide that are generated from the host immune response in combination with selected microbial species of the microbiome (Aja, Mangar, et al., 2021; Moffatt et al., 2011; Tóthová & Celec, 2017). Periodontal disease affects approximately 65 million individuals in the United States (Aja, Mishra, et al., 2021; Eke et al., 2015; Mehrotra & Singh, 2019) and is the sixth most prevalent infectious disease (Eke et al., 2015). The advancement and availability of sequencing techniques allowed for more in-depth microbiome analysis, identifying novel pathogens such as *Filifactor alocis* which demonstrates correlation with the onset of disease (Schlafer et al., 2010a). *Filifactor alocis*, a gram-positive, rod-shaped, assaracholytic bacteria, with trypsin-like qualities, was first isolated from the human gingival crevice in 1985 (Schlafer et al., 2010a), and can survive in an oxidative stress-rich environment. The unavailability of genetic tools in *F. alocis* has made it difficult to carry out the genetic manipulation required to characterize the pathogen's functional genes. *F. alocis* has been observed to be more prevalent than the "Red complex" species, *Porphyromonas gingivalis*; the keystone pathogen, *Treponema denticola* and *Tanerella forsythia* (A Wilson Aruni et al., 2015; Chen et al., 2015a; G. Hajishengallis & Lamont, 2012), in patients with periodontal disease, but is relatively undetectable in healthy patients. This

implies that *F. alocis* may have the characteristics to be used as a diagnostic indicator of periodontal disease. Despite ongoing progress in understanding the virulence mechanism of *F. alocis*, there is still a gap in our understanding of its oxidative stress resistance mechanism and its ability to survive in a dysbiotic environment. Previous observation utilizing proteome analysis techniques, determined there are several genes believed to be involved in oxidative stress resistance were upregulated in *F. alocis*, during co-infection of epithelial cells with *P. gingivalis* (A. W. Aruni et al., 2014).

The interaction between the host and pathogen results in the recruitment of phagocytes to the site of infection. Phagocytosis results in the generation of free radicals including O_2^- , H_2O_2 and $O\bullet$, as the by-products of mitochondrial respiratory burst in polymorphonuclear neutrophils. This generated ROS can have a detrimental effect on lipids, nucleic acids and other cellular structures (Szczepanik et al., 2020). Anaerobic bacteria can dispose of ROS using methods such as superoxide dismutase and peroxidase. These antioxidant defense mechanisms are important to host and pathogen alike, under oxidative stress conditions (Flohé, Jaeger, Pilawa, & Sztajer, 2003). Studies conducted by Mishra et al determined that *F. alocis* has a superoxide reductase (SOR), which catalyzes the reduction of superoxide to hydrogen peroxide in anaerobic species (Arunima et al., 2020). It is however unlikely that SOR is the only mechanism *F. alocis* uses to combat oxidative stress. To further elucidate a likely mechanism for oxidative stress resistance in *F. alocis* the recombinant protein expressed by the HMPREF0389_01654 (*FAI654*) gene was characterized.

The primary habitat of *F. alocis* is the periodontal pocket, hence it is consistently exposed to oxidative stress, fluctuations in nutrients, changes in pH and the microbial

population, despite these conditions *F. alocis* is still able to tolerate its environment (Arunima et al., 2020). Recent studies have indicated that *F. alocis* is relatively more resistant to hydrogen peroxide-induced oxidative stress (W. Aruni et al., 2014), can form biofilm (Schlafer et al., 2010a), subvert the complement system (Jusko et al., 2016b), and invade and adhere to epithelial cells, TGIKs and NOKs (Aja, Mishra, et al., 2021; A. W. Aruni et al., 2014; Arunima et al., 2020). Taken together these observations suggest that *F. alocis* can likely detoxify the oxidative stress microenvironment of the periodontal pocket. A previous study examining the regulation of the proteome during coinfection of epithelial cells with *F. alocis* and *P. gingivalis* has demonstrated the upregulation of several *F. alocis* genes believed to be induced under oxidative stress, including *FA1654* (A. W. Aruni et al., 2014). To further examine the regulation of *FA1654* under oxidative stress conditions, *F. alocis* in monoculture and coculture with *P. gingivalis* was treated with 0.25mM hydrogen peroxide for 15 minutes and qPCR analysis was carried out. *FA1654* was shown to be upregulated in monoculture under the previously described conditions.

The amino acid sequence of the FA1654 protein indicated homology to DNA starvation and stationary phase protection protein (DPS). *In silico* analysis demonstrated that the FA1654 protein carries a putative “di-iron binding” domain (obtained from NCBI). Di-iron binding domains are common across DPS and DPS-like proteins, across bacterial species (de Alcântara et al., 2020; Huergo et al., 2013; Karas et al., 2015; Stillman et al., 2005). Iron binding domains contain two iron binding motifs; an A site and a B site, with site A having a higher affinity for iron binding (Bellapadrona et al., 2010a). These proteins have been associated with the regulation of Fenton-mediated

oxidative stress. It is noteworthy, that even though *in silico* analysis indicates that FA1654 carries an iron-binding domain, it does not have any predicted DNA binding motifs, both of which are characteristic of established DPS proteins.

F. alocis ability to combat oxidative stress is critical to its survival in the oral cavity. Prior to this study, our lab has established two genes (*FA796* and *FA519*) that are a part of the complex oxidative stress regulatory machinery. In this study, we have shown that the rFA1654 protein protected DNA from Fenton reaction- mediated damage. Consistent with the lack of any predicted DNA binding motif in FA1654, there was no observable DNA binding, suggesting that the protein's DNA protection is via another mechanism. Using circular dichroism and differential scanning fluorimetry, the rFA1654 protein was demonstrated to have the ability to bind iron in its native state. This, suggests that FA1654 has a likely iron sequestering function, which should reduce free iron and minimize the *in vivo* generation of the lethal ROS.

Anaerobic bacteria possess hydrogen peroxide-scavenging enzymes including catalases, peroxidases and alkyl hydroperoxide reductase (Arunima et al., 2020). The ability to scavenge and/or degrade hydrogen peroxide is critical to the survival of bacteria. Since rFA1654 shows the ability to protect DNA against reactive oxygen species, we tested for the ability to detoxify hydrogen. The rFA1654 protein was unable to detoxify hydrogen peroxide, indicating it has no intrinsic peroxidase activity. Thus, we cannot rule out the possibility that hydrogen peroxide may play a role as an oxidant in the iron-binding activity of the protein. Hydrogen peroxide may be involved in the multistep oxidation process of iron conversion from its 2+ to 3+ oxidation state (de Alcântara et al., 2020), eventually forming iron(iii) oxide hydroxide (FeOOH), a possible non-reactive

by-product of iron oxidation (Haikarainen & Papageorgiou, 2010; Xiaoke Yang et al., 1998; Xiaoke Yang et al., 2000). The sequestration of iron in a non-reactive form may limit its interaction with hydrogen peroxide, thus reducing the generation of free radicals.

The data obtained in the study have expanded our understanding of the survival strategy and likely pathogenicity of *F. alocis* in a dysbiotic microbial community. To summarize, this study has shown that *F. alocis* HMPREF0389_01654 (FA1654), as a member of the ferritin super-family, may function as a DPS protein that can modulate the Fenton reaction-mediated oxidative stress-induced damage of macromolecules via iron sequestration. Taken together, this function could protect the bacterium against oxidative stress-induced DNA damage and is a likely vital component of a more complex damage protection pathway(s). Recently, the *F. alocis* recombinant FA519 protein with an intrinsic ability to reduce hydrogen peroxide and disulfide bonds was shown to protect DNA from Fenton-mediated damage (Aja, Mishra, et al., 2021). Coordinate regulation of FA1654 and FA519 could support an oxidative stress-induced damage protection mechanism that involves the sequestration of iron with its conversion to non-reactive form by FA1654, whereas, FA519 as a peroxidase, can detoxify excess hydrogen peroxide.

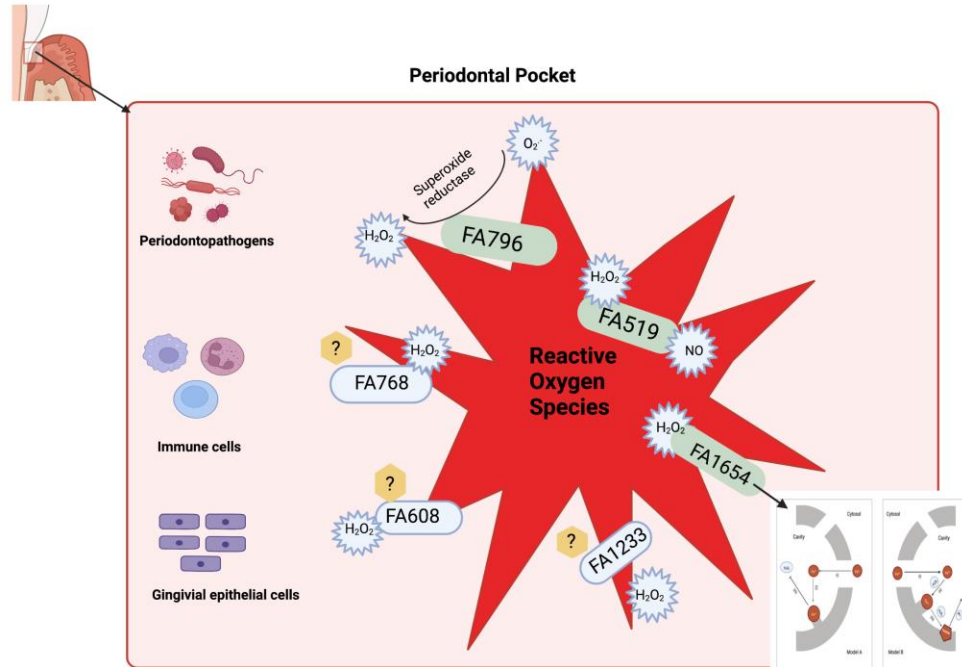


Fig. 3.1. Depicting a possible oxidative stress mechanism of *F. alocis*. Adapted from Aja, et al 2021

Several unsuccessful attempts have been made to inactivate the *FA1654* gene in *F. alocis*, however we will continue the efforts to generate a *FA1654* deficient mutant. Also, despite being able to elucidate rFA1654's ability to protect DNA from Fenton-mediated damage by iron-binding, gaps remain in our understanding of the protein's mechanism of action. Moving forward we intend to generate site-directed mutants of the protein to determine the amino acid residues involved in iron binding. We will also explore the kinetics of iron binding (Xiaoke Yang et al., 2000) in an attempt to answer the following questions:

- 1) What is the role of FA1654 in the homeostasis and regulation of iron of *F. alocis*?
- 2) What is the oxidation state of iron after it is bound to the protein's active site?

- 3) The phases of iron binding and formation of
- 4) Can sequestered iron be released and recycled in times of iron deficiency in the bacteria?

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