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Characterization of Oral Pathogen, *Filifactor Alocis*, and its Virulence Factors that Contribute to the Progression of Periodontitis

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ABSTRACT
Periodontitis is among one of the most common chronic diseases afflicting nearly half of all adults in the USA. The immune cells patrolling the oral cavity typically exist in harmony with symbiotic bacteria residing in the oral biofilms. Certain risk factors out individuals at higher risk for the colonization of pathogenic bacteria in the oral cavity and thus the alteration of the composition of the once symbiotic biofilm. These pathogens induce inflammation mediated by the host immune system, but certain virulence factors allow the pathogens to manipulate the immune response to be deleterious to the host and beneficial to themselves. The dysregulation of the host immune response leads to irreversible chronic inflammation, the hallmark of periodontitis. New sequencing technologies have allowed for the identification of bacterial species associated with periodontitis including the gram-positive anaerobic rod, *Filifactor alocis*. This literature review aims to synthesize the current characterizations of *F. alocis* and its potential virulence factors that may ultimately lead to the progression of periodontitis.

KEYWORDS: Periodontitis, oral pathogens, *Filifactor alocis,* inflammation

One of the most prevalent diseases in human health is an affliction of the oral cavity called periodontitis. Epidemiological studies have estimated that periodontitis afflicts 20 – 50% of the global population and nearly half of the adult population in the United States [1, 2]. Periodontitis puts its hosts at increased risk for the development of other systemic diseases such as cardiovascular disease, diabetes, and arthritis among many others (reviewed in [3]). Specifically, this disease affects the periodontium, the structure that supports the tooth in the oral cavity. The periodontium is composed of different types of tissues subject to microbial colonization such as the alveolar bone, periodontal ligament, and gingival tissues. This structure has other important functions, namely, acting as a barrier between microbes and the underlying structures [4]. The oral cavity is under constant exposure to microbes from the environment. Basic activities such as eating, drinking, and breathing bring foreign microbes into the oral cavity where the host must adequately neutralize any threats posed by pathogens.

While the colonization of bacteria in the oral cavity may seem deleterious to the host, the reality is that there has always been a diverse community of bacteria in the oral cavity. These bacteria form biofilms on the teeth and gingival tissue and usually live symbiotically with the host and are thought to aid in outcompeting exogenous pathogens to subdue colonization attempts [5]. The host immune system has evolved to be tolerant of specific species in the oral microbiome while also maintaining high surveillance of the oral cavity to immediately recognize pathogens in this constantly exposed cavity of the body. Some pathogens have evolved mechanisms that allow them to evade or manipulate the host immune response to colonize the tissues and become pathogenic towards the host. The colonization of the oral cavity by certain pathogens alters its microbial community and therefore alters the relationship between the host and the microbes. A once symbiotic relationship between host and microbes can quickly become dysbiotic upon colonization by keystone pathogens of periodontitis, that is, pathogens whose colonization of the oral cavity is crucial to the alteration of the microbiome in the mouth that leads to periodontitis [6]. This dysbiosis evokes a response from the host immune system which attempts to subdue the pathogens creating the dysbiosis. However, the virulence factors possessed by the pathogens allow for subversion of the immune system leading to prolonged inflammation in the gingival tissue. This chronic, irreversible inflammation is beneficial to the pathogens because it leads to the eventual destruction of the host tissue which releases nutrients to pathogens as well as allowing them to colonize further into the periodontium [6]. Initially, it was thought that only a handful of species were responsible for the cause of the onset and progression of periodontitis, but new technologies have altered the current understanding of the onset and progression of this disease. It is now hypothesized that colonization of specific keystone pathogens sets the exposition for periodontitis, and they have been coined “the red complex” [7]. Once these pathogens are introduced, the environment of the oral microbiome changes to allow for the colonization of other periodontal pathogens that then contribute to the progression of periodontitis.

New sequencing technologies have identified several newly appreciated species associated with periodontitis including *Filifactor alocis* [8]. Oral biofilm sequencing of those afflicted with periodontitis has revealed a strong

association of periodontitis with *F. alocis* having a higher prevalence in diseased individuals compared to healthy controls [9]. *F. alocis* is a gram-positive, rod-shaped bacterium with surface projections that may aide in its attachment to sites in the periodontium [8]. *F. alocis* is quite fastidious by being an obligate anaerobe and asaccharolytic (i.e., unable to metabolize carbohydrates for energy) [10]. Rather than using carbohydrates as a primary source of energy, *F. alocis* metabolizes amino acids. *F. alocis* specifically prefers arginine, lysine, and cystine which have been shown to significantly increase its growth [10]. Arginine may be a preference of *F. alocis* due to the metabolic pathway used for arginine catabolism. The arginine metabolic pathway utilized by *F. alocis* is predicted to generate ammonia and ornithine via arginine deaminase. These basic compounds could be useful in the keeping the pH of the environment around *F. alocis* favorable to its growth due to the metabolic products of other bacteria in the periodontal pocket producing acidic metabolites. In the periodontium these amino acids can be obtained from proteins produced by other microbes as well as from host tissues that are degraded from the prolonged inflammation associated with periodontitis [10].

Bacteria in the oral microbiome do not exist independently but rather in communities. *F. alocis* utilizes quorum-sensing to determine the other members of the communities it colonizes which potential affects its interactions [11]. Some of these interactions are strain specific as with the oral pathogen *Aggregaatibacter actinomycetemcomitans* which only showed synergistic effects with specific strains while other interactions were more generalized such as increased biofilm formation when co-cultured with the periodontitis keystone pathogen *Porphyromonas gingivalis* [11]. There are many strong associations between the presence of *P. gingivalis* and *F. alocis* in diseased sites and the mechanism between the synergistic effects may reveal more about the interactions between *F. alocis* and other oral pathogens. The current understanding is that *P.gingivalis* produces minor fimbriae that, when expressed, inhibit the growth of F. alocis. However, metabolites generated by arginine deaminase which is possessed by *F. alocis* cause the suppression of these fimbriae and a synergistic effect occurs where *F. alocis* and *P. gingivalis* may provide nutritional and adhesion support for each other [11]. *F. alocis* has been shown to be a complex organism both with its abnormal metabolism and fastidious nature as well as its interactions with the many other microbes that make up the oral microbiome.

One of the key factors that play into the ability of *F. alocis* to survive and grow in the host is its ability to resist oxidative stress. Oxidative stress comes from many sources, including the environment it inhabits being exposed to open air, oxygen in the bloodstream, and reactive oxygen species released by immune cells in attempt to kill the pathogen. Aruni et al. found that *F. alocis* is significantly more resistant to oxidative stress than other obligate anaerobes in the oral microbiome [12]. Interestingly, although *F. alocis* is an obligate anaerobe, its growth is stimulated when placed under oxidative stress. These characteristic allows for *F. alocis* to outcompete other more oxygen-intolerant species in the oral microbiome [12]. The mechanism by which *F. alocis* can detoxify oxygen in its environment was unknown until its genome was sequenced which gave researchers insight into what enzymes *F. alocis* possesses that would allow for it to be so resilient in oxidative environments compared to other anaerobic oral microbes [13].

A protein believed to be key to this characteristic is the protein FA796. This protein is a superoxide reductase that can convert superoxide radicals (which can be produced by immune cells fighting infection) into hydrogen peroxide [13]. While hydrogen peroxide is less detrimental to cells than oxygen radicals such as superoxide, it is still toxic and poses a threat to cells in its vicinity. However, *F. alocis* possesses yet another mechanism to detoxify its environment. FA519 is a hypothetical protein that has been shown to play a significant role in the ability of *F. alocis* to survive and reproduce under hydrogen peroxide-induced stress, but the exact mechanism that causes this is unknown [14]. Oxidative stress resistance is also hypothesized to play a role in the interactions between *F. alocis* and other members of the oral microbiome. It was found that the expression of FA519 was significantly increased when *F. alocis* was co-cultured with *P. gingivalis* but not with other bacteria species [14]. FA796 has also shown a similar importance in hydrogen peroxide-induced stress resistance in addition to its superoxide reductase function [14].

The genome sequencing of *F. alocis* revealed another surprising mystery in its ability to counteract oxidative stress. Within the genome there exists a gene encoding for alkyl hydroperoxide reductase subunit C (AhpC) which could potentially aid in clearance of hydrogenperoxide. However, the partner to this subunit, AhpF, is missing completely from the genome in all strains analyzed [14]. This could be a consequence of evolution although the cause for the species to abandon an enzyme

that could theoretically further promote its survival is unclear.

While these mechanisms do aid in the clearance of sources of oxidative stress, there is a limit at which these enzymes can function and especially in the presence of an onslaught of immune cells. It is still a mystery as to why *F. alocis* is so resilient under extremely oxidative conditions, especially when considering close relatives and similar oral pathogens do not possess this ability. It is hypothesized that *F. alocis* has over time developed a more efficient mechanism to repair cellular and DNA damage caused by oxidative stress than the other microbes in the oral microbiome, allowing it to outcompete other inhabitants of this space including symbiotic organisms [12]. It is important to consider how this resistance to oxidative stress plays into the interactions between *F. alocis* and immune cells which commonly use reactive oxygen species as a defense against pathogens. This bacterium's ability to detoxify these agents makes it a formidable competitor against the immune system and plays a role in its virulence and pathogenesis.

Another important characteristic of bacteria is the ability to release cellular contents into the extracellular matrix. Extracellular vesicles function to export cellular contents outside of the cell that produces them and are found in all domains of life. In Gram-positive bacteria such as *F.alocis* these cellular contents are packaged and released in structures known as membrane vesicles. Extracellular vesicles can function locally or be widespread to deliver their contents to furthersites. Extracellular vesicles can carry a wide variety of bioactive molecules used for functions such as cell-to-cell communication and pathogenesis. *F. alocis* membrane vesicles have been identified as a virulence factor of the pathogen.

Upon analysis of the purified proteins derived from *F. alocis* membrane vesicles, 28 proteins were found [15]. The proteins included in these vesicles included glycoproteins, autolysins, ribosomal proteins, metabolism-related proteins, transporter-related proteins, and *F. alocis* compliment inhibitor protein ("FACIN"). FACIN has been identified as a virulence factor of *F. alocis* due to its inhibition of the complement system. The complement system is family of proteins that functions in antimicrobial responses of the host. The complement system has many mechanisms by which it aids in microbial clearance. Two of these functions are opsonization and the formation of the membrane attack complex ("MAC"). Opsonization involves the proteins of the complement system binding to the surface of microbes which then bind to receptors on host immune cells allowing the microbe to be more efficiently phagocytosed by the immune cell. Phagocytosis is detrimental to a microbe due to the microbicidal environment created in phagosomes upon fusion with lysosomes and antimicrobial granules. FACIN functions to prevent opsonization by inhibiting C3 cleavage into C3b, a protein that mediates phagocytosis by immune cells [16]. Additionally, three different pathways are used by the complement system to form the MAC. The formation of the MAC is detrimental to pathogens by creating pores in the membrane of the pathogen which may cause them to lyse. FACIN inhibits MAC formation by inhibiting the C3 protein. The C3 protein is central to all three pathways that lead to MAC formation and its inhibition results in a total loss of MAC formation ability by the complement system [16].

Also found in the membrane vesicles of *F. alocis* are lipoprotein-like molecules that have interesting effects on osteoclasts and osteoblasts. Osteoclasts are cells that are responsible for the resorption of bone tissue with age whereas osteoblasts are cells that are responsible for the formation of new bone

A e tissue. The lipoprotein-like molecules found in *F. alocis* have been found to stimulate osteoclastogenesis which creates more osteoclasts that cause increased bone resorption in the host characteristic of periodontitis [17]. Furthermore, the lipoprotein-like molecules inhibit osteogenesis which decreases the amounts of osteoblasts and inhibits the host's ability to create new bone tissue in the presence of increased osteoclasts due to the lipoprotein-like moleculeinduced enhanced osteoclastogenesis [18]. The resorption of bone may be beneficial to *F. alocis* by freeing nutrients stored in the bones such as proteins and minerals that allow for its further growth. These effects on osteoclastogenesis and osteogenesis are mediated through a family of pattern recognition receptors ("PRRs") on innate immune cells called Toll-like receptors ("TLRs"). These receptors are used by immune cells to determine a course of response to pathogen challenge; however, some pathogens have evolved to exploit host defenses to their own benefit [17, 18]. Cytokines are small molecules released by cells to evoke a response and allow for communication between other systems and the immune system and within the immune system. Chemokines are cytokines that evoke chemotaxis of immune cells. Proinflammatory cytokines and chemokines cause the responding immune cells to employ antimicrobial defenses.*F. alocis* membrane vesicles have also been found to be recognized by other PRRs on cells and cause the release of proinflammatory cytokines and chemokines such as TNF, IL-8, IL-6, CXCL1, and many others (14 in total) [15]. Although proinflammatory

defenses are often seen as beneficial to the host by aiding in the clearance of pathogens, pathogens such as *F. alocis* have virulence factors (such as oxidative stress resistance and complement inhibition) that allow for the evasion of the inflammatory response causing prolonged inflammation that is detrimental to the host.

As a non-symbiont of the oral microbial community, *F. alocis* is recognized as a threat by the host immune system. Polymorphonuclear leukocytes (neutrophils) are most likely to encounter *F. alocis* in the oral cavity as they are the most populous white blood cell in circulation [19]. Neutrophils have many antimicrobial defenses to subdue pathogen infection such as generation of reactive oxygen species (ROS), granule recruitment, neutrophil extracellular traps (NETs), and releasing of pro-inflammatory cytokines to recruit other immune cells. *F. alocis*, however, has been shown to possess virulence factors that modulate the neutrophil response and delay its death in the presence of these normally microbicidal immune cells. As previously mentioned, although *F. alocis* is an obligate anaerobe it is very resistant to oxidative stress via proteins that convert oxygen to more benign compounds. Furthermore, *F. alocis* has been shown to induce very minimal ROS responses from neutrophils, meaning what little ROS is produced by the neutrophils in response to *F. alocis* can easily be tolerated and not significantly affect the organism's integrity [20]. This decrease in ROS production may, in part, be because of the effect *F. alocis* has on granule recruitment. Edmisson et al. observed specific granule recruitment in the neutrophil towards the periphery of the neutrophil rather than the *F. alocis*-containing phagosome, as well as preventing the fusion of azurophilic granules to the phagosome [20]. These granule recruitment. Edmisson et al. observed specific granule recruitment in the neutrophil towards the periphery of the neutrophil rather than the *F. alocis*-containing phagosome, as well as preventing the fusion of azurophilic granules to the phagosome [20]. These granule subsets are known to carry antimicrobial peptides and subunits of the NADPH oxidase complex [21]. Without these subunits, the NADPH oxidase complex cannot assemble and therefore cannot produce ROS. Decreased ROS production along with decreased antimicrobial peptides in *F. alocis*-containing phagosomes culminates in its increased survival within the neutrophil observed up to 20 hours post-challenge [20].

NETs are antimicrobial defenses that involve the neutrophil extruding its chromatin into the extracellular space where it is coated in antimicrobial peptides that function to trap and/or kill pathogens. Armstrong et al. observed that *F. alocis* does not induce NET release from neutrophils upon challenge, suggesting yet another virulence factor that allows this organism to persist in the presence of neutrophils [22].

F. alocis does, however, stimulate neutrophils to release several pro-inflammatory cytokines [23]. Increasing inflammation serves to benefit the pathogen rather than lead to its detriment. Because the neutrophils are unable to effectively clear the pathogen which further creates a pro-inflammatory environment, the sustained inflammation culminates into tissue damage which releases nutrients for the pathogen to benefit from and further evoke dysbiosis in the oral microbiome and the progression of periodontitis [6]. The manipulation of immune cells by *F. alocis* is key to the progression of periodontitis and speaks to the importance of further investigating the virulence factors possessed by this organism.

F. alocis has been identified as a key organism in the progression of periodontitis and its complex interactions with other periodontal pathogens, its own virulence factors, and its interactions with immune cells all culminate in its virulence towards potential hosts. Periodontitis is currently regarded as an irreversible condition; however, therapeutics targeting pathogens that contribute to the onset and progression of the disease could be a viable avenue for research to treat and hopefully reverse the progression of the disease as well as the prevention of periodontitis. Knowledge of the virulence factors possessed by *F. alocis* is limited due to its more recent discovery thanks to improved sequencing technologies. Fully understanding these virulence factors could be key in developing therapeutics that target this organism in hopes to rid the host of its presence. The oral microbiome is very complex, and more research needs to be done to understand how these pathogens of the periodontium, including *F. alocis*, interact with each other to create such a detrimental dysbiotic environment. Hopefully, with increased knowledge, this pathogen can be more well understood, and it can be less of a potential detriment to human health.

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