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Filifactor alocis — a new emerging periodontal pathogen

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Abstract

Filifactor alocis, a previously unrecognized Gram-positive anaerobic rod, is now considered a new emerging pathogen that may play a significant role in periodontal disease. *F. alocis*' unique characteristics and variations at the molecular level that may be responsible for the functional changes required to mediate the pathogenic process are discussed.

Keywords

Filifactor alocis; Polymicrobial infection; Periodontitis; Community dynamics

1. Introduction

Recent technological advances that have more clearly defined the oral microbiome have yielded novel insights and a paradigm shift in the etiology of periodontal diseases. Periodontitis, characterized by chronic inflammation, alveolar bone loss and destruction of gingival and periodontal ligament attachments to the teeth, affects approximately 65 million people in the United States [1]. Its occurrence is also associated with systemic diseases such as cardiovascular disease [2], rheumatoid arthritis [3], Alzheimer's disease [4]. With more than 650 species of bacteria identified in the human oral cavity, only a subset of these microbes which now includes previously unrecognized and yet-unculturable species [5] are associated with the disease. In addition to the important "red complex" bacteria along with other cultivable bacterial species such as Prevotella intermedia, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Selenomonas noxia, and *Eubacterium nodatum* are associated with periodontitis, organisms such as *Selenomonas, Synergistes, Desulfobulbus*, TM7 (new candidate bacterial division) and *Filifactor alocis* have now been identified as new potential pathogens in a number of independent studies [6,7]. Moreover, with 20–60% of the phylotypes identified in the oral cavity are yet-to-be cultivated [6,7] raises questions on the relative significance of these microbes in the disease process. This

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Conflict of interest

The authors disclose no conflict of interest.

review focuses on the new organism *F. alocis* and explores its unique characteristics, virulence potential and capacity to influence the oral microbiome in community dynamics and a role in periodontitis.

2. Incidence

Multiple studies have documented the increasing incidence and importance of *F. alocis* (reviewed in Ref. [8]) (Table 1). In comparison to the other traditional periodontal pathogens, the high incidence of *F. alocis* in the periodontal pocket compared to its absence in healthy or periodontitis resistant patients has highlighted its importance in the infectious disease process [9]. *F. alocis* has also been discovered in the canals of root-filled teeth with periapical lesions and is associated with signs and symptoms of endodontic infections [10]. It has also been identified as one of the prevalent phylotypes in cases of failed endodontic treatment [11]. There is documented evidence that *F. alocis* is associated with peri-implantitis [12]. Tamura et al. [12] have shown that the sulcus around oral implants with peri-implantitis harbors high levels of asaccharolytic anaerobic Gram-positive rods (AAGPRs) including *F. alocis* which is one of the most prominent in that environment [12]. Collectively, these studies implicate *F. alocis* as one of few organisms associated with multiple oral infections.

3. Morphological and cultural characteristics

F. alocis is a non-spore forming, Gram-positive obligate anaerobic rod that is slow growing and generally unreactive to conventional biochemical tests, hence difficult to identify [13]. The main habitat of *F. alocis* is the gingival sulcus [14]. *F. alocis* was first isolated in 1985 from the gingival sulcus in gingivitis and periodontitis patients and originally classified as *Fusobacterium alocis*, [14], but later reclassified into the genus *Filifactor* [13]. *In silico* analysis of *F. alocis* has shown close relatedness to *Clostridium* and *Fusobacterium* [15]. Common to these genera is their asaccharolytic nature thus an ability to utilize specific amino acids including arginine. Consistent with this observation, arginine has been shown to stimulate the growth of *F. alocis* [16].

4. Virulence factors

F. alocis appears to have unique properties such as resistance to oxidative stress with its stimulated growth under these condition and genes coding for a well-developed amino acid metabolic pathway that can allow it to colonize and survive with other traditional periodontal pathogens in the stress environment of the periodontal pocket [15,17]. These unique properties of *F. alocis* in addition to its ability to interact with other microbial species forming a polymicrobial synergistic relationship can enhance its invasive capacity [15] and cause chronic inflammation [18] in prevailing adverse conditions including fluctuations in nutrient availability, temperature, pH and oxygen tension. Additionally, an impact of *F. alocis* on the host is its ability to induce proinflammatory cytokines triggering apoptosis of gingival epithelial cells [19]. Other interactions with the host have triggered in *F. alocis* the upregulation of several proteins (e.g. proteases, proteins involved in secretion systems and proteins with cell wall anchor motifs) that are considered to have virulence

properties in other bacteria [20]. Collectively, these observations implicate specific *F. alocis* that may be of significance in the pathogenic process.

4.1. Proteases

Proteases play a significant role in virulence among the major oral pathogens. Similarities in virulence attributes among these organisms have been notably due to the presence of a battery of proteases. In Gram-positive bacteria, proteolysis plays a pivotal role in major biological processes such as post-translational regulation of gene expression, processing and maturation of proteins. Also, expression of various surface proteins involved in virulence modulation depends on proteolysis which could strongly influence the levels of activity of proteases and their cellular mobility.

The *F. alocis* genome possesses at least 15 different proteases [20]. Moreover, there are variations in the expression of some of these proteases among strains of *F. alocis* when a low passaged strain was compared to the type strain [20]. Several of these proteases may have functional similarity to those of other periodontal pathogens. A comparison of proteases between *F. alocis* and the red complex bacteria is given in Table 2. Membrane bound proteases of *F. alocis*, Caax protease (HMPREF0389_00590) could be involved in protein and/or peptide modification and secretion [21]. There was higher expression of Caax proteases during *F. alocis* co-culture with *Porphyromonas gingivalis* [20]. Other than their metalloprotease activity, the Caax amino-terminal proteases in other oral bacteria such as *Streptococcus gordonii*, have been shown to play an important role in transport of proteins and also protect the bacteria against bacteriocins. Additionally, the Xaa-pro-dipeptidase (HMPREF0389_01538), O-sialoendo-peptidase (HMPREF0389_01445), Nlp/P60 family protein (peptidase M23/37) (HMPREF0389_00239) and oligo endo-peptidase M3 family (HMPREF0389_00926), were shown to be present only in the membrane fraction of *F. alocis*. However, the protease (HMPREF0389_00122) was identified only in the extracellular fraction. Additionally, this protease is predicted to possess a collagen peptidase function. The role of this enzyme could be important in *F. alocis* pathogenesis since several oral pathogens are known to produce or induce host-derived collagenases that are implicated in tissue destruction in periodontal diseases [22].

4.2. Adhesion

Attributes such as adherence and invasion of host cells, are considered important to the success of a pathogen. Genes encoding for proteins such as CaaX aminopeptidases may be crucial for masking the host ubiquitin system and facilitate invasion of host cells [23].

F. alocis was shown to adhere and invade epithelial cells [15]. These attributes were enhanced in the presence of *P. gingivalis* [15]. While a similar enhancement of invasion was observed between *P. gingivalis*, *F. nucleatum* and *P. intermedia* [24], *F. nucleatum* and *Streptococcus cristatus* [25], and *F. nucleatum* and *Pseudomonas aeruginosa* [26], the exact mechanism for *F. alocis* is unclear.

Co-infection of *F. alocis* with *P. gingivalis* showed filapodial projections on the surface of the host cell that were believed to mediate the organism's internalization. Also, vesicle-

mediated internalization of *P. gingivalis* and *F. alocis* was observed during invasion of epithelial cells in co-infection studies [15]. This process may protect the pathogen after invasion, facilitating its pathogenic potential. The mechanism of membrane-ruffling commonly noted among Gram-positive bacteria during invasion strategies, were not noted in *F. alocis*. Since vesicle-mediated endocytic internalization of Gram-positive bacteria are generally mediated by type II and III exotoxins [27], it is likely that such exotoxins may contribute to the enhancement of internalization observed during *F. alocis–P. gingivalis* co-infection. It is worthy to note that several exotoxins have been identified in *F. alocis* but their role in invasion remains unclear.

Proteomic analysis of *F. alocis* during co-infection of epithelial cells with *P. gingivalis* using tandem mass tagging technique revealed increase in several membrane adhesion proteins and Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs). *In silico* analysis of the mass spectrometry data using database search and domain prediction revealed some of these adhesion proteins to be known virulence factors in other systems, however, the functions of several unique hypothetical proteins with transmembrane domains needs to be elucidated. Taken together, this suggests that community dynamics through the interaction of *F. alocis* and *P. gingivalis* may result in the upregulation of specific factor(s) that may enhance their virulence potential [15]. In our preliminary studies, *F. alocis* co-cultured with *P. gingivalis* showed adhesion to epithelial cells and alteration in their morphology leading to cell death. This was in contrast to mono-infections with either *F. alocis* or *P. gingivalis* which did not trigger the same morphological alteration, although these bacteria were still able to induce cell death over a longer time period [15].

4.2.1. MSCRAMMs—MSCRAMMs are known to play an important role in Gram-positive bacterial virulence by mediating adherence to and colonization of host tissues as an early step toward clinically manifested infection. There is also evidence to suggest that these extracellular matrix adhesion proteins can be regulated by quorum-sensing, which implies that some environmental signals can modulate their expression and hence promote adhesion and colonization. MSCARMMs are evidently important in the pathogen attachment and virulence modulation. Expression of *F. alocis* MSCRAMMs have been identified in the cell membrane and cell wall fractions [20]. Genome annotations of putative *F. alocis* MSRAMMs compared with the three red complex bacteria are given in Table 2. Among the MSCRAMMs of *F. alocis*, the collagenolytic MSCRAMMs and collagenase related proteins could be important to bring about initial pathogen interaction and host adherence due to proteolysis of the common extracellular matrix (ECM)-collagen. The role of collagenases in periodontal pathogenesis has been well documented in other pathogens [28-30]. Our preliminary studies based on *in silico* analysis of *F. alocis* collagenases also reveal molecular relatedness to some of the collagenolytic MSCRAMMs of other pathogenic bacteria. It has been reported over the past decade that many organisms that express cell surface adhesins can mediate microbial adhesion to the ECM of the host tissues. Hence, *F. alocis* can also target the ECM in a similar manner through interaction with collagenolytic MSCRAMMs.

5. Unique amino acid metabolism

Even though *F. alocis* showed low gingipain-type activity, it had increased non-gingipain protease activity [15]. The amino acids mostly utilized by *F. alocis* include arginine and lysine, followed by cysteine. The *F. alocis* arginine metabolic pathway predicts the enzymatic degradation of arginine by arginine deiminase, leading to the conversion of arginine to ornithine and ammonia [16]. Arginine degradation could favor increase in the pH that would counteract acidic conditions generated from carbohydrate catabolism in a mixed bacterial oral flora. In the periodontal pocket, these amino acids can also be made available from the degradation of various protein substrates by other bacteria and host-derived proteases for nutritional support, survival and virulence [31].

In silico analysis of the *F. alocis* genome predicts a well-developed amino acid metabolism pathway that should be functional to catabolize protein and amino acids. One among the well-developed pathways identified in *F. alocis* is the arginine deiminase pathway which is involved in arginine metabolism leading to citrulline and ornithine (Fig. 1). Earlier studies have shown that *F. alocis* can convert arginine to ornithine through an enzymatic process without the intermediate step of citrulline production [16]. This process may be vital for its survival as studies from our laboratory have shown that the *arg* operon consisting of 5 genes involved in arginine metabolism were highly upregulated during co-infection studies with *P. gingivalis*. While the three genes involved in arginine deiminase pathway namely the arginine deiminase (*HMPREF0389_ 001584*), ornithine carbamoyltransferase (*HMPREF0389_ 00791*) and carbamate kinase (*HMPREF0389_ 00535*) were annotated in the genome of *F. alocis, in silico* analysis of ornithine carbamoyltransferase the enzyme involved in conversion of L-citrulline to L-Ornithine show an ornithine/aspartate carbamoyltransferase domain inferring a dual role of the enzyme. *F. alocis* also possess glutamate N acetyl transferase (*HMPREF0389_ 001568*) an enzyme know to have dual role in other bacteria that converts glutamate one of the important intermediary metabolites during energy metabolism to Ornithine.

Certain oral bacteria like *F. nucleatum* lack essential amino acid synthetic pathways and rely on the ability to import and degrade di and oligo peptides [32]. Consistent with the assacharolytic properties of *F. alocis*, several proteins that play an important role in amino acid metabolism (e.g. Arginine degradation pathway proteins referred above) including many that may contribute to protein degradation (such as peptidases and proteases) are encoded in its genome (http://www.ncbi.nlm.nih.gov/bioproject/46625). Even if many inherent amino acid synthesis pathways may be nonfunctional, the occurrence of a wide range of such dipeptidases, metalloproteases and o-sialoglycoproteases, could likely provide *F. alocis* with the appropriate substrates to compensate for its nutritional needs. Additionally, certain proteins, such as the oxy acyl carrier protein (HMPREF0389_ 01112) which is involved in fatty acid metabolism and not usually identified among the oral biofilm forming pathogens, fibronectin binding protein (HMPREF0389_00575) and dipicolinate reductase (HMPREF0389_01077) which are involved in amino acid metabolism and virulence [33], were also identified in *F. alocis* [20]. Taken together, it is likely that *F. alocis* may be well adapted to provide for its own nutritional needs. However, the role these systems play in bacterial community dynamics should be further elucidated.

6. Co-existence, polymicrobial synergy and biofilm formation

Oral bacterial composition varies during the progression of disease from a scanty biofilm forming Gram-positive bacteria to increased number of Gram-negative bacteria. Oral biofilms are primary initiating factors of periodontal disease. Biofilm formation involving *F. alocis* has been demonstrated both in periodontic and endodontic cases [34]. While some interspecies interaction can inhibit biofilm formation, *P. gingivalis* ATCC 33277 co-cultured with *F. alocis* showed significant increase in biofilm formation [15]. This enhanced biofilm forming capacity may be due to the ability of both species to auto aggregate and express unique components. This could indicate a commensal relationship between *F. alocis* and *P. gingivalis*. Thus, *F. alocis* and *P. gingivalis*, each with different growth rates, could form a mixed species biofilm and coexist [15]. As a result, *F. alocis* proteins could enable *P. gingivalis* to proliferate and disseminate from these biofilms thus facilitating its virulence.

A recent *in vitro* study, evaluating the community interactions of two strains of *F. alocis* with *S. gordonii*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans*, which are organisms of differing pathogenic potential in the oral cavity, suggests that *F. alocis* is likely to interact with a variety of oral bacteria and participate in community development [35]. Further, *F. alocis* colonization seemed influenced by the spatial composition of microbial microenvironments through organism preferentially accumulating at sites rich in *F. nucleatum*. *S. gordonii* was antagonistic to the accumulation of *F. alocis* in a dual species community. This was consistent with the observation that streptococcal rich dental plaques were resistant to colonization by *F. alocis* [35]. In three species communities of *S. gordonii*, *F. nucleatum* and *F. alocis*, the antagonistic effects of *S. gordonii* superseded the synergistic effects of *F. nucleatum* toward *F. alocis* [35]. The interaction between *A. actinomycetemcomitans* and *F. alocis* was strain specific. It was also noted that *A. actinomycetemcomitans* could either stimulate *F. alocis* accumulation or have no effect, depending on the strain. *P. gingivalis* and *F. alocis* formed heterotypic communities, with the abundance of *P. gingivalis* being enhanced in the presence of *F. alocis* [35]. While the mechanism of the interaction is complex with inhibitory and counterbalancing measures, It is likely that *F. alocis* proteins induced under those conditions may facilitate adhesion and nutrient support for *P. gingivalis*. Also the question of how arginine deiminase affects the community dynamics can be raised. The inhibitory effect of *P. gingivalis* on *F. alocis* was observed to be partially dependent on the minor fimbriae [35]. The arginine deiminase of *S. cristatus* is known to suppress fimbrial production in *P. gingivalis* [36]. Based on the relative abundance of *F. alocis* in the periodontal pocket compared to *P. gingivalis*, it is unclear if the *F. alocis* arginine deiminase is induced in that microenvironment and could have an effect on *P. gingivalis* fimbrial expression. However, the primary role of a well-developed arginine degradation pathway leads to the concept of reduction in pH and increased CO_2 production that might have an effect on the expression of virulence factors.

7. Oxidative stress resistance

Our earlier studies have shown that *F. alocis* is relatively resistant to oxidative stress compared to *P. gingivalis* and that its growth is stimulated under those conditions [15]. These observations may indicate an important attribute for the survival and relative

abundance of *F. alocis* compared to other organisms in the inflammatory microenvironment of the periodontal pocket. It is also likely that *F. alocis* may play a role as an "oxidative sink" to stabilize the microbial community in the microenvironment of the periodontal pocket. It was also noted that survival of *P. gingivalis* under hydrogen peroxide-induced oxidative stress is enhanced in the presence of *F. alocis*. A likely mechanism could be due to the presence of sialidase activity in *F. alocis* that not only satisfy its asaccharolytic property by breakdown of sialated glycoproteins found in saliva, but the released sialic acid can also act as a reactive oxygen species (ROS) scavenger to reduce the oxidative stress in the inflammatory environment of the periodontal pocket. *F. alocis* possesses a superoxide reductase (GenBank accession no. EFE28874) that could help to facilitate its growth in the presence of hydrogen peroxide (http://www.ncbi.nlm.nih.gov/bioproject/46625).

The interaction of *F. alocis* with other organisms can also enhance its oxidative stress resistance and hence its virulence potential. In co-culture with *P. gingivalis*, an upregulation of many proteins involved in oxidative stress resistance such as superoxide reductase, iron–sulfur cluster protein, iron permease, rubrerythrin, ferrous hydrogenase family protein and thioredoxin family proteins were observed in *F. alocis* [20]. One of the key attributes of *F. alocis* is the 3-methyladenine DNA glycosylase (HMPREF0389_1529), an enzyme reported to be involved in oxidative and nitrosative stress resistance in other pathogenic bacteria; although, its function under those conditions is unclear. The genome of *F. alocis* also includes genes that encode for a well-developed group of iron sulfur cluster proteins and a ferrous iron transport system which are unique to this organism compared to other "red complex" bacteria. Additionally, *F. alocis* seems to possess a well-developed protein sorting/transport system which is evident by the presence of a large number of membrane proteins [20]. It is likely that surface and secretory proteins from *F. alocis* may play a role in this protein transport process. With a well-developed arginine deiminase pathway leading to Ornithine production, hence similar to other bacteria, ornithine production could also favor oxidative stress resistance in *F. alocis*. Together, these systems could possibly facilitate the efflux of ROS.

8. CRISPR-associated genes

Regions of unusual DNA composition in the bacterial genome such as the clustered regularly interspaced short palindromic repeats (CRISPR) locus together with the CRISPR-associated genes are thought to act as adaptive immunity systems in bacteria and recently found to play a role in bacterial virulence [37]. The virulence attribute may partly be due to genome rearrangement and/or regulation of gene expression that will facilitate host environment adaptation.

CRISPRs are widely distributed amongst bacteria and archaea [38] and show some sequence similarities [39], however their most notable characteristic is their repeating spacers and direct repeats which is the basis for their division into three groups. The three groups are further divided into subgroups depending on a specific combination of Cas genes. Genes encoding Cas 1 and 2 are present in each group. Of the three major groups, type II is the most studied. Type I CRISPR–Cas system is based on the ubiquitous presence of a signature protein, the Cas3 helicase/nuclease [40]. The Type II CRISPR/Cas locus contains Cas1,

Cas2 and Cas9, as well as a predicted trans-activating crRNA (tracrRNA), and a small CRISPR/Cas-associated RNA (scaRNA) [41]. The Type III CRISPR/Cas system is typified by the presence of Cas10 [40]. Based on phylogenetic analyses of *Cas9* (member of type II system) and its orthologues, *F. alocis* is predicted to be a member of the Type II-A CRISPR–Cas system (Fig. 2) [42]. The locus architecture of Type II CRISPR–cas system consists of *cas9, cas1, cas2, csn2a* in other bacteria (Fig. 3A) [42]. The orientation of CRISPR–Cas system in *F. alocis* is found to be localized in two clusters (Fig. 3B). The upregulation of CRISPR/Cas system components during the co-infection of epithelial cells with *F. alocis* and *P. gingivalis* (Table 3) could suggest their role in virulence and pathogen synergy. The virulence of *F. alocis* was enhanced in the presence of *P. gingivalis* [17]. The significance of the CRISPR–Cas system in this process needs further investigation.

9. Host cell modulation

Various studies have demonstrated the ability of *F. alocis* to impact host cells, enabling its persistence in the periodontal pocket. *F. alocis* infection demonstrated its ability to induce the secretion of the proinflammatory cytokines IL-1β, IL-6 and TNF-α from epithelial cells, while also inducing their apoptosis through pathways involving caspase-3 [19]. The *in vivo* pathogenicity of *F. alocis* tested using a mouse subcutaneous chamber model induced a local inflammatory response as observed by the influx of neutrophils in addition to an increase in the IL-1β, IL-6 and TNF-α proinflammatory cytokine levels [43]. Overall, with respect to the various mechanisms by which *F. alocis* could possibly impact host cells, proteome analysis of *F. alocis* in co-infection of epithelial cells with *P. gingivalis* revealed proteins that could contribute to several functions including host cell signaling, metabolic host response, cell–cell interaction, and activation of oncogenes, among others [17].

Host responses to bacterial infections may favor survival and play a role in pathogenesis through modulation of metabolic processes. High amounts of arginine in the periodontal pocket and the abundance of *F. alocis* proteins involved in arginine metabolism and citrulline synthesis, such as arginine deiminase (HMPREF 0389_01584), acetyl ornithine transferase (HMPREF0389_01570) [20], aminotransferases (HMPREF0389_01352 and HMPREF0389_01353), amino-transferase family protein (HMPREF 0389_00349), arginine – tRNA ligase (HMPREF0389_00390), and arginine decarboxylase (HMPREF0389_00102) (http://www.ncbi.nlm.nih.gov/bioproject/46625), indicate that the nutritional needs of the bacterium could be adequately met during infection and vital for its survival in the harsh microenvironment of the periodontal pocket. Furthermore, its interaction with other microbes may collectively enhance their survival. Ammonia production from arginine metabolism has been identified as an important mechanism by which oral bacteria are protected against acid killing. Among the three key enzymes, namely, arginine deiminase, ornithine carbamoyltransferase and carbamate kinase important in arginine metabolism [44], *F. alocis* genome shows genes coding for arginine deiminase, and carbamate kinase but not the ornithine carbamoyltransferase. It is noteworthy that *P. gingivalis* and *F. alocis* interspecies interaction resulted in the upregulation of arginine deiminase (HMPREF_0389_01584) and carbamate kinase (HMPREF_0389_00535) in *F. alocis*. While it may use a novel arginine catabolic pathway compared to other AAGPRs in the periodontal pocket [16], its relative abundance in the periodontal pocket and it's enhanced ability to

produce ammonia could promote species co-habitation and survival. Because butyrate is a metabolic end product from arginine, it could likely also have an impact on other microbial interactions including viruses in the oral cavity. Butyric acid produced by periodontopathic bacteria including *P. gingivalis* can lead to viral reactivation. The impact of viral infection on periodontal disease is now being recognized as the active inflamed lesion appears to be a major site for re-activation and accumulation of Herpes virus resulting in enhanced tissue breakdown.

Interrogation of the *F. alocis* genome also revealed templates for a well-developed citrulline synthesis mechanism using arginine [16]. Citrullination of proteins is understood to be an important posttranslational modification with systemic implications. Previous studies have shown that upregulation of peptidylarginine deiminase (PAD) expression and the associated increase in citrullinated proteins were found in patients with rheumatoid arthritis [45]. A mechanistic link between periodontal infection and rheumatoid arthritis has been established, collagen-induced arthritis was dependent on the expression of a unique *P. gingivalis* peptidylarginine deiminase (PPAD) [46]. The arginine deiminase from pathogens was shown to possess multiple regulatory roles similar to PAD function [47]. Bioinformatic analyses indicate that *P. gingivalis* PAD has major sequence and structural homology with the *F. alocis* arginine deiminase enzyme (unpublished data). It is likely that in *F. alocis*, arginine deiminase could have citrullination-induced systemic implications.

It is also important to note that the proteins ornithine transaminase (HMPREF0389_01570), acetyl glutamate kinase (HMPREF0389_01569), glutamate racemase (HMPRE F0389_00100) and aminotransferase (HMPREF0389_00478) involved in ornithine biosynthesis were identified in *F. alocis*. In fact, arginine deiminase (HMPREF0389_01584) involved in ornithine catabolism and urea breakdown was found both in the membrane and the extracellular fractions of *F. alocis* [20] suggesting a well-developed nitrogen assimilatory pathway that may play a role as an alternative mode of amino acid synthesis in *F. alocis*.

10. Conclusion

F. alocis is one of a few bacteria that is associated with multiple oral diseases including periodontitis, localized aggressive periodontitis, endodontitis and peri-implantitis. Its relative abundance in the periodontal pocket of patients with periodontitis may support the hypothesis to include *F. alocis* as a diagnostic marker organism. This organism has unique potential virulence characteristics such as resistance to oxidative stress and genes coding for a well-developed amino acid metabolic pathway that can modulate multiple changes to the oral microbial community and the host cell proteome, that collectively can lead to the disease process. Most of the potential virulence attributes of this assacharolytic bacteria is unexplored and deserves further extensive study.

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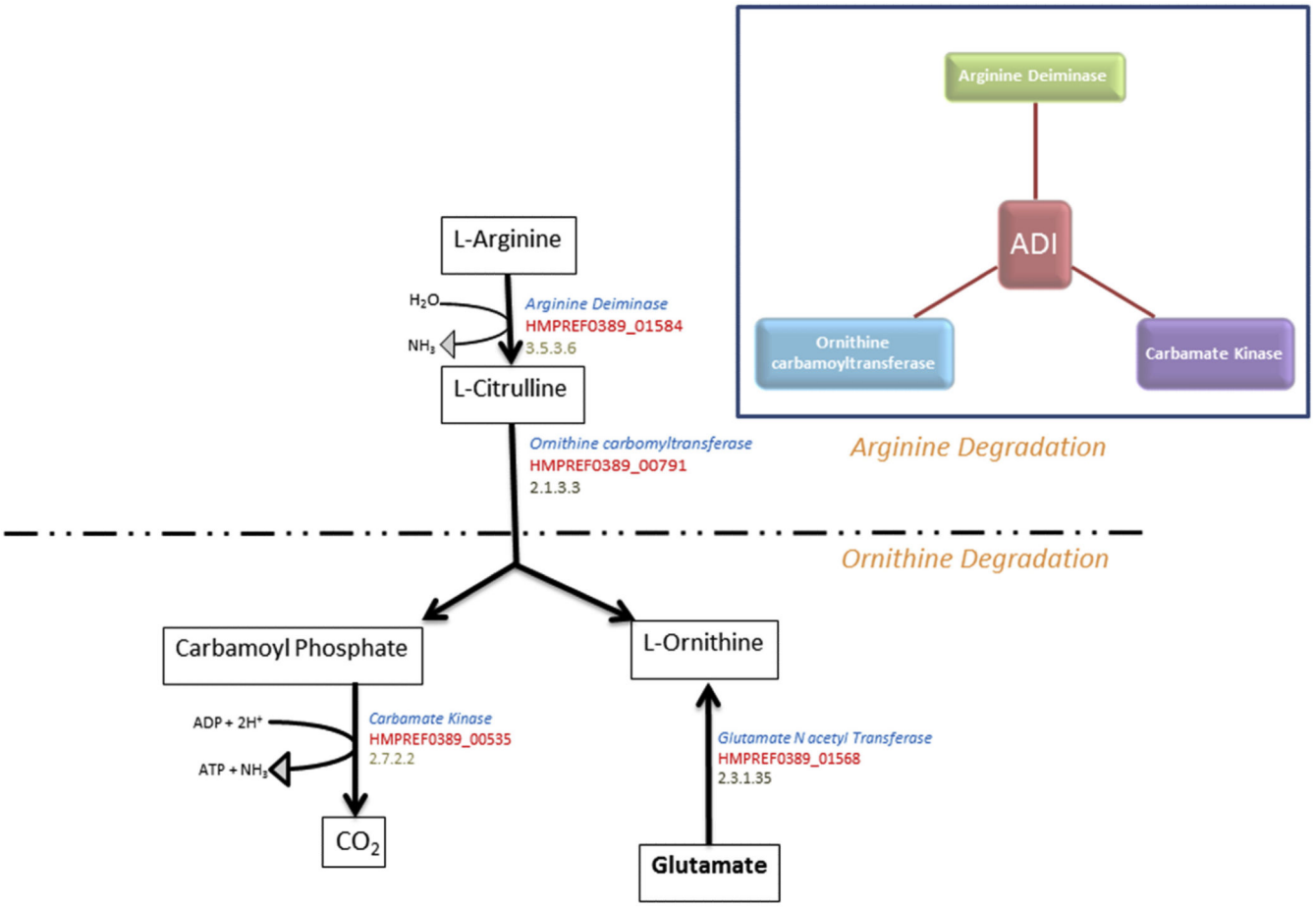

Fig. 1.

Arginine degradation in *F. alocis* through the arginine deiminase pathway. The boxes show the metabolites. The NCBI annotations are given in red, gene descriptions are given in blue and enzyme nomenclature (EC number) is given in gray. The inset showing the three enzymes of the arginine deiminase pathway.

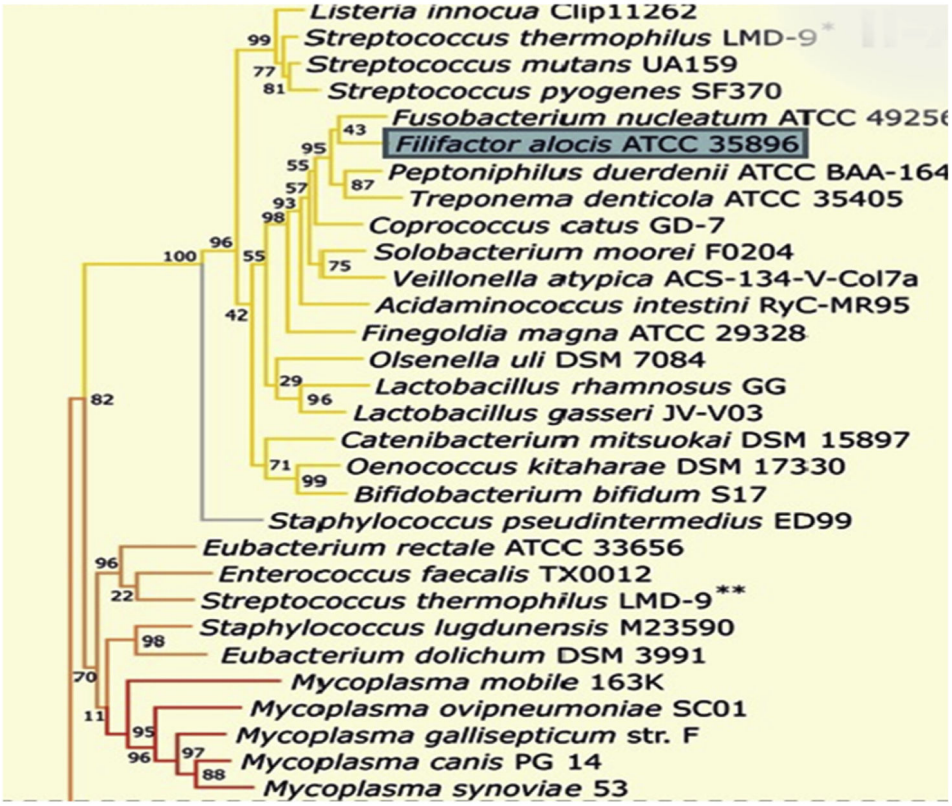

Fig. 2.

Phylogenetic tree of representative Cas9 sequences. Bootstrap values calculated for each node are indicated. Blue box shows the location of *F. alocis*. Subclusters of similar Cas9 orthologs are indicated by the same branch colors.

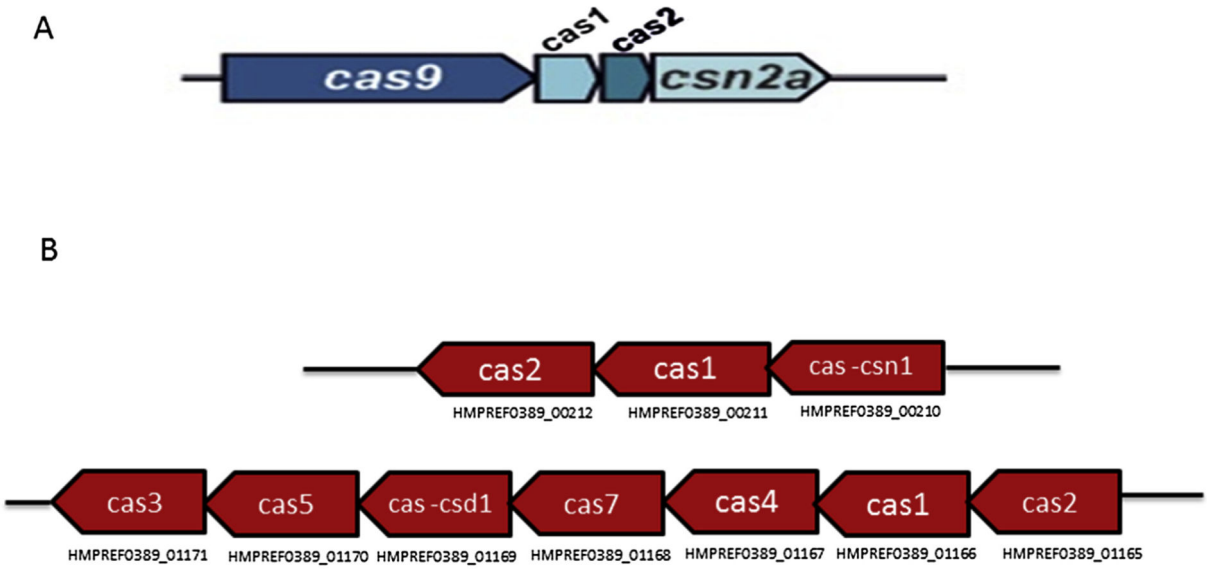

Fig. 3.

A. Genome organization of Type II CRISPR–Cas system [42]. B. *F. alocis* genome organization of CRISPR–Cas system genes located in two clusters.

Table 1

Pubmed information on *F. alocis*.

Relevant information on *F. alocis*	**Reference**
F. alocis a common taxa noted in the subgingival microbiome of smokers compared to non-smokers	[47]
F. alocis one among the four predominant bacteria in subgingival plaque samples that could produce hydrogen sulphide	[48]
F. alocis showed variation in proteome modulation during co-infection with *P. gingivalis*	[17]
F. alocis showed strain variation in virulence and expression of proteins. Showed major virulence factors common to other bacteria.	[20]
Incidence of *F. alocis* in root canals and adjacent periodontal pockets in combined periodontal-endodontic lesions.	[49]
F. alocis virulence in mouse subcutaneous chamber model	[19]
F. alocis was more frequently identified at higher levels in the salivary bacterial profile in periodontitis patients than in control samples	[50]
F. alocis was one of the predominant bacterial species present in peri-implantitis cases.	[12]
F. alocis showed variations in dual species community interactions. Showed synergism with *F. nucleatum* and was antagonistic to the accumulation of *S. gordonii. A. actinomycetemcomitans* showed accumulation or no effect and *F. alocis* with *P. gingivalis* formed synergistic relationship and *P. gingivalis* was shown benefited from the relationship.	[35]
Investigation of specific anaerobic species in necrotic teeth in the pulp chamber and root canal showed the frequency of incidence of *F. alocis* to be 73.3%.	[51]
Prevalence of *F. alocis* in aggressive periodontitis	[52]
Prevalence of *F. alocis* in endodontitis	[53-57]
Prevalence of *F. alocis* in subgingival microbiota	[7,58]
F. alocis virulence attributes	[15,18,34]
Incidence of *F. alocis*	[9,10,59]
F. alocis classification, isolation and phylogeny	[60-62]

Table 2

Comparison of proteases and MSCRAMMs between *F. alocis* and the red complex bacteria.

No.	Protease	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
1.	RIP metalloprotease	RIP metalloprotease RseP (HMPREF0389_00112)	Membrane-associated zinc metalloprotease (PGTDC60_1498) 97 Zinc metalloprotease (PGN_1582) *[RIP metalloprotease domain]* Membrane-associated zinc metalloprotease (PG0383) *[RIP metalloprotease domain]*	Membrane-associated zinc metalloprotease (TDE2341) *[RIP metalloprotease domain]*	Putative RIP metalloprotease RseP (BFO_2027) *[RIP metalloprotease domain]*
2.	Protease	Protease (HMPREF0389_00122) *[Collagenase domain]*	Protease (PG0753) *[Collagenase domain]*	Protease II (TDE2140) *[Peptidase_S9; Prolyl oligopeptidase family]*	Protease 2 (BFO_3078) *[Peptidase_S9; Prolyl oligopeptidase family]*
3.	ATP-dependent protease La	ATP-dependent protease La (HMPREF0389_00279)	ATP-dependent protease La (PG0620) ATP-dependent protease La (PGN_0662) Lon ATP-dependent protease La (PGTDC60_1748)	ATP-dependent protease La (TDE0670)	Lon endopeptidase La (BFO_3077) *[LON; ATP-dependent protease La (LON) domain]*
4.	Zinc protease	Zinc protease (HMPREF0389_00298)	Zinc protease (PGN_0303) Membrane-associated zinc metalloprotease (PGTDC60_1498) *[S2P-M50; Site-2 protease (S2P) class of zinc metalloproteases]* ATP-dependent protease ATP-binding subunit ClpX (PG0417) *[zf-C4_ClpX; ClpX C4-type zinc finger domain]* ATP-dependent protease ATP-binding subunit ClpX (PGTDC60_1529) *[zf-C4_ClpX; ClpX C4-type zinc finger]* Zinc metalloprotease (PGN_1582) *[RIP metalloprotease RseP domain]* Membrane-associated zinc metalloprotease (PG0383) *[RIP metalloprotease RseP domain]* ATP-dependent protease ATP-binding subunit ClpX (PGN_1550) *[zf-C4_ClpX;*	Membrane-associated zinc metalloprotease (TDE2341) *[PDZ_metalloprotease; PDZ domain of bacterial and plant zinc metalloproteases]* ATP-dependent protease ATP-binding subunit ClpX (TDE1673) *[clpX; ATP-dependent protease ATP-binding subunit ClpX; Provisional]* Hypothetical protein (TDE2485) *[zf-ribbon_3; zinc-ribbon domain]*	ATP-dependent Clp protease ATP-binding subunit ClpX (BFO_1501) *[zf-C4_ClpX; ClpX C4-type zinc finger]* Putative RIP metalloprotease RseP (BFO_2027) *[zinc metalloproteases]* Thermolysin metallopeptidase, catalytic domain-containing protein (BFO_0703) *[LasB; Zinc metalloprotease]* Hypothetical protein BFO_2238 *[S2P-M50; Site-2 protease (S2P) class of zinc metalloproteases]*

No.	Protease	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
			ClpX C4-type zinc finger]		
5.	ATP-dependent zinc metalloprotease FtsH (neutral zinc metallopeptidase family protein)	Neutral zinc metallopeptidase family protein (HMPREF0389_01001) *[Zn_peptidase_2; Putative neutral zinc metallopeptidase]*	ftsH transmembrane AAA-metalloprotease FtsH (PGTDC60_0044) *[FtsH_fam; ATP-dependent metalloprotease FtsH]* Transmembrane AAA-metalloprotease FtsH(PGN_0043) *[FtsH_fam; ATP-dependent metalloprotease FtsH]* Cell division protein FtsH(PG0047) *[FtsH_fam; ATP-dependent metalloprotease FtsH domain]*	FtsH cell division protein FtsH (TDE0470) *[ATP-dependent metalloprotease FtsH domain]*	hflB ATP-dependent metallopeptidase HflB (BFO_2507) *[FtsH_fam; ATP-dependent metalloprotease FtsH domain]* Putative neutral zinc metallopeptidase (BFO_1527) *[Zn_peptidase_2; Putative neutral zinc metallopeptidase domain]* Thermolysin metallopeptidase, catalytic domain-containing protein (BFO_0703) Zinc [metalloprotease (elastase) [Amino acid transport and metabolism] domain]
6.	Caax amino protease family protein	CAAX amino protease family protein (HMPREF0389_00590)	Abortive infection protein (PGN_1454) *[Abi; CAAX protease self-immunity domain]* Putative abortive infection protein (PGTDC60_1637) *[Abi; CAAX protease self-immunity]* PG0518 hypothetical protein *[Abi; CAAX protease self-immunity]*	CAAX amino terminal protease (TDE0275) CAAX amino terminal protease (TDE1288) CAAX amino terminal protease (TDE0716) CAAX amino terminal protease (TDE1870) Hypothetical protein (TDE1873) *[Abi; CAAX protease self-immunity]* Hypothetical protein (TDE1871) *[Abi; CAAX protease self-immunity]* Hypothetical protein (TDE1317) *[Abi; CAAX protease self-immunity]*	CAAX amino terminal protease family protein (BFO_1873) CAAX amino terminal protease family protein (BFO_0185) Pseudo (BFO_1506) *[CAAX amino terminal protease family protein]*
7.	Caax amino protease	CAAX amino protease (HMPREF0389_00677)	Abortive infection protein (PGN_1454) *[Abi; CAAX protease self-immunity domain]* Putative abortive infection protein (PGTDC60_1637) *[Abi; CAAX protease self-immunity]* PG0518 hypothetical protein *[Abi; CAAX protease self-immunity]*	CAAX amino terminal protease (TDE0275) CAAX amino terminal protease (TDE1288) CAAX amino terminal protease (TDE07160) CAAX amino terminal protease (TDE1870) Hypothetical protein(TDE1873) *[Abi; CAAX protease self-immunity]* Hypothetical protein (TDE1871) *[Abi; CAAX protease self-immunity]* Hypothetical protein (TDE1317) *[Abi; CAAX protease self-*	CAAX amino terminal protease family protein (BFO_1873) CAAX amino terminal protease family protein (BFO_0185) Pseudo (BFO_1506) *[CAAX amino terminal protease family protein]*

No.	Protease	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
				immunity]	
8.	Metalloprotease	Metalloprotease (HMPREF0389_00692)	Membrane-associated zinc metalloprotease (PGTDC60_1498) Transmembrane AAA-metalloprotease FtsH (PGTDC60_0044) Zinc metalloprotease (PGN_1582) Membrane-associated zinc metalloprotease (PG0383) Transmembrane AAA-metalloprotease FtsH (PGN_0043)	Metalloprotease (TDE1242) Membrane-associated zinc metalloprotease (TDE2341) (TDE2337) aminopeptidase *[Peptidase_M29; Thermophilic metalloprotease (M29)]*	Hypothetical protein (BFO_1297) *[Zinc-dependent metalloprotease]* Hypothetical protein (BFO_2661) *[Zinc-dependent metalloprotease]*
9.	Glycoprotease family protein	Glycoprotease family protein (HMPREF0389_01443)	Putative glycoprotease (PGTDC60_1893) Glycoprotease (PGN_0802) Hypothetical protein (PG0778) *[Peptidase_M22; Glycoprotease family]* DNA-binding/iron metalloprotein/AP endonuclease (PGN_0393) *[PRK09604; UGMP family protein]*	Glycoprotease (TDE1468) DNA-binding/iron metalloprotein/AP endonuclease (TDE2504) *[COG1214; Inactive homolog of metal-dependent proteases]*	Putative glycoprotease GCP (BFO_0842) Hypothetical protein (BFO_2190) *[Peptidase_M22; Glycoprotease family]*
10.	Xaa pro dipeptidase	Xaa-Pro dipeptidase (HMPREF0389_01538)	PepD-1 aminoacyl-histidine dipeptidase (PGTDC60_0414) *[aa-his-dipept; Xaa-His dipeptidase]* PepD-2 aminoacyl-histidine dipeptidase (PGTDC60_1655) *[a-his-dipept; Xaa-His dipeptidase]* Peptidase M24 family (PGTDC60_1183) *[PepP; Xaa-Pro aminopeptidase]* M24 family peptidase (PGTDC60_0816) *[PepP; Xaa-Pro aminopeptidase]* Aminoacyl-histidine dipeptidase (PGN_0250) *[aa-his-dipept; Xaa-His dipeptidase]* Peptidase M24 family Aminoacyl-histidine (PGN_0914) *[PepP; Xaa-Pro aminopeptidase]* Dipeptidase (PGN_1434) *[aa-his-dipept; Xaa-His dipeptidase]* M24 family peptidase (PG0889) *[PepP; Xaa-Pro aminopeptidase]* pepD-2 aminoacyl-histidine	TDE2228 aminoacyl-histidine dipeptidase *[aa-his-dipept; Xaa-His dipeptidase]* TDE1482 peptidase, M24 *[PepP; Xaa-Pro aminopeptidase]*	pepD_1 Xaa-His dipeptidase (BFO_1038) *[aa-his-dipept; Xaa-His dipeptidase]* pepD_2 Xaa-His dipeptidase (BFO_2543) *[aa-his-dipept; Xaa-His dipeptidase]* Aminopeptidase P, N-terminal domain-containing protein (BFO_2955) *[Prolidase; Prolidase. E.C. 3.4.13.9. Also known as Xaa-Pro dipeptidase]* **Creatinase (BFO_2658)** *[PepP; Xaa-Pro aminopeptidase]* Putative glycoprotease GCP (BFO_0842) YeaZ hypothetical protein (BFO_2190) *[Peptidase_M22; Glycoprotease family]*

No.	Protease	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
			dipeptidase(PG0537) *[aa-his-dipept; Xaa-His dipeptidase]* pepD-1 aminoacyl-histidine dipeptidase(PG0137) *[aa-his-dipept; Xaa-His dipeptidase]* M24 family peptidase (PG1210) *[Xaa-Pro aminopeptidase]* Hypothetical protein (PGN_1050) *[Xaa-Pro aminopeptidase]*		
11.	O-sialoglycoprotein endopeptidase	O-sialoglycoprotein endopeptidase (HMPREF0389_01445)	gcp putative DNA-binding/iron metalloprotein/AP endonuclease (PG1724) Hypothetical protein (PG0778) *[Glycoprotease family]* Glycoprotease (PGN_0802) *[Glycoprotease family]*	–	Putative glycoprotease GCP (BFO_0842) yeaZ hypothetical protein (BFO_2190) *[Peptidase_M22; Glycoprotease family]*
12.	Serine protease HtrA	Serine protease HtrA (HMPREF0389_01460)	HtrA protein (PG0593) *[PDZ_serine_protease]* Heat shock-related protease htrA (PGTDC60_1718) *[PDZ_serine_protease]* Heat shock-related protease htrA protein (PGN_0637) *[PDZ_serine_protease]* PDZ domain-containing protein (PGTDC60_0574) *[PDZ_serine_protease]* Hypothetical protein (PGN_0391) *[PDZ_serine_protease]*	Trypsin domain/PDZ (TDE1966) *[PDZ_serine_protease domain]* Trypsin domain/PDZ (TDE2300) *[PDZ_serine_protease]* Trypsin domain/PDZ (TDE1343) *[PDZ_serine_protease]* Membrane-associated zinc metalloprotease (TDE2341) *[PDZ_serine_protease]*	DegP peptidase Do (BFO_0430) *[PDZ_serine_protease]*
13.	ATP-dependent Clp protease	ATP-dependent Clp protease ATP-binding subunit ClpX (HMPREF0389_01648)	ATP-dependent Clp protease, proteolytic subunit (PGTDC60_1530) ATP-dependent Clp protease proteolytic subunit (PG0418) ATP-dependent Clp protease, ATP-binding subunit ClpC (PGTDC60_0010) ATP-dependent Clp protease, ATP-binding subunit ClpC (PG0010) ATP-dependent Clp protease proteolytic subunit (PGN_1549) ATP-dependent Clp protease ATP-binding subunit ClpC (PGN_0008)	TP-dependent Clp protease, ATP-binding subunit ClpA (TDE2124) ATP-dependent Clp protease, ATP-binding subunit ClpB (TDE2327) ATP-dependent Clp protease proteolytic subunit (TDE2388) ATP-dependent Clp protease proteolytic subunit (TDE1672) Hypothetical protein (TDE2123) *[ClpS; ATP-dependent Clp protease adaptor protein ClpS]*	ATP-dependent Clp protease ATP-binding subunit ClpX (BFO_1501) ATP-dependent Clp endopeptidase, proteolytic subunit ClpP (BFO_1502) Hypothetical protein (BFO_2017) *[Clp_protease_like; Caseinolytic protease (ClpP) is an ATP-dependent protease]*

No.	Protease	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
14.	Carboxy-processing protease	Carboxy- processing protease (HMPREF0389_00522)	Zinc carboxypeptidase (PG0232) *[Peptidase_M14_like; M14 family of metallocarboxypeptidases and related proteins]* Hypothetical protein (PGN_0335) *[Peptidase_M14_like; M14 family of metallocarboxypeptidases and related proteins]* Carboxyl-terminal protease (PGTDC60_0120) *[C-terminal peptidase domain]* Carboxyl-terminal protease (PGTDC60_1149) *[C-terminal processing peptidase domain]* Carboxyl-terminal protease (PGTDC60_0515) *[C-terminal processing peptidase domain]* Carboxyl-terminal protease-like protein (PGTDC60_0655) Carboxyl-terminal processing protease (PGN_1788) *[C-terminal processing peptidase domain]* Carboxyl-terminal protease (PG1855) *[C-terminal processing peptidase domain]* Carboxyl-terminal processing protease) (PGN_0340) *[C-terminal processing peptidase domain]* Carboxyl-terminal processing protease (PGN_0952) *[C-terminal processing peptidase domain]* Carboxyl-terminal processing protease (PGN_1914) *[C-terminal processing peptidase domain]* Carboxyl-terminal protease (PG1060) *[C-terminal processing peptidase domain]* Carboxyl-terminal protease	–	–

No.	Protease	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
			(PG0235) *[C-terminal processing peptidase domain]* Carboxyl-terminal protease-like protein (PG1620)		
15.	Oligoendopeptidase F	Oligoendopeptidase F (HMPREF0389_00926) Oligoendopeptidase F (HMPREF0389_00527)	–	Oligoendopeptidase F (TDE2001) Oligoendopeptidase F (TDE2639) Oligoendopeptidase F (TDE2738)	–

No.	MSCRAMMs (putative)	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
1.	Fibronectin-binding protein	Fibronectin-binding protein (HMPREF0389_00575) *[Fibronectin binding domain]*	Hypothetical protein (PGN_1211) – *[Fibronectin binding domain]*	Fibronectin/fibrinogen-binding protein, internal deletion (TDE1579) *[Fibronectin/ fibrinogen binding domain]* Fibronectin type III (TDE0446) *[Fibronectin 3 domain]*	Fibronectin type III domain-containing protein (BFO_0565) *[Fibronectin 3 domain]* Fibronectin type III domain-containing protein (BFO_0035) *[Fibronectin 3 domain]* Hypothetical protein (BFO_2860) *[Fibronectin 3 domain]* Hypothetical protein (BFO_2865) *[Fibronectin type 3 domain]* Hypothetical protein (BFO_2862) *[Fibronectin type 3 domain]* Hypothetical protein (BFO_2644) *[Fibronectin type 3 domain]* *Hypothetical protein* (BFO_0575) *[Fibronectin type 3 domain]* Glycosyl hydrolase family 3, C-terminal domain-containing protein (BFO_0699) *[Fibronectin type 3-like domain]* Glycosyl hydrolase family 3, N-terminal domain-containing protein (BFO_2182) *[Fibronectin type 3-like domain]*
2.	Heparin-binding protein	50S ribosomal protein L29(HMPREF0389_00839) *[Ribosomal_L29_HIP; Ribosomal L29 protein/HIP* heparin/ heparan sulfate interacting protein (HIP)]*	RpmC 50S ribosomal protein L29 (PGTDC60_0200) *[Ribosomal_L29_HIP; Ribosomal L29 protein/ HIP]* RpmC 50S ribosomal protein L29 (PGN_1860) *[Ribosomal_L29_HIP;*	50S ribosomal protein L29 (TDE0775) *[Ribosomal_L29_HIP; Ribosomal L29 protein/HIP]*	RpmC 50S ribosomal protein L29 (BFO_1554) *[Ribosomal_L29_HIP; Ribosomal L29 protein/ HIP]*

No.	MSCRAMMs (putative)	*F. alocis*	*P. gingivalis*	*T. denticola*	*T. forsythia*
			Ribosomal L29 protein/ HIP] 50S ribosomal protein L29 (PG1930) *[Ribosomal_L29_HIP; Ribosomal L29 protein/ HIP.]*		
3.	Fibrinogen-binding protein	–	–	Fibronectin/fibrinogen-binding protein, internal deletion (TDE1579)	–
4.	Collagenase	Protease (HMPREF0389_00122) *[Collagenase domain]* Peptidase, U32 family (HMPREF0389_00504) *[Collagenase domain]*	Collagenase (PG1542) Collagenase (PGN_0567) PrtC collagenase (PGTDC60_0756) PrtQ PrtQ, protease (PGTDC60_1870) PrtQ PrtQ, protease (PGN_0780) Protease (PG0753) *[All have Collagenase domains]*	U32 family peptidase (TDE0071) *[Collagenase domain]* U32 family peptidase (TDE2262) *[Collagenase domain]*	PrtC collagenase (BFO_1352) Peptidase, (U32 family BFO_0839) *[Collagenase domain]*
5.	Collagen adhesin/ binding protein	Collagen adhesin protein (HMPREF0389_01006) *[Collagen binding domain]* Gram positive anchor (HMPREF0389_01336) *[Collagen binding domain]* Hypothetical protein (HMPREF0389_01750) *[Collagen binding domain]*	–	–	–
6.	Laminin binding protein	–	–	Fibronectin type III (TDE0446) *[Fibronectin type 3 domain]* Hypothetical protein (TDE1139) *[Laminin_G_3; Concanavalin A-like lectin/ glucanases superfamily]*	Putative lipoprotein (BFO_1193) *[Laminin_G_3; Concanavalin A-like lectin/glucanases superfamily]*

( ) Gene annotation from NCBI.

[*Italics*] conserved domains.

Table 3

Relative abundance of CRISPR associated proteins of *F. alocis* strains during co-infection with *P. gingivalis* [17].

Gene ID	Annotation	Fold change	
		D-62D	ATCC-35896
HMPREF 0389_00212	CRISPR-associated protein (Cas2)	2.12	1.40
HMPREF 0389_00211	CRISPR-associated protein (Cas1)	1.4	0.70
HMPREF 0389_00210	CRISPR-associated protein (Csn1)	1.046	1.00
HMPREF 0389_01169	CRISPR-associated protein (Csd1)	0.960	0.95
HMPREF 0389_01170	CRISPR-associated protein (Cas5)	0.748	0.78
HMPREF 0389_01165	CRISPR-associated protein (Cas2)	1.30	1.20
HMPREF 0389_0167	CRISPR-associated protein (Cas4)	1.01	1.00