Tobacco-gene activity profile in Porphyromonas gingivalis, Filifactor alocis and Treponema denticola.

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TOBACCO - GENE ACTIVITY PROFILE IN PORPHYROMONAS GINGIVALIS, FILIFACTOR ALOCIS AND TREPONEMA DENTICOLA

By

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University of Louisville, School of Dentistry
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A Thesis approved on
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Smoking is an established risk factor for periodontitis. Prior studies have shown that cigarette smoke extract (CSE) can induce profound phenotypic changes in Porphyromonas gingivalis and alters the virulence of this important periodontal pathogen. We hypothesized that CSE might also alter gene expression in established periodontal pathogens, Porphyromonas gingivalis and Treponema denticola, as well as in the emerging pathogen, Filifactor alocis. Oral bacteria were grown in CSE-conditioned medium (1000 ng/ml nicotine equivalents) or in unconditioned control medium. Total RNA was extracted and CSE-regulated genes were identified by comparison of the mRNA profiles of CSE with control cultures using RNA-Seq analysis. Approximately, 30% of genes in the P. gingivalis genome and 5% of genes in the F. alocis genome were found to be differentially expressed when exposed to cigarette smoke. Several genes responsible for DNA replication and repair, transfer (tra) genes, ABC transporter genes and several metabolic genes were found to be differentially expressed in both F. alocis and P.
*P. gingivalis*. Validation of RNA-Seq differentially expressed genes was done by qPCR analysis for selected genes and similar results were found. More in depth study of these genes could provide some of the first insights into how cigarette smoke changes the *P. gingivalis* and *F. alocis* phenotype in a manner likely to promote their colonization and infection.
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CHAPTER 1: INTRODUCTION

Tobacco and Disease

Tobacco is the single biggest preventable reason for death and illness in the United States. According to the World Health Organization, there were around 1.3 billion smokers worldwide in 2003, and that number is relied upon to increment to 1.7 billion by 2020 (1). Cigarette smoking kills more than 480,000 Americans each year, with more than 41,000 of these deaths from exposure to secondhand smoke (2). In addition, smoking-related illness in the United States costs more than $300 billion a year (2, 3).

Cigarette smoke is a complex mixture of chemical compounds. Researchers have estimated that cigarette smoke has > 7000 chemical compounds from many different classes (4). Components of smoke are contained in either the particulate phase or the gas phase. The particulate phase ingredients include tar, polynuclear hydrocarbon phenol, cresol, catechol and trace elements which are carcinogens; indol, carbazole (tumor accelerators) and 4-aminobiphenyl (known to cause hepatocellular carcinoma) (5). The gas phase contains carbon monoxide, hydrocyanic acid, acetaldehyde, acrolein, ammonia, formaldehyde and oxides of nitrogen, nitrosamines, hydrazine, and vinyl chloride that have carcinogenic activity (6,7,8,9). Nicotine in cigarette smoke is highly addictive. In
little doses nicotine goes about as a stimulant to the brain. In substantial doses, it's a depressant, repressing the signals between nerve cells. In considerably bigger doses, it's a deadly toxic substance, influencing the heart, veins, and hormones (10).

There is a positive association between tobacco smoking and cancers of the lung, oral cavity, pharynx, larynx, esophagus, pancreas, bladder, kidney, pelvis, nasal cavities, paranasal sinuses, nasopharynx, liver, stomach, kidney and cervix (12). Smoking accounts for at least 30% of all cancer deaths and 87% of lung cancer deaths. In the United states, tobacco use is responsible for nearly 1 in 5 deaths (11). In 2012, the estimated percentage of new lung cancers in males and females was 14% each. Among these lung cancers, 29% of male and 26% of female cases were estimated to be fatal (11).

Smoking and Infectious diseases

Smokers are more susceptible to multitude of infectious diseases compared to non-smokers (13). These include respiratory tract infections, pneumonia, tuberculosis, meningitis, sexually transmitted bacterial infections and bacterial induced periodontal diseases (14-20). The specific mechanisms by which cigarette smoking increases the risk of systemic infections are incompletely understood. They are multifactorial and can be due to Mechanical and Structural or Immunologic changes caused by smoking (21). Cigarette smoke and many of its components like acrolein, acetaldehyde, formaldehyde, free radicals and nitric oxide, are believed to be responsible for structural alterations in the airway
epithelial cells, which is thought to predispose to the development of upper and lower respiratory tract infections (22,23).

Cigarette smoking alters various cellular and humoral immune system functions. These alterations include a (i) decreased level of circulating immunoglobulins. Several studies have found that smokers had serum immunoglobulin levels (IgA, IgG, and IgM) 10% to 20% lower than those of nonsmokers (24-27), (ii) depression of antibody responses to certain antigens, such as influenza virus infection (28) and Aspergillus fumigatus infection (29), (iii) decrease in CD4+ lymphocyte counts, an increase in CD8+ lymphocyte counts. Since CD4+ cells facilitate B-cell proliferation and differentiation and immunoglobulin synthesis, decrease in the CD4 count seen in heavy smokers (≥50 pack-year) might contribute to the increased susceptibility to infections in this population. Increase in CD8+ cells in heavy smokers (≥50 pack-year) has also been found to be associated with infection (30,31,32), (iv) depressed phagocyte activity. There is reduced migration and chemotaxis of Polymorphonuclear leukocytes in the peripheral blood of smokers compared with PMNs from nonsmokers (33,34). Also motility and chemotaxis of PMNs are depressed in the oral cavity of smokers compared with nonsmokers (33), and (v) decreased release of proinflammatory cytokines. The release of cytokines from macrophages may also be altered in smokers. Studies showed that there is decrease in IL-1, IL-6 and TNF (35,36).
Figure 1: Diseases related to smoking

Image showing association between smoking and various cancers and chronic diseases. The conditions in red are the new diseases that have been linked to smoking in the 2014 report by surgeon general and conditions in black are linked to smoking by USDHHS in 2004, 2006 and 2012.


Smoking and chronic inflammatory diseases

Tobacco smoking is known to substantially increase the risk for chronic, inflammatory diseases (14,15,37,38), such as acute vascular diseases (40,41), inflammatory bowel disorders (42,43) renal disease (15), pancreatitis (16) and periodontal diseases (44). Smoking is a major cause of cardiovascular diseases...
and the risk for these diseases increases with the quantity of cigarettes smoked per day and the duration of smoking history (2). Chemicals in cigarette smoke can increase vascular permeability and activate adhesion molecule expression, resulting in increased leukocyte adhesion and, eventually, platelet aggregation. This can narrow the blood vessels and lead to cardiovascular conditions like atherosclerosis, acute myocardial infarction and stroke (45,46). Also, smoking increases blood pressure and decreases tolerance to exercise (46).

Smoking increases risk for lung diseases like chronic bronchitis (47,48), a long-term inflammation of the bronchi (large airways). The chemicals in cigarette smoke irritate and activate macrophages and epithelial cells. This causes the cells to release multiple types of cytokines which lead to thickening and inflammation of the airway lining. This persistent inflammation caused by cigarette smoke can change the structure of the airways and make them narrower through a cycle of injury and repair (49). Smoking is also associated with other chronic inflammatory renal diseases like inflammatory bowel disease and chronic kidney disease (50,51).

**Periodontal diseases**

Periodontal diseases are one of the most predominant diseases all through the world (52). They represent a group of infectious inflammatory diseases affecting the supporting and surrounding tissues of teeth (53). They are second to dental caries as a cause of tooth loss among adults in developed countries (54), affecting 47.2% adults aged 30 years and older in the US (55). Periodontal diseases happen as a consequence of mixed microbial infections within which
specific groups of bacteria coexist. In a healthy mouth there are more than 350 species of microorganisms and periodontal infections are linked to less than 5% of these organisms (56). *Porphyromonas gingivalis, Treponema denticola, Prevotella intermedia, Aggregatibacter actinomycetemcomitans, Tannerella forsythia,* and *Fusobacterium nucleatum* are believed to play prominent roles in the etiology of periodontal diseases (56). Recent studies have identified a wide range of bacteria associated with disease status like *Filifactor alocis, Selenomonas, Synergistes, Desulfobulbus* and *TM7* (57,58). These bacteria exist as an organized biofilm on the tooth surface. Extension of the biofilm into the gingival sulcus begins a series of events that mediate periodontal disease (59).

In the gingival sulcus, pathogenic bacteria and their metabolic products initiate the inflammatory response in host cells (neutrophils, epithelial cells and macrophages). This results in an influx of an inflammatory infiltrate which is rich in neutrophils. These neutrophils attempt to phagocytose the bacteria (60). However, some periodontal pathogens have developed ways to resist phagocytosis using virulence factors like capsules, or avoid phagocytosis by gaining entry into host cells (61). Another method of fighting periodontal pathogens is neutrophil degranulation. When neutrophils degranulate, they release granular enzymes such as elastase, and matrix metalloproteinases (MMPs) as well as superoxide and oxygen radicals, and nitric oxides. These products do not discriminate between the bacteria and the host tissues. Thus, periodontal tissue destruction will result from prolonged exposure (62).

**Smoking and periodontal diseases**
Compared to non-smokers, tobacco smokers are more susceptible to plaque-induced gingivitis and periodontitis. There is a negative, dose-dependent relationship between smoking and periodontal health (63,64). Smokers are also more refractory to periodontal treatment than non-smokers (64).

Smoking has been shown to affect various aspects of the host immune response. It has adverse effects on fibroblast function (65), chemotaxis and phagocytosis by neutrophils (66), and immunoglobulin production (67). Macrophages play important roles in both cell mediated and humoral immunity as antigen-presenting cells. However, antigens are presented in the context of class 1 major histocompatibility complex (MHC) surface molecules. There might be a gradual reduction in the humoral immune response in smokers because of reduced expression of class I MHC by the alveolar macrophages in smokers (68,69).

Smokers show increased gingival recession and alveolar bone loss, greater periodontal ligament (PDL) attachment loss and deeper gingival pocket formation that is responsible for increased tooth mobility and tooth loss at an earlier age than non-smokers (70,71). Smoking has also been shown to reduce the concentration of serum IgG (25, 72,73). Smoking is also known to alter the host inflammatory response to plaque bacteria. According to several studies, nicotine activates the anti-inflammatory pathway and suppresses pro-inflammatory cytokine production (74-78). Also there is reduced levels of pro-inflammatory cytokines, such as IL-1 (84,85,86) in the gingival crevicular fluid (GCF) of smokers with periodontitis compared to non-smokers with periodontitis, whereas anti-inflammatory cytokines like IL-10 and TGF-β1 are increased in the GCF of smokers (79,80,81).
Despite being more susceptible to periodontitis and exhibiting faster disease progression and severity, chronic smokers lack the clinically overt inflammatory response to bacterial plaque that non-smokers exhibit such as redness, swelling, bleeding on probing making diagnosis of the disease more complicated in smokers (45,82).

*Porphyromonas gingivalis*

*Porphyromonas gingivalis* is a Gram-negative, proteolytic, asaccharolytic anaerobe. Although this bacterium is a natural member of the oral microbiome, it can proliferate to high numbers in periodontal lesions and can be highly destructive (83,84,85). *P. gingivalis* is found in significantly higher numbers in smokers compared to non-smokers and the infection is more persistent (70,86).

The “red complex” bacteria *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, are frequently isolated together and are strongly associated with advanced periodontal lesions (56,87-89), but according to recent studies periodontal diseases are caused by synergistic and dysbiotic microbial community rather than “select periopathogens” such as “red complex” (88). So the concept of “red complex” has been superseded by the “Keystone pathogen” hypothesis, at least in mice. This indicates that certain low-abundance microbial pathogens can cause inflammatory disease by increasing the quantity of the normal microbiota and by changing its composition (88). For instance, *Porphyromonas gingivalis* has been shown to manipulate the native immune system of the host (90). By doing so, it was hypothesized that it not only facilitates its own survival and multiplication, but also that of the entire microbial community.
Intensive study has revealed multiple virulence factors which are responsible for the survival and pathogenesis of *P. gingivalis*.

**Major fimbrial antigen (FimA)**

The major fimbriae of *P. gingivalis* are long, hair-like, peritrichous, adhesive, filamentous structures that project away from the cell surface (91). They are primarily comprised of a 41 kD protein (FimA, fimbrillin) encoded by the *fimA* gene (92). *P. gingivalis* fimbriae adhere to a wide variety of molecules and oral substrates, which include salivary molecules, such as proline-rich proteins, proline-rich glycoproteins, statherins, oral epithelial cells, fibrinogen, fibronectin, lactoferrin, and bacteria, such as oral streptococci and *Actinomyces* species (93-96). Long fimbriae interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) allowing for localization of *P. gingivalis* on the streptococcal surface (97). Human GAPDH has also been shown to bind to long fimbriae (98).

Based on its nucleotide sequence variation, the *fimA* gene has been classified into six types (I, Ib, II, III, IV, V) (99,100). *fimA* genotypes II, Ib, and IV were shown to cause inflammatory changes in animal models (101,102,103). Recombinant protein of *fimA* genotype II is known to adhere to and invade human epithelial cells than *fimA* from other genotypes (104). In human Gingival epithelial cells (GECs) long fimbriae are also known to induce cytokines involved in bone resorption, such as tumor necrosis factor (TNF), interleukin-1b (IL-1b), IL-8, and IL-6 by stimulating nuclear factor-kB (NF-kB) via TLR 2 and CD14 (105, 106, 107).

**Minor fimbrial antigen (Mfa1)**
Short fimbriae (Mfa1 fimbriae) are homopolymers of a subunit protein encoded by the *mfa1* gene, with a molecular mass of 75 kDa (92,108). They are shorter than the major fimbriae and can be easily seen when the latter are absent (101). Short fimbriae mediate co-adhesion between *P. gingivalis* and *S. gordonii* via adhesin-receptor interactions with streptococcal SspA and SspB surface proteins (109). Studies have reported that short fimbriae induced the expressions of cytokines, including IL-1α, IL-1β, IL-6, and TNF, in both human monocytic cell lines and mouse macrophages by interacting with TLR2 and CD14 (110,111).

**Capsule**

The *P. gingivalis* capsule is composed of glycosamionglycans and plays an important role in providing resistance to stressful conditions (112). It can help shield the microbe from the host defense and modulate host physiology (113) by providing resistance against neutrophils (114) and complement-mediated lysis or opsonization (115). The higher virulence potential of encapsulated strains compared to that of non-encapsulated ones, evaluated using a mouse abscess model, suggests that the capsule plays a significant role in the pathogenesis of this bacterium (116,117).

*P. gingivalis* capsule allows bacteria to escape host immune defenses by inhibiting the host immune response, thus promoting bacterial survival and growth (118). Recent studies show that encapsulated *P. gingivalis* strains trigger different host responses than non-encapsulated mutant strains (119,120). *P. gingivalis* K1 serotype capsular polysaccharide is capable of eliciting chemokine production from macrophages that in turn promote cell migration (121). Defensins, a small
antimicrobial peptides produced by host cells have a bactericidal effect. *P. gingivalis* capsule reduces the bactericidal effect of these peptides contributing to increased survival of the organism (122). The non-encapsulated PgC strain is less virulent than the encapsulated W50 strain, demonstrating that capsule plays a role in the virulence of *P. gingivalis* in the mouse abscess model (111). However, the role of capsule in the virulence of *P. gingivalis* is still vague.

**Lipopolysaccharides (LPS)**

The outer leaflet on the outer membrane of Gram-negative bacteria is comprised of lipopolysaccharide (123), which is at least 10 kDa in size (84). Components of lipopolysaccharide are O-antigen, core, and lipid A. Lipid A is responsible for endotoxic activity, while the O-antigen is responsible for significant immunobiological activity (124) and is the easiest target for the humoral response of the host. The O antigen is recognized by the innate immune response and participates in complement activation (125,126). Many studies have established the immunobiological importance of the lipopolysaccharide of Gram negative cell wall envelope (127,128). It has the ability to activate the host inflammatory responses and disrupt bone remodeling process (129,130). *P. gingivalis* LPS binds to the CD14 and TLR-4, activating macrophages, epithelial cells respectively, leading to secretion of pro-inflammatory cytokines (131,132).

*P. gingivalis* is known to show an unusual amount of lipid A heterogenicity containing both tetra-acylated and penta-acylated structures (134). Compared to *E. coli*, *P. gingivalis* Lipid A is heterogenic with varying numbers and positions of phosphate and fatty acid groups (134). When human monocytes were stimulated
with *P. gingivalis* LPS, the level of TNF, IL-1β and IL-6 was enhanced (133). LPS in *P. gingivalis* also plays a critical role in inducing cells to secrete pro-inflammatory cytokines, which mediate inflammation and participate in periodontal connective tissue destruction and alveolar bone resorption. *P. gingivalis* LPS is significantly less inflammatory than other Gram negative bacterial LPS such as that of *E. coli*. This might be because of differences in lipid A structure, reduced 4’ phosphorylation, reduced acylation at the 3 and 3’ positions on the back bone and the absence of acyloxyacyl group at the 3’ position (135).

**Proteases including gingipains**

The classification of the proteases has relied upon their catalytic mechanisms. There are four categories of *P. gingivalis* proteases: serine, cysteine and metalloproteinase. These include gingipains (Arg- or Lys-), periodontain (cysteine endopeptidase), PrtT proteinase, Tpr proteinase, collagenase (*prtC* gene), prolyl tripeptidyl peptidase (serine exopeptidase), dipeptidyl-peptidase IV (serine exopeptidase known as glycy1prolyl peptidase, a product of the *dppIV* gene), dipeptidyl-peptidase VI (putative cysteine exopeptidase), amino-peptidase P, oligo-peptidase O and gelatinase (a proteinase degrading type IV collagen, gelatin, low-molecular-mass-kininogen and transferrin) (136). Of these, the collagenases, aminopeptidases, and the trypsin-like proteases are critical to *P. gingivalis* pathogenesis (137).

Gingipains are cysteine proteases that can cleave the proteins at arginine and lysine specific sites (138). All the extracellular and cell-associated protease activity with specificity for arginine peptide bonds is derived from two genes, *rgpA*
and \textit{rgpB}. All the extra-cellular activity with specificity for lysine peptide bonds is derived from a single gene, \textit{kgp} (139). These are important etiological agents in periodontal diseases.

\textit{P. gingivalis} proteases degrade and metabolise the extracellular matrix proteins (137). Gingipains have collagenolytic activity and degrade or inactivate inflammatory cytokines IL-6, IL-8, TNF and IFN. Proteases (Trp, PrtT) have MMP-activating or hemagglutinating properties. Dipeptidyl amino-peptidase IV (DPPIV) may also act as a virulence factor by contributing to the degradation of connective tissue (140,141).

\textit{Filifactor alocis}

\textit{F. alocis} is a Gram-positive, slow-growing, asaccharolytic, obligate anaerobic rod (142). It was first isolated in 1985 in the gingival sulcus of gingivitis and periodontitis patients and was classified as \textit{Fusobacterium alocis} (143). Later, based on phylogenetic analysis of 16s rRNA, it was reclassified into the genus \textit{Filifactor} (144). The size of the \textit{F. alocis} genome is 1.93 Mb (www.broadinstitute.org). Cultivable strains of \textit{F. alocis} include, ATCC 35896, D-62D (clinical strain) (145) and CCUG 47790 T (www.straininfo.net). \textit{F. alocis} is present in diseased periodontal pockets in higher numbers than in healthy mouths (146,147). Arginine, which increases the growth of \textit{F. alocis in vitro}, is abundantly present in periodontal pockets (148). This has been hypothesized to help explain the presence of \textit{F. alocis} in high numbers in periodontal pockets (149).

The gingival crevice is lined by epithelial cells, the first cells to be encountered by periodontal bacteria (150). Epithelial cells produce of the
chemoattractant cytokines like IL-8 which are responsible for signaling of the underlying tissues (151). Neutrophil migration into the gingival crevice is thought to be the first line of defense against plaque bacteria (152,153). *F. alocis* adheres to and invades the surface of gingival epithelial cells (GECs) (143). The secretion of proinflammatory cytokines IL-1, IL-6 and TNF by GECs are increased when infected with *F. alocis* (154). Such cytokines can induce osteoclastic activity and, thus, increased bone resorption (155). The effect of *F. alocis* on cell viability was investigated by observing the levels of apoptotic and necrotic cells after infection and it was confirmed that *F. alocis* stimulates apoptosis in GECs through the extrinsic apoptotic pathway, as it increases caspase-3 production (154). Transient activation of MEK1/2 and long term inhibition of MEK activity is caused by *F. alocis*. MEK1/2 activates MAPK pathways which can control cell proliferation and differentiation (154). Inhibition of MEK leads to induction of apoptosis in various cell types (156). Thus the pro-apoptotic effect of *F. alocis* is a result of its ability to inhibit MEK activity. All these effects of *F. alocis* on gingival cells may be responsible for the tissue destruction caused in periodontitis.

**Virulence factors of *F. alocis***

**Oxidative stress resistance**

Periodontitis is characterized by the generation of reactive oxygen species (ROS) (166) by activated phagocytes at the gingival sulcus (157,158). ROS have the ability to initiate the destruction of connective tissue, and increasing ROS levels may kill different pathogens. So, oxidative stress resistance is important for the survival of an organism in the periodontal pocket (149). In broth culture, the
generation time was approximately 13 hours for *F. alocis* 35896 and 3 hours for *P. gingivalis*. When grown with 0.25mM of hydrogen peroxide to test their adaptation to oxidative stress (142), the generation time of *F. alocis* was 6 to 7 hours compared to 10 hours for *P. gingivalis*, which shows that *F. alocis* is more resistant to oxidative stress conditions than *P. gingivalis*. Also the growth of *F. alocis* appeared to be stimulated under such conditions (142). This might be another reason that helps explain the ability of *F. alocis* to thrive in the periodontal pocket (146). The exact mechanism underlying the oxidative stress resistance of *F. alocis* is not known. *F. alocis* has sialidase activity (142) which is important for the survival and pathogenesis of periodontal pathogens (160). This sialidase activity results in release of sialic acids that act as an ROS scavenger to reduce oxidative stress in the periodontal pocket (152). *F. alocis* has a putative neutrophil activating protein A (NapA). *H. pylori* NapA co-localizes with DNA, causing it to accumulate in one area of the bacterial cell protecting its DNA from damage by free radicals (161). There is a speculation that this neutrophil activating protein A may be an important virulence factor in *F. alocis*. It may also be responsible for the survival and stimulated growth of the bacteria under oxidative stress conditions (142) using a mechanism similar to that of *H. pylori*. Therefore, it is likely that the ability of *F. alocis* to survive oxidative stress in the periodontal pocket contributes to its ability to establish itself in this niche.

**Proteases**

Proteases are important virulent factors of several oral pathogens (163). In *Streptococcus mutans* (164), *Porphyromonas gingivalis* (165) and *Fusobacterium*
proteome analysis has been used to understand the molecular mechanisms of bacterial invasion, survival and pathogenesis. When similar proteome analysis was performed in *F. alocis* ATCC 35896 and D-62D, strain-specific variation in their protein profiles was observed and a few proteases that could potentially play an important role in the pathogenesis were identified (167). The *F. alocis* genome has a putative total of 15 different proteases. Both strains of *F. alocis* have CaaX proteases (167) which are known in *S. gordonii* for their role in protein transportation and protection from bacteriocins produced by other similar bacteria (168). Protease HMPREF0389_00122 is present in the extracellular fraction of the D-62D strain and is known to have a collagen peptidase function. This protease might be responsible for the damage of the connective tissue which leads to tissue destruction in periodontitis (167). So this could be important in *F. alocis* pathogenesis. Proteins that play a crucial role in the amino acid metabolism are seen in *F. alocis* (142). Although *F. alocis* lacks some inherent amino acid synthesis pathways, the release of required amino acids through protein degradation with the help of these proteases and peptidases may be important for *F. alocis* survival. *F. alocis* has proteins responsible for ornithine catabolism and urea breakdown (167) and this well-developed nitrogen assimilatory pathway may also be involved in alternative amino acid synthesis pathways (169). So we can conclude that *F. alocis* has mechanisms to provide for its nutritional needs. One of the major virulence mechanisms in bacteria is their ability for extracellular secretion of proteins (170). Proteins involved in type-II secretory pathway, namely, Type IV pilus assembly protein (HMPREF0389_00426) and trigger factor
(HMPREF0389_01646), were also identified in the membrane fraction of the *F. alocis* ATCC 35896 (167). *F. alocis* D62-D proteins, leucotoxin translocation ATP-binding protein, fibronectin-binding protein, type IV pilus assembly protein, fimbrial assembly protein, hemolysin III type calcium-binding protein, toxin-antitoxin component protein and Na +/H + antiporter protein (NAPA) homologous to K+/H + antiporter (171), are considered virulence factors in other microorganisms (167). *F. alocis* also has glycolytic enzymes, such as phospho-glycerate mutase and glyceraldehyde 3-phosphate dehydrogenase, that are responsible for energy metabolism (167). These virulence proteins and glycolytic proteins might have protein moonlighting functions such as mediating binding of bacteria to proteins of the extracellular matrix (ECM) like fibronectin (172), which is important for bacterial virulence in several human pathogens. Moonlighting proteins are multifunctional proteins which perform multiple autonomous, often unrelated, functions (173). These proteins add another dimension to cellular complexity and benefit cells in several ways (174). In conclusion, all of these proteins seen in *F. alocis* may contribute to its virulence but the exact role of these systems in the bacterial community is not clearly known.

*Treponema denticola*

*Treponema denticola* is a helically shaped Gram-negative Spirochete that is motile and flexible. It has periplasmic flagella, which allow for mobility by using a proton motive force to cause thrusting through rotation (175).

Oral treponemes are a part of the polymicrobial biofilm (176). They play an important role in the etiology of several chronic diseases like chronic periodontitis,
acute necrotizing ulcerative gingivitis and dental abscesses (177-180). Treponemes are present normal healthy individuals in low numbers (181).

*T. denticola* has an ability to bind with *Fusobacterium* (182,183), early colonizing *Streptococcus crista* (184), *P. gingivalis* and *T. forsythia* (185,186,187). When co-cultured, *P. gingivalis* and *T. denticola* form significantly increased biofilm formation compared with monoculture (185). These interactions may be important for *T. denticola* to colonize and persist during health.

Virulence factors of *T. denticola*

Little is known about the virulence factors of *T. denticola* but they are believed to have features needed for adherence, invasion and damage of the periodontal tissues.

Leucine-rich repeat proteins (Lrr)

A *T. denticola* leucine-rich repeat protein (LrrA) has recently been shown to play a role in binding to *T. forsythia*. Also leucine-rich repeat protein in *T. forsythia* has been shown to be important for epithelial cell invasion and virulence in a mouse alveolar bone loss model (186-190) and is believed to have similar function in *T. denticola*.

Dentilisin

Dentilisin is proposed to be a major virulence factor of *T. denticola*. It contributes to disease progression by disrupting or modulating intercellular host signaling pathways and degrading host cell matrix proteins (191). It also allows for penetration of epithelial cell layers by *T. denticola* through degradation of intercellular adhesion proteins (191) and modulates host cell immune responses.
through degradation of interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF), and monocyte chemo attractant protein 1 (192,193).

**Major sheath protein (Msp)**

Msp is an abundant protein in the *T. denticola* outer membrane. It has surface-exposed loops that are able to bind to a variety of host proteins (181). Msp has been proposed to mediate colonization of host tissues (194,195). It is also one of the immunodominant *T. denticola* antigens recognized by human serum antibodies (196). Msp interaction leads to actin remodeling and reorganization in host cells, which is likely how it impairs neutrophil chemotaxis and phagocytic activity (197-200).

**Lipoproteins**

Lipoproteins are the most abundant membrane-associated proteins found in spirochetes, and *T. denticola* 35405 is predicted to have 166 of them, the highest number for any of the sequenced spirochetes (201). The role of these in *T. denticola* virulence is as yet unresolved, but they are likely to be responsible for epithelial cell binding and invasion, subversion of the complement cascade, or tissue invasion (202,203).

**Outer Membrane Vesicles (OMVs)**

Initially, OMV were thought to be the result of random blebbing of the outer membrane, or sheath, producing small spherical vesicles of 50-100 nm in diameter. However, recent studies have revealed that OMVs are formed by a highly regulated process which may increase the fitness of the bacterium in response to environmental stress (204,205,206). OMVs are considered potent
virulence factors, since they possess adhesins, toxins, and proteolytic enzymes that can mediate bacterial aggregation and invasion and can modulate the host immune response (205). *T. denticola* outer sheath vesicles (OSVs) can penetrate tissues more readily than the bacterium itself (207,208,209). Application of *T. denticola* OSVs to Hep-2 epithelial cell monolayers disrupted the tight junctions, which could facilitate penetration into underlying tissues (210). However, the involvement of treponemal OSVs in disease remains to be properly explored.

**Smoking and Periodontal Pathogens**

Tobacco-induced susceptibility to periodontitis was also believed to be associated with shifts in the microbial composition from one that is mainly constituted of Gram positive, aerobic, commensal bacteria to one that contains more Gram negative, anaerobic pathogens (211-218). Haffajee and Socransky (219) showed an increased prevalence of eight species, including *P. gingivalis*, in current smokers, while Eggert et al. (216) have shown a higher prevalence and proportion of *T. forsythia*, *C. rectus*, *P. gingivalis* and *P. micros* in plaque samples from smokers. A recent study done by Camello Castello et al. et al... (221), showed that there is an increased presence of *Filifactor*, *Tanerella*, *Schwartzia*, *Bulleida* and *Anaeroglobus* in chronic periodontitis patients who smoke compared to non-smoking chronic periodontitis patients and non-smoking healthy individuals. Also molecular studies have demonstrated that smokers with periodontitis have a diverse sub gingival microbial profile compared to non-smokers with periodontitis (198,200,201), which was contradicted in a recent study which showed that smokers with periodontitis has decreased bacterial diversity (221).
According to Bagaitkar et al. (222), several functionally-related genes including multiple genes in the major fimbrial and capsular polysaccharide operons, as well as genes encoding transcriptional regulators; efflux pump and transport proteins; proteases and cell envelope proteins, were dysregulated when *P. gingivalis* W83 was exposed to cigarette smoke extract conditioned media using Microarray analysis (222). Also she found that cell surface or outer membrane proteins, i.e., RagA, RagB and PG0179 were shown by biochemical approaches to be present at higher levels after CSE treatment. But the genes encoding for these components were not identified as differentially expressed in her microarray experiments (222). This reflects inherent limitations in the microarray approach. However, till today, very little is known about how tobacco smoke affects the phenotype of periodontal pathogens.
HYPOTHESIS

Cigarette smoke extract (CSE) represents an environmental stressor to which bacteria may adapt by several different mechanisms, one of which is by altering their gene expression. So, we hypothesize that established periodontal pathogens *P. gingivalis*, *T. denticola* and emerging periodontal pathogen *F. alocis* adapt to this environmental stress caused by cigarette smoke extract by altering their gene expression.
Bacterial Culture and *in Vitro* Modelling of Tobacco Exposure

*Porphyromonas gingivalis* ATCC 33277, *Filifactor alocis* ATCC 35896 and *Treponema denticola* ATCC 35405 were purchased from the American Type Culture Collection (Manassas, VA) and maintained as frozen stocks. Growth medium for *P. gingivalis* - Gifu anaerobic medium (GAM), was purchased from Nissui Pharmaceutical (Tokyo, Japan). Growth medium for *F. alocis* - Brain heart infusion (BHI), was purchased from Becton, Dickinson and Company (Sparks, MD) and infused with L-cysteine (0.1%) and arginine (20%) purchased from Sigma-Aldrich (St. Louis, MO). Growth medium for *T. denticola* - Tryptone-yeast extract-gelatin-volatile fatty acids-heat inactivated rabbit serum (TYGVS) (223). 3R4F standard reference cigarettes were obtained from the Kentucky Tobacco Research and Development Center (Lexington, KY). All three media (GAM, BHI and TYGVS) were conditioned with cigarette smoke extract by drawing cigarette smoke through 50ml of the medium by using a three-way stopcock and a syringe, with 35ml drags performed every 20 seconds. This cigarette smoke extract conditioned medium was then filtered (0.2mm). Nicotine content was determined by gas-liquid chromatography and adjusted to 7.2 pH and 1000 ng/ml nicotine concentration.
*P. gingivalis*, *F. alocis*, and *T. denticola* were grown in their respective control and cigarette smoke extract conditioned media under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂) at 37°C. For all experiments, bacteria were grown either in control or CSE conditioned media and bacterial cells were harvested at mid to late log phase (*P. gingivalis* - O.D 600 nm =1.0, corresponding to 10⁹ cells per ml; *F. alocis* - O.D 600nm =0.35, corresponding to 3×10⁹ cells per ml; *T. denticola* - O.D 600 nm = 1.6, corresponding to 8×10⁹ cells per ml).

**Isolation of Total RNA**

Total RNA was isolated from bacterial cells according to manufacturer’s instructions using one of two kits. Perfect Pure™ RNA Cultured Cell Kit by 5 Prime (Gaithersburg, MD) was used for *P. gingivalis* and *T. denticola*. RiboPure™ – Bacteria by Ambion life sciences (Thermofisher, Waltham, MA) was used for the Gram positive *F. alocis*. Gram positive bacteria require an additional step to break the thick peptidoglycan layer. The quantity and quality of RNA was measured by performing a Nanodrop on a ND-1000 Spectrophotometer and then samples of RNA were stored at -80°C.

**RNA-seq Analysis**

Total RNA samples of *P. gingivalis*, *F. alocis* and *T. denticola* were sent to University of Michigan for RNA-Seq analysis. They first removed rRNA using a Ribo-zero rRNA removal kit by Epicentre (Chicago, IL). TruSeq RNA (non-stranded) kit by Illumina (Madison, WI) was used to generate m-RNA focused libraries. Once the libraries were generated, they were sequenced on 2 lanes using 50bp single end reads. These raw reads were then sent to University of Michigan.
Bioinformatics core, where quality of the reads was checked using FastQC (version 0.10.1). Tuxedo Suite software was used for alignment, differential expression analysis and post-analysis diagnostics. Bowtie2 (version 2.1.0) was used to align reads to the respective reference genome. FastQC was used for a second round of quality control (post-alignment), to ensure that only high quality data would be input to expression quantitation and differential expression analysis. Cufflinks/CuffDiff (version 2.1.1) was used for expression quantitation and differential expression analysis and CummeRbund was used to generate diagnostic plots. Genes having ≥1.5-fold change were classified as up regulated genes and genes with ≤0.6-fold change were classified as down regulated genes.

**Enrichment Analysis of RNA-Seq data**

KEGG and DAVID enrichment analysis were done on the differentially expressed *P. gingivalis* and *F. alocis* genes to identify significantly enriched functional categories.

**Validation of RNA-Seq data**

Differentially expressed genes of interest were confirmed by quantitative PCR analysis using an Applied Biosystems 7500 Real Time PCR system. Primers were designed using the qPCR primer design software, Primer Quest, provided by Integrated DNA technologies (http://www.idtdna.com/Primerquest/Home/Index).

**Table 1: *P. gingivalis* oligonucleotide primers used for quantitative PCR**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGN_1047</td>
<td>TTCCATAGCCAACGTGTAGAG</td>
<td>CTGAGGCAACCCGATCATATT</td>
</tr>
<tr>
<td>PGN_0295</td>
<td>GGGTTCACTCAGTGCTCAAA</td>
<td>GAGCCATCCAACACTCGATAG</td>
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<td>kgp</td>
<td>AGGACAGGGTGAAGTTGTAATC</td>
<td>GCCTGCTTGAATGGAATAC</td>
</tr>
<tr>
<td>PGN_1367</td>
<td>CTCCCGGTTAGGCTTGGTAATG</td>
<td>CAGCCACCTGCTGCTTCTTT</td>
</tr>
<tr>
<td>PGN_1740</td>
<td>TGAATGAGGAGGAGGAGGATAC</td>
<td>ATGGGAATGGCTGCTTGAG</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Forward primer 5'-3'</td>
<td>Reverse primer 5'-3'</td>
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<td>-------------------------------</td>
<td>-------------------------------</td>
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<td>HMPREF0389_00184</td>
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<td>GATGGCGGGGAAAGTTGTTTG</td>
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<td>upp</td>
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<td>GGGTCACTCTTGTGCGATCT</td>
</tr>
<tr>
<td>HMPREF0389_0134</td>
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<td>GCCGAAAGAAGGTCCGATT</td>
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<td>HMPREF0389_00865</td>
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<td>CGAAGGCGTGGTGGGAA</td>
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<td>HMPREF0389_0545</td>
<td>CCTTCTGCTTGTGACATACTCT</td>
<td>AGACTGGACATACGCTTGTACT</td>
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<td>RgpA</td>
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<td>Dps</td>
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<td>HMPREF0389_0173</td>
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<td>HMPREF0389_0175</td>
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<td>HMPREF0389_1080</td>
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<td>CAAAGGCGGAGGCGCATACC</td>
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<td>HMPREF0389_0724</td>
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<td>GGGTCACTCTTGTGCGATCT</td>
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<tr>
<td>HMPREF0389_0660</td>
<td>GACCGCGGAAAGGAGGATGATTA</td>
<td>GGGTCACTCTTGTGCGATCT</td>
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</table>

Table 2: *F. alocis* oligonucleotide primers used for quantitative PCR

<table>
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<tr>
<th>Gene ID</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
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<td>PGN_1644</td>
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<td>GGGTCACTCTTGTGCGATCT</td>
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<tr>
<td>PGN_0134</td>
<td>TCCATCAACACGCAAGAGAAGATGATTA</td>
<td>GCCGAAAGAAGGTCCGATT</td>
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<td>PGN_0865</td>
<td>CACCTATGCTTGGCCTTCT</td>
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</tr>
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<td>PGN_0545</td>
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</tr>
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<td>RgpA</td>
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</tr>
<tr>
<td>PGN_1695</td>
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<td>GAAAGGCGGAGGGAAGAAGTACATCC</td>
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<td>RpoC</td>
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<td>Dps</td>
<td>GCCGACAAATGCTGACCTAGAAGAAG</td>
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<td>PGN_0173</td>
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<td>ATGGAAGAAGGAGGCTGTAAGG</td>
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<tr>
<td>PGN_0175</td>
<td>CGGTCCCTCCTCGGCTTGGTATTT</td>
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<td>PGN_1080</td>
<td>GTAGACATCATGCGGTTGATGAAAA</td>
<td>CAAAGGCGGAGGCGCATACC</td>
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<td>PGN_0724</td>
<td>GACCGCGGAAAGGAGGATGATTA</td>
<td>GGGTCACTCTTGTGCGATCT</td>
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<tr>
<td>PGN_0660</td>
<td>GACCGCGGAAAGGAGGATGATTA</td>
<td>GGGTCACTCTTGTGCGATCT</td>
</tr>
</tbody>
</table>

Primers were designed for the species specific 16s rRNA gene, which was our reference gene.
*P. gingivalis* 16s rRNA - F: TGTAGATGACTGATGGTGAAAACC  
R: ACGTCATCCCCACCTTCCTC  

*F. alocis* 16s rRNA - F: CAGGTGGTTAACAAGTATGCGG  
R: CTAAGTGTCTTCTAGCTCTCG  

Primers were ordered from Biosynthesis (www.biosyn.com) and were reconstituted and stored at -20°C. Total RNA (up to 1 μg) was reverse transcribed to cDNA using Superscript ® III- First Strand Synthesis Super Mix by Invitrogen (Waltham, MA) following manufacturer’s instructions and stored at -20°C. SYBR Green Master Mix for qPCR analysis was ordered from Quanta Biosciences (Gaithersburg, MD) and the manufacturer’s instructions were followed to set up a reaction. Reaction conditions used for qPCR were 50°C for 2 minutes, 95°C for 10 minutes, and 95°C for 15 seconds – 45 repetitions and 60°C for 1 minute. An additional dissociation stage was added to check for validity of primers which included 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds and 60°C for 15 seconds.

**Statistical Analysis**

All experiments were done in triplicate unless otherwise mentioned. Statistical significance between groups was evaluated by one-way nonparametric ANOVA and Fisher multiple comparison test. A probability value < 0.001 was considered statistically significant.
CHAPTER 3: RESULTS

Growth of (P. gingivalis, F. alocis and T. denticola) in CSE-conditioned media

We compared the growth of bacteria in CSE-conditioned and non-conditioned medium in order to determine if 1000 ng/ml nicotine equivalency was toxic to P. gingivalis, T. denticola or F. alocis. Similar growth characteristics were observed for all three species at this concentration of CSE. As shown in Figures 2, 3 and 4, the bacteria can tolerate this dose of CSE. All further experiments were done at 1000 ng/ml nicotine equivalency, a dose that is relevant to the concentration of nicotine found in the periodontal pockets of cigarette smokers (224).

Figure 2: Effect of CSE on P. gingivalis growth
Growth curve of *P. gingivalis* in unconditioned GAM and CSE conditioned GAM. Triangles represent the growth of *P. gingivalis* in CSE conditioned media and squares represent the growth in GAM. Error bars represent the mean Standard deviation (SD) of 3 experiments. There were no statistically significant differences in the growth curves between experimental conditions.

**Figure 3: Effect of CSE on *F. alocis* growth**

Growth curve of *F. alocis* in unconditioned BHI and CSE conditioned BHI. Triangles represent the growth of *F. alocis* in CSE conditioned media and squares represent the growth in BHI. Error bars represent the mean Standard deviation (SD) of 3 experiments. There were no statistically significant differences in the growth curves between experimental conditions.
Figure 4: Effect of CSE on T. denticola growth

Growth curve of T. denticola in unconditioned TYGVS and CSE conditioned TYGVS. Triangles represent the growth of T. denticola in CSE conditioned media and squares represent the growth in TYGVS. Error bars represent the mean Standard deviation (SD) of 3 experiments. There were no statistically significant differences in the growth curves between experimental conditions.

P. gingivalis differentially expressed genes

RNA-Seq analysis was performed in order to determine the differentially expressed genes of P. gingivalis when exposed to CSE. A total of 644 were found to be differentially expressed (P < 0.005). 54 genes were up regulated and 590 genes were down regulated.
Up regulated genes (> 1.5 fold) include the arginine and lysine gingipain encoding genes, $kgp$, $rgpA$ and $rgpB$; genes encoding arginine and proline metabolism (PGN_1367, PGN_0504 and PGN_1434); genes encoding DNA binding (PGN_1740, dps and rpoC); a group of genes responsible for carbohydrate and energy metabolism, these include nitrogen metabolism (PGN_1047 and PGN_1367) and several genes encoding carbohydrate metabolism (PGN_1695, PGN_0173, PGN_1753, PGN_0504, PGN_1529 and PGN_1755).

**Table 3: List of up regulated genes in *P. gingivalis* upon CSE exposure**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Gene name</th>
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<tr>
<td>PGN_1962</td>
<td>hypothetical protein</td>
<td>PGN_0504</td>
<td>methylmalonyl-CoA decarboxylase beta subunit</td>
<td>PGN_1172</td>
<td>acyl-CoA dehydrogenase</td>
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<tr>
<td>PGN_1047</td>
<td>hydroxyamine reductase</td>
<td>PGN_1670</td>
<td>conserved hypothetical protein with predicted lysozyme domain</td>
<td>PGN_1000</td>
<td>glycine cleavage system protein H</td>
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<tr>
<td>PGN_0295</td>
<td>C-terminal domain of Arg- and Lys- gingipain proteinase</td>
<td>PGN_0731</td>
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<td>PGN_0099</td>
<td>peptidase</td>
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<td>$kgp$</td>
<td>Lys-gingipain</td>
<td>PGN_0503</td>
<td>biotin carboxyl carrier protein</td>
<td>PGN_0301</td>
<td>outer membrane protein</td>
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<tr>
<td>PGN_1367</td>
<td>glutamate dehydrogenase</td>
<td>PGN_1048</td>
<td>hypothetical protein</td>
<td>PGN_1014</td>
<td>elongation factor G</td>
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<td>PGN_1740</td>
<td>RNA polymerase ECF-type sigma factor</td>
<td>ustA</td>
<td>upregulated in stationary phase protein A</td>
<td>PGN_1752</td>
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<td>$rgpA$</td>
<td>RgpAc, glycosyltransferase</td>
<td>PGN_1755</td>
<td>2-oxoglutarate oxidoreductase subunit beta</td>
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<td>PGN_2065</td>
<td>Lys- and Rgp- gingipain domain protein</td>
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<td>$rgpB$</td>
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<td>TonB protein</td>
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<td>PGN_0806</td>
<td>MotA/TolQ/ExbB proton channel protein</td>
<td>PGN_1120</td>
<td>NADPH-NAD transhydrogenase</td>
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<td>PGN_1418</td>
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<td>PGN_0604</td>
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<td>mfa1</td>
<td>Mfa1 fimbrilin</td>
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<td>PGN_0724</td>
<td>NAD-dependent 4-hydroxybutyrate dehydrogenase</td>
<td>PGN_1880</td>
<td>malate dehydrogenase</td>
<td>PGN_1655</td>
<td>electron transport complex RsxE subunit</td>
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</table>
Table shows gene ID number and name of all *P. gingivalis* genes that were upregulated (> 1.5 fold) when exposed to CSE conditioned media.

Down regulated genes (< 0.6 fold) include genes encoding proteins that may be involved in DNA replication, recombination and repair (e.g., PGN_1216, PGN_2011, and PGN_0644); the transfer gene cluster (*traJ*, *traK*, *traM*, *traA* and *traG*); Several genes in the ABC transporter operons (e.g., PGN_1325, PGN_1324, PGN_0706, PGN_0707 and PGN_0708); minor fimbrial operon (*mfa1*); several operons of transposases and partial transposases; genes encoding nucleotide excision, repair and metabolism (PGN_0327, PGN_1706, PGN_0195); putative endonuclease gene (PGN_1801) and genes in the capsular biosynthesis locus (PGN_110 and PGN_1072).

**Table 4: List of down regulated genes in *P. gingivalis* upon CSE exposure**

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<tr>
<th>Gene ID</th>
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<td>transposase in ISPg1</td>
<td>PGN_0385</td>
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<td>PGN_0196</td>
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<td>PGN_1461</td>
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<td>putative lipoprotein</td>
<td>PGN_1478</td>
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<td>PGN_1291</td>
<td>conserved hypothetical protein related to phage</td>
<td>PGN_0028</td>
<td>calcium-transporting ATPase</td>
<td>PGN_1333</td>
<td>ISPg1, transposase component I</td>
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<td>partial transposase Orf1 in ISPg5</td>
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<td>DNA-binding protein histone-like family</td>
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<td>hypothetical protein</td>
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<td>porR protein</td>
<td>PGN_1109</td>
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<td>PGN_0948</td>
<td>hypothetical protein</td>
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<td>PGN_0000</td>
<td>4</td>
<td>tRNA-Val</td>
<td>PGN_1265</td>
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</tbody>
</table>
Table shows gene ID number and name of all *P. gingivalis* genes that were downregulated (< 0.6 fold) when exposed to CSE.

**Figure 5: *P. gingivalis* ATCC 33277 differentially expressed genes in CSE**

Genes differentially expressed in *P. gingivalis* were grouped into different functional categories using DAVID and KEGG enrichment analysis software, A.
Metabolism; B. Genetic information processing; C. Environmental information processing; D. Transposases or partial transposases; E. DNA replication, recombination and repair; F. Others and G. hypothetical or conserved hypothetical proteins. Closed boxes represent up regulated genes in *P. gingivalis* (> 1.5 fold), when exposed to CSE and open boxes represent down regulated genes in *P. gingivalis* (< 0.6 fold), when exposed to CSE.

**KEGG analysis for differentially expressed *P. gingivalis* genes**

KEGG analysis was done on differentially expressed *P. gingivalis* genes to group them into various functional categories. Genes in several essential pathways were found to be differentially expressed. KEGG orthology system was used to generate these pathways with increased number of differentially expressed genes, which include glycolysis, citrate cycle, pyruvate metabolism, biosynthesis of amino acids, butanoate metabolism, ABC transporters and CAMP resistance pathway genes. Several genes related to citrate cycle (PGN_1418, PGN_1529, PGN_1753, PGN_1755, PGN_1880, PGN_0496, PGN_0498, PGN_1752, and PGN_1756), Figure 6; pyruvate metabolism (PGN_0504, PGN_1755, PGN_1753, PGN_1529, PGN_1418, PGN_1880, Figure 7); glycolysis (PGN_1695, PGN_0173, PGN_1418, PGN_1529, PGN_1753, PGN_1755, Figure 8); butanoate metabolism (PGN_0496, PGN_0498, PGN_0724, PGN_0727, PGN_1172, PGN_1418, PGN_1755, PGN_1753, PGN_1529, Figure 9); and cationic antimicrobial peptide (CAMP) resistance (rgpB and rgpA) (Figure 10) pathways were found to be up regulated in *P. gingivalis* upon CSE exposure. Genes related to ABC transporter pathway (PGN_1325, PGN_1324, PGN_1025, PGN_1387, PGN_1471,
PGN_0706, PGN_0686, PGN_0707, PGN_0708, Figure 11) were found to be down regulated when *P. gingivalis* was exposed to Cigarette smoke. In amino acid biosynthesis pathway a few genes (PGN_0173, PGN_1695 and PGN_1080) were up regulated and a few genes (PGN_1475, PGN_0230, PGN_1474 and PGN_1495) were down regulated (Figure 12).

**Figure 6: Citrate cycle pathway of *P. gingivalis***

Generated using KEGG orthology system and red boxes show differentially regulated *P. gingivalis* genes involved in Citrate cycle when exposed to CSE.
Figure 7: Pyruvate metabolism pathway of *P. gingivalis*

Generated using KEGG orthology system and red boxes show differentially regulated *P. gingivalis* genes involved in Pyruvate metabolism when exposed to CSE.
Figure 8: Glycolysis pathway of *P. gingivalis*
Generated using KEGG orthology system and red boxes show differentially regulated \textit{P. gingivalis} genes involved in Glycolysis when exposed to CSE.

Figure 9: Butanoate metabolism pathway of \textit{P. gingivalis}

Generated using KEGG orthology system and red boxes show differentially regulated \textit{P. gingivalis} genes involved in Butanoate metabolism when exposed to CSE.
Figure 10: CAMP resistance pathway of *P. gingivalis*

Generated using KEGG orthology system and red box shows differentially regulated *P. gingivalis* genes RgpA and RgpB when exposed to CSE.
Figure 11: ABC transporter pathway of *P. gingivalis*
Generated using KEGG orthology system and red boxes show differentially regulated *P. gingivalis* ABC transporter genes when exposed to CSE.

**Figure 12: Biosynthesis of amino acids pathway of *P. gingivalis***

Generated using KEGG orthology system and red boxes show differentially regulated *P. gingivalis* genes involved in amino acid biosynthesis when exposed to CSE.

*F. alocis* differentially expressed genes
When *F. alocis* was exposed to CSE, 83 genes were found to be differentially expressed (P < 0.005); 72 genes were up regulated (> 1.5 fold) and 11 genes were down regulated (< 0.6 fold). Many of the 83 genes that were differentially expressed encode hypothetical proteins whose functions have yet to be determined.

Up regulated genes include DNA replication gene (HMPREF0389_0155); Transfer gene cluster (*traE* and *traG*); Genes in the ABC transporter operon (HMPREF0389_00895, HMPREF0389_00896, HMPREF0389_00897, HMPREF0389_01591, HMPREF0389_01592, HMPREF0389_01190, HMPREF0389_01191 and HMPREF0389_01281); gene encoding fimbrial assembly protein (HMPREF0389_00415); transcription regulator genes (HMPREF0389_00643, HMPREF0389_01102 and HMPREF0389_01590); gene encoding cell wall serine protease (HMPREF0389_00110); nucleotide metabolism gene (HMPREF0389_00826); genes responsible for Carbohydrate metabolism (HMPREF0389_00473 and HMPREF0389_00883) and energy metabolism (HMPREF0389_01302 and HMPREF0389_01303); gene encoding type IV pilus protein (HMPREF0389_00416); several genes for processing genetic information (e.g., HMPREF0389_00822, HMPREF0389_00821, HMPREF0389_00820, HMPREF0389_00819, HMPREF0389_00831, HMPREF0389_00830, HMPREF0389_00829, HMPREF0389_00828)

**Table 5: List of up regulated genes in *F. alocis* upon CSE exposure**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPREF0389_00155</td>
<td>DNA replication protein</td>
<td>HMPREF0389_01736</td>
<td>rpmJ; psM; Ribosomal protein L36</td>
<td>HMPREF0389_01728</td>
<td>hypothetical; Copper amine oxidase domain protein</td>
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<tr>
<td>HMPREF0389_00186</td>
<td>NipC/P60 family protein</td>
<td>HMPREF0389_01591</td>
<td>ABC transporter permease</td>
<td>HMPREF0389_01281</td>
<td>ABC transporter ATP-binding protein</td>
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</tbody>
</table>
Table shows gene ID number and name of all *F. alocis* genes that were upregulated (> 1.5 fold) when exposed to CSE.

Down regulated genes include gene encoding nitrate/nitrite response regulator protein (HMPREF0389_00802); signal peptidase gene
(HMPREF0389_00799) and a gene responsible for glycolysis (HMPREF0389_00226).

**Table 6: List of down regulated genes in *F. alocis* upon CSE exposure**

<table>
<thead>
<tr>
<th>Gene ID</th>
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<tr>
<td>HMPREF0389_00226</td>
<td>acetyl coenzyme A synthetase</td>
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<tr>
<td>HMPREF0389_00802</td>
<td>Nitrate/nitrite response regulator protein</td>
</tr>
<tr>
<td>HMPREF0389_01749</td>
<td>hypothetical</td>
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<td>HMPREF0389_01188</td>
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<td>HMPREF0389_01353</td>
<td>amidinotransferase</td>
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<td>HMPREF0389_00486</td>
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<td>HMPREF0389_00798</td>
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<td>HMPREF0389_00799</td>
<td>signal peptidase I</td>
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<tr>
<td>HMPREF0389_00801</td>
<td>hypothetical</td>
</tr>
<tr>
<td>HMPREF0389_00800</td>
<td>low density lipoprotein receptor 2</td>
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</table>

Table shows gene ID number and name of all *F. alocis* genes that were down regulated (< 0.6 fold) when exposed to CSE.

**Figure 13: *F. alocis* ATCC 35896 differentially expressed genes in CSE**

Genes differentially expressed in *F. alocis* were grouped into different functional categories using KEGG enrichment analysis software, A. Carbohydrate metabolism; B. Energy metabolism; C. Lipid metabolism; D. Nucleotide
metabolism; E. Metabolism of Cofactors and vitamins; F. Transcription; G. Translation; H. Folding, sorting and degradation; I. Membrane transport; J. Hypothetical proteins and K. Others. Closed boxes represent up regulated genes in *F. alocis* (> 1.5 fold), when exposed to CSE and open boxes represent down regulated genes in *F. alocis* (< 0.6 fold), when exposed to CSE.

**KEGG analysis for differentially expressed *F. alocis* genes**

KEGG analysis was done on differentially expressed *F. alocis* genes to group them into various functional categories. Genes in several essential pathways were found to differentially expressed. KEGG orthology system was used to generate these pathways with increased number of differentially expressed genes, which include pyruvate metabolism, oxidative phosphorylation, ABC transporter, protein export and bacterial secretory system pathways. Genes related to oxidative phosphorylation (HMPREF0389_01302 and HMPREF0389_01303, Figure 14), ABC transporter (HMPREF0389_00896, HMPREF0389_00897 and HMPREF0389_00895, Figure 15) and bacterial secretory system (HMPREF0389_01581 and HMPREF0389_00827, Figure 16) pathways were up regulated when *F. alocis* was exposed to Cigarette smoke. In protein export pathway gene (HMPREF0389_00827) was up regulated and gene (HMPREF0389_00799) was down regulated (Figure 17). Similarly, in pyruvate metabolism pathway gene (HMPREF0389_00473) was up regulated and gene (HMPREF0389_00226) was down regulated (Figure 18) in *F. alocis* upon CSE exposure.
Figure 14: Oxidative phosphorylation pathway of *F. alocis*

Generated using KEGG orthology system and red boxes show differentially regulated *F. alocis* genes involved in oxidative phosphorylation when exposed to CSE.
Figure 15: ABC transporter pathway of *F. alocis*
Generated using KEGG orthology system and red boxes show differentially regulated *F. alocis* ABC transporter genes when exposed to CSE.

**Figure 16: Bacterial secretory system pathway of *F. alocis***

Generated using KEGG orthology system and red boxes show differentially regulated *F. alocis* genes involved in secretion system when exposed to CSE.
Figure 17: Protein export in *F. alocis*

Generated using KEGG orthology system and red boxes show differentially regulated *F. alocis* genes involved in protein export when exposed to CSE.
Figure 18: Pyruvate metabolism pathway of *F. alocis*

Generated using KEGG orthology system and red boxes show differentially regulated *F. alocis* genes involved in Pyruvate metabolism when exposed to CSE.

*T. denticola* differentially expressed genes

We were unable to efficiently isolate RNA from *T. denticola*, therefore differentially regulated genes could not be identified.

Validation of RNA-Seq data by qPCR analysis

To validate the RNA-Seq analysis, qPCR was performed on selected up regulated and down regulated genes for both *F. alocis* and *P. gingivalis*. As seen in Table 7
and Table 8, most of the selected genes showed trends similar to that of RNA-Seq analysis. However differential expression of only a small number of genes was statistically significant in the qPCR experiments.

**Table 7: qPCR expression values for selected *P. gingivalis* genes**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Control qPCR expression value (Mean CT)</th>
<th>CSE qPCR expression value (Mean CT)</th>
<th>P- value (T- test)</th>
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<td>16s rRNA</td>
<td>Reference gene</td>
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Selected *P. gingivalis* up-regulated genes by qPCR analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>ΔCt Value (control)</th>
<th>ΔCt Value (CSE)</th>
<th>Delta Delta Ct (DDCt) Value</th>
<th>Fold change (2-DDCt)</th>
<th>P-value (T-test)</th>
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<tbody>
<tr>
<td>PGN_1047</td>
<td>hydroxylamine reductase</td>
<td>4.77</td>
<td>3.09</td>
<td>-1.68</td>
<td>3.2</td>
<td>0.16</td>
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<tr>
<td>PGN_0295</td>
<td>C-terminal domain of Arg- and Lys-gingipain proteinase</td>
<td>5.41</td>
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<td>-2.03</td>
<td>4.08</td>
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<tr>
<td>Kgp</td>
<td>Lys-gingipain</td>
<td>4.37</td>
<td>2.04</td>
<td>-2.33</td>
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<td>PGN_1367</td>
<td>glutamate dehydrogenase</td>
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<td>2.86</td>
<td>-1.89</td>
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<td>PGN_1740</td>
<td>RNA polymerase ECF-type sigma factor</td>
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<td>RgpA</td>
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<td>PGN_0727</td>
<td>4-hydroxybutyryl-CoA dehydratase</td>
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<td>PGN_1695</td>
<td>fructose-1,6-bisphosphate aldolase</td>
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<td>RpoC</td>
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<td>-2.89</td>
<td>7.41</td>
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Selected *P. gingivalis* down-regulated genes by qPCR analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>ΔCt Value (control)</th>
<th>ΔCt Value (cse)</th>
<th>Delta Delta Ct (DDCt) value</th>
<th>Fold change (2-DDCt)</th>
<th>P-value (T-test)</th>
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<tbody>
<tr>
<td>PGN_1047</td>
<td>hydroxylamine reductase</td>
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<td>3.09</td>
<td>-1.68</td>
<td>3.2</td>
<td>0.16</td>
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<tr>
<td>PGN_0295</td>
<td>C-terminal domain of Arg- and Lys-gingipain proteinase</td>
<td>5.41</td>
<td>3.38</td>
<td>-2.03</td>
<td>4.08</td>
<td>0.23</td>
</tr>
<tr>
<td>Kgp</td>
<td>Lys-gingipain</td>
<td>4.37</td>
<td>2.04</td>
<td>-2.33</td>
<td>5.02</td>
<td>0.58</td>
</tr>
<tr>
<td>PGN_1367</td>
<td>glutamate dehydrogenase</td>
<td>4.75</td>
<td>2.86</td>
<td>-1.89</td>
<td>3.70</td>
<td>0.06</td>
</tr>
<tr>
<td>PGN_1740</td>
<td>RNA polymerase ECF-type sigma factor</td>
<td>3.61</td>
<td>2.50</td>
<td>-1.11</td>
<td>2.15</td>
<td>0.2</td>
</tr>
<tr>
<td>RgpA</td>
<td>RgpA; glycosyltransferase</td>
<td>3.87</td>
<td>1.23</td>
<td>-2.64</td>
<td>6.23</td>
<td>0.125</td>
</tr>
<tr>
<td>PGN_0727</td>
<td>4-hydroxybutyryl-CoA dehydratase</td>
<td>3.29</td>
<td>0.98</td>
<td>-2.31</td>
<td>4.95</td>
<td>0.013</td>
</tr>
<tr>
<td>PGN_1695</td>
<td>fructose-1,6-bisphosphate aldolase</td>
<td>3.52</td>
<td>1.12</td>
<td>-2.4</td>
<td>5.27</td>
<td>0.56</td>
</tr>
<tr>
<td>RpoC</td>
<td>DNA-directed RNA polymerase subunit beta</td>
<td>4.25</td>
<td>1.28</td>
<td>-2.97</td>
<td>7.83</td>
<td>0.01</td>
</tr>
<tr>
<td>Dps</td>
<td>DNA-binding protein from starved cells Dps</td>
<td>4.07</td>
<td>1.17</td>
<td>-2.9</td>
<td>7.46</td>
<td>0.0009</td>
</tr>
<tr>
<td>PGN_0173</td>
<td>glyceraldehyde 3-phosphate dehydrogenase type I</td>
<td>3.77</td>
<td>1.13</td>
<td>-2.64</td>
<td>6.23</td>
<td>0.035</td>
</tr>
<tr>
<td>PGN_0175</td>
<td>2-ketoisovalerate ferredoxin reductase</td>
<td>4.01</td>
<td>1.13</td>
<td>-2.88</td>
<td>7.36</td>
<td>0.022</td>
</tr>
<tr>
<td>PGN_1080</td>
<td>branched-chain amino acid aminotransferase</td>
<td>3.66</td>
<td>1.1</td>
<td>-2.56</td>
<td>5.89</td>
<td>0.126</td>
</tr>
<tr>
<td>PGN_0660</td>
<td>alkyl hydroperoxide reductase</td>
<td>3.94</td>
<td>1.12</td>
<td>-2.82</td>
<td>7.06</td>
<td>0.0001</td>
</tr>
<tr>
<td>PGN_0724</td>
<td>NAD-dependent 4-hydroxybutyrate dehydrogenase</td>
<td>4.13</td>
<td>1.24</td>
<td>-2.89</td>
<td>7.41</td>
<td>0.008</td>
</tr>
</tbody>
</table>
The table shows all *P. gingivalis* up-regulated and down-regulated genes from RNA-Seq selected for qPCR analysis. Positive DDC\(_t\) values indicate more PCR cycles and therefore less targeted gene after CSE exposure, meaning suppression of targeted gene expression. Conversely, negative DDC\(_t\) values indicate more targeted gene after CSE exposure, indicating induction of gene expression.

a. Differentially expressed genes in qPCR which correlate with RNA-Seq results.
b. Represents *P. gingivalis* genes that showed statistically significant (P < 0.05) difference in their expression when exposed to cigarette smoke in qPCR analysis.

**Table 8: qPCR expression values for selected *F. alocis* genes**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Control qPCR expression value (Mean C(_T))</th>
<th>CSE qPCR expression value (Mean C(_T))</th>
<th>(P)-value (T-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16srRNA Reference gene</td>
<td></td>
<td>10.54</td>
<td>11.23</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Selected *F. alocis* up-regulated genes by qPCR analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>(\Delta Ct) Value (control)</th>
<th>(\Delta Ct) Value (CSE)</th>
<th>Delta Delta Ct (DDC(_t)) Value</th>
<th>Fold change (2^{\Delta \Delta Ct})</th>
<th>(P)-value (T-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPREF0389_00155</td>
<td>DNA replication protein DnaC</td>
<td>12.97</td>
<td>14.90</td>
<td>1.93</td>
<td>0.26</td>
<td>0.01</td>
</tr>
<tr>
<td>HMPREF0389_00186</td>
<td>Nlpc/P60 family protein</td>
<td>15.47</td>
<td>14.7</td>
<td>-0.77</td>
<td>1.70</td>
<td>0.64</td>
</tr>
<tr>
<td>HMPREF0389_00184</td>
<td>Type IV conjugative transfer protein TraE</td>
<td>15.79</td>
<td>14.65</td>
<td>-1.14</td>
<td>2.20</td>
<td>0.04</td>
</tr>
<tr>
<td>HMPREF0389_00178</td>
<td>TraG family protein</td>
<td>15.66</td>
<td>15.74</td>
<td>0.08</td>
<td>0.94</td>
<td>0.03</td>
</tr>
<tr>
<td>HMPREF0389_00166</td>
<td>TnpX site-specific recombinase</td>
<td>14.38</td>
<td>13.28</td>
<td>-1.1</td>
<td>2.14</td>
<td>0.13</td>
</tr>
<tr>
<td>HMPREF0389_00246</td>
<td>Antirestriction protein (ArdA)</td>
<td>14.24</td>
<td>13.43</td>
<td>-0.81</td>
<td>1.75</td>
<td>0.50</td>
</tr>
<tr>
<td>HMPREF0389_00154</td>
<td>Replication initiation protein</td>
<td>12.21</td>
<td>11.32</td>
<td>-0.89</td>
<td>1.85</td>
<td>0.71</td>
</tr>
<tr>
<td>HMPREF0389_00069</td>
<td>Nitrate transporter NirC</td>
<td>8.41</td>
<td>9.1</td>
<td>0.69</td>
<td>0.61</td>
<td>0.01</td>
</tr>
<tr>
<td>HMPREF0389_00162</td>
<td>Conjugation protein</td>
<td>13.26</td>
<td>12.58</td>
<td>-0.68</td>
<td>1.60</td>
<td>0.98</td>
</tr>
<tr>
<td>HMPREF0389_01096</td>
<td>MATE efflux family protein</td>
<td>9.06</td>
<td>8.18</td>
<td>-0.88</td>
<td>1.84</td>
<td>0.84</td>
</tr>
<tr>
<td>HMPREF0389_00644</td>
<td>FtsK/SpoIIIE family protein</td>
<td>11.18</td>
<td>10.42</td>
<td>-0.76</td>
<td>1.69</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Table shows all *F. alocis* up regulated and down regulated genes from RNA-Seq selected for qPCR analysis. Positive DDC\(_t\) values indicate more PCR cycles and therefore less targeted gene after CSE exposure, meaning suppression of targeted gene expression. Conversely, negative DDC\(_t\) values indicate more targeted gene after CSE exposure, indicating induction of gene expression.

a. Differentially expressed genes in qPCR which correlate with RNA-Seq results.
b. Represents the *F. alocis* genes that showed statistically significant (P<0.5) difference in their expression when exposed to cigarette smoke in qPCR analysis.

### Selected *F. alocis* down-regulated genes by qPCR analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>ΔCt Value (control)</th>
<th>ΔCt Value (CSE)</th>
<th>Delta Delta Ct (DDC(_t)) Value</th>
<th>Fold change (2^{\text{DDC}_t})</th>
<th>P-value (T-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPREF0389_00226(^a)</td>
<td>Acetyl coenzyme A synthetase</td>
<td>4.61</td>
<td>4.71</td>
<td>0.1</td>
<td>0.93</td>
<td>0.02</td>
</tr>
<tr>
<td>HMPREF0389_00802</td>
<td>Nitrate/nitrite regulator protein</td>
<td>9.95</td>
<td>9.49</td>
<td>-0.46</td>
<td>1.37</td>
<td>0.27</td>
</tr>
<tr>
<td>HMPREF0389_01353</td>
<td>Amidinotransferase</td>
<td>13.17</td>
<td>12.54</td>
<td>-0.63</td>
<td>1.54</td>
<td>0.75</td>
</tr>
<tr>
<td>HMPREF0389_00799(^a)</td>
<td>Signal peptidase I</td>
<td>8.39</td>
<td>8.77</td>
<td>0.38</td>
<td>0.98</td>
<td>0.02</td>
</tr>
<tr>
<td>HMPREF0389_00800</td>
<td>Low density lipoprotein receptor 2</td>
<td>8.93</td>
<td>8.72</td>
<td>-0.21</td>
<td>1.15</td>
<td>0.09</td>
</tr>
</tbody>
</table>

### ABC transporter permease, ATP-binding protein

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>ΔCt Value (control)</th>
<th>ΔCt Value (CSE)</th>
<th>Delta Delta Ct (DDC(_t)) Value</th>
<th>Fold change (2^{\text{DDC}_t})</th>
<th>P-value (T-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPREF0389_00823</td>
<td>Translation initiation factor IF-1</td>
<td>4.12</td>
<td>4.21</td>
<td>0.09</td>
<td>0.93</td>
<td>0.11</td>
</tr>
<tr>
<td>HMPREF0389_00879(^a)</td>
<td>Riboflavin biosynthesis protein RibD</td>
<td>10.78</td>
<td>10.41</td>
<td>-0.37</td>
<td>1.29</td>
<td>0.5</td>
</tr>
<tr>
<td>HMPREF0389_01079</td>
<td>Iron-sulfur cluster-binding protein</td>
<td>10.56</td>
<td>11.66</td>
<td>1.1</td>
<td>0.46</td>
<td>0.1</td>
</tr>
</tbody>
</table>
CHAPTER 4: DISCUSSION

Cigarette smoke is an important environmental risk factor for periodontal diseases. Also cigarette smoke is known to increase vulnerability to oral bacterial infection, but with reduced clinical signs of overt inflammation. The underlying mechanism for this response is not clearly established. However, we can hypothesize that cigarette smoke causes alterations in the gene expression in periodontal bacteria. In our RNA-Seq experiments we were able to find the differentially expressed genes in an established periodontal pathogen, \textit{P. gingivalis}, and an emerging periodontal pathogen, \textit{F. alocis}. Approximately, 30\% of genes in \textit{P. gingivalis} genome and 5\% of genes in \textit{F. alocis} genome were found to be differentially expressed when exposed to cigarette smoke.

In \textit{P. gingivalis} several functionally related genes were found to be up regulated, including genes encoding arginine and lysine gingipains (\textit{kgp}, \textit{rgpA} and \textit{rgpB}), DNA binding genes and genes responsible for carbohydrate and energy metabolism. Gingipains play an important role in multiple virulence mechanisms in \textit{P. gingivalis}, which are responsible for the growth and survival of the bacterium. They protect \textit{P. gingivalis} from phagocytosis by PMN's by degrading macrophage CD14, thus inhibiting activation of leucocytes through the LPS receptor (138) and by degrading complement factor C3, preventing deposition of C3b on the bacterial cell surface (225,226). Also in addition to providing energy through degradation...
and metabolism of extracellular matrix proteins (227), they subvert the host response by degrading inflammatory cytokines, IL-6, IL-8 and TNF (136,138). DNA binding gene PGN_1740 is known to play a key role in biofilm formation by \textit{P. gingivalis} (228). It has been demonstrated that the Dps (DNA-binding protein from starved cells) protein in \textit{E. coli} plays an important role in the protection of cells from peroxide stress and is believed to have similar kind of function in \textit{P. gingivalis} (229,230,231). Carbohydrate and amino acid metabolism provide \textit{P. gingivalis} with energy necessary for its growth.

Several genes encoding proteins involved in DNA replication, recombination and repair which are essential genes for \textit{P. gingivalis}; transfer (tra) genes which might be responsible for genomic diversity in \textit{P. gingivalis} strains (232,233); ABC transport genes required for optimal entry of \textit{P. gingivalis} into GECs (234); genes in the capsular biosynthesis locus (PGN_110 and PGN_1072) needed for capsule synthesis; minor fimbrial operon gene (\textit{mfa1}) which is known to play key role in \textit{P. gingivalis} auto-aggregation (235) and interspecies interactions with oral streptococci that facilitate biofilm formation (97,236), were found to be down regulated when \textit{P. gingivalis} was exposed to cigarette smoke extract.

Many of the differentially expressed genes in \textit{F. alocis} when exposed to CSE conditioned media encode hypothetical proteins whose function is yet to be determined. Several ABC transporter genes were found to be up regulated which are believed to provide resistance to \textit{P. gingivalis} from potentially harmful chemicals in cigarette smoke (222) and might have similar function in \textit{F. alocis}.  

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Transfer (tra) genes necessary for non-sexual transfer of genetic material in both Gram-positive and Gram-negative bacteria (237) and several genes processing genetic information were induced when exposed to cigarette smoke, suggesting the potential for increased genomic diversity among F. alocis strains. A fimbrial assembly gene (HMPREF0389_00415) which is known for its virulence in other bacteria like P. gingivalis, E. coli and also several carbohydrate and lipid metabolism genes that provide energy to the organism were found to be upregulated. Nitrate/nitrite response regulator protein (HMPREF0389_00802) needed for alternative mode of amino acid synthesis (169) was seen to be down regulated when exposed to cigarette smoke.

Even though all selected up regulated P. gingivalis genes from RNA-Seq showed up regulation (fold change > 1.5) in their expression when exposed to CSE in qPCR analysis, there were only a few genes with significant (P≤0.01) differences, which include PGN_0724, dps, rpoC and PGN_0660. DNA-binding genes (dps and rpoC) were seen to show increased expression in both RNA-Seq and qPCR analysis when exposed to CSE. These proteins bind DNA and are known as histone-like proteins and are believed to have diversity of functions responsible for survival of the organism. Also as said earlier Dps (DNA-binding protein from starved cells) protein in E. coli plays an important role in the protection of cells from peroxide stress and might show similar function in P. gingivalis (222,223,224). PGN_0660 and PGN_0724 are oxidoreductases and increase in the expression of these gene may be involved in protecting the bacteria from oxidative stress generated in periodontal diseases.
In contrast with our RNA-Seq data, only one gene Upp (uracil phospho-
ribosyl transferase) which might be responsible for cell wall organization and
regulation of cell wall shape was found to be down regulated (Fold change < 0.6)
in qPCR analysis.

Similar to RNA-Seq results most of the selected up regulated _F. alocis_
genes from were found to be up regulated (Fold change >1.5) in qPCR analysis,
but only expression of few genes was statistically significant (P≤0.01) difference,
which include HMPREF0389_00969 and HMPREF0389_01592. Formate/ Nitrate
transporter protein (HMPREF0389_00969) may be necessary for the anaerobic
respiration of _F. alocis_. ABC transporter protein (HMPREF0389_01592) was seen
to be up regulated in both qPCR and RNA-Seq analysis of _F. alocis_ genes. As said
earlier, this gene is believed to responsible for protecting _P. gingivalis_ from harmful
chemicals of cigarette smoke (222) and it might have similar function in _F. alocis_.

In contrast with our RNA-Seq data, most of the selected _F. alocis_ down
regulated genes remain unaltered in qPCR analysis. One gene
HMPREF0389_01353 (amidino transferase) found to be up regulated in qPCR and
downregulated in RNA-Seq. It’s function in the virulence of _F. alocis_ was yet to be
determined.

As stated earlier, expression of most of the selected genes for qPCR was similar
to that of RNA-seq but not statistically significant (P≤0.01). This might be due to
limitations in qPCR, like intra- and inter- assay variability (238) and/or complicated
RNA-Seq technique. As statistical power is closely linked to sample size, a long
transcript is more likely to be found differentially expressed during RNA-Seq than
a short transcript (240). So expression levels of short genes are not accurate with RNA-Seq analysis (239).

Also our RNA-seq data for *P. gingivalis* is not in agreement with the study done by Bagaitkar et al. (222). Several genes responsible for growth and survival of *P. gingivalis* like genes responsible for DNA-replication, recombination and repair; ABC transporter genes which were found to be up regulated in her study, were down regulated in our study. Study done by Bagaitkar et al. (222) was on *P. gingivalis* W83 strain, by using microarray analysis and at a CSE concentration of 500 ng/ml nicotine equivalents. We used *P. gingivalis* ATCC 33277 and a CSE concentration of 1000 ng/ml nicotine equivalency and RNA sequencing. Therefore, genetic, dose and technical differences may each and all have contributed to variation in results between studies.

Future studies can be done on the key *P. gingivalis* and *F. alocis* differentially expressed genes (validated by qPCR), when exposed to CSE conditioned media, using site-directed mutagenesis and complement assays. These procedures provide better understanding of specific genes as well as for developing novel variants of gene of interest. These studies might also provide some of the first insights into how tobacco smoke changes the *P. gingivalis* and *F. alocis* phenotype in a manner likely to promote their colonization and infection.

In summary, smokers are more prone to bacterial infection and to develop periodontitis, yet exhibit reduced clinical inflammation. Our experimental results showed that several genes essential for growth and survival of *P. gingivalis* and *F. alocis* were differentially expressed when exposed to cigarette smoke. These
results may explain in part the altered virulence and host-pathogen interactions.
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