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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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ABSTRACT

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

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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.*

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).

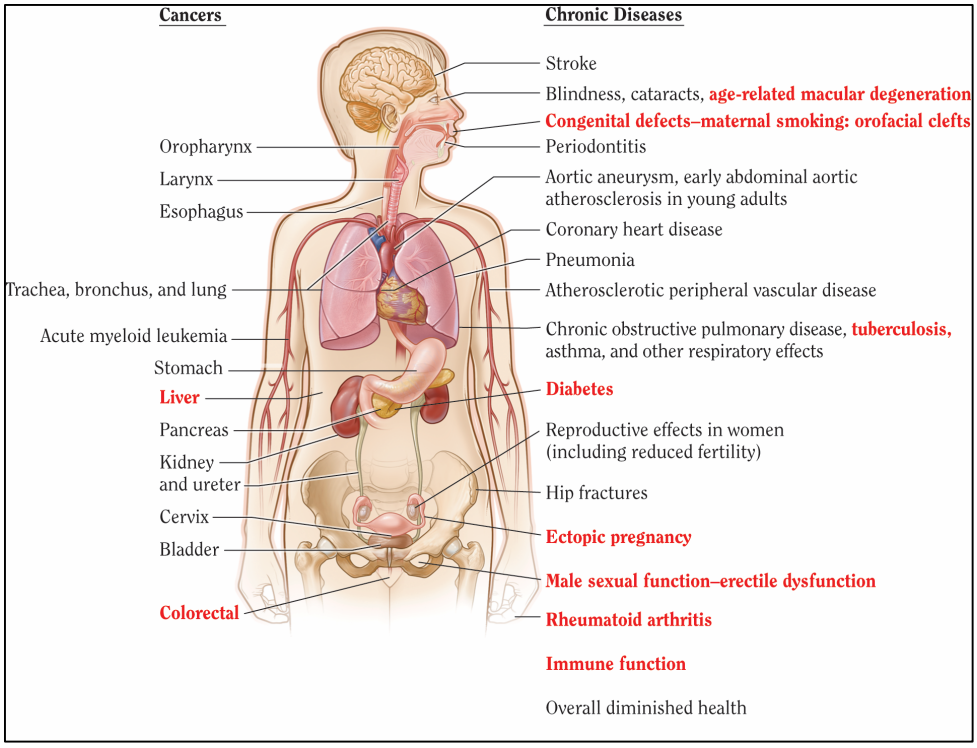

Figure 1: Diseases related to smoking

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.

Photo courtesy of: U.S. Department of Health and Human Services (USDHHS). *The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General*. Atlanta (GA): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2014.

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 atleast 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-infammatory 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 glycylprolyl 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 *rgpB.* All the extra- cellular activity with specificity for lysine peptide bonds is derived from a single gene, *kgp* (139). These are important etiological agents in periodontal diseases.

P. gingivalis proteases degradae 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).

Filifactor alocis

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 *Fusobacterium alocis* (143). Later, based on phylogenetic analysis of 16s rRNA, it was reclassified into the genus *Filifactor* (144). The size of the *F. alocis* genome is 1.93 Mb (www.broadinstitute.org). Cultivable strains of *F. alocis* include, ATCC 35896, D-62D (clinical strain) (145) and CCUG 47790 T (www.straininfo.net). *F. alocis* is present in diseased periodontal pockets in higher numbers than in healthy mouths (146,147). Arginine, which increases the growth of *F. alocis in vitro,* is abundantly present in periodontal pockets (148). This has been hypothesized to help explain the presence of *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*

nucleatum (166), 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*, *Bullleida* 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.

CHAPTER 2: MATERIALS AND METHODS

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% N_2, 10% H_2, 10% CO_2) at 37^0c. 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 10^9 cells per ml; *F. alocis*- O.D 600nm =0.35, corresponding to 3×10^9 cells per ml; *T. denticola*- O.D 600 nm = 1.6, corresponding to 8×10^9 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 ^{TM} RNA Cultured Cell Kit by 5 Prime (Gaithersburg, MD) was used for *P. gingivalis* and *T. denticola.* RiboPure ^{TM} – 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 -80^0C.

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

Gene ID	Forward primer 5'-3'	Reverse primer 5'-3'
PGN_1047	TTCCATAGCCAAACGTGTAGAG	CTGAGAGCCAACCGATCATATT
PGN_0295	GGGTTCACTCAGTGCTCAAA	GAGCCATCCAAACACTCGATAG
kgp	AGGACAGGGTGAAGTTGTAATC	GCCTGCTTCGAATGTGAAATC
PGN_1367	CTCCGGGTAAGGCTGTTAATG	CAGCCACTTGTCCACTTCTT
PGN_1740	TGAATGAGGGAGGAGAGGATAC	ATGGAGAATGGCTGCTTGAG

PGN_1644	GGCTGAAGATGGAAGAGGTATC	GATGGCGGGAAAGTTGTTTG
upp	AGATGCGCGATGTCACTATTC	GGGTCATCTTCTTGCTGATCTC
PGN_0134	TCCATCATCAACGCCAAGAG	GGCCGAAAGAAGTCCGTATT
PGN_0885	CACCCTATGCTTTGCCTCTT	CGAAGGCTCATGGGTGAATAA
PGN_0545	CCTCTTGCTTTGTACGACTATCT	AGACTGGATCACTGCTTCTATTC
RgpA	GGACCGACGAAAGAAGATGATTA	CTTCCACCACCTTCGCTTATAG
PGN_0727	CCCTTTATGCTTGCGGTATTG	AAGGAAGGCGGGTGATATTT
PGN_1695	TAGGAGCATTGGATCAGAGTGGTG	GAAAGCCGGAGAAGTAATCATCC
RpoC	CGAAGGTGTAGTGGAGAATGT	CATAGGGAGCCATCGTCTTATC
Dps	CGCTGAAGAATGTGACCGATA	TCAAATCCACCGTTACCTCATC
PGN_0173	GGATCAAGTCGGAGTGGAATAC	CTTTAGAGGGAGCCGACATAAC
PGN_0175	GCGTATCCTCTCTGAAGTTGTT	GGTATCCTCCGATGCGATATG
PGN_1080	GTAACTATGCAGCCGGTATGA	CGGCTTCGTCTATGTACTTCTT
PGN_0724	AACTTCTCGGATGCCTTCTTAC	CGCAAAGCCTCTTACCTCTT
PGN_0660	TGGCTTATCGTGGCTCTTTC	GGAGGATCTCTTCTGCATCAC

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

Gene ID	Forward primer 5'-3'	Reverse primer 5'-3'
HMPREF0389_00184	CCATAGAGGCGGAGGACTTA	GCTCCTCACCATCAAGTACATAG
HMPREF0389_00178	GATGCCTGCTTGATGAGTTTG	GCTGAGATTGTGCCTGAAGTA
HMPREF0389_00166	CTGACACCGACCATCATCAA	GCGGTTCATAGTTCCCGATAA
HMPREF0389_00226	GAAGGACCTGTGGCTATGATTT	AGCCTTATTACCGAGTCCTACA
HMPREF0389_00802	GAACAGTGGAAGAGGCGATAAG	AAGCCACTCTCCTTCCCTAA
HMPREF0389_01353	ATGGAAGAAGAAGGCTGTAAGG	ACTCTCTATGCAACGGACAAG
HMPREF0389_00799	CTAAGGGTCTGTTGCTGAATCT	CTATGGCGAACCTCCTGTATTT
HMPREF0389_00800	CCGACTCAGATTGTAGTGGAAA	CAGCAAGCCATCTCCTTCTAA
HMPREF0389_00155	ACATCATAGACAGCAGATATAGGG	GTTGCCATTTCGAGAAGTCTG
HMPREF0389_00186	GGGCAAATGGACCGAATAAC	GCTTGGTGAAACGGGATTAC
HMPREF0389_01079	TTGTCACCTTGCCGTTTCT	CCAAGTGCCGCTCTGATATT
HMPREF0389_00246	GCACCCTTTGAAGCCTTTATC	CTCCTGTAGACTTTCACGATCC
HMPREF0389_00154	ACTATCCATTATCTACAATGCTCCT	TCTTGCCATATTCTTTCATCATCAG
HMPREF0389_00969	TCGTTTCGGGAGCATTGG	AAAGTTCTCCGCCTACCATAAC
HMPREF0389_00162	AAGCAGAAGAGTGGCGAAA	CCCATGTGAATTGTCGGTATTTG
HMPREF0389_01096	GTTCTGGAGATGGGTGTTTCT	CCCTCTGCCCTTATTACCATATT
HMPREF0389_00644	AGGTCGTGGCTATGTTGATG	GACTGTCTGCTGTTGGTTAGT
HMPREF0389_01592	ACAGCCTTAATCGGTTCGAG	CTCACTTATATCTTCTCCGCCTATC
HMPREF0389_00823	GGCACATATTTCCGGTAAACTTC	CTTTCTCCATGTGATACGACCT
HMPREF0389_00879	TCGCAAGTCACTCAGGAAAG	CTGTTCCGACACCTACCATAAT

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: CAGGTGGTTTAACAAGTTAGTGG

R: CTAAGTTGTCCTTAGCTGTCTCG

Primers were ordered from Biosynthesis (www.biosyn.com) and were reconstituted and stored at -20^{0}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^{0}C. SYBYR 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^{0}C for 2 minutes, 95^{0}C for 10 minutes, and 95^{0}C for 15 seconds – 45 repetitions and 60^{0}C for 1 minute. An additional dissociation stage was added to check for validity of primers which included 95^{0}C for 15 seconds, 60^{0}C for 1 minute, 95^{0}C for 15 seconds and 60^{0}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).

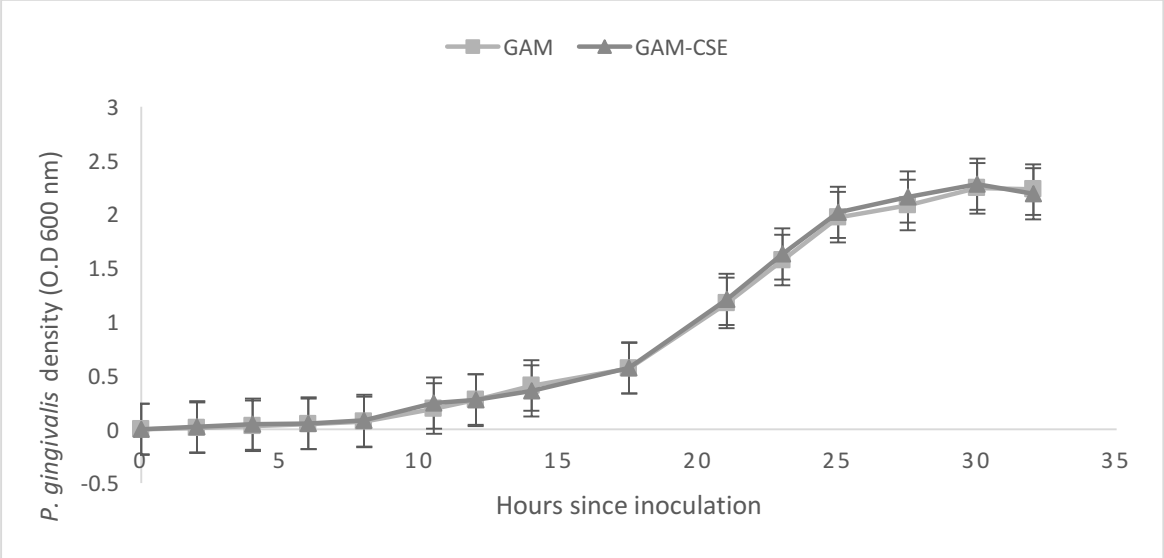

Figure 2: Effect of CSE on *P. gingivalis* growth

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.

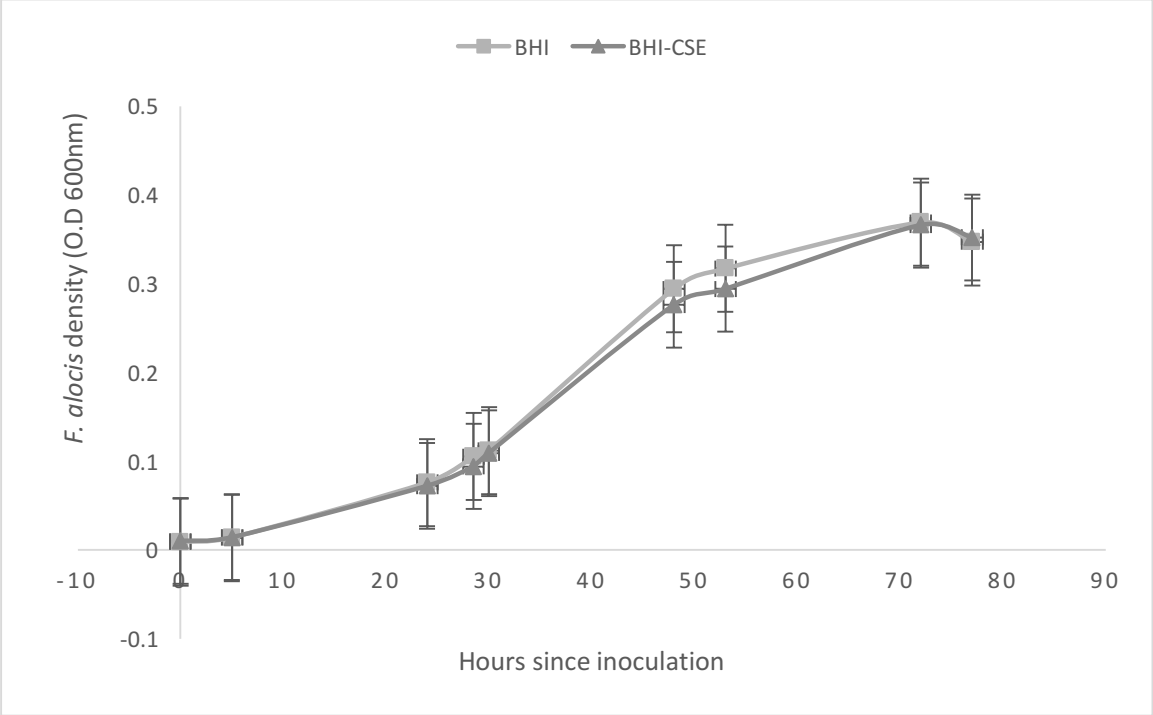

Figure 3: Effect of CSE on *F. alocis* growth

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.

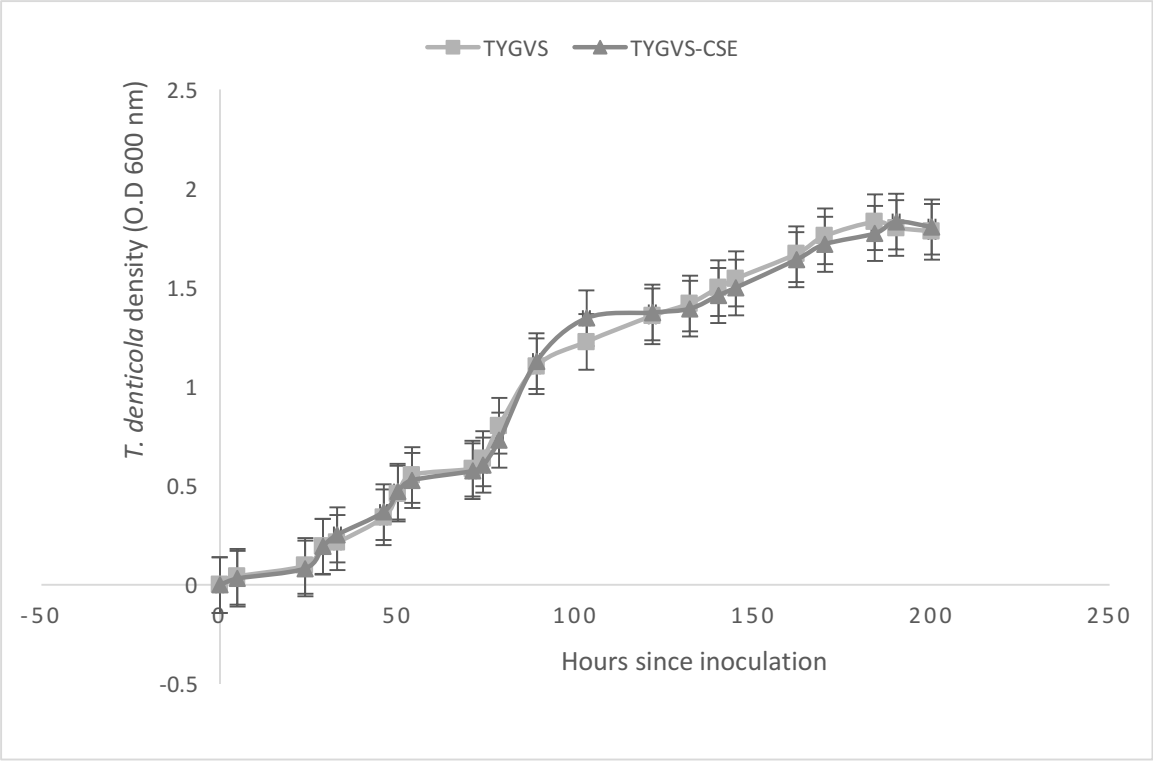

Figure 4: Effect of CSE on *T. denticola* growth

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 r*gpB*; 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

Gene ID	Gene name	Gene ID	Gene name	Gene ID	Gene name
PGN_1962	hypothetical protein	PGN_0504	methylmalonyl-CoA decarboxylase beta subunit	PGN_1172	acyl-CoA dehydrogenase
PGN_1047	hydroxylamine reductase	PGN_1670	conserved hypothetical protein with predicted lysozyme domain	PGN_1000	glycine cleavage system protein H
PGN_0295	C-terminal domain of Arg- and Lys-gingipain proteinase	PGN_0731	hypothetical protein	PGN_0099	peptidase
kgp	Lys-gingipain	PGN_0503	biotin carboxyl carrier protein	PGN_0301	outer membrane protein
PGN_1367	glutamate dehydrogenase	PGN_1048	hypothetical protein	PGN_1014	elongation factor G
PGN_1740	RNA polymerase ECF-type sigma factor	ustA	upregulated in stationary phase protein A	PGN_1752	ferredoxin 4Fe-4S
rgpA	RgpAc; glycosyltransferase	PGN_1755	2-oxoglutarate oxidoreductase subunit beta	PGN_1174	electron transfer flavoprotein alpha subunit
PGN_0727	4-hydroxybutyryl-CoA dehydratase	PGN_1529	2-oxoglutarate ferredoxin oxidoreductase subunit beta	PGN_1419	hypothetical protein
PGN_1695	fructose-1,6-bisphosphate aldolase	PGN_0033	thioredoxin	PGN_2065	Lys- and Rgp- gingipain domain protein
rpoC	DNA-directed RNA polymerase subunit beta'	rgpB	arginine-specific cysteine proteinase RgpB	PGN_1756	2-oxoglutarate oxidoreductase subunit gamma
dps	DNA-binding protein from starved cells Dps	PGN_0496	cytochrome B subunit	PGN_1578	elongation factor Tu
PGN_0173	glyceraldehyde 3-phosphate dehydrogenase type I	PGN_1434	aminoacyl-histidine dipeptidase	PGN_0498	succinate dehydrogenase/fumarate reductase iron-sulfur subunit
PGN_1753	2-ketoisovalerate ferredoxin reductase	PGN_0805	hypothetical protein	PGN_0809	TonB protein
PGN_1080	branched-chain amino acid aminotransferase	PGN_0806	MotA/TolQ/ExbB proton channel protein	PGN_1120	NADPH-NAD transhydrogenase
PGN_0649	hypothetical protein	PGN_1418	pyruvate-flavodoxin oxidoreductase	PGN_1739	hypothetical protein (lipoprotein)
PGN_0660	alkyl hydroperoxide reductase	PGN_0604	ferritin	mfa1	Mfa1 fimbrilin
PGN_0724	NAD-dependent 4-hydroxybutyrate dehydrogenase	PGN_1880	malate dehydrogenase	PGN_1655	electron transport complex RsxE subunit

PGN_1995	hypothetical protein	aspA	aspartate ammonia-lyase	PGN_1352	hypothetical protein

Table shows gene ID number and name of all *P. gingivalis* genes that were up regulated (> 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

Gene ID	Gene name	Gene ID	Gene name	Gene ID	Gene name	Gene ID	Gene name
PGN_1644	transposase in ISPg1	PGN_0210	transposase in ISPg1	PGN_0385	integrase/recombinase XerD	PGN_0196	xanthine/uracil permease
uvrC	excinuclease ABC subunit C	PGN_0706	exported periplasmic protein	PGN_0405	alpha-1,2-mannosidase	PGN_0439	hypothetical protein
PGN_1526	hypothetical protein	PGN_0206	lipid A disaccharide synthase	PGN_2013	cation efflux system protein	PGN_1024	ribosome-binding factor A
PGN_0820	hypothetical protein	PGN_1627	4-amino-4-deoxy-L-arabinose transferase	PGN_0549	dTDP-glucose 4,6-dehydratase	PGN_1324	ABC transporter membrane protein
PGN_1713	PAP2 superfamily protein	PGN_0524	PAP2 superfamily protein	PGN_1669	transposase in ISPg1	PGN_0738	phosphoglycerate mutase
upp	uracil phosphoribosyltransferase	ispD	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	traJ	conjugate transposon protein TraJ	PGN_1491	hypothetical protein
PGN_0134	biotin synthetase	PGN_1901	transposase in ISPg1	PGN_0324	transposase in ISPg1	PGN_1586	hypothetical protein
PGN_1177	transposase in ISPg1	PGN_1132	transposase in ISPg1	PGN_0056	conjugate transposon protein	PGN_0688	hypothetical protein
PGN_0885	nitroimidazole resistance protein	PGN_1984	hypothetical protein	PGN_1794	hypothetical protein (glycerate kinase)	PGN_1259	histidinol-phosphate aminotransferase
PGN_0216	hypothetical protein	PGN_1216	transposase in ISPg1	PGN_1337	hypothetical protein	PGN_t0049	tRNA-Ala
PGN_0545	sulfatase	PGN_0153	hypothetical protein	PGN_1957	transposase in ISPg1	PGN_t0014	tRNA-Ala
PGN_0113	hypothetical protein	PGN_0195	xanthine phosphoribosyltransferase	PGN_0260	hypothetical protein	PGN_0959	transcriptional regulator
PGN_0220	partial transposase in ISPg1	PGN_1511	hemolysin	PGN_0971	transposase in ISPg1	PGN_1929	hypothetical protein
PGN_0148	hypothetical protein	PGN_0058	conjugate transposon protein	PGN_1899	hypothetical protein (TPR domain protein)	murQ	N-acetylmuramic acid-6-phosphate etherase
PGN_0966	partial transposase in ISPg1	PGN_1886	NAD dependent epimerase	PGN_1831	ribosome-associated GTPase	PGN_1950	hypothetical protein

PGN_1077	transposase in ISPg1	PGN_1455	hypothetical protein	PGN_1309	ferrous iron transport protein B	PGN_0777	glycosyl transferase
PGN_1456	hypothetical protein	PGN_0490	DNA-damage-inducible protein F	PGN_0858	ABC transporter ATP-binding protein	PGN_0317	decarboxylating precorrin-6Y C5,15-methyltransferase
PGN_1706	phosphoribosylglycinamide formyltransferase	PGN_1241	hypothetical protein (glycosyl transferase family protein)	PGN_t0028	tRNA-Arg	PGN_1276	transposase in ISPg1
PGN_0807	DNAse related protein	PGN_2009	hypothetical protein	PGN_1110	partial transposase in ISPg1	trmE	tRNA modification GTPase TrmE
PGN_0810	hypothetical protein	PGN_1568	hypothetical protein	PGN_0832	gliding motility protein SprA	PGN_1724	glycosyl transferase group 2 family protein
PGN_2030	hypothetical protein (membrane protein)	PGN_1645	dipeptidyl-peptidase III	PGN_1300	transcriptional regulator	rnhB	ribonuclease HII
PGN_1044	alpha-amylase	PGN_0967	partial transposase in ISPg1	PGN_1734	nucleoside permease NupG	PGN_0406	alpha-1,2-mannosidase family protein
PGN_1302	hypothetical protein (O-antigen polymerase)	PGN_1050	M24 family peptidase	PGN_0145	hypothetical protein	PGN_0089	Hypothetical protein (DNA-binding helix-turn-helix protein)
PGN_0797	hypothetical protein	PGN_1025	hypothetical protein	PGN_1668	glycosyl transferase group 2 family protein	PGN_1161	transposase in ISPg1
PGN_1583	hypothetical protein	PGN_1383	hypothetical protein (DNA alkylation repair protein)	PGN_0587	transposase in ISPg1	PGN_1213	ATP-binding protein
PGN_0104	transposase in ISPg1	PGN_0379	hypothetical protein	traM	conjugate transposon protein TraM	PGN_1365	hypothetical protein
PGN_1471	hypothetical protein	PGN_0240	ferrochelatase	PGN_1539	ABC transport system exported protein	PGN_t0052	tRNA-Gly
PGN_1798	UbiE/COQ5 family methlytransferase	PGN_0372	hypothetical protein	PGN_0702	hypothetical protein	PGN_0700	oxidoreductase Gfo/Idh/MocA family
traK	conjugal transfer protein TraA	PGN_1822	hypothetical protein	PGN_t0053	tRNA-Cys	PGN_0716	ABC transporter permease
PGN_0242	glycosyl transferase family 1	PGN_1039	alpha-1,2-mannosidase	PGN_1636	hypothetical protein	PGN_1723	hypothetical protein
PGN_0602	transposase in ISPg1	PGN_1312	transcriptional regulator	PGN_1131	hydrolase	PGN_0007	hypothetical protein
PGN_0036	hypothetical protein (transposase family protein)	PGN_0070	hypothetical protein	gmk	guanylate kinase	PGN_1207	transport multidrug efflux protein
PGN_0956	hypothetical protein	PGN_1258	cobalamin biosynthesis protein	PGN_t0022	tRNA-Gly	PGN_1797	hypothetical protein
PGN_1061	hypothetical protein	PGN_0267	riboflavin biosynthesis protein	PGN_1815	hypothetical protein(selenium metabolism protein YedF)	PGN_1325	ABC transporter membrane protein
PGN_1086	hypothetical protein	PGN_0045	hypothetical protein	PGN_0973	hypothetical protein (outer membrane protein)	PGN_t0034	tRNA-Pro
PGN_t0046	tRNA-Asp	PGN_1211	hypothetical protein	PGN_1967	sulfatase	lacZI	beta-galactosidase
PGN_t0018	tRNA-Leu	PGN_0212	transposase in ISPg1	PGN_2050	ATP-dependent helicase	PGN_0285	pyridine nucleotide-disulphide oxidoreductase
PGN_1897	transport related membrane protein	PGN_1956	putative DNA methylase	PGN_1838	partial transposase in ISPg1	PGN_2090	hypothetical protein
PGN_1378	replicative DNA helicase	PGN_1389	acetyltransferase	PGN_1215	hypothetical protein	PGN_1952	hypothetical protein
PGN_1198	sodium-solute transporter	PGN_0435	partial hemagglutinin-related protein	PGN_1896	sugar transferase	PGN_0770	ribonuclease Z
PGN_0707	iron ABC transporter permease	PGN_1629	hypothetical protein	traG	conjugate transposon protein TraG	PGN_1009	calcium-transporting ATPase
PGN_1708	magnesium chelatase subunit ChlI	PGN_1487	dephospho-CoA kinase	PGN_0425	partial transposase in ISPg3	PGN_1821	putative integrin subunit alpha
PGN_1421	hypothetical protein	PGN_1474	S-ribosylhomocysteinase	PGN_0798	hypothetical protein	PGN_1747	hypothetical protein
PGN_0332	hypothetical protein	PGN_0855	hypothetical protein	PGN_t0038	tRNA-Leu	PGN_0194	hypothetical protein
PGN_1538	cation efflux system	PGN_1528	NOL1/NOP2/sun family protein	PGN_1145	hypothetical protein	PGN_1074	DNA methyltransferase
PGN_1033	polysaccharide transport protein	PGN_1639	hypothetical protein (cupin domain-containing protein)	PGN_1185	acetyltransferase	PGN_1195	hypothetical protein
PGN_2066	ABC transporter ATP-binding protein	PGN_2001	sensor histidine kinase	PGN_1690	exported fucosidase	PGN_0633	yadS protein
PGN_2028	hypothetical protein	PGN_0575	transposase in ISPg1	PGN_0686	iron ABC transporter permease	PGN_0859	ABC transporter permease protein
PGN_0644	transposase in ISPg1	PGN_1087	hypothetical protein	PGN_1766	hypothetical protein	PGN_0519	competence protein
PGN_0325	hypothetical protein	PGN_0986	hypothetical protein	PGN_1584	potassium/proton antiporter	PGN_1295	hypothetical protein
PGN_0870	rod shape-determining protein RodA	PGN_0162	hypothetical protein	PGN_1765	hypothetical protein	prtQ	PrtQ, protease
PGN_1438	hypothetical protein	PGN_1790	hypothetical protein	PGN_1661	hypothetical protein	PGN_1939	D-tyrosyl-tRNA(Tyr) deacylase
PGN_0030	beta-mannosidase	PGN_1481	polysaccharide biosynthesis protein	PGN_0617	hypothetical protein	PGN_0689	hypothetical protein

PGN_1214	RHS repeat-associated core domain protein	PGN_1222	hypothetical protein	PGN_1499	hypothetical protein	PGN_1719	Appr-1-p processing protein
PGN_0708	iron ABC transporter ATP-binding protein	PGN_0074	hypothetical protein	PGN_1332	para-aminobenzoate synthase component I	PGN_1194	bifunctional UDP-N-acetylmuramoyl-tripeptide:D-alanyl-D-alanine ligase/alanine racemase
PGN_1322	hypothetical protein	PGN_0130	partial transposase in ISPg1	PGN_0311	hypothetical protein	PGN_1731	hypothetical protein
PGN_0681	hypothetical protein	PGN_1475	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	PGN_1653	thiamine biosynthesis lipoprotein ApbE	PGN_1462	hypothetical protein
PGN_0718	ABC transporter permease protein	PGN_1923	hypothetical protein	PGN_1535	hypothetical protein	PGN_1459	hypothetical protein
PGN_1029	GtrA family protein	PGN_1949	hypothetical protein	PGN_0005	hypothetical protein	PGN_1279	partial transposase in ISPg3
PGN_0878	hypothetical protein	PGN_0940	partial transposase in ISPg2	lacZII	beta-galactosidase	PGN_0111	partial transposase in ISPg6
PGN_0512	biotin--acetyl-CoA-carboxylase ligase	PGN_0980	alpha-1,2-mannosidase	PGN_1227	TPR domain protein	PGN_1351	TetR family transcriptional regulator
PGN_0877	SNF2-related helicase	PGN_1386	conjugative transposon TraJ protein	PGN_0849	hypothetical protein	PGN_0786	hypothetical protein
PGN_1928	CRISPR-associated Cmr5 family protein	PGN_1190	RNA methyltransferase	PGN_0506	hypothetical protein	PGN_0013	two-component system sensor histidine kinase
PGN_1681	ABC transporter ATP-binding protein	PGN_0307	TatD family protein	PGN_0908	hypothetical protein	PGN_0032	hypothetical protein
PGN_1083	hypothetical protein	PGN_0003	hypothetical protein	PGN_0703	hypothetical protein	PGN_1679	outer membrane protein
PGN_2011	helicase	PGN_0006	Na+driven multidrug efflux pump	PGN_1801	putative endonuclease	PGN_1192	DNA-binding protein histone-like family
PGN_0750	copper homeostasis protein CutC	PGN_1072	glycosyl transferase family 2	PGN_1709	hypothetical protein	PGN_0205	AraC family transcriptional regulator
PGN_1898	transport protein	PGN_1310	glycogen synthase	PGN_1100	capsule biosynthesis protein CapA	PGN_1983	ion transporter
PGN_1101	hypothetical protein	PGN_1944	hypothetical protein	PGN_1725	polysaccharide deacetylase	PGN_1013	putative Fe-S oxidoreductase
PGN_0270	amidophosphoribosyl-transferase	PGN_0314	formate/nitrite transporter	PGN_1404	hypothetical protein	PGN_0869	penicillin-binding protein 2
PGN_1985	N-acetylmuramoyl-L-alanine amidase	PGN_0699	hypothetical protein	PGN_1559	hypothetical protein	PGN_1267	SerB family protein
PGN_0327	DNA polymerase III epsilon chain	PGN_1008	partial transposase in ISPg1	PGN_0230	serine acetyltransferase	PGN_1084	hypothetical protein
PGN_0844	hypothetical protein	PGN_t0016	tRNA-Arg	PGN_0651	hypothetical protein	PGN_1915	conjugative transposon TraJ protein
PGN_1931	CRISPR-associated Cmr3 family protein	PGN_0554	hypothetical protein	PGN_0436	partial hemagglutinin-related protein	traA	conjugal transfer protein TraA
PGN_t0020	tRNA-Pro	PGN_0147	putative lipoprotein	PGN_0071	PF11888 domain protein	PGN_0979	hypothetical protein
PGN_1483	hypothetical protein	PGN_1608	sialidase	PGN_0776	hypothetical protein	PGN_0556	cobalamin biosynthesis-related protein
PGN_0478	partial transposase in ISPg4	PGN_0910	hypothetical protein	PGN_0719	ABC transporter permease protein;	PGN_t0015	tRNA-His
PGN_1076	DNA methylase	PGN_0579	hypothetical protein	PGN_0132	hypothetical protein	PGN_1427	hypothetical protein
PGN_0961	hypothetical protein	PGN_1919	hypothetical protein	PGN_1307	hypothetical protein	PGN_0926	hypothetical protein
PGN_1720	hypothetical protein	PGN_0076	mobilization protein TraG family	PGN_0286	pyridine nucleotide-disulfide oxidoreductase	PGN_1540	ABC transport membrane protein
PGN_0981	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	PGN_0848	hypothetical protein	PGN_0606	glucosamine-6-phosphate deaminase-like protein	PGN_1289	hypothetical protein
PGN_1463	phosphoribose diphosphate:decaprenyl-phosphate phosphoribosyltransferase	PGN_0720	ABC transporter permease protein	PGN_1440	vancomycin B-type resistance protein VanW	PGN_0352	hypothetical protein
PGN_0052	hypothetical protein	PGN_0061	putative conjugative transposon protein	PGN_0339	hypothetical protein	PGN_1494	oxygen-independent coproporphyrinogen III oxidase
truA	tRNA pseudouridine synthase A	PGN_1290	hypothetical protein	PGN_0107	partial transposase in ISPg3	PGN_0835	hypothetical protein
PGN_1932	CRISPR-associated Csm1 family protein	PGN_0943	alginate O-acetyltransferase	PGN_0677	multi antimicrobial extrusion protein MatE	PGN_0430	ABC transporter ATP-binding protein
PGN_0539	metallo-beta-lactamase superfamily protein	PGN_1925	CRISPR-associated Cas1 family protein	PGN_1248	hypothetical protein	PGN_0745	hypothetical protein
PGN_0164	hypothetical protein	PGN_0340	carboxyl-terminal processing protease	PGN_0479	hypothetical protein	PGN_1133	hypothetical protein
PGN_1738	long-chain-fatty-acid-CoA ligase	PGN_0542	partial transposase in ISPg2	PGN_0596	conjugate transposon protein	PGN_0098	hypothetical protein
PGN_0951	conjugative transposon TraJ protein	PGN_0553	hypothetical protein	PGN_1464	HAD-superfamily subfamily IB hydrolase	PGN_0222	conjugative transposon TraJ protein

PGN_0166	hypothetical protein (transposase)	PGN_1073	hypothetical protein	PGN_1730	hypothetical protein	porS	membrane protein PorS
PGN_1940	MazG nucleotide pyrophosphohydrolase	PGN_0771	NLP/P60 family protein	PGN_1665	hypothetical protein	PGN_1147	hypothetical protein
PGN_0014	hypothetical protein	PGN_1070	hypothetical protein	xseA	exodeoxyribonuclease VII large subunit	PGN_1824	putative integrin subunit alpha
PGN_0582	DNA topoisomerase I	PGN_1071	methylthioribose kinase	PGN_0308	hypothetical protein	PGN_0018	hypothetical protein
PGN_1527	tetrapyrrole methylase	PGN_0146	hypothetical protein	PGN_0431	hypothetical protein	PGN_0863	DNA methylase N-4/N-6
PGN_0046	hypothetical protein	PGN_1250	hypothetical protein	PGN_1682	ABC transporter permease protein	PGN_1387	ABC transporter permease protein;
PGN_0342	uracil-DNA glycosylase	PGN_1680	ABC transporter permease protein	PGN_1495	low-specificity L-threonine aldolase	PGN_1336	putative lipoprotein
PGN_0151	hypothetical protein	PGN_0923	DNA primase	PGN_0469	biotin synthesis protein	PGN_t0023	tRNA-Asp
PGN_1030	hypothetical protein	PGN_1201	tRNA (adenine-N6)-methyltransferase	PGN_0536	hypothetical protein	PGN_0338	hypothetical protein
PGN_0862	Type III restriction enzyme, res subunit	PGN_0845	hypothetical protein	PGN_1907	hypothetical protein	PGN_0480	partial transposase in ISPg4
PGN_0086	DNA methylase	PGN_1953	TonB-dependent outer membrane receptor	PGN_t0005	tRNA-Val	PGN_1534	hypothetical protein
PGN_0417	hypothetical protein	PGN_1683	ABC transporter permease protein	PGN_0565	hypothetical protein	PGN_0822	hypothetical protein
PGN_1223	uracil permease	PGN_0922	Virulence-associated protein E	PGN_0924	mobilization protein	PGN_1306	hypothetical protein
PGN_0051	hypothetical protein	PGN_1082	hypothetical protein	PGN_1920	hypothetical protein	PGN_1064	transposase in ISPg3
PGN_0584	topoisomerase	PGN_1473	hypothetical protein	PGN_0600	conjugal transfer protein TraG	PGN_t0002	tRNA-Asn
PGN_0581	topoisomerase	PGN_t0041	tRNA-Ser	traQ	conjugate transposon protein TraQ	PGN_0538	hypothetical protein
PGN_0362	hypothetical protein	PGN_1046	DNA repair protein	PGN_0787	hypothetical protein	PGN_1144	hypothetical protein
PGN_1234	hypothetical protein	PGN_2084	TraJ family protein conjugative transpos	PGN_1918	hypothetical protein	PGN_0048	PcfK-like protein
PGN_0852	immunoreactive 47 kDa antigen PG97	PGN_2063	hypothetical protein	PGN_0588	hypothetical protein	PGN_0555	hypothetical protein
PGN_0050	hypothetical protein	PGN_1461	spore maturation protein A/B	PGN_0105	hypothetical protein	PGN_0957	hypothetical protein
PGN_1394	transposase in ISPg2	PGN_1292	anti-restriction protein	PGN_0946	hypothetical protein	PGN_1068	oxidoreductase domain protein
PGN_0152	immunoreactive 61 kDa antigen PG91	traO	conjugate transposon protein TraO	PGN_1916	ABC transporter ATP-binding protein	PGN_1609	hypothetical protein
PGN_0839	transposase in ISPg2	PGN_1017	hypothetical protein	PGN_0075	relaxase/mobilization nuclease domain p	PGN_1662	partial transposase in ISPg3
PGN_1924	CRISPR-associated Cas2 family protein	PGN_1257	hypothetical protein	PGN_0652	hypothetical protein	PGN_0402	hypothetical protein
PGN_0851	hypothetical protein	PGN_1707	putative lipoprotein	PGN_1478	hypothetical protein	PGN_0442	transposase in ISPg3
PGN_0513	hypothetical protein	PGN_0559	hypothetical protein	PGN_1075	ISPg2, transposase	PGN_0949	ABC transporter ATP-binding protein
PGN_0947	hypothetical protein	PGN_1291	conserved hypothetical protein related to phage	PGN_0028	calcium-transporting ATPase	PGN_1333	para-aminobenzoate synthase component I
PGN_0383	transporter	PGN_1069	hypothetical protein	PGN_0306	hypothetical protein	PGN_0953	transposase in ISPg3
PGN_1825	hypothetical protein	PGN_1217	hydroxylamine reductase	PGN_0958	hypothetical protein	hmuR	TonB-dependent receptor HmuR
PGN_1823	hypothetical protein	PGN_0455	partial transposase Orf1 in ISPg5	PGN_1476	hypothetical protein	PGN_1371	hypothetical protein
PGN_1353	DNA alkylation repair enzyme	PGN_0460	DNA-binding protein histone-like family	PGN_1059	conjugative transposon TraJ protein	PGN_0601	hypothetical protein
PGN_1089	methyltransferase	PGN_1067	hypothetical protein	PGN_0978	hypothetical protein	PGN_0072	hypothetical protein
PGN_1277	hypothetical protein	PGN_1368	hypothetical protein	PGN_1726	transposase in ISPg3	PGN_1917	ABC transporter ATP-binding protein
PGN_1624	hypothetical protein	PGN_1160	transposase in ISPg2	PGN_0441	htpG; heat shock protein 90	PGN_r0006	5S ribosomal RNA
PGN_0825	hypothetical protein	PGN_0950	ABC transporter ATP-binding protein	PGN_t0008	tRNA-Pro	PGN_2036	hypothetical protein
PGN_1293	PcfK-like protein	PGN_1927	CRISPR-associated Cas1 family protein	PGN_0899	hypothetical protein	PGN_1748	cytochrome c biogenesis protein CcsA
PGN_0909	transposase in ISPg3	PGN_1922	transposase in ISPg3	PGN_1921	transcriptional regulator	PGN_0796	hypothetical protein
PGN_1065	transposase in ISPg3	porR	porP protein	PGN_1109	hypothetical protein	PGN_0363	hypothetical protein
PGN_1490	precorrin-2 C20-methyltransferase	PGN_1060	transposase in ISPg3	PGN_0856	A/G-specific adenine glycosylase	PGN_0552	hypothetical protein
PGN_0948	hypothetical protein	PGN_0954	partial transposase in ISPg6	PGN_0920	partial excisionase	PGN_t0017	tRNA-Ser
PGN_0049	anti-restriction protein	PGN_1684	hypothetical protein	PGN_t0004	tRNA-Val	PGN_1265	hypothetical protein

PGN_0454	transposase in ISPg3	PGN_1335	hypothetical protein	PGN_1006	transposase in ISPg3	PGN_1228	hypothetical protein
PGN_1911	transposase in ISPg3	PGN_t0024	tRNA-Phe	PGN_1063	partial transposase Orf2 in ISPg5	PGN_0846	hypothetical protein
PGN_1592	hypothetical protein	PGN_0879	transposase in ISPg3	PGN_0843	hypothetical protein	PGN_t0045	tRNA-Lys
PGN_0213	hypothetical protein	PGN_0459	transposase in ISPg3	PGN_1334	hypothetical protein	PGN_0578	conjugate transposon protein
PGN_0790	transposase in ISPg3	PGN_0106	partial transposase in ISPg3	PGN_0305	hypothetical protein	PGN_1294	hypothetical protein
PGN_0586	hypothetical protein	PGN_1826	hypothetical protein	PGN_0945	TetR family transcriptional regulator	PGN_r0012	5S ribosomal RNA
PGN_t0027	tRNA-Met	PGN_1729	acetyltransferase	PGN_0401	hypothetical protein	PGN_0921	hypothetical protein
PGN_0326	DNA-binding protein histone-like family	PGN_2059	hypothetical protein	PGN_0850	hypothetical protein	PGN_1237	hypothetical protein
PGN_0934	transposase in ISPg3	PGN_0925	mobilization protein	PGN_t0025	tRNA-Leu	PGN_1477	hypothetical protein
PGN_1066	transposase in ISPg3	PGN_0847	hypothetical protein	PGN_0019	hypothetical protein	PGN_t0006	tRNA-Met
PGN_0955	transposase in ISPg3	PGN_0475	hypothetical protein	PGN_t0007	tRNA-Met	PGN_0364	hypothetical protein
PGN_0558	transposase in ISPg3	PGN_0337	hypothetical protein	PGN_0474	hypothetical protein	PGN_1778	hypothetical protein
PGN_1278	partial transposase in ISPg3	PGN_1428	transposase in ISPg3	PGN_0574	hypothetical protein	PGN_t0019	tRNA-Arg
PGN_0576	hypothetical protein	PGN_1913	transposase in ISPg3	PGN_1414	hypothetical protein	PGN_0551	hypothetical protein
PGN_0944	transposase in ISPg3	PGN_1560	hypothetical protein	PGN_1266	hypothetical protein	PGN_0853	hypothetical protein
PGN_0585	transposase in ISPg3	PGN_1912	partial transposase in ISPg6	PGN_r0007	5S ribosomal RNA	PGN_0821	hypothetical protein
PGN_1836	transposase in ISPg3	PGN_0304	hypothetical protein	PGN_r0003	5S ribosomal RNA	PGN_0047	hypothetical protein
PGN_0864	transposase in ISPg3	PGN_0589	hypothetical protein				

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

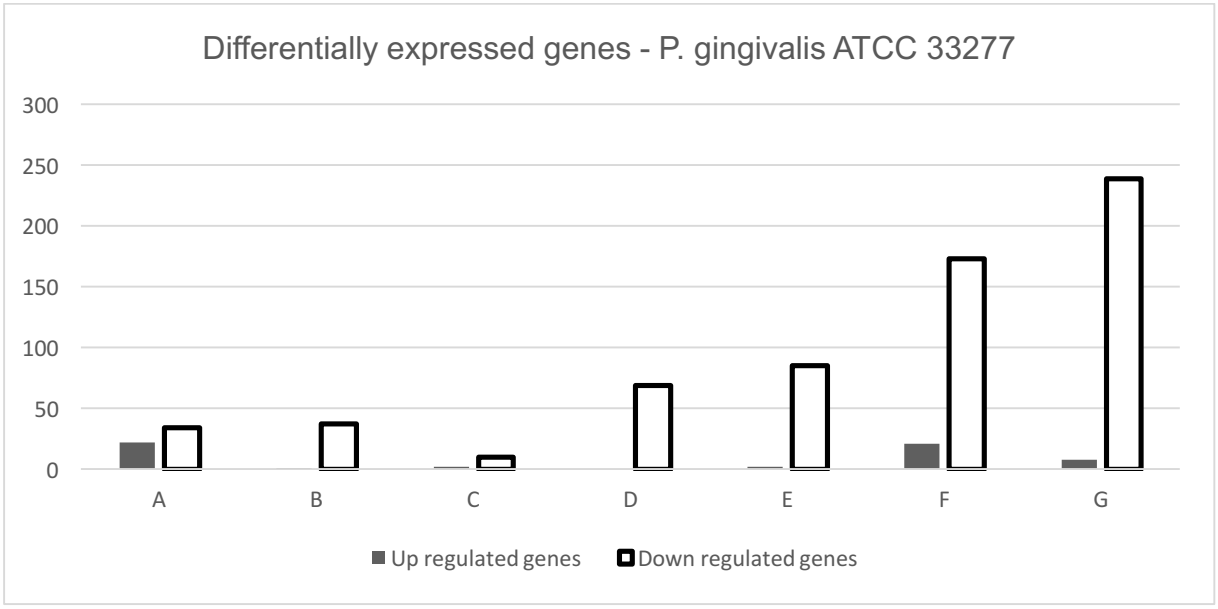

Figure 5: *P. gingivalis* ATCC 33277 differentially expressed genes in CSE

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, biosyntehsis 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).

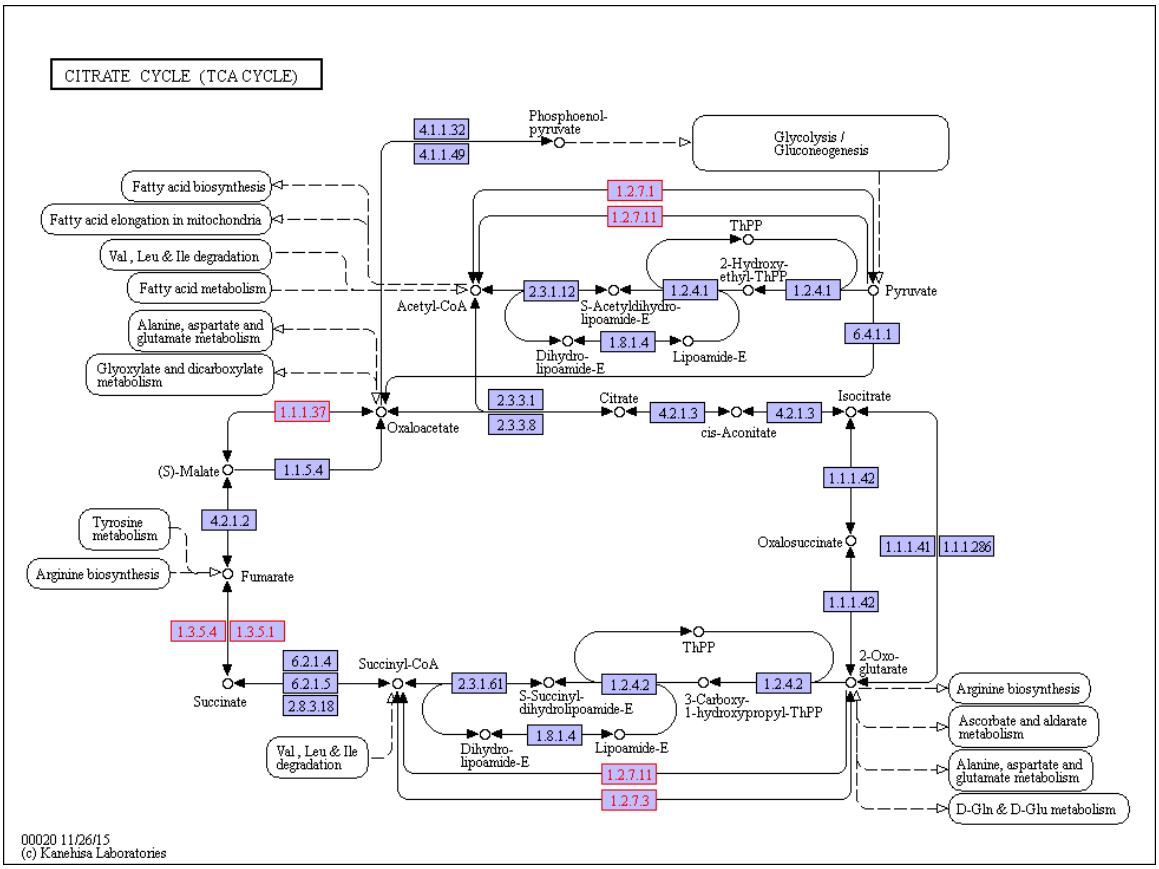

Figure 6: Citrate cycle pathway of *P. gingivalis*

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

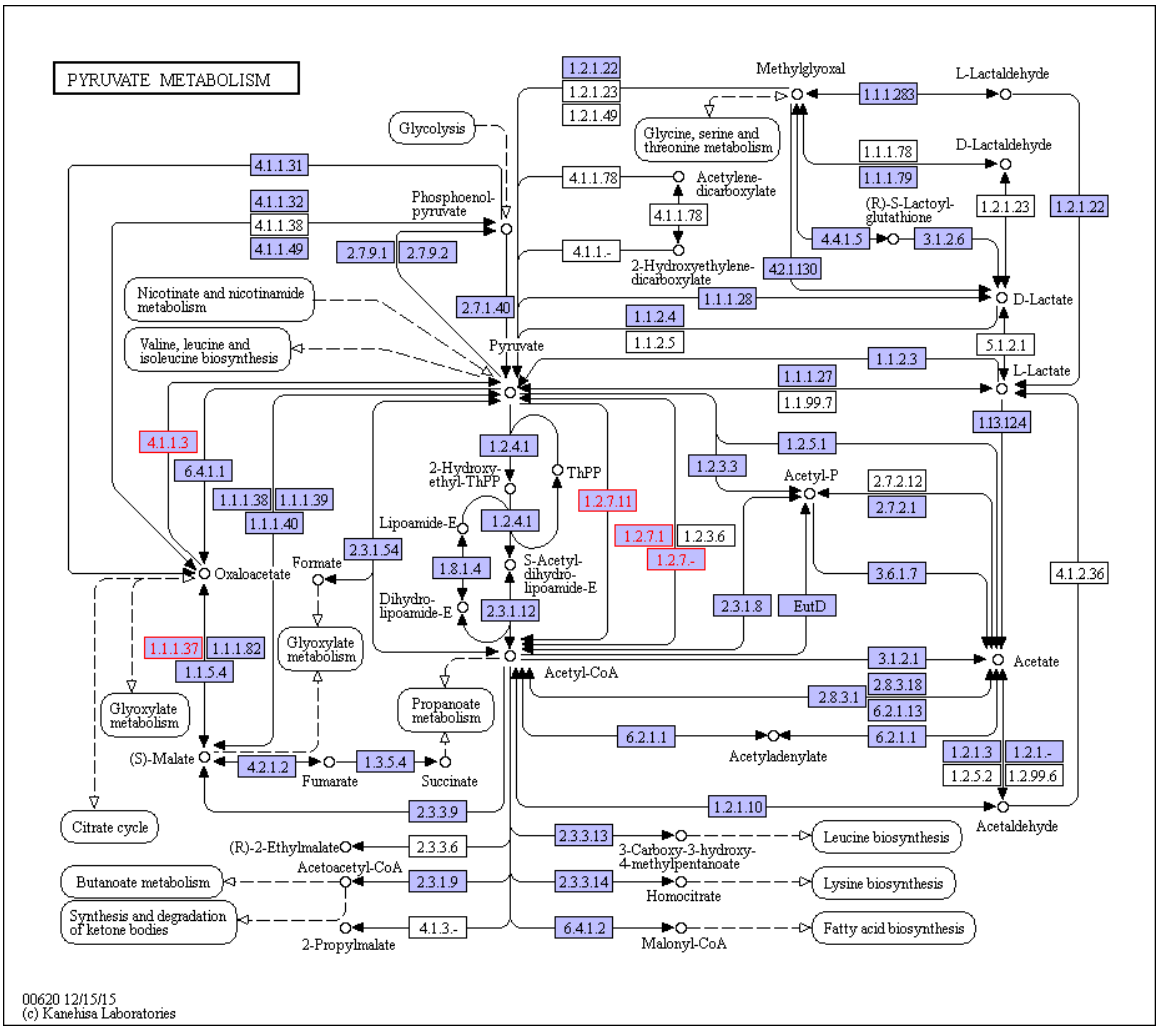

Figure 7: Pyruvate metabolism pathway of *P. gingivalis*

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

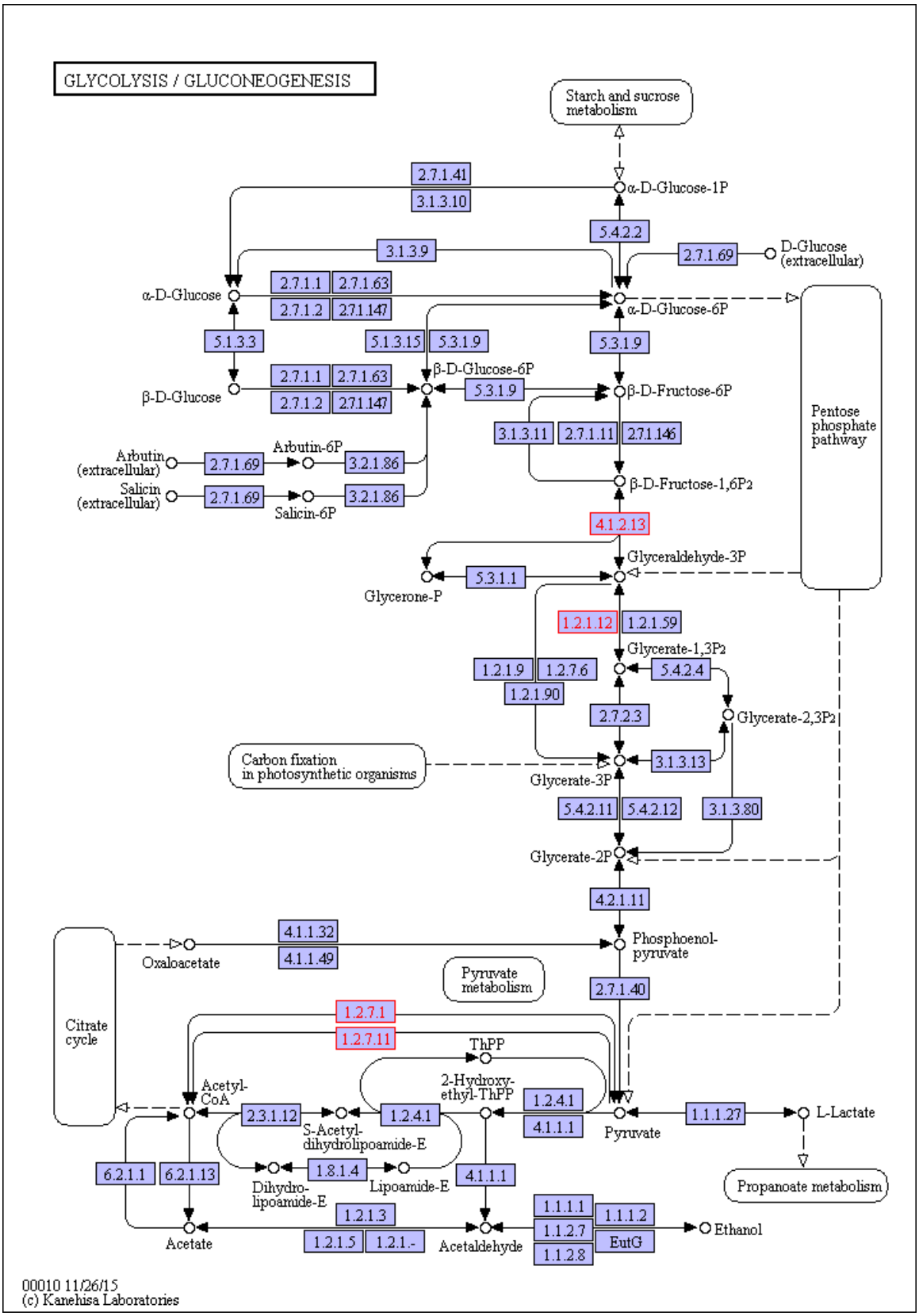

Figure 8: Glycolysis pathway of *P. gingivalis*

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

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

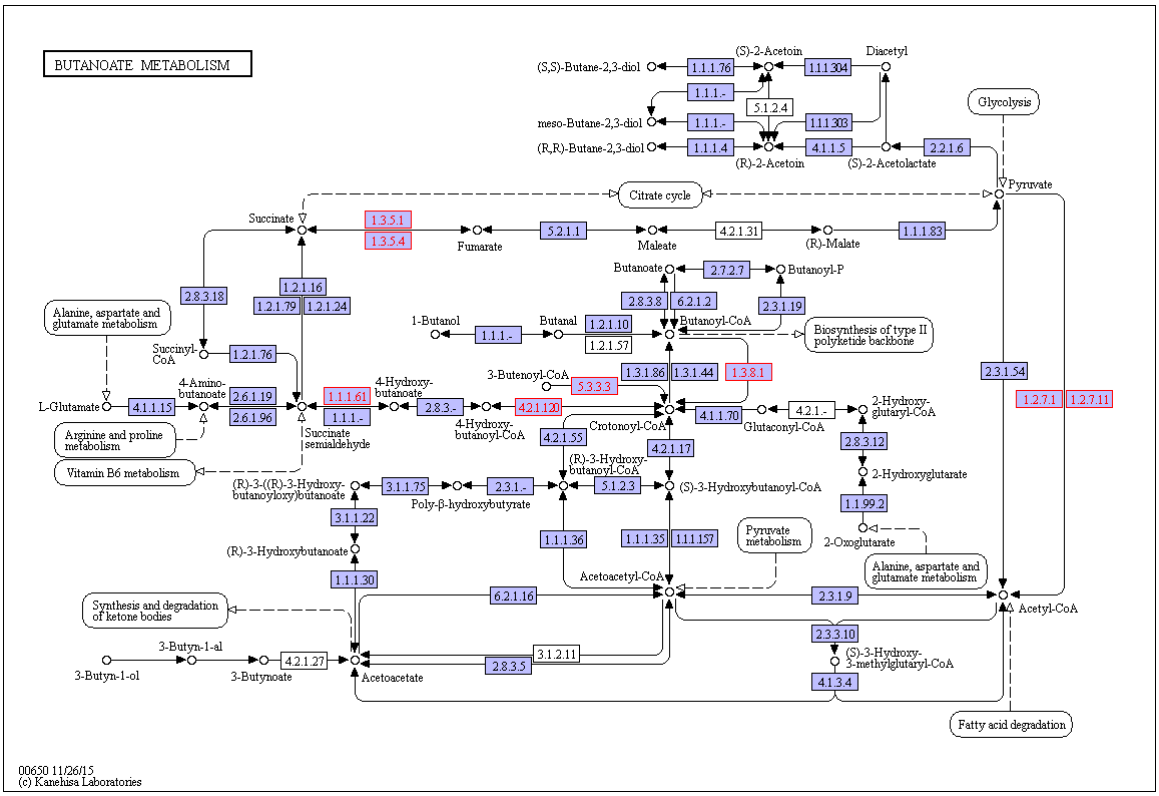

Figure 9: Butanoate metabolism pathway of *P. gingivalis*

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

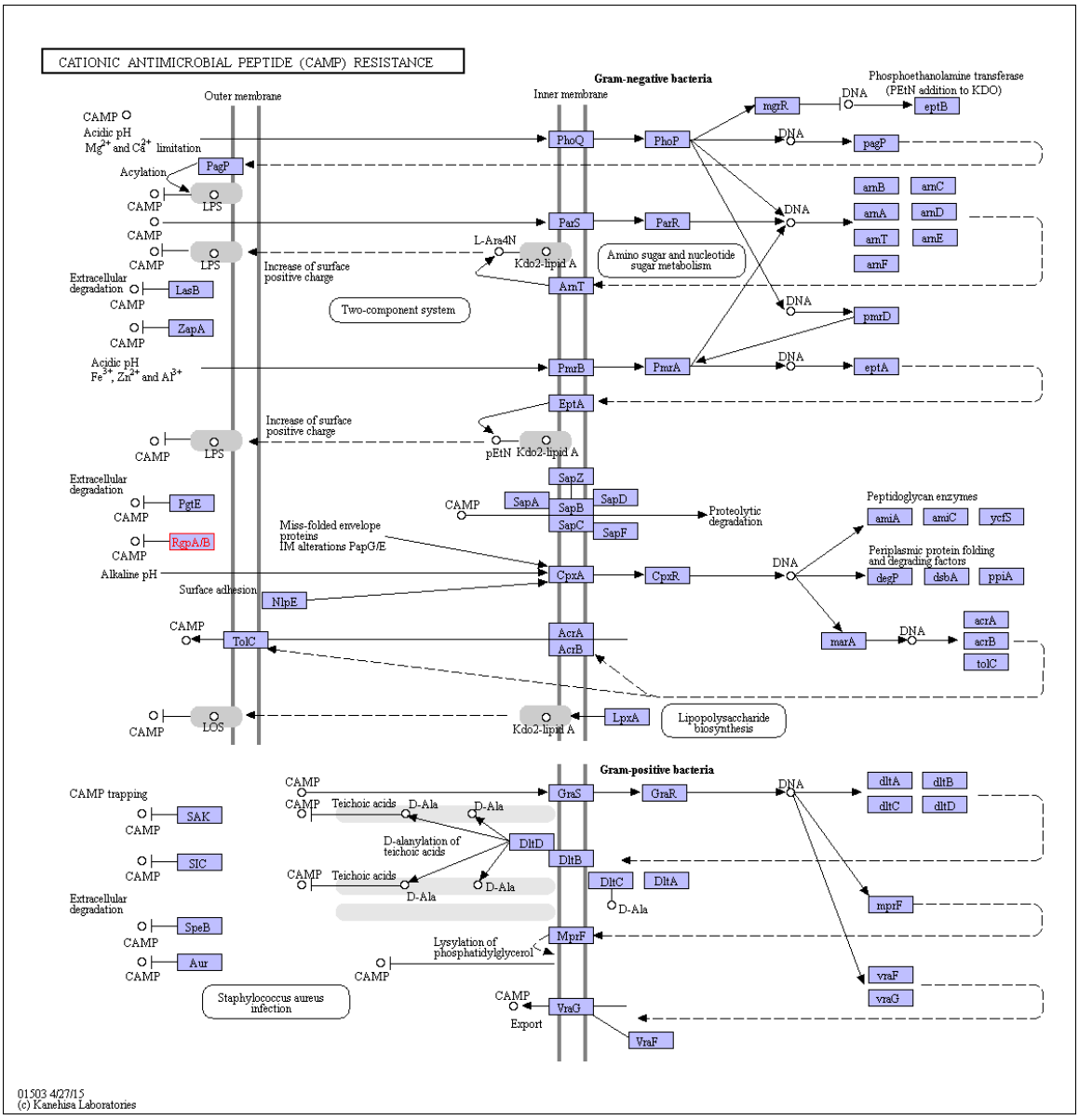

Figure 10: CAMP resistance pathway of *P. gingivalis*

Generated using KEGG orthology system and red box shows differentially regulated *P. gingivalis* genes RgpA and RgpB when exposed to CSE.

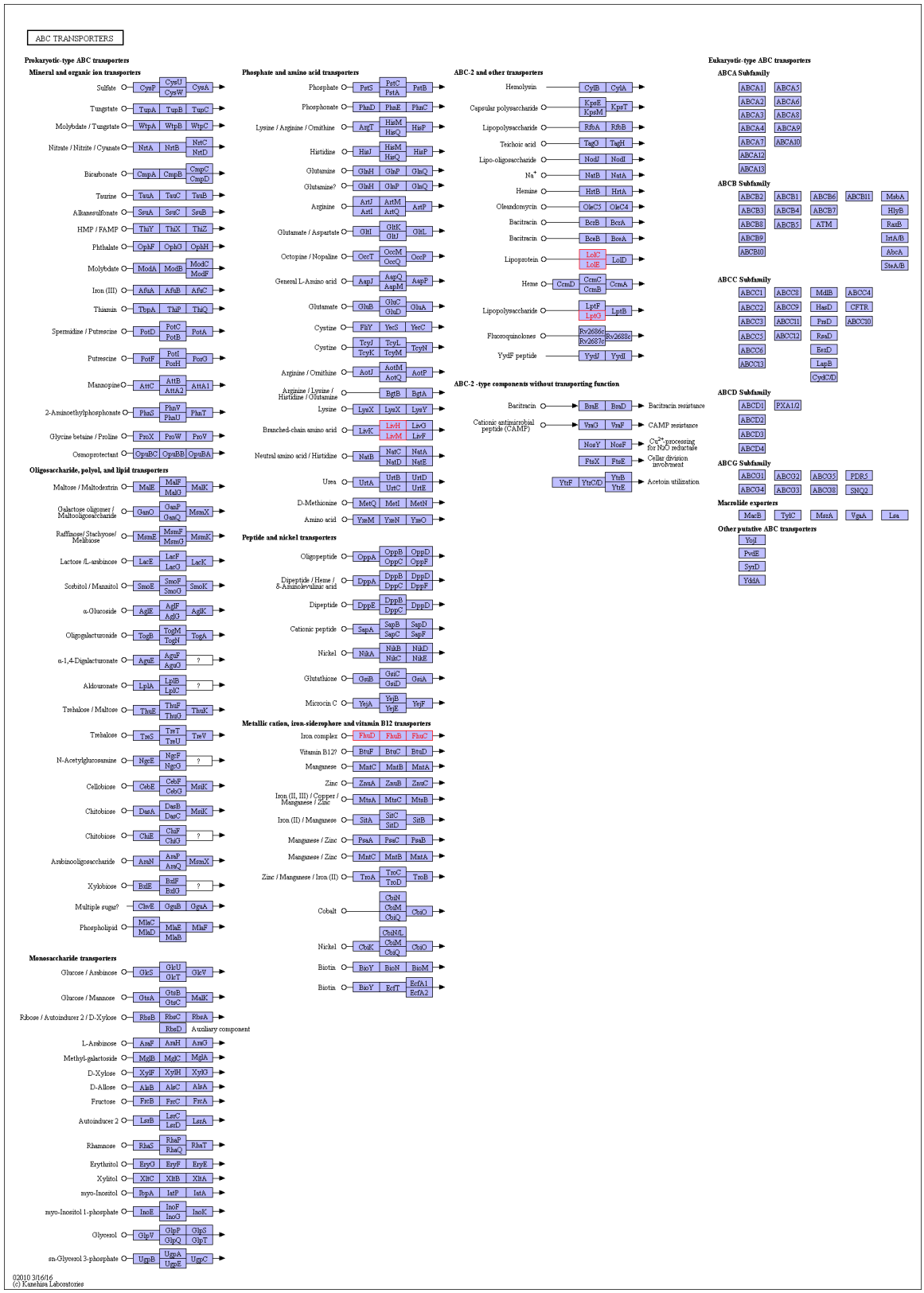

Figure 11: ABC transporter pathway of *P. gingivalis*

Generated using KEGG orthology system and red boxes show differentially regulated *P. gingivalis* ABC transporter genes when exposed to CSE.

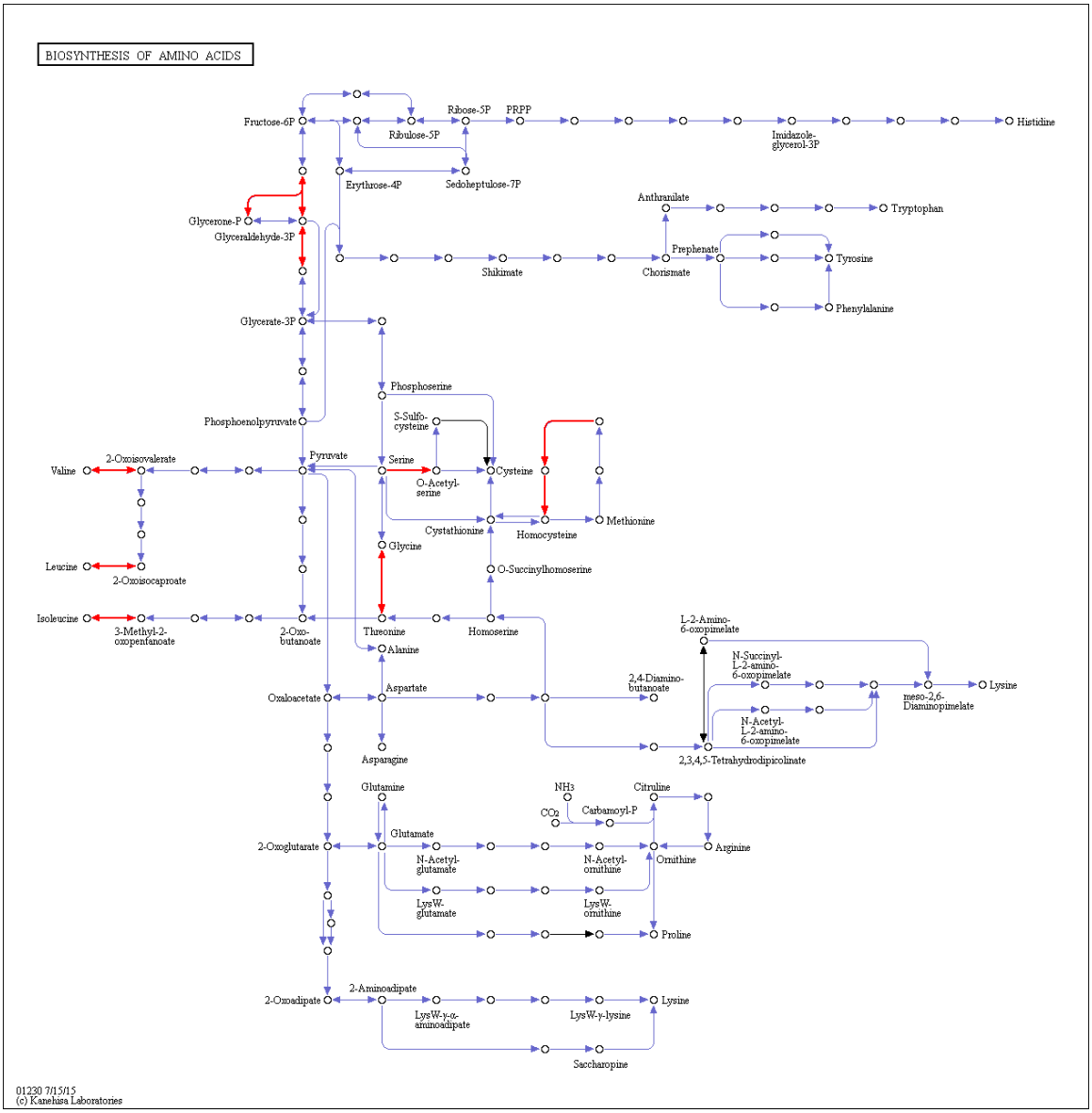

Figure 12: Biosynthesis of amino acids pathway of *P. gingivalis*

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

Gene ID	Gene name	Gene ID	Gene name	Gene ID	Gene name
HMPREF0389_00155	DNA replication protein DnaC	HMPREF0389_01736	rpmJ; psM; Ribosomal protein L36	HMPREF0389_01728	hypothetical; Copper amine oxidase domain protein
HMPREF0389_00186	NlpC/P60 family protein	HMPREF0389_01591	ABC transporter permease	HMPREF0389_01281	ABC transporter ATP-binding protein

HMPREF0389_00184	type IV conjugative transfer system protein TraE	HMPREF0389_01302	V-type ATP synthase beta chain 2	HMPREF0389_00412	hypothetical
HMPREF0389_00178	TraG family protein	HMPREF0389_00820	DNA-directed RNA polymerase subunit alpha	HMPREF0389_01273	46unctional46l membrane protein
HMPREF0389_00166	TnpX site-specific recombinase	HMPREF0389_00825	map; Methionine aminopeptidase	HMPREF0389_01581	hlyD; hemolysin D
HMPREF0389_00246	antirestriction protein (ArdA)	HMPREF0389_00826	adk; adenylate kinase	HMPREF0389_00830	rpsE: 30S ribosomal protein S5
HMPREF0389_00154	replication initiator protein	HMPREF0389_00896	zinc ABC transporter permease	HMPREF0389_00415	fimbrial assembly protein PilN
HMPREF0389_00969	nitrite transporter NirC	HMPREF0389_00414	hypothetical	HMPREF0389_03101	23S ribosomal RNA
HMPREF0389_01685	hypothetical	HMPREF0389_01102	transcriptional regulator, TetR	HMPREF0389_00831	50S ribosomal protein L18
HMPREF0389_00162	conjugation protein	HMPREF0389_01448	calcium-binding acidic-repeat protein	HMPREF0389_01537	hypothetical
HMPREF0389_01096	MATE efflux family protein	HMPREF0389_01590	Transcriptional regulator, AraC	HMPREF0389_03107	23S ribosomal RNA
HMPREF0389_00644	FtsK/SpoIIIE family protein	HMPREF0389_00882	ribH; 6,7-dimethyl-8-ribityllumazine synthase	HMPREF0389_00413	hypothetical
HMPREF0389_01592	ABC transporter permease, ATP-binding protein	HMPREF0389_00881	ribBA; 3,4-dihydroxy-2-butanone 4-phosphate synthase	HMPREF0389_00416	pilM; type IV pilus assembly protein
HMPREF0389_00823	Translation initiation factor IF-1	HMPREF0389_00827	preprotein translocase subunit SecY	HMPREF0389_00505	O-methyltransferase family protein
HMPREF0389_00879	riboflavin biosynthesis protein RibD	HMPREF0389_00819	rplQ; 50S ribosomal protein L17	HMPREF0389_01169	CRISPR-associated protein, Csd1 family
HMPREF0389_00824	hypothetical protein	HMPREF0389_00895	zinc ABC transporter ATP-binding protein	HMPREF0389_01190	efflux ABC transporter permease
HMPREF0389_01079	Iron-sulfur cluster-binding protein	HMPREF0389_01191	ABC transporter ATP-binding protein	HMPREF0389_00883	HAD-superfamily hydrolase (46unctional partner with ribBA and ribH)
HMPREF0389_01303	V/A-type H+-transporting ATPase subunit D	HMPREF0389_00828	rplO; 50S ribosomal protein L15	HMPREF0389_03104	23S ribosomal RNA
HMPREF0389_00897	Environmental sensor; manganese/zinc/iron transport system permease protein	HMPREF0389_03110	23S ribosomal RNA	HMPREF0389_01135	hypothetical
HMPREF0389_01744	hypothetical	HMPREF0389_00829	rpmD; 50S ribosomal protein L30	HMPREF0389_00928	fabZ; beta-hydroxyacyl-(acyl-carrier-protein) dehydratase
HMPREF0389_00643	transcriptional regulator	HMPREF0389_01138	GTP binding protein	HMPREF0389_00473	pyruvate carboxylase
HMPREF0389_00822	rpsM; 30S ribosomal protein S13	HMPREF0389_01110	prtA: cell wall serine protease	HMPREF0389_01189	RND family efflux transporter MFP subunit
HMPREF0389_00898	GNAT family acetyltransferase	hypothetical		HMPREF0389_01137	thiH; thiazole biosynthesis protein
HMPREF0389_00821	rpsK; 30S ribosomal protein S11	HMPREF0389_00660	NlpC/P60 family protein	HMPREF0389_00959	NADH oxidase, water-forming

Table shows gene ID number and name of all *F. alocis* genes that were up regulated (> 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

Gene ID	Gene name
HMPREF0389_00226	acetyl coenzyme A synthetase
HMPREF0389_00802	Nitrate/nitrite response regulator protein
HMPREF0389_01749	hypothetical
HMPREF0389_01748	hypothetical
HMPREF0389_01188	hypothetical
HMPREF0389_01353	amidinotransferase
HMPREF0389_00486	hypothetical
HMPREF0389_00798	hypothetical
HMPREF0389_00799	signal peptidase I
HMPREF0389_00801	hypothetical
HMPREF0389_00800	low density lipoprotein receptor 2

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. Trancription; 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.

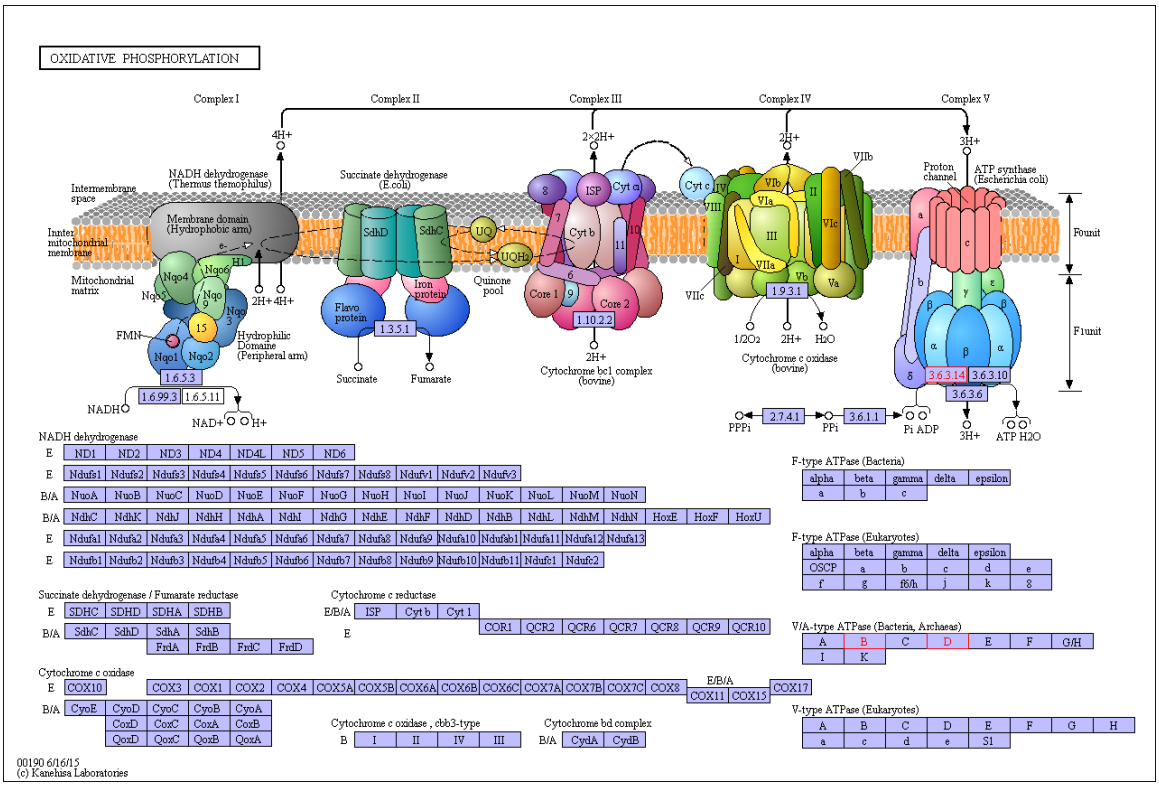

Figure 14: Oxidative phosphorylation pathway of *F. alocis*

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

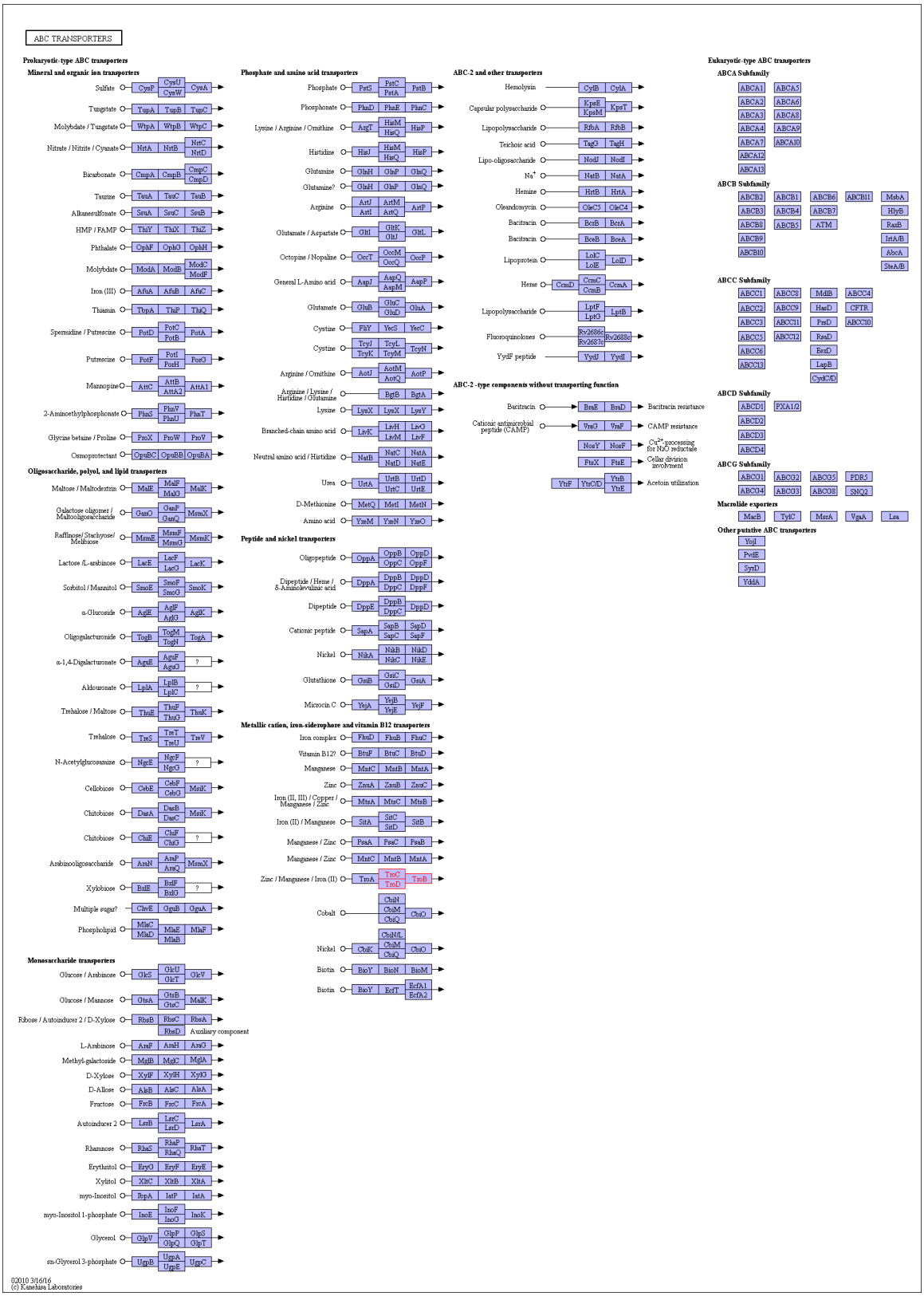

Figure 15: ABC transporter pathway of *F. alocis*

Generated using KEGG orthology system and red boxes show differentially regulated *F. alocis* ABC transporter genes when exposed to CSE.

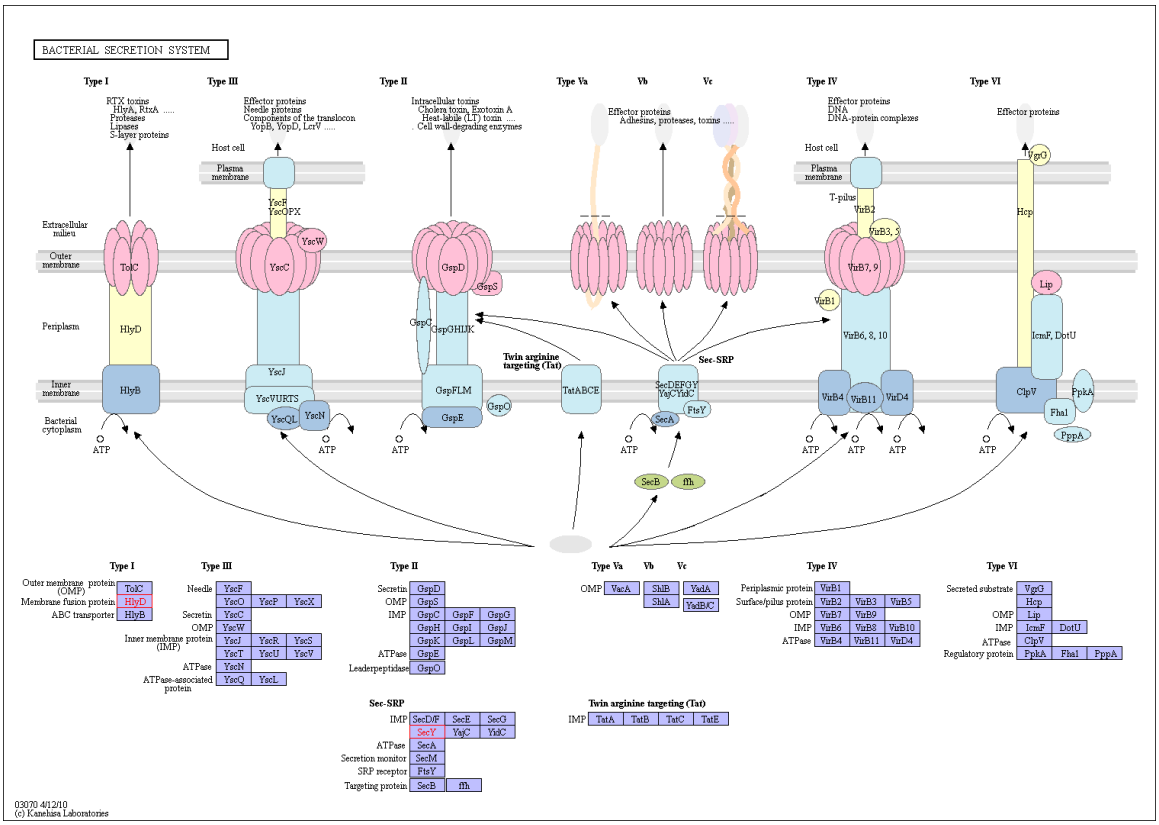

Figure 16: Bacterial secretory system pathway of *F. alocis*

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

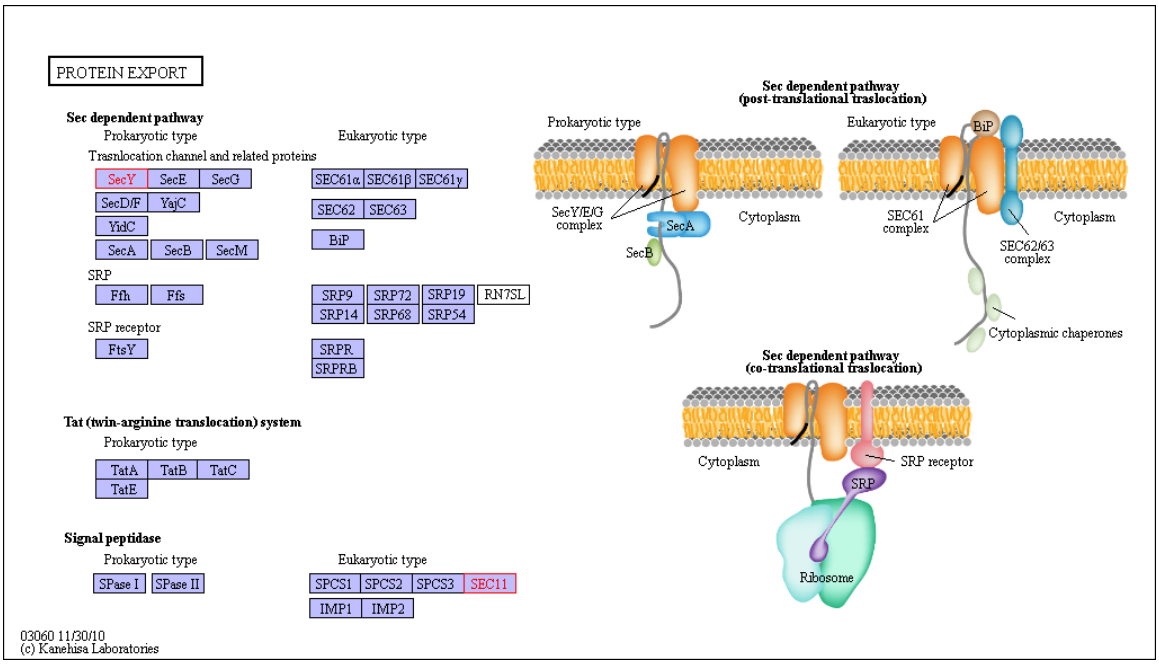

Figure 17: Protein export in *F. alocis*

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

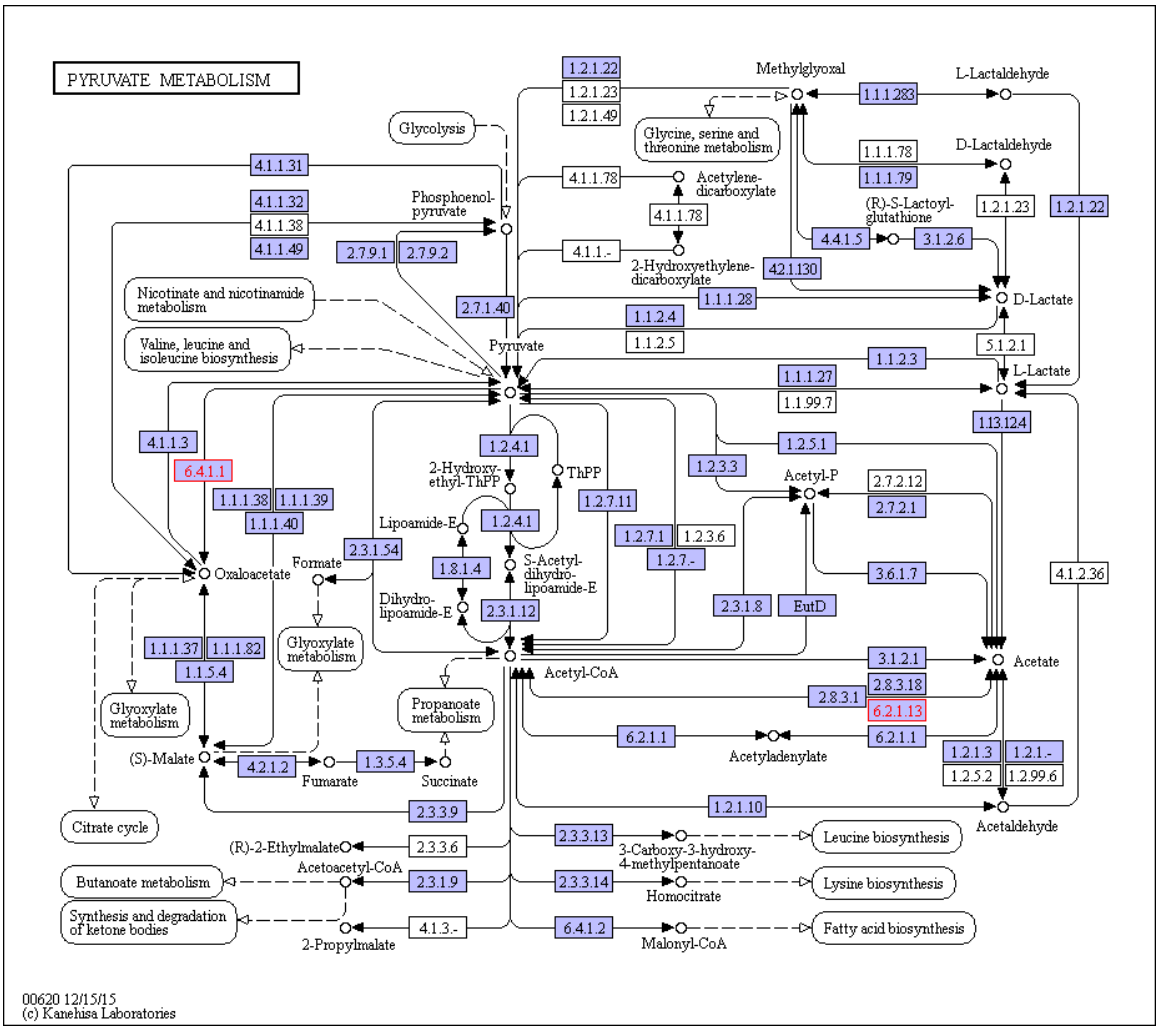

Figure 18: Pyruvate metabolism pathway of *F. alocis*

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

Gene ID	Gene name	Control qPCR expression value (Mean C_T)	CSE qPCR expression value (Mean C_T)	P- value (T- test)
16s rRNA	Reference gene	10.73	13.17	0.0001

Selected *P. gingivalis* up-regulated genes by qPCR analysis

Gene ID	Gene name	ΔCt Value (control)	ΔCt Value (CSE)	Delta Delta Ct (DDC_t) Value	Fold change (2^{-DDCt})	P- value (T- test)
PGN_1047[a]	hydroxylamine reductase	4.77	3.09	-1.68	3.2	0.16
PGN_0295[a]	C-terminal domain of Arg- and Lys-gingipain proteinase	5.41	3.38	-2.03	4.08	0.23
Kgp[a]	Lys-gingipain	4.37	2.04	-2.33	5.02	0.58
PGN_1367[a]	glutamate dehydrogenase	4.75	2.86	-1.89	3.70	0.06
PGN_1740[a]	RNA polymerase ECF-type sigma factor	3.61	2.50	-1.11	2.15	0.2
RgpA[a]	RgpAc; glycosyltransferase	3.87	1.23	-2.64	6.23	0.125
PGN_0727[a]	4-hydroxybutyryl-CoA dehydratase	3.29	0.98	-2.31	4.95	0.013[b]
PGN_1695[a]	fructose-1,6-bisphosphate aldolase	3.52	1.12	-2.4	5.27	0.56
RpoC[a]	DNA-directed RNA polymerase subunit beta	4.25	1.28	-2.97	7.83	0.01[b]
Dps[a]	DNA-binding protein from starved cells Dps	4.07	1.17	-2.9	7.46	0.0009[b]
PGN_0173[a]	glyceraldehyde 3-phosphate dehydrogenase type I	3.77	1.13	-2.64	6.23	0.035
PGN_0175[a]	2-ketoisovalerate ferredoxin reductase	4.01	1.13	-2.88	7.36	0.022
PGN_1080[a]	branched-chain amino acid aminotransferase	3.66	1.1	-2.56	5.89	0.126
PGN_0660[a]	alkyl hydroperoxide reductase	3.94	1.12	-2.82	7.06	0.0001[b]
PGN_0724[a]	NAD-dependent 4-hydroxybutyrate dehydrogenase	4.13	1.24	-2.89	7.41	0.008*

Selected *P. gingivalis* down-regulated genes by qPCR analysis

Gene ID	Gene name	ΔCt Value (control)	ΔCt Value (cse)	Delta Delta Ct (DDC_t) value	Fold change (2^{-DDCt})	P- value (T- test)

PGN_1644	transposase in ISPg1	2.54	1.51	-1.03	2.04	0.01[b]
Upp[a]	uracil phosphoribosyltransferase	4.97	5.87	0.9	0.53	0.0003[b]
PGN_0134[a]	biotin synthetase	5.53	5.89	0.36	0.77	0.001[b]
PGN_0885	nitroimidazole resistance protein	5.83	5.79	-0.04	1.02	0.022[b]
PGN_0545	sulfatase	5.88	5.63	-0.25	1.18	0.056

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

Gene ID	Gene name	Control qPCR expression value (Mean C_T)	CSE qPCR expression value (Mean C_T)	P-value (T-test)
16srRNA	Reference gene	10.54	11.23	0.16

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

Gene ID	Gene name	ΔCt Value (control)	ΔCt Value (CSE)	Delta Delta Ct (DDC_t) Value	Fold change (2^{-DDCt})	P-value (T-test)
HMPREF0389_00155	DNA replication protein DnaC	12.97	14.90	1.93	0.26	0.01[b]
HMPREF0389_00186[a]	Nlpc/P60 family protein	15.47	14.7	-0.77	1.70	0.64
HMPREF0389_00184[a]	Type IV conjugative transfer protein TraE	15.79	14.65	-1.14	2.20	0.04[b]
HMPREF0389_00178	TraG family protein	15.66	15.74	0.08	0.94	0.03[b]
HMPREF0389_00166[a]	TnpX site-specific recombinase	14.38	13.28	-1.1	2.14	0.13
HMPREF0389_00246[a]	Antirestriction protein (ArdA)	14.24	13.43	-0.81	1.75	0.50
HMPREF0389_00154[a]	Replication initiation protein	12.21	11.32	-0.89	1.85	0.71
HMPREF0389_00969	Nitrate transporter NirC	8.41	9.1	0.69	0.61	0.01[b]
HMPREF0389_00162[a]	Conjugation protein	13.26	12.58	-0.68	1.60	0.98
HMPREF0389_01096[a]	MATE efflux family protein	9.06	8.18	-0.88	1.84	0.84
HMPREF0389_00644[a]	FtsK/SpoIIIE family protein	11.18	10.42	-0.76	1.69	0.88

HMPREF0389_01592[a]	ABC transporter permease, ATP-binding protein	8.46	5.51	-2.95	7.72	0.01[b]
HMPREF0389_00823	Translation initiation factor IF-1	4.12	4.21	0.09	0.93	0.11
HMPREF0389_00879[a]	Riboflavin biosynthesis protein RibD	10.78	10.41	-0.37	1.29	0.5
HMPREF0389_01079	Iron-sulfur cluster-binding protein	10.56	11.66	1.1	0.46	0.1

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

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

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.

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, *P. gingivalis*, and an emerging periodontal pathogen, *F. alocis*. Approximately, 30% of genes in *P. gingivalis* genome and 5% of genes in *F. alocis* genome were found to be differentially expressed when exposed to cigarette smoke.

In *P. gingivalis* several functionally related genes were found to be up regulated, including genes encoding arginine and lysine gingipains (*kgp*, *rgpA* and *rgpB*), DNA binding genes and genes responsible for carbohydrate and energy metabolism. Gingipains play an important role in multiple virulence mechanisms in *P. gingivalis*, which are responsible for the growth and survival of the bacterium. They protect *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 *P. gingivalis* (228). It has been demonstrated that the Dps (DNA-binding protein from starved cells) protein in *E. coli* plays an important role in the protection of cells from peroxide stress and is believed to have similar kind of function in *P. gingivalis* (229,230,231). Carbohydrate and amino acid metabolism provide *P. gingivalis* with energy necessary for its growth.

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

Many of the differentially expressed genes in *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 *P. gingivalis* from potentially harmful chemicals in cigarette smoke (222) and might have similar function in *F. alocis*.

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 up regulated. 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 ($P \leq 0.01$) 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 ($P \leq 0.01$) 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 ($P \leq 0.01$). 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. gingvalis* 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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