EXPLORATION OF ENDOTHELIAL CELL INVASION AND RESPONSES TO NICOTINE AND ARGININE BY *STREPTOCOCCUS MUTANS* SEROTYPE *K* STRAINS IN A SUCROSE-INDUCED BIOFILM LIFESTYLE

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DEDICATION

To my parents for instilling in me the importance and power of education.

In memory of John A. McIntyre, Ph.D. who encouraged inquisitive pursuit of life and allowed me the freedom to explore.

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EXPLORATION OF ENDOTHELIAL CELL INVASION AND RESPONSES TO NICOTINE AND ARGININE BY STREPTOCOCCUS MUTANS SEROTYPE K STRAINS IN A SUCROSE-INDUCED BIOFILM LIFESTYLE

Streptococcus mutans, an inhabitant of oral biofilm or dental plaque, adheres to the tooth surface via protein antigen I/II (PA I/II). Pathologic lesions of atherosclerosis (AT) and infective endocarditis (IE) harbor *S. mutans*. Serotypes *f* and *k* strains with collagen binding protein genes *cbm* and *cnm* are uncommon in the mouth, but these are the most prevalent *S. mutans* strains in AT and IE tissues and can invade endothelial cells (EC) *in vitro*. Tobacco use increases the risk for cardiovascular and oral diseases. Oral *S. mutans* encounter many substances including nicotine. Arginine is present in saliva and the EC glycocalyx that coats and protects ECs from shear forces of blood flow. Prior studies demonstrated arginine alters *S. mutans* biofilm. This work characterizes *S. mutans* serotype *k* strains and serotype *c* strains, the most prevalent in the mouth. The effects of nicotine and arginine on biofilm mass, metabolic activity and EC invasion were investigated. Biofilm production by serotypes *c* and *k* strains did not differ; there were no differences in responses to nicotine and arginine between these serotypes. Increased production of biofilm was associated with the *cbm* and *cnm* genes. Nicotine increased biofilm for all strains whereas arginine plus nicotine reduced bacteria and the extracellular polymeric substances. Previous EC invasion studies were performed with planktonic cultures of *S. mutans*; therefore, EC invasion by biofilm was evaluated. Significant factors for EC invasion by *S. mutans* are presence of the *cbm* gene and lack of PA I/II expression on the bacterial cell surface. Presence of the *cnm* gene increased EC invasion by biofilm but not planktonic cells. Planktonic cells of six strains invaded better than biofilm, whereas four strains showed increased invasion by biofilm cells. Neither nicotine nor arginine significantly altered the ability of *S. mutans* biofilm cells to invade ECs. Not all strains with *cbm* or *cnm* and no PA I/II expression invaded EC. A strain with PA I/II expression and without *cbm* and *cnm* genes invaded EC. While *cbm*, *cnm* and PA I/II expression are predictors of EC invasion, additional mechanisms for EC invasion by *S. mutans* remain to be revealed.

Richard L. Gregory, Ph.D., Chair

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LIST OF ABBREVIATIONS

AT	Atherosclerosis
BAEC	Bovine aortic endothelial cells
Cbm	Collagen binding protein product of *cbm* gene
cbm	Gene for Cbm
CBP	Collagen binding protein
cfu	Colony forming unit
CLSM	Confocal laser scanning microscopy
Cnm	Collagen binding protein product of *cnm* gene
cnm	Gene for Cnm
CSC	Cigarette smoke condensate
CSE	Cigarette smoke extract
CV	Cardiovascular
CVD	Cardiovascular disease
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
e-cig	Electronic cigarette
ECM	Extracellular matrix
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FimA	Major fimbrial protein of *Pseudomonas gingivalis*
Gbp	Glucan binding protein
GLIC	*Gloeobacter violaceus*
Gtf	Glucosyltransferase
HUVEC	Human umbilical vein endothelial cells
IE	Infective endocarditis
LDH	Lactate dehydrogenase
MBIC	Minimum biofilm inhibitory concentration
MIC	Minimum inhibitory concentration
nAChR	Nicotinic acetylcholine receptor
OD 595nm	Optical density determined at a wavelength of 595nm
PA I/II	*Streptococcus mutans* surface protein antigen I/II
PD	Periodontal disease
pLGIC	Pentameric ligand-gated ion channel
PTS	Phosphotransferase system
RT-PCR	Reverse transcriptase-polymerase chain reaction
SpaP	*Streptococcus mutans* surface protein antigen I/II
TSB	Tryptic soy broth
TSBS	Tryptic soy broth plus 1% sucrose
WpaA	Cell wall protein of *S. mutans* that mediates binding to collagen
XTT	2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

Chapter One
GENERAL INTRODUCTION

CARDIOVASCULAR DISEASE

Cardiovascular diseases (CVD), including atherosclerosis (AT), are a leading cause of death in developed countries. Tobacco use is a well-documented risk factor for CVD and has been linked with oral diseases such as periodontitis and dental caries (Friedewald, Kornman et al. 2009, Benedetti, Campus et al. 2013). A direct causal link between AT-CVD and periodontal disease (PD) has not been established but an indirect link is supported by the many shared risk factors of the two diseases, i.e., diabetes mellitus, systemic inflammation, family history, advanced age, male gender, and smoking (Friedewald, Kornman et al. 2009). Demonstrating the importance of this association, a recent Editors' consensus simultaneously published in the American Journal of Cardiology and Journal of Periodontology informed both medical and dental practitioners of this significant link (Friedewald, Kornman et al. 2009, Friedewald, Kornman et al. 2009). A 2012 statement by the American Heart Association after review of 537 peer-reviewed publications supported an association between periodontal disease and atherosclerotic vascular disease but the reported observational studies failed to support a causative relationship (Lockhart, Bolger et al. 2012).

Longitudinal population-based studies with large numbers of participants further support an association, if not a causal relationship between CVD and oral health. Studies of a Swedish cohort of 8999 subjects with a median of 17-year follow-up found an increased incidence of CVD for subjects with a poor response to periodontal treatment compared with responders ($p<0.001$) (Holmlund, Lampa et al. 2017). Within this cohort, oral health represented by the number of remaining teeth was related to incident myocardial infarction and heart-failure (Holmlund, Lampa et al. 2017). Inasmuch as the measure of this study was tooth loss, it should be noted that tooth loss can be related to dental caries as well as PD. Evidence from the case-control Periodontitis and Its Relation to Coronary Artery Disease (PAROKRANK) study supported an association between periodontitis and first myocardial infarction even after adjustment for confounding factors including participant's smoking status (Ryden, Buhlin et al. 2016). In a broader evaluation of oral health, a 44-year epidemiological study of the oral health of 1393 randomly selected subjects in Stockholm reported that the quality of oral health is a risk indicator for death by CVD (Jansson, Kalkali et al. 2018).

Inflammation is a common key factor in all stages of both CVD and PD. The incidence of CVD increases in the presence of chronic inflammatory diseases such as Lupus erythematosus, rheumatoid arthritis and PD (Frostegard 2013). Injury of the vascular endothelium is likely an early pathophysiological event in the development of an atherosclerotic plaque. Inception of the atherosclerotic plaque is marked by activation of the endothelial cells and production of pro-inflammatory cytokines leading to recruitment of immune cells and triggering additional cytokine production at the endothelium (Spagnoli, Bonanno et al. 2007, Libby 2012).

Considerable evidence suggests that infection is a risk factor for AT and plays a role in the chronic inflammatory processes of the disease (Rosenfeld and Campbell 2011, Campbell and Rosenfeld 2015). Indeed the molecular footprints of oral bacteria have been identified in extra-oral tissues including atherosclerotic plaques (Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009, Reyes, Herrera et al. 2013, Lanter, Sauer et al. 2014). While many studies of AT and CVD have focused on periodontal pathogens, *Streptococcus mutans*, the causative agent of dental caries has also been identified in atherosclerotic plaques and in heart valves from cases of infective endocarditis (IE) (Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009). Kozarov, et al. detected *S. mutans* DNA in 22.5% of atherosclerotic plaques obtained from a young population (mean age 26.8 ± 8.2 years) and found twice as many atheromatous plaques (44%) contained evidence of *S. mutans* DNA in an elderly population (mean age 67 years) (Kozarov, Sweier et al. 2006). Nakano and colleagues reported detection of *S. mutans* in 74% of atheromatous plaques from elderly Japanese patients (average age 71 years) (Nakano, Inaba et al. 2006, Nomura, Nakano et al. 2006). These data are suggestive of interactions between *S. mutans* and the endothelial cells.

In addition to reports of *S. mutans* in atheromatous plaques and heart valves, this species of bacteria is implicated in intra-cerebral hemorrhage, cerebral microbleeds and cognitive impairment (Nakano, Hokamura et al. 2011, Miyatani, Kuriyama et al. 2015, Watanabe, Kuriyama et al. 2016, Inenaga, Hokamura et al. 2018). *S. mutans* has also been correlated with IgA nephropathy and non-alcoholic steatohepatitis (Naka S Fau - Nomura, Nomura R Fau - Takashima et al. 2014, Misaki, Naka et al. 2015, Misaki, Naka et al. 2016, Naka, Hatakeyama et al. 2016). Ulcerative colitis and inflammatory bowel disease are reported to be correlated with or aggravated by *S. mutans* (Kojima, Nakano et al. 2012, Kojima, Nomura et al. 2014). Undeniably, *S. mutans* survives outside the oral cavity and is involved in extra-oral infection of several organ systems.

STREPTOCOCCUS MUTANS

Strains of *S. mutans* are serotyped according to specific polysaccharides found on the cell wall. Cell surface polysaccharides of *S. mutans* are comprised of a rhamnose backbone with glucose side chains. The ratio of glucose to rhamnose is drastically decreased in serotype *k* strains compared to strains of serotypes *c, e* and *f*. As a consequence, until recently serotype *k* strains were identified as non-serotype *c/e/f S. mutans* (Nakano and Ooshima 2009). Serotype *c* strains are the most prevalent strains of *S. mutans* found in the oral cavity. Serotype *k* strains comprise 1.5% of the *S. mutans* isolated from the oral cavities of healthy subjects. In comparison, for patients with cardiovascular (CV) disease, serotype *k* strains are significantly increased in frequency within the oral cavity (10.3%, $p<0.05$) (Nakano, Nemoto et al. 2007). Patients with CV disease are more likely to harbor multiple *S. mutans* serotypes in the oral cavity compared to healthy subjects (53.8% versus 25.0%, respectively) (Nakano, Nemoto et al. 2007). In addition, Nakano et al. reported that *S. mutans* serotype *k* strains were present in 9.1% of heart valve and 25% of atheromatous tissue specimens and were increased in prevalence in diseased CV tissue compared to the oral cavities of the same patients (Nakano, Nemoto et al. 2007). These data suggest that *S. mutans* serotype *k* strains may adhere to, or interact with, endothelial cells at increased frequency compared to serotype *c* strains which are the most prevalent in the oral cavity.

The presence of *S. mutans* in atheromatous plaque may result from phagocytosis by monocytes at the endothelial cell surface of blood vessels followed by extravagation of the monocytes through the endothelial cell layer. Alternatively, collagen binding proteins (CBP) on the surface of *S. mutans* may facilitate binding directly to the endothelium. Two CBP, Cnm and Cbm, have been described in *S. mutans* (Abranches, Miller et al. 2011, Lapirattanakul, Nomura et al. 2013). These proteins are the products of *cnm* and *cbm* genes, which are found primarily in serotype *f* and *k* strains of *S. mutans* (Lapirattanakul, Nomura et al. 2013). Interestingly, serotypes *f* and *k* are increased in prevalence in the atherosclerotic plaques compared to the oral cavity of the same subjects with CVD and the oral cavities of normal, healthy controls (Nakano, Nemoto et al. 2007). In addition to binding collagen, *cnm* and *cbm* genes confer *S. mutans* with the ability to adhere to and invade endothelial cells (Abranches, Miller et al. 2011, Nomura, Nakano et al. 2012). *S. mutans* strains that express *cnm* and *cbm* are able to invade non-phagocytic cells, a trait directly related to chronic and systemic

infections (Nomura, Nakano et al. 2009, Nomura, Nakano et al. 2012, Aviles-Reyes, Miller et al. 2017).

Previous studies of endothelial cell invasion by *S. mutans* strains with CBP were performed with *S. mutans* growing in planktonic or a free-living bacterial cell conditions (Abranches, Miller et al. 2011, Lapirattanakul, Nakano et al. 2011, Nomura, Nakano et al. 2012, Lapirattanakul, Nomura et al. 2013). The natural habitat of *S. mutans* is within dental plaque which is a multi-species oral biofilm formed on hard surfaces of teeth (Lemos, Palmer et al. 2019). Indeed, the ability to form and reside in biofilms is an important virulence factor for *S. mutans* in dental caries (Matsumoto-Nakano 2018). Within the biofilm, three core attributes of *S. mutans* are the ability to synthesize extracellular polymers of glucan from sucrose, metabolize carbohydrates into organic acids, and flourish under low pH conditions and other environmental stresses (Lemos, Palmer et al. 2019). Synthesis of the biofilm extracellular matrix by *S. mutans* requires upregulation of many genes (i.e., glucosyltransferases, glucan biding proteins, etc.) that serve no purpose for free-living cells except as receptors for future surface attachment. An *in vitro* comparative transcriptome analysis found 12% of *S. mutans* genes showed significant differential expression between *S. mutans* biofilm and free-living cells (Shemesh, Tam et al. 2007). Indeed, differential gene expression was observed based upon the carbohydrate(s) and surface provided for *S. mutans* biofilm production (Shemesh, Tam et al. 2007, Shemesh, Tam et al. 2010). These data suggest that endothelial cell invasion may not be the same for *S. mutans* in free-living and biofilm environments.

SMOKING

Smoking is a well-known risk factor for both CVD and oral diseases, including dental caries. The direct mechanisms for smoking induced disease are yet to be fully elucidated, but the indirect effects of smoking are many. For example, mutans streptococci are increased in the saliva of smokers compared to non-smokers (Sakki and Knuuttila 1996). An increase in streptococcus 16S rRNA in oral washes of smokers compared to never smokers suggests that smoking alters the oral biome to the advantage of streptococcal species (Wu, Peters et al. 2016). Nicotine, a component of tobacco, causes the addictive behavior of smoking and increases biofilm growth of *S. mutans* serotypes *c*, *e* and *f* (Huang, Li et al. 2012). The effects of nicotine on *S. mutans* serotype *k* have not been investigated. *In vivo* and *in vitro* studies demonstrate an

increase in *S. mutans* in the oral cavity of smokers. This increased bacterial cell count enhances the opportunity for *S. mutans* to enter the blood stream through injury caused by mastication and daily oral hygiene practices.

ARGININE

The basic amino acid, arginine, is abundant in a wide range of foods and in saliva. In addition, relevant to this project, arginine is present in the glycoprotein-polysaccharide covering (glycocalyx) at the endothelial cell surface (Tarbell and Pahakis 2006, Flam, Eichler et al. 2007). Arginine alters *S. mutans* biofilm formation and structure by reducing or altering adhesion properties of the extracellular polysaccharides (Sharma, Lavender et al. 2014, Zheng, Cheng et al. 2015). These properties have prompted addition of arginine to toothpaste to augment the benefit of fluoride in suppressing *S. mutans* growth in oral biofilm (Zheng, Cheng et al. 2015). Arginine induces changes in biofilm elicited by an increase in biomass (bacteria cell number) and decrease in production of extracellular polymeric substances (EPS) by the bacteria (Zheng, Cheng et al. 2015). The combination of fluoride and arginine may be of greater benefit to smokers than to non-smokers as it potentially can reduce nicotine enhanced *S. mutans* biofilm growth. The presence of arginine in the oral cavity and at the endothelial cell surface as well as its effects on reducing *S. mutans* biofilm leads to speculation whether arginine might negate the biofilm enhancing effects of nicotine.

RESEARCH SUMMARY

The following chapters will first review the effects of nicotine on oral bacteria and tissues. Since serotype *k* strains of *S. mutans* have been implicated in CVD and other extra-oral infections, my work focuses on 33 serotype *k* strains of *S. mutans* and the biofilm lifestyle of these strains. The effects of nicotine and arginine on biofilm production by serotype *k* strains will be presented and discussed. In addition, endothelial cell invasion by both free-living *S. mutans* and *S. mutans* growing in biofilm will be compared. Finally, the effects of nicotine and arginine on the ability of *S. mutans* biofilm cells to invade endothelial cells will be explored. I hope these studies expand the literature about the effects of lifestyle on *S. mutans* responses to certain environmental exposures and endothelial cell invasion. Moreover, I am optimistic that the reader will find food for thought, speculation, and inspiration for additional experiments within the following pages.

Chapter Two

EFFECTS OF NICOTINE ON ORAL MICROORGANISMS, HUMAN TISSUES AND THE INTERACTIONS BETWEEN THEM

INTRODUCTION

Cigarette smoking is a preventable risk factor for many cancers and chronic diseases. The 2014 Surgeon General's report: The Health Consequences of Smoking - 50 Years of Progress found sufficient evidence to add liver and colorectal cancers to the long list of cancers causally related to cigarette smoking (National Center for Chronic Disease, Health Promotion Office on et al. 2014). Moreover, chronic diseases such as stroke, cardiovascular disease, respiratory diseases and periodontitis have long been linked to smoking. The 2014 report found sufficient evidence to add age related macular degeneration, diabetes, rheumatoid arthritis, ectopic pregnancy and erectile dysfunction to the catalog of chronic conditions caused by cigarette smoking. Additionally, the 2014 report found sufficient evidence to link maternal smoking to structural birth defects of oral-facial clefts (National Center for Chronic Disease, Health Promotion Office on et al. 2014).

The burning of tobacco, as in smoking, produces a complex mixture of over 7000 compounds (National Center for Chronic Disease, Health Promotion Office on et al. 2014). Nicotine, an alkaloid, is the primary bioactive and addictive agent in tobacco. Nicotine and its metabolites are found in blood, saliva, urine and breast milk of smokers (Shafagoj, Mohammed et al. 2002, Jacob, Golmard et al. 2015). Salivary nicotine levels vary by mode and duration of tobacco exposure such as cigarette smoking (median 27 ug/ml, range 4 – 207 ug/ml) (Jacob, Golmard et al. 2015), water pipe smoking (283.5 ng/ml ± 75 ng/ml) (Shafagoj, Mohammed et al. 2002), and electronic cigarette (e-cig) smoking (541 ug/L, range 291-2553 ug/L) (Papaseit, Farre et al. 2017). Levels of nicotine in the blood and saliva vary depending upon the number and type of cigarettes smoked. Smokers of menthol cigarettes had slower rates of salivary nicotine metabolism than smokers of non-menthol cigarettes (Fagan, Pokhrel et al. 2016). Levels of nicotine and it's metabolites are reported to be 100-fold greater in oral fluid compared to blood, and breast milk contains 25% of the nicotine concentrations found in blood (Jacob, Golmard et al. 2015, Papaseit, Farre et al. 2017). The nicotine concentrations in breast milk were significantly associated with salivary nicotine concentration and cigarette consumption (Jacob, Golmard et al. 2015). This suggests that in addition to potential

second-hand smoke exposure, breast-fed infants of mothers who smoke are directly and systemically exposed to nicotine from an early age.

Significant increases in the use of non-conventional tobacco products such as e-cigs and hookah are reported among high school students (Marshall, Lotfipour et al. 2016). Despite a drop in the use of conventional tobacco products, the rate of tobacco consumption remained constant in the school-age population between 2011 and 2014 (Marshall, Lotfipour et al. 2016). After conventional cigarette smoking, blood plasma nicotine concentrations ranged from 2.9 to 16.4 ug/L compared to 0 – 14.5 ug/L following e-cig use (Papaseit, Farre et al. 2017). A small crossover study of cigarette smokers naïve to e-cig use reported oral nicotine concentrations higher after cigarette smoking compared to e-cig smoking (728 ug/L, range 241 – 3960 ug/L and 541 ug/L, range 291-2553 ug/L, respectively) (Papaseit, Farre et al. 2017). The concentration of nicotine in e-cig cartridges varies by product; thus, plasma and salivary nicotine concentrations may be higher depending upon the e-cig product chosen.

The oral microbiome of smokers is directly exposed to nicotine at high levels, and for sustained periods. Recent literature on the effects of nicotine on the oral bacteria associated with dental diseases such as periodontitis and caries will be summarized in this review. In addition, the roles of nicotine and oral bacteria in oral and cardiovascular diseases will be explored.

PERIODONTAL PATHOGENS

Many studies report that smoking is a risk factor for PD (Friedewald, Kornman et al. 2009, National Center for Chronic Disease, Health Promotion Office on et al. 2014). PD involves pathological inflammation of the gingiva, alveolar bone, periodontal ligament and cementum (How, Song et al. 2016). In the United States, PD affects about 50% of adults over 30 years of age is the primary cause of tooth loss in this age group (Bostanci and Belibasakis 2012). Smoking is associated with increased dental plaque formation as well as shifts in the composition of the oral bacteria within periodontal plaque, both of which may increase risk for PD (Zambon, Grossi et al. 1996, Kamma, Nakou et al. 1999, Eggert, McLeod et al. 2001, Haffajee and Socransky 2001). Smoking increases the pathogenicity of certain microorganisms and elevates the amount of free iron in the mouth thereby supporting increased growth of the oral bacteria (Zambon, Grossi et al. 1996, Kamma, Nakou et al. 1999, Weinberg 2000, Eggert, McLeod et al. 2001, Haffajee and Socransky 2001, Weinberg 2009). In addition, smoking decreases oxygen tension

and induces changes in the gingival vasculature (Kumar 2012). All of these factors can potentially modify cytokines, chemokines and growth factors in the oral cavity thereby altering subgingival bacterial composition (Kumar 2012). Moreover, it was proposed that smoking increased the amount of bacterial pathogen load by inducing physiological changes in the body, increasing bacterial virulence or compromising the immune system (Bagaitkar, Demuth et al. 2008).

In a study of 1426 subjects, Zambon et al. used indirect immunofluorescence microscopy to detect subgingival infection by select periodontal pathogens. Smokers had higher prevalence of *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and *Porphyromonas gingivalis* compared to non-smokers (Zambon, Grossi et al. 1996). The prevalence of *Treponema denticola* was also increased in smokers (Umeda, Chen et al. 1998). Other studies support increased prevalence of *P. gingivalis* and *T. denticola* in smokers compared to non-smokers (Umeda, Chen et al. 1998, Eggert, McLeod et al. 2001, Socransky and Haffajee 2005). Van Winkelhoff et al. anaerobically cultured samples from the deepest site of each quadrant and found increased prevalence of *Prevotella intermedia* and *Prevotella nigrescens* from smokers (P=0.05) compared to non-smokers (van Winkelhoff, Bosch-Tijhof et al. 2001). Subgingival plaque samples obtained from 200 subjects free of systemic disease and PD revealed that dental plaque from smokers harbored significantly more diverse, and commensal-poor, anaerobic bacteria than non-smokers (Mason, Preshaw et al. 2015). The pathogens found in oral plaque are more likely to be associated with future disease for smokers than for non-smokers (Mason, Preshaw et al. 2015). Joshi et al. also concluded that smokers are more likely to have gingival inflammation than non-smokers due to greater microbial diversity in the oral biofilm communities from smokers (Joshi, Matthews et al. 2014). Similar changes in biofilm composition secondary to smoking are reported in the peri-implant microbiome (Tsigarida, Dabdoub et al. 2015). Further, smoking alters the oral microbial flora without increasing the amount of dental plaque suggesting that the bacterial composition rather than the quantity of dental plaque is an important factor for PD (Palmer, Wilson et al. 2005). Smoking not only increases the risk for periodontitis but it also increases resistance to treatment; however, healthier biofilm composition can be achieved six months after smoking cessation (Palmer, Wilson et al. 2005, Fullmer, Preshaw et al. 2009).

P. gingivalis, an obligate anaerobic bacterium, along with *T. denticola* and *T. forsythia* form the red bacterial complex, which has been shown to cause PD

(Mahanonda, Seymour et al. 1991, How, Song et al. 2016). Cigarette smoke extract (CSE) and nicotine increase the pathogenicity of *P. gingivalis* by enhancing adhesion of *P. gingivalis* to epithelial cell monolayers (Cogo, Calvi et al. 2009, Bagaitkar, Demuth et al. 2010). Major fimbrial protein (FimA) facilitates *P. gingivalis* adherence to epithelial cell monolayers and is upregulated by CSE (Bagaitkar, Demuth et al. 2010). Bagaitkar et al. reported that CSE augments *P. gingivalis* biofilm formation by increasing FimA, which then promotes interspecies interactions. For example, *P. gingivalis* adherence to *Streptococcus gordonii* increases three-fold in the presence of CSE (Bagaitkar, Daep et al. 2011) . Zeller et al. found that smoking increased the pathogenicity of *P. gingivalis* by impairing host IgG response to the pathogen (Zeller, Hutcherson et al. 2014). This study reported significant increases of oral and systemic *P. gingivalis* infections in smokers, which may be a consequence of decreased host IgG response to *P. gingivalis* (Zeller, Hutcherson et al. 2014). Moreover, synergistic effects between nicotine and toxins produced by *P. gingivalis* lead to PD (Sayers, Gomes et al. 1997). In contrast, other studies showed no correlation between smoking and changes in the supragingival and subgingival microflora (Preber, Bergstrom et al. 1992, Van der Velden, Varoufaki et al. 2003).

In addition to its effects on the oral microflora, smoking has adverse effects on tissues of the oral cavity. A 3-year longitudinal study of 81 subjects compared the outcome of regular maintenance therapy for chronic periodontitis for smokers and non-smokers. Although not statistically significant, smokers experienced greater clinical attachment loss and deeper periodontal pockets than non-smokers. (Fisher, Kells et al. 2008). After just 24 hours of *in vitro* exposure to millimolar concentrations of nicotine, gingival fibroblast adhesion to the root surface was significantly reduced and fibroblasts that attached to the root surface failed to proliferate (Esfahrood, Zamanian et al. 2015). Nicotine has been shown to induce apoptosis of gingival fibroblasts through generation of reactive oxygen species and activation of the caspase-3 dependent pathway (Kang, Park et al. 2011). In contrast, nicotine through the endoplasmic reticulum stress pathway induces necrosis of human periodontal ligament cells and periodontal tissue destruction by means of Akt, ERK, p38, JNK MAPK and NF-κβ pathways (Lee, Kang et al. 2012). Nicotine significantly inhibits migration of human gingival fibroblast and periodontal ligament cells, induces production of pro-fibrotic molecules (CCN2/CTGF and TGF-β1) and increases production of tissue inhibitor of metalloproteinase-1. These actions alter the extra cellular matrix (ECM) remodeling systems of periodontal cells resulting in

periodontal fibrosis due to increased accumulation of type I collagen in the ECM (Takeuchi-Igarashi, Kubota et al. 2016).

Nicotine also alters inflammatory responses to bacterial infection. Contrary to the expected inflammatory response, CSE-exposed *P. gingivalis* induced a lower pro-inflammatory response (TNF-α, IL-6, IL-12) and higher anti-inflammatory response (IL-10) from human monocytes and peripheral blood mononuclear cells than unexposed *P. gingivalis* (Bagaitkar, Williams et al. 2009, Bagaitkar, Demuth et al. 2010). Increased production of the pro-inflammatory cytokines, IL-1β and IL-8, was reported subsequent to nicotine treatment of human gingival epithelial and periodontal ligament cells (Wu, Zhou et al. 2014). These findings are consistent with the clinical observations that smokers with severe PD often do not manifest obvious signs of inflammation.

S. MUTANS AND DENTAL CARIES

Dental caries is caused by acid induced mineral loss from the tooth structure (Hayes, Cheng et al. 2017). In 2007, 91% of American adults aged more than twenty experienced dental caries, and 27% had untreated carious teeth (Dye, Thornton-Evans et al. 2015). *S. mutans* and *Lactobacillus* species are considered the main causative agents of dental caries. The oral cavities of caries-affected subjects harbor 70 times more *S. mutans* than caries-free subjects (Peterson, Snesrud et al. 2013). The cariogenicity of *S. mutans* is related to its ability to produce lactic acid from available carbohydrates leading to demineralization of the tooth structure (Moynihan and Petersen 2004). Not only does *S. mutans* produce acid, but it survives in highly acidic environments (Moynihan and Petersen 2004). *S. mutans* attaches to the tooth structure either by glucosyltransferase (Gtf) enzymes which convert sucrose to glucan or by a unique interaction with salivary proteins and agglutinin (Ahn and Burne 2007, Wen, Yates et al. 2010). Biofilm on the tooth surface is established by bacterial interaction with salivary components and with other microorganisms (Zijnge, van Leeuwen et al. 2010). Many studies indicate that the incidence of dental caries is higher for smokers (Aguilar-Zinser, Irigoyen et al. 2008, Avsar, Darka et al. 2008, Campus, Cagetti et al. 2011). *In vitro* studies demonstrate that nicotine and cigarette smoke condensate (CSC) increase the cariogenicity and growth of *S. mutans*. Huang et al. investigated the effects of nicotine on seven *S. mutans* strains including UA159, UA130, 10449, A32-2, NG8, LM7, and OMZ175 and found that cultures grown in trypticase soy broth (TSB) plus 1% sucrose had increased metabolic activities and biofilm quantity in the presence of

nicotine (0.5 – 8.0 mg/ml). Most strains exhibited maximum metabolic activity in 8 mg/ml nicotine; A32-2 peaked at 16 mg/ml nicotine. Peak biofilm mass was found at 4 or 8 mg/ml nicotine except for LM7 biofilm which peaked at 1 mg/ml nicotine. Thirty-two mg/ml nicotine was bactericidal for all strains (Huang, Li et al. 2012). Confocal scanning laser microscopy of *S. mutans* biofilm demonstrated that both bacterial cell numbers and extracellular polysaccharides (EPS) increased with increasing nicotine dose (Huang, Li et al. 2015). Nicotine upregulates Gtf and glucan binding protein (Gbp) A expression by planktonic cells (2.2 and 4-fold, respectively) but had no effect on GbpB. In contrast, GbpB was decreased by 75% in biofilm grown with 1% sucrose and nicotine, while Gtf and GbpA were unchanged (Huang, Li et al. 2015). Nicotine did not directly affect specific lactate dehydrogenase (LDH) activity of the biofilm; however, the overall concentration of lactate was significantly higher due to the larger number of biofilm cells (Huang, Li et al. 2015).

In dual-species experiments, Li et al. demonstrated that nicotine did not have a significant effect on *Streptococcus sanguinis* in either mono- or dual-species biofilm, whereas nicotine significantly increased *S. mutans* bacterial cell mass and EPS in both mono- and dual-species biofilm (Li, Huang et al. 2014). By using a sortase-defective strain of *S. mutans*, Li and colleagues demonstrated decreased sucrose and saliva-induced biofilm formation in the presence of nicotine compared to the wild-type strain. Biofilm formation increased significantly with low concentrations of nicotine compared to biofilm without nicotine (Li, Huang et al. 2013). In the presence of 1 mg/ml nicotine, the biofilm was more dense; *ldh*, *nlmC* and phosphotransferase system (PTS)-associated virulence genes were upregulated; and sucrose metabolism increased lactic acid production leading to a reduction in pH creating an environment favoring caries development (Li, Huang et al. 2016). A recent study in Dr. Rich Gregory's lab that investigated *S. mutans* isolates from oral washes collected from 10 smokers and 10 non-smokers supports the previous study. El-ezmerli and Gregory found increased biofilm formation from *S. mutans* isolates in oral washes from smokers compared to isolates from non-smokers (El-ezmerli & Gregory, unpublished).

Experiments done in my lab investigated the effects of CSC and nicotine on *S. mutans* and the efficacy of certain agents to inhibit nicotine-enhanced *S. mutans* biofilm formation. Mutacins are bacteriocins produced by *S. mutans* to favor its growth over other bacterial species in the biofilm. Nicotine at doses of 2 and 4 mg/ml in the culture broth significantly increased *S. mutans* production of bacteriocins (Shepherd et. al.,

unpublished). Naturally occurring dietary substances exert inhibitory effects on nicotine enhanced *S. mutans* biofilm formation. A polyphenol found in green tea inhibited nicotine-induced *S. mutans* biofilm formation (Foltz et al. and Taylor et. al., unpublished). Sodium chloride, potassium chloride and potassium iodide salts cause a decrease in nicotine stimulated *S. mutans* biofilm as measured by crystal violet (Balhaddad & Gregory, unpublished). In addition, 90 mg/mL of garlic significantly decreased the amount of nicotine-induced *S. mutans* biofilm (Dietl et. al., unpublished). These data suggest the potential of dietary supplementation to combat increased risk of dental caries in smokers.

OTHER BACTERIAL SPECIES

Aside from those bacteria associated with PD and caries, nicotine also affects other oral microbes (Table 2.1). Dubois et al. investigated the stimulating and inhibitory effect of nicotine on biofilm growth of *Candida albicans* and five species of oral bacteria, *Lactobacillus casei*, *Actinomyces viscosus*, *Actinomyces naeslundii*, *Rothia dentocariosa* and *Enterococcus faecalis*. The minimum biofilm inhibitory concentration (MBIC) for these species was 16 mg/ml of nicotine except for *E. faecalis*, which was 32 mg/ml. The MBIC for all species was 16 mg/ml nicotine. The total amount of growth increased through 8 mg/ml of nicotine for *L. casei*, *A. viscosus*, *R. dentocariosa*, and *C. albicans*, while the total growth for *E. faecalis* increased up to 16 mg/ml. *A. naeslundii* growth was diminished by 8 mg/ ml of nicotine and completely inhibited by 16 mg/ml (Dubois, Bennett et al. 2014). Huang and colleagues found that *S. gordonii* had significantly increased planktonic and biofilm growth when 1 to 4 mg/ml of nicotine was added to the culture media (Huang, Li et al. 2014).

Scardovia wiggsiae, discovered in 2011, is significantly associated with severe early childhood caries (Tanner, Mathney et al. 2011). As in other species of oral bacteria, biofilm formation by *S. wiggsiae* increased in a nicotine dose dependent manner from 0.5 to 8 mg/ml of nicotine. The MBIC of *S. wiggsiae* was 16 mg/ml of nicotine, and as observed in other species, 32 mg/ml of nicotine was bactericidal. The amount of nicotine-induced *S. wiggsiae* biofilm formation on human enamel was greater than on human dentin (Balhaddad and Gregory, unpublished). These findings suggest that nicotine load in saliva acquired from first- or second-hand smoke might increase the risk of early childhood caries.

CANDIDA ALBICANS AND STOMATITIS

Denture stomatitis, characterized by inflammation and erythema in the oral cavity of denture wearers, occurs in 11% to 67% of patients with complete dentures (Arendorf and Walker 1987, Webb, Thomas et al. 1998, Webb, Thomas et al. 1998). *C. albicans* infection of the denture is the primary cause of stomatitis; however, other factors including dietary habits, denture trauma, lack of oral hygiene, allergy and systemic diseases contribute to the condition (Arendorf and Walker 1987, Webb, Thomas et al. 1998, Webb, Thomas et al. 1998). Nicotine increases the total growth and biofilm formation of *C. albicans*, which may increase the risk of denture stomatitis for tobacco users (Dubois, Bennett et al. 2014). Ashkanane et al. demonstrated that 4 mg/ml of nicotine significantly increased co-aggregation of *C. albicans* and *S. mutans* (Ashkanane, Gomez et al. 2017). Upregulation of *S. mutans* production of GtfB in the presence of nicotine was proposed as the mechanism for the increased co-aggregation (Huang, Li et al. 2015). GtfB increased co-aggregation of *S. mutans* and *C. albicans* by converting *C. albicans* to de facto glucan producers, thus increasing the cariogenicity of *S. mutans* (Ashkanane, Gomez et al. 2017).

ORAL BACTERIA AND CARDIOVASCULAR DISEASE

CVDs, including AT, are a leading cause of death in developed countries, and tobacco use is a well-documented risk factor for CVD. A causal link between AT-CVD and PD has not been established, but AT-CVD and PD have shared risk factors such as diabetes mellitus, systemic inflammation, family history, advanced age, male gender, and smoking (Friedewald, Kornman et al. 2009). Inflammation is a key factor in all stages of both CVD and PD. The incidence of CVD increases in the presence of chronic inflammatory diseases such as Lupus, rheumatoid arthritis and PD (Frostegard 2013). Vascular endothelial injury is proposed as an early pathophysiological event of AT plaque formation which is marked by activation of the endothelial cells and production of pro-inflammatory cytokines leading to recruitment of immune cells and additional cytokine production (Spagnoli, Bonanno et al. 2007, Libby 2012).

Considerable evidence suggests that chronic infection is a risk factor for AT and plays a role in the chronic inflammatory processes of the disease (Rosenfeld and Campbell 2011, Campbell and Rosenfeld 2015). The molecular footprints of oral bacteria have been identified in CV tissues including atherosclerotic plaques (Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009, Reyes, Herrera et al. 2013, Lanter, Sauer et al.

2014). *P. gingivalis* was identified by molecular and histological methods in excised aortic aneurysm specimens (Nakano, Wada et al. 2011). *S. mutans* has been identified in excised atherosclerotic plaques and in heart valves from cases of IE (Nakano, Inaba et al. 2006, Nomura, Nakano et al. 2006, Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009, Kozarov 2012). These data are suggestive of interactions between oral bacteria and vascular endothelial cells.

Strains of *S. mutans* are serotyped as *c, e, f* and *k* according to specific polysaccharides found on the cell wall. *S. mutans* serotype *c* strains are the most prevalent serotype in oral biofilm, whereas serotype *k* strains comprise only 1.5% of the *S. mutans* isolated from the oral cavities of healthy subjects. In contrast, patients with CVD have significantly increased frequency of serotype *k* strains in the oral cavity (10.3%, $p<0.05$) and are more likely to harbor multiple *S. mutans* serotypes compared to healthy subjects (53.8% versus 25.0%, respectively) (Nakano, Nemoto et al. 2007). Nakano et al. reported *S. mutans* serotype *k* strains in 9.1% of excised heart valves and 25% of atheromatous tissue specimens (Nakano, Nemoto et al. 2007). Further, serotype *k* strains were increased in prevalence in the diseased CV tissue compared to the oral cavities of the same patients (Nakano, Nemoto et al. 2007). These data suggest that serotype *k* may interact with endothelial cells at increased frequency compared to serotype *c,* the most prevalent in the oral cavity.

Smoking is a well-accepted risk factor for both CVD and oral diseases, including dental caries. In addition to the *in vitro* biofilm findings with nicotine previously discussed, a clinical study reported increased mutans streptococci in the saliva of smokers compared to non-smokers (Sakki and Knuuttila 1996). An increase in streptococcal 16S rRNA in oral washes of smokers compared to never smokers suggests that smoking alters the oral biome to the advantage of streptococcal species (Wu, Peters et al. 2016). Nicotine increases biofilm growth of *S. mutans* serotypes *c, e* and *f* (Huang, Li et al. 2012). The effects of nicotine on *S. mutans* serotype *k* are not different from serotype *c* strains (manuscript in preparation). These effects may be associated with increased oral biofilm in smokers. Increased biofilm growth enhances the opportunity for *S. mutans* to enter the blood stream through injury caused by mastication or daily oral hygiene practices.

S. mutans found within the AT plaque may be a consequence of monocyte phagocytosis and extravagation through the endothelial cell layer of vascular tissues. Alternatively, collagen binding proteins (CBP), Cnm and Cbm, on the surface of *S.*

mutans may facilitate direct binding to the endothelium (Abranches, Miller et al. 2011, Lapirattanakul, Nomura et al. 2013). A large multi-locus typing study of clinical *S. mutans* isolates found the *cnm* gene primarily in serotype *f* and *k* strains, whereas the *cbm* gene was predominately found in serotype *k* strains (Lapirattanakul, Nomura et al. 2013). Cnm and Cbm confer upon *S. mutans* the ability to adhere to and invade human coronary artery and umbilical vein endothelial cells (HUVEC) (Abranches, Miller et al. 2011, Nomura, Nakano et al. 2012, Lapirattanakul, Nomura et al. 2013). In addition, *S. mutans* strains expressing Cnm invade non-phagocytic cells, a trait directly related to chronic and systemic infections (Aviles-Reyes, Miller et al. 2017). *In vitro* studies demonstrated various levels of collagen binding by *S. mutans* strains expressing either Cnm or Cbm, whereas strains lacking *cnm* and *cbm* genes exhibited very low levels of collagen binding (Lapirattanakul, Nomura et al. 2013). Compared to *cnm*-positive strains, *cbm*-positive strains demonstrated increased binding to collagen as well as enhanced adhesion to, and invasion of, HUVEC (Nomura, Naka et al. 2013). This was supported by the increased detection rate of *cbm*-positive versus *cnm*-positive strains in heart valve tissue from subjects with *S. mutans*-positive IE (62.5% versus 12.5%). The high rate of *cbm*-positive strains of *S. mutans* in IE tissues lead to speculation that Cbm may play a key role in the pathogenesis of IE (Nomura, Naka et al. 2013). Similarly, *S. mutans* strains expressing CBP are associated with increased risk for cerebrovascular dysfunction (hemorrhagic stroke and microbleeds) and ulcerative colitis (Nakano, Hokamura et al. 2011, Kojima, Nakano et al. 2012, Miyatani, Kuriyama et al. 2015). Given evidence in a variety of other vascular diseases, the CBP of *S. mutans* may play a key role in evolution of atherosclerotic plaques.

NICOTINIC ACETYLCHOLINE RECEPTORS

The addictive power of nicotine is mediated through neuronal nicotinic acetylcholine receptors (nAChR). Nicotine stimulates endothelial and vascular smooth muscle cells on the vessel wall through activation of nAChR (Heeschen, Weis et al. 2002, Lee and Cooke 2011, Lee and Cooke 2012, Cooke 2015). Specifically, nAchRs are activated by endogenous acetylcholine or exogeneous nicotine to form ion channels in the cell membrane (Lee and Cooke 2011). At clinically relevant concentrations nicotine enhances endothelial cell growth, migration and survival – all key processes of angiogenesis (Heeschen, Jang et al. 2001, Heeschen, Weis et al. 2002, Heeschen, Weis et al. 2003, Cooke 2015). In $ApoE^{-/-}$ mice, clinically relevant doses of nicotine

enhanced progression of atherosclerotic plaque and neovascularization (Heeschen, Jang et al. 2001). Activation of nAchR in smooth muscle cells results in proliferation and migration into the vascular intima where the smooth muscle cells phenotypically change to myofibroblasts and osteoblast-like cells that contribute to the extracellular collagen matrix and osteopontin in the atherosclerotic plaque (Cooke 2015). Mast cells in the aortic adventitia are activated through nAChR leading to degranulation and pro-inflammatory cytokine production and release, which may be yet another mechanism by which nicotine accelerates AT (Wang, Chen et al. 2017).

The prototypic member of the pentameric ligand-gated ion channel (pLGIC) family, nAChR is widely expressed in eukaryotic cells. Prokaryotic homologs of nAChR predicted by eukaryotic-prokaryotic genome comparisons have been described in several bacterial species including *Gloeobacter violaceus* (GLIC), *Erwinia chrysanthemi* and *Escherichia coli* (Bocquet, Prado de Carvalho et al. 2007, Corringer, Baaden et al. 2010, Lepore, Indic et al. 2011). Bocquet, et al. suggested in 2007 that the GLIC receptor might contribute to bacterial adaptation to pH change, and indeed crystal structures reveal that GLIC is an open channel at pH 4 and at pH 7 the proton channel is closed (Bocquet, Prado de Carvalho et al. 2007, Cecchini and Changeux 2015). Although not specifically studied in *S. mutans* and other oral bacteria, one might speculate that similar prokaryotic pLGIC functions allow these species to exist and thrive in acidic environments. Taking the speculation further, much as nicotine stimulates endothelial and smooth muscle cell proliferation through nAChR, a prokaryotic homologue may play a role in nicotine stimulation of biofilm and planktonic proliferation in oral bacteria (Figure 2.1).

CONCLUSIONS

This review revealed how smoking and nicotine alter microbial composition in the oral cavity and increase the pathogenicity of several oral microorganisms. Pathological interactions between oral microorganisms and human tissues after nicotine exposure are associated with multiple oral and systematic diseases. It is important to mention that bacterial interaction with other microbial species or human tissues is a complicated process. Future studies are needed to demonstrate how the interactions between bacterial pathogens and human tissues are affected by nicotine and how these interactions alter supra and subgingival bacterial composition. Several studies in this review did not discuss or speculate on the mechanism for nicotine to increase biofilm

formation. Future studies are needed to define the mechanism(s) by which nicotine enhances growth of oral bacteria.

Table 2.1 Summary of the effects of nicotine and cigarette smoke condensate (CSC) on different species of oral microorganisms.

Study	Microorganism	Effects of Nicotine/CSC	MIC	MBIC
Sayers et al., 1997	*P. gingivalis*	Synergistic effects between nicotine and bacterial toxins cause periodontal disease	-	-
Bagaitkar et al., 2010		Upregulated FimA upregulation and promoted biofilm formation	-	-
Bagaitkar et al., 2011		Increased *P. gingivalis* adherence to *Streptococcus gordonii* by three-fold and promoted biofilm formation	-	-
Zeller et al., 2014		Suppressed the production of human IgG anti-*P. gingivalis*	-	-
Huang et al., 2012	*S. mutans*	Enhanced biofilm formation and biofilm metabolic activity	16 mg/ml	16 mg/ml
Li et al., 2013		Increased metabolic activity and sucrose-induced biofilm formation	-	-
Li et al., 2014		Enhanced biofilm formation by *S. mutans* more than *S. sanguinis*	-	-
Huang et al., 2015		Upregulated Gtf and glucan binding protein (GbpA) expression	-	-
Li et al., 2016		Upregulated *Idh*, *nlmC* and phosphotransferase system (PTS)-associated genes and increased lactate production	-	-
Huang et al., 2014	*S. gordonii*	Stimulated planktonic growth, biofilm formation, aggregation and gene expression (abpA, scaA, ccpA, and srtA)	-	-

Dubois et al., 2014	*L. casei*	Increased total growth and promoted biofilm formation	16 mg/ml	16 mg/ml
Dubois et al., 2014	*A. viscosus*	Increased total growth and promoted biofilm formation	16 mg/ml	16 mg/ml
Dubois et al., 2014	*R. dentocariosa*	Increased total growth and promoted biofilm formation	16 mg/ml	16 mg/ml
Dubois et al., 2014	*E. faecalis*	Increased total growth and promoted biofilm formation	32 mg/ml	16 mg/ml
Dubois et al., 2014	*A. naeslundii*	Decreased total growth and biofilm formation	16 mg/ml	16 mg/ml
Dubois et al., 2014	*C. albicans*	Increased total growth and promoted biofilm formation	16 mg/ml	16 mg/ml

Table 2.1 was previously published in (Wagenknecht, Balhaddad et al. 2018)

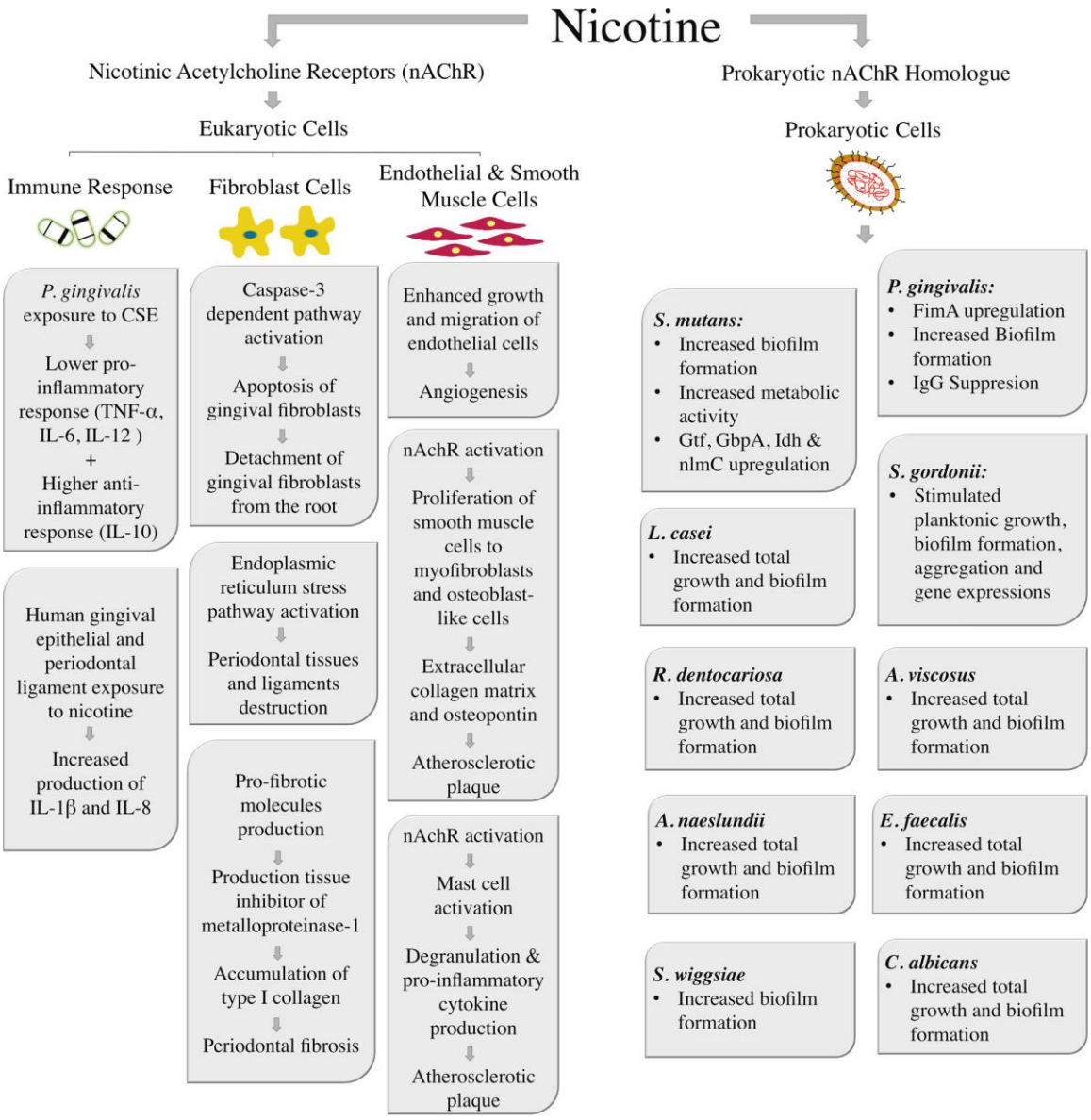


Figure 2.1 Nicotine has wide-ranging effects on many different eukaryotic cells through activation of nicotinic acetylcholine receptors (nAChR). This figure was designed by A. A. Balhaddad and was previously published (Wagenknecht, Balhaddad et al. 2018).

Chapter Three

SURVEY OF THE EFFECTS OF ARGININE AND NICOTINE ON SUCROSE-INDUCED BIOFILM DEVELOPMENT OF 33 STRAINS OF *S. MUTANS*

INTRODUCTION

S. mutans, a gram-positive oral bacterium key to development of dental caries, is classified by serotype based upon the composition and structure of cell wall polysaccharides. Serotypes *c, e* and *f* have glucose side chains on a rhamnose backbone (Nakano and Ooshima 2009). In contrast, for strains of serotype *k* there is a dearth of glucose side chains on the rhamnose backbone (Nakano, Nomura et al. 2004). Serotype *c* strains of *S. mutans* are most prevalent. Strains of serotypes *f* and *k* comprise less than 5% of the *S. mutans* isolated from the oral cavities of healthy individuals, but these serotypes are significantly increased in oral isolates from patients with CVD and IE (Nakano, Nemoto et al. 2007, Nakano and Ooshima 2009, Yamamoto and Takada 2011). Diseased heart valves and atherosclerotic plaque tissues harbor a higher prevalence of serotypes *f* and *k S. mutans* strains than the oral cavities of the same subjects (Nakano, Nemoto et al. 2007). The presence of the CBP Cbm and Cnm has been shown to facilitate *S. mutans* invasion of endothelial cells and heart valve tissues (Nakano, Inaba et al. 2006, Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009, Abranches, Miller et al. 2011, Aviles-Reyes, Miller et al. 2017). The genes for these CBP, *cbm* and *cnm*, are found predominantly in *S. mutans* serotype *f* and *k* strains.

Tobacco use is associated with increased risk for CVD and oral diseases, including caries (Wagenknecht, Balhaddad et al. 2018). Nicotine, a bioactive agent in tobacco, has been shown to increase *in vitro* growth and metabolism of *S. mutans* serotypes *c, e* and *f* strains grown in planktonic and biofilm cultures. Huang et. al. found minor enhancement of planktonic growth in seven strains of *S. mutans* upon addition of 1 and 2 mg/ml nicotine (Huang, Li et al. 2012). In contrast, biofilm growth of the same seven strains increased in a nicotine dose dependent manner until toxicity was reached at 16 or 32 mg/ml nicotine depending upon the individual strain. Metabolism of established *S. mutans* biofilm increased, and the cellular morphology changed from the usual ellipsoid shape to a more spherical shape with increasing nicotine exposure (Huang, Li et al. 2012). Confocal laser scanning microscopy of *S. mutans* biofilm revealed that both bacterial volume and EPS volume were increased in the presence of

nicotine (Huang, Li et al. 2015). Nicotine had significant effects on biofilm growth of serotype *c, e* and *f* strains of *S. mutans*. The effects of nicotine on serotype *k* strains of *S. mutans* have not been investigated.

The basic amino acid, arginine, is present in whole saliva and in ductal saliva collected from parotid glands (Van Wuyckhuyse, Perinpanayagam et al. 1995, Sharma, Lavender et al. 2014). The concentration of arginine in parotid saliva is increased in caries-free compared to caries-susceptible individuals, suggesting that arginine plays a role in the control of caries development (Van Wuyckhuyse, Perinpanayagam et al. 1995). Free arginine is also found in the endothelial glycocalyx of endothelium at the site of *S. mutans* invasion into cardiovascular tissues (Tarbell and Pahakis 2006, Flam, Eichler et al. 2007). *In vitro* studies have demonstrated that the adhesive and structural properties of *S. mutans* biofilm are altered in the presence of arginine compared to biofilm grown without supplemental arginine. By using atomic force microscopy, Sharma et. al. demonstrated that both adhesion force and rupture length for *S. mutans* biofilms were decreased with addition of arginine in a dose dependent manner, leaving the biofilm with a more fragile structure (Sharma, Lavender et al. 2014). *In vitro* studies of serotype *c* strains demonstrated that arginine increased biomass and decreased extracellular polysaccharides (EPS) compared to *S. mutans* biofilm cultured in the absence of arginine (Zheng, Cheng et al. 2015). Other studies demonstrated that arginine causes downregulation of genes encoding virulence factors responsible for attachment, competence development and bacteriocin production (Chakraborty and Burne 2017). In addition, arginine decreased tolerance to environmental acid and oxidative stresses (Chakraborty and Burne 2017). Arginine synergizes with fluoride to suppress *S. mutans* planktonic and biofilm cultures *in vitro* and increase protection against caries lesions in clinical trials of dentifrices (Kraivaphan, Amornchat et al. 2013, Zheng, Cheng et al. 2015). Analogous to the alterations of *S. mutans* biofilm in the presence of nicotine, arginine also provokes alterations in *S. mutans* biofilm production.

Studies of the effects of nicotine and arginine on *S. mutans* biofilm have been carried out with serotype *c, e* and *f* strains of *S. mutans*. The serotype *k* strains of *S. mutans* are more prevalent in the oral cavities of CVD patients compared to healthy subjects and are predominant in diseased CV tissue. Most studies of the effects of nicotine and arginine have been carried out with single reference strains. No published studies have included serotype *k* strains or strains which have been typed for CBP. I investigated the effects of arginine and nicotine, separately and in combination, on

biofilm growth and structure using a large panel of serotyped and CBP genotyped strains of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. mutans strains used in this study are listed in Table 3.1. The serotyped and genotyped strains were kind gifts of Drs. Noel Childers and Stephanie Momeni at University of Alabama School of Dentistry; Dr. Kazuhiko Nakano, Osaka University Graduate School of Dentistry, Osaka, Japan and Kazuko Takada, Nihon University School of Dentistry at Matsudo, Chiba, Japan. Frozen stock of each strain was inoculated into TSB (Becton, Dickinson and Co., Sparks, MD, USA), and then incubated at 37^{o}C, 5% CO_2 for 18 to 48 hours, depending on bacteria growth rate, prior to each experiment.

Biofilm experiments

Experiments were performed in 96-well microtiter plates using TSB with 1% sucrose (TSBS) to promote biofilm formation. A stock solution of TSBS containing 200 mg/ml arginine (Sigma-Aldrich, St. Louis, MO, USA) in TSB with 1% sucrose (TSBS) was adjusted to the pH of TSBS (pH 7.2) prior to sterilization. TSBS was used to dilute the stock arginine-TSBS to the desired concentration. Nicotine (Sigma-Aldrich) was added to TSBS immediately prior to each experiment. Briefly, 100 µl of TSBS without or with double the desired concentration of nicotine and/or arginine was added to quadruplicate wells of a 96-well microtiter plate. Next, 100 µl of overnight *S. mutans* culture diluted 1:50 in TSBS (10^6 *S. mutans* cells) was added to each well, resulting in the stated final concentrations of nicotine and arginine. Biofilms were grown separately in nicotine and arginine, in the absence of either and in the presence of both and in quadruplicate wells for each treatment. The microtiter plates were incubated for 24 hours at 37^{o}C in 5% CO_2. Each experiment was repeated three times.

Crystal violet assay

In order to assess biofilm mass after 24-hours, the biofilm was stained with crystal violet. Briefly, after removing the growth media, each biofilm was washed three times with sterile distilled H_2O (dH_2O); the plates were then blotted on absorbent paper prior to 30-minute room temperature incubation with 10% formaldehyde (Sigma-Aldrich).

After three additional dH_2O washes, a 0.5% crystal violet solution (Sigma-Aldrich) was placed on the biofilm for 30 minutes followed by additional dH_2O washes to remove excess crystal violet. Next, 2-propanol was added for 60 minutes to extract the crystal violet from the bacterial cells prior to reading the optical density of each well at 490 nm on a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

XTT assay

S. mutans cellular metabolism within the biofilm was measured by a 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay as previously described (Huang, Li et al. 2012). Briefly, the biofilm was washed three times with sterile 0.9% NaCl (Sigma-Aldrich) prior to reaction with 0.2 mg/ml XTT salt (Sigma-Aldrich) in 10% ethanol with 3 µg/ml menadione (Sigma-Aldrich) for 2 hours at 37^{o}C in the dark. After the incubation, the XTT solution from each well was transferred to the same relative well in a fresh microtiter plate, and the optical density at 490 nm was read in a spectrophotometer.

Confocal laser scanning microscopy (CLSM)

Biofilm was grown directly on 8-chamber cover glasses (Nunc Lab-Tek™II, ThermoFisher Scientific, Waltham, MA, USA) according to the method of Huang, Li et al. (Huang, Li et al. 2012) by inoculating 18-hour planktonic cultures into TSBS containing the appropriate additive and 1.0 µM Alexa Fluor 568 labeled dextran (ThermoFisher Scientific), to label developing EPS. The biofilms were incubated at 37^{o}C in 5% CO_2 for 24-hours, and then washed 3 times with sterile dH_2O. Next, bacterial cells within the biofilm were stained with Syto9 (1:5000, ThermoFisher Scientific) for 15 minutes in the dark at room temperature before three additional washes with sterile dH_2O. After the final wash, the biofilms were air-dried, then covered with Prolong™ Gold Antifade Mountant (ThermoFisher Scientific). Fluorescent images were obtained by using an Olympus FV1000 MPE confocal laser scanning microscope with Olympus FV10-ASW software (Olympus Corp., Center Valley, PA, USA) at the Indiana Center for Biological Microscopy, Indiana University School of Medicine. Full-depth Z-stacks were collected from at least three distinct fields from each cover glass grown biofilm. Up to 7 fields per biofilm were collected when inspection through the CLSM revealed visually distinct regions within a biofilm. All Z-stacks from the total depth of each biofilm field were processed. The total volumes of EPS (red, Alexa Fluor 568) and bacteria (green, Syto9)

in the processed images were analyzed by Imaris Image Analysis Software version 8 (Bitplane, South Windsor, CT, USA), and the volumes of EPS and bacteria were expressed in pixels.

Statistical analyses

Each crystal violet and XTT experiment was performed in quadruplicate microtiter wells and independently repeated three times. For the CLSM experiments, a minimum of three separate microscopic fields were collected for each strain and treatment combination. Z-stacks were collected from the full-thickness observed in each field from the first visible fluorescence on the surface of the cover glass to the last visible fluorescence at the top of the biofilm. Two-way ANOVA with interaction and random effect for multiple measures was used for statistical analyses. When non-normality was present, a rank transformation was performed prior to the analysis.

RESULTS

Titration of nicotine and arginine in biofilm growth media

Working under the observation that serotype *k* strains of *S. mutans* differ from serotype *c* strains in terms of prevalence in the oral cavity and in association with extra-oral tissue infection, I selected 10 strains of each serotype to determine whether serotype influenced biofilm growth. In previous studies, nicotine enhanced *S. mutans* biofilm growth. Variability was found among the strains; however, biofilm growth peaked between 4 and 8 mg/ml nicotine for serotype *c, e* and *f* strains (Huang, Li et al. 2012, Huang, Li et al. 2015). Previous studies from my lab showed that the metabolic activity of *S. mutans* biofilm decreased with 16 mg/ml and was absent at 32 mg/ml nicotine (Huang, Li et al. 2012). Nicotine dose titration curves of the 20 selected strains were consistent with published data (Figure 3.1). Based upon these experiments, doses of 0 and 4 mg/ml of nicotine were selected for the present study of biofilm growth.

In order to determine the arginine concentration for subsequent studies, a dose titration was performed for the same 20 strains (Figure 3.2). There was no difference in biofilm mass between 0 and 5 mg/ml arginine doses measured by crystal violet staining ($p=0.9325$). Compared to biofilm grown without arginine, increases in biofilm mass occurred in the presence of 10 mg/ml and 20 mg/ml arginine ($p=0.0339$ & 0.0253, respectively). Biofilm grown in 10 mg/ml arginine was not different from 20 mg/ml arginine ($p=0.9078$). Doses of 40 and 80 mg/ml arginine resulted in significant

decreases in biofilm mass compared to baseline ($p<0.001$). Similarly, bacterial metabolism measured by the XTT assay was increased by doses of 10 and 20 mg/ml arginine ($p=0.0133$ and 0.0054, respectively) and decreased by 40 and 80 mg/ml doses ($p=0.0016$ and <0.0000). When analyzed individually, several strains displayed lower biofilm mass and metabolism with 20 mg/ml than with 10 mg/ml arginine. Collectively, these observations lead us to select 10 mg/ml arginine for subsequent experiments.

Effects of arginine and nicotine on S. mutans biofilm

The effects of nicotine and arginine, separately and in combination, were assessed in parallel biofilm experiments with 10 strains of serotype *c* and 23 serotype *k* strains. Biofilm grown in TSBS served as the biofilm baseline control for the same strain grown concurrently in 10 mg/ml arginine, 4 mg/ml nicotine, or 10 mg/ml arginine plus 4 mg/ml nicotine. Each biofilm was grown in quadruplicate wells and in three separate experiments. Figure 3.3 summarizes the biofilm biomass measured by crystal violet staining and metabolic activity measured by XTT for the 33 strains. Biofilm grown in 10 mg/ml arginine was not different from biofilm grown in TSBS alone. Addition of 4 mg/ml nicotine significantly increased the biomass and metabolic activity of 24-hour *S. mutans* biofilm ($p<0.0001$). Similarly, the combination of nicotine and arginine increased *S. mutans* biofilm mass and metabolic activity compared to nicotine alone ($p=0.0028$ and 0.0021, respectively).

When analyzed by serotype, neither the biomass nor metabolic activity was significantly different between serotypes *c* and *k* across all treatments ($p=0.6093$ and 0.2650, respectively). Individual treatments did not affect the biomass of serotype *c* strains differently than serotype *k* strains as shown in Table 3.2. Biofilm from serotype *k* strains was not significantly different from that produced by serotype *c* strains regardless of treatment group.

We next asked whether CBP genotype influenced biofilm biomass or metabolic activity. The CBP genotype was known for 30 of the 33 strains tested; 13 strains were *cbm*+/*cnm*-, seven were *cbm*-/*cnm*+, three had both genes and seven strains had neither gene. The biomass of *cbm*-/*cnm*+ strains was significantly increased compared to strains lacking CBP genes, *cbm*-/*cnm*- ($p=0.0032$, Table 3.3 and Figure 3.4). The *cbm*+/*cnm*- strains showed a non-significant increase in biomass compared to *cbm*-/*cnm*- and *cbm*+/*cnm*+ strains. Compared to TSBS, arginine had a significant effect only on *cbm*+/*cnm*- strains which demonstrated decreased biomass. In contrast, nicotine

significantly increased the biomass of cbm+/*cnm*-, *cbm*-/*cnm*+ and *cbm*-/*cnm*- stains. A modest, non-significant increase was seen when nicotine was added to the cultures of strains with both CBP. Strains with the both genes demonstrated increased biomass when arginine was added to the nicotine (p=0.0001).

Table 3.4 displays the metabolic activity data. When grown in TSBS, the metabolic activity of *cbm*-/*cnm*+ strains was not significantly different from that of the *cbm*+/*cnm*- strains (p=0.1702) but was increased compared to strains lacking CPB (p<0.0001) and *cbm*+/*cnm*+ strains (p=0.0040). In general, neither arginine nor nicotine altered the aforementioned observations. When arginine and nicotine were combined, the metabolic activity of *cbm*-/*cnm*+ strains was increased compared to *cbm*+/*cnm*- strains (p=0.0172) and even more so compared to *cbm*-/*cnm*- and *cbm*+/*cnm*+ strains (p<0.0001 and =0.0032, respectively).

Confocal laser scanning microscopy for measurement of bacteria and EPS in biofilm

Crystal violet stains the bacterial cells as well as the EPS produced by the *S. mutans* during biofilm formation. By comparison, the XTT assay detects metabolic activity of *S. mutans*, and the amount of EPS production is not directly measured in this assay. I therefore used CLSM to separately measure *S. mutans* nucleic acid (Syto9, green) and EPS (Alexa Fluor 568, red) in the biofilm. Images of the full biofilm thickness were analyzed with Imaris image analysis software to create full-depth three-dimensional images of the biofilm in order to separately quantitate the amount of *S. mutans* and EPS in each field. Six strains representing serotype *c* (n=3) and *k* (n=3) and three CBP genotypes *cbm*-/*cnm*- (n=3), *cbm*+/*cnm*- (n=2) and *cbm*-/*cnm*+ (n=1) were selected for CLSM analyses (see Table 3.1). Representative images of single CLSM fields are shown in Figure 3.5. In most images, *S. mutans* and EPS were found interspersed throughout the biofilm. Images of strain 219-1-PP30-07-07 contained towers of *S. mutans* with little to no EPS visible within the towers. Analyses of the red and green pixels in each image revealed that neither serotype nor genotype had a significant effect on the amount of *S. mutans* in the 24-hour biofilms (p=0.8325 and 0.3391, respectively). In contrast, the effects of serotype and genotype on EPS production were significant (p=0.0145 and <0.0001, respectively). As shown in Table 3.5, the *cbm*-/*cnm*+ strain produced significantly more EPS than the *cbm*+/*cnm*- and *cbm*-/*cnm*- strains (p<0.0001 and <0.0001). The serotype *c* strains produced significantly more EPS than the serotype *k* strains (p=0.0145); however, this observation is confounded because the single *cnm*

positive strain studied by CLSM is serotype *c*. The amount of EPS produced by the strains lacking the *cnm* gene was not different for strains with or without the *cbm* gene (p=0.9396).

CLSM analyses revealed that treatment of the biofilm with arginine and nicotine had significant effects on both *S. mutans* (p=0.0069) and EPS production (p=0.0032). Addition of 4 mg/ml nicotine to TSBS resulted in increased volume of *S. mutans* compared to biofilm grown in TSBS alone and arginine supplemented media with or without nicotine (Figure 3.6A). Addition of arginine to nicotine decreased *S. mutans* bacterial cell volume compared to nicotine alone (p=0.0404, Table 3.6) and was not significantly different from biofilm grown in TSBS or arginine only. Similarly, nicotine increased EPS production but addition of arginine with nicotine decreased EPS (p=0.0006). In order to assess the combined effects on *S. mutans* volume and EPS production, the ratio of EPS to *S. mutans* was evaluated for each genotype and treatment (Figure 3.6B). The ratio of EPS to *S. mutans* volume was increased for the strain with the *cnm* gene compared to strains with *cbm* and those lacking both genes. Addition of nicotine, arginine, or the combination to TSBS decreased the EPS:*S. mutans* volume ratio significantly indicating that the bacterial volume increased disproportionally to EPS production in the presence of nicotine and/or arginine.

DISCUSSION

Previous studies have reported that compared to serotype *c* strains of *S. mutans*, serotype *k* strains produce less glucose-induced acid, and demonstrate low levels of sucrose-dependent adhesion and dextran-binding activity (Lapirattanakul, Nakano et al. 2009, Yamamoto and Takada 2011). Additionally, serotype *k* strains are less susceptible to phagocytosis by human polymorphonuclear leukocytes, which may explain the increased prevalence of serotype *k* strains in infected mammalian soft tissues (Lapirattanakul, Nakano et al. 2009). I report a large survey of the biofilm growth characteristics of *S. mutans* serotype *k* strains in comparison to *S. mutans* serotype *c* strains. There was an insignificant increase in biomass of *S. mutans* serotype *c* strains compared to serotype *k* strains when grown in TSBS alone. In contrast, the metabolic activity of the serotype *k* strains was increased compared to the serotype *c* strains studied but this also was not significant. CLSM analyses of 3 strains of each serotype did not reveal statistical differences between the serotypes for volume of either bacterial cells or EPS. These observations expand on an earlier study of serotype *k* strains NUM-

Smk-51, NUM-Smk52 and NUM-Smk89, which were also used in the present study, wherein sucrose-induced glucan synthesis and dental plaque formation by these three serotype *k* strains were similar to serotype *c* strains (Yamamoto and Takada 2011). Yamamoto and Takada reported lower acid production in the presence of glucose by these serotype *k* strains compared to serotype *c* strains, which may relate to the idea that serotype *k* strains are less cariogenic than serotype *c* strains. The serotype of the *S. mutans* strains used in this study did not have a significant effect on sucrose-induced biofilm growth.

Thirty of the strains were previously genotyped for CBP genes allowing analysis of the effects of the *cbm* and *cnm* genes on development of sucrose-induced biofilm. The CBP genotype of the strain had significant effects on biofilm production. When grown in TSBS alone, *cbm*-/*cnm*+ strains produced significantly more biofilm and metabolic activity was increased compared to *cbm*-/*cnm*- strains. The *cbm*+/*cnm*- strains produced moderately increased biofilm, but the metabolic activity of these strains was significantly higher than that of the strains lacking both genes. While *cbm*+/*cnm*- and *cbm*-/*cnm*+ strains are not statistically different from each other, there does not appear to be a synergistic effect of *cbm* and *cnm* on biofilm production as both biomass and metabolic activity were decreased for *cbm*+/*cnm*+ strains compared to strains possessing just one of the CBP genes. In fact, the biomass and metabolic activity of *cbm*+/*cnm*+ strains were not statistically different from the strains lacking both genes. In the CLSM studies, the *cbm*-/*cnm*+ strain produced significantly more EPS than the *cbm*+/*cnm*- and *cbm*-/*cnm*- strains. Due to the small number of strains used for the CLSM experiments, caution must be used in interpretation of the results; however, data are sufficiently intriguing to warrant investigation with additional strains to substantiate this observation.

The addition of 4 mg/ml of nicotine to TSBS significantly increased both the biomass and the metabolic activity of *S. mutans* biofilms. This is the first report of the effects of nicotine on serotype *k S. mutans*, and I report no significant differences in biomass, metabolic activity or EPS production compared to serotype *c* strains upon exposure to nicotine. My findings are consistent with previous studies using *S. mutans* serotypes *c, e* and *f*, wherein *S. mutans* biofilm growth and metabolism were increased in a nicotine dose dependent manner (Huang, Li et al. 2012, Li, Huang et al. 2013, Huang, Li et al. 2015). In the present study, nicotine was added at the beginning of biofilm formation, whereas in the 2012 study by Huang et al. nicotine was added to

established 24-hour *S. mutans* biofilms, and the metabolic activity was measured 24 hours later. Given that similar nicotine dose response profiles were reported for nascent and established biofilm, comparatively these studies indicate that nicotine can increase *S. mutans* biofilm growth regardless of when nicotine exposure occurs.

The CLSM studies with six *S. mutans* stains revealed that 4 mg/ml nicotine increased *S. mutans* bacterial volume. My findings are supported by previous studies of *S. mutans* UA159 (serotype *c*) biofilm, which reported increased bacterial volume with nicotine doses of 2 and 4 mg/ml in single- and dual-species biofilms (Li, Huang et al. 2014, Huang, Li et al. 2015). In studies of cigarette smoke exposure, another *S. mutans* strain (ATCC 25175) displayed increased colony size as the nicotine content of the cigarette increased (Zonuz, Rahmati et al. 2008). Zonuz, et al. reported a 51% greater increase in *S. mutans* colony size compared to *Streptococcus sanguinis*. In this study of 6 strains, nicotine alone did not have a significant effect on EPS production ($p=0.3669$), whereas other single- and dual-species biofilm studies found increased EPS production in the presence of nicotine. Single-species studies of sucrose-induced UA159 biofilm found that the EPS volume was significantly increased by addition of 2 and 4 mg/ml nicotine (Huang, Li et al. 2015). In dual-species biofilm with *S. sanguinis*, EPS production by UA159 in 2 mg/ml nicotine was significantly increased compared to biofilm grown without nicotine (Li, Huang et al. 2014). In both studies, only UA159 was investigated. My data suggest that increased EPS production in the presence of nicotine may be a characteristic of UA159 and may not be attributable to all strains of *S. mutans*.

Overall, addition of 10 mg/ml arginine to sucrose-induced biofilm had no effect on the metabolic activity of *S. mutans* biofilm. Collectively, the biomass was unchanged by addition of 10 mg/ml arginine for the strains investigated. In contrast, *cbm*+/*cnm*- strains produced significantly less biomass in the presence of 10 mg/ml arginine compared to TSBS alone. Significantly more biomass was produced by *cbm*-/cnm+ strains than *cbm*+/*cnm*- and *cbm*-/*cnm*- genotypes in the presence of arginine. The metabolic activities of the *cbm*-/cnm+ strains were greater than those of the strains lacking both genes but were not statistically different from *cbm*+/*cnm*- strains. CLSM studies showed that strains with a CBP gene treated with arginine had greater proliferation. In contrast, EPS production was decreased by addition of arginine. There was no significant change in either *S. mutans* or EPS volume in the presence of arginine; however, the EPS:*S. mutans* bacteria ratios were significantly lower when compared to biofilm grown in TSBS alone indicating a decrease in EPS relative to *S. mutans* volume. These data are

consistent with previous studies of single type strains of *S. mutans*,, which have shown that addition of arginine results in increased biomass with decreased EPS production (UA159) and a more fragile sucrose-induced *S. mutans* biofilm (UA140) compared to biofilm grown in the absence of arginine (Sharma, Lavender et al. 2014, Zheng, Cheng et al. 2015, He, Hwang et al. 2016, Huang, Zhang et al. 2017). Using two different nutrient-rich media, Chakraborty and Burne demonstrated that 1.5% arginine induced decreases in overall biofilm production and differences in UA159 growth curves (Chakraborty and Burne 2017). The relative amount of L-arginine and the growth media used in these experiments vary thereby demonstrating that the effect of arginine is not dependent upon the specific growth media and that arginine exerts its effect over a range of concentrations (0.5 to 10%).

Nicotine upregulates *S. mutans* UA159 virulence genes, glucosyltransferases, glucan binding proteins and bacteriocin production (Huang, Li et al. 2015, Li, Huang et al. 2016, Liu, Qiu et al. 2017) (Shepherd et al., unpublished). *S. mutans* antigen I/II was upregulated in strain NG8 biofilm in the presence of nicotine (Li, Huang et al. 2013). In contrast, Chakraborty and Burne demonstrated that arginine downregulates genes encoding virulence factors responsible for attachment, competence development and bacteriocin production (Chakraborty and Burne 2017). *S. mutans* displayed lower tolerance to environmental acid and oxidative stress in the presence of arginine (Chakraborty and Burne 2017). Because nicotine and arginine exert opposite effects on *S. mutans* UA159, I investigated the combined effects on biofilm production by UA159 and 32 additional strains of *S. mutans*. My results support published observations in that nicotine enhanced biofilm production and arginine decreased biofilm. Furthermore, combined arginine and nicotine enhanced biomass and metabolic activities compared to nicotine alone as shown by the crystal violet and XTT experiments. The CLSM experiments with a limited number of strains demonstrated that addition of arginine to nicotine resulted in significantly decreased volume of *S. mutans* for the *cbm*+/*cnm*- strains and decreased EPS volume for all strains compared to biofilm grown with nicotine alone. These data indicate that arginine diminishes nicotine enhanced EPS production. The nicotine enhanced *S. mutans* bacterial growth of strains bearing the *cbm* gene was reduced by adding arginine to nicotine. Additional studies are required to determine the mechanism(s) by which arginine reduces nicotine increased EPS production; however, one might speculate that arginine acts upon regulatory processes or upstream of the point of nicotine interaction with genes that control EPS production.

Two clinical studies found that tobacco smokers do not have increased *S. mutans* in saliva compared to non-smokers (Sheth, Makda et al. 2016, Nakonieczna-Rudnicka and Bachanek 2017). These studies did not type for CBP in oral isolates. I found nicotine enhanced biofilm production for all strains. The biofilm metabolic activities for strains bearing *cbm* and *cnm* genes were increased compared to strains without genes for CBP. Previous studies have shown that the *cnm* gene contributes to oral colonization by *S. mutans*, and is a predictor of development and severity of dental caries lesions (Nomura, Nakano et al. 2009, Miller, Aviles-Reyes et al. 2015, Esberg, Sheng et al. 2017). The *cbm* gene has 78% identity with the *cnm* gene, and *cbm*+ strains exhibit greater collagen binding activity than strains bearing the *cnm* gene (Nomura, Nakano et al. 2012). There is controversy over the relationship between the two genes, which have been reported to be located within the same locus, whereas others have reported multiple strains bearing both genes simultaneously (Aviles-Reyes, Miller et al. 2017, Momeni, Ghazal et al. 2019). My data from multiple strains indicate similarities between *cbm* and *cnm* in biomass and metabolic activities for strains with either gene. Strains bearing both genes do not demonstrate synergistic effects of the genes and were found to have lower responses to nicotine than strains with either gene alone. These observations are indicative of *cbm* and *cnm* being separate genes with significant sequence identity.

In conclusion, serotype *c* and serotype *k* strains are not significantly different in terms of biofilm production or responses to nicotine and arginine. The presence of *cbm* and *cnm* affected biofilm growth and responses to nicotine such that strains with a CBP produced more biomass and were more metabolically active. Addition of nicotine resulted in increased biofilm for all strains. Arginine added with nicotine reduced the quantity of bacteria and EPS in the biofilm. In as much as arginine has been added to fluoride in dentifrices, this combination may be especially beneficial for tobacco users due to the ability of arginine to reduce nicotine induced EPS production.

Table 3.1 *S. mutans* strains used in this Chapter.

Strain	Serotype	*cbm*	*cnm*	Source
106-1-PP3-06-01*	*k*	-	-	S. Momeni, N. Childers[1]
151-1-PP19-07-05*	*k*	-	-	S. Momeni, N. Childers
505-1-PBB-05-06	*k*	-	-	S. Momeni, N. Childers
548-1-PBT-05-04*	*k*	+	-	S. Momeni, N. Childers
573-1-PBS-05-03	*k*	-	-	S. Momeni, N. Childers
578-1-PBB-06-01	*k*	+	-	S. Momeni, N. Childers
608-1-PBB-06-03	*k*	+	-	S. Momeni, N. Childers
AT1	*k*	+	-	K. Nakano[2]
FT1	*k*	-	-	K. Nakano
LJ23	*k*	-	+	K. Nakano
NN2193-1	*k*	+	-	K. Nakano
NN2323M-1	*k*	+	-	K. Nakano
NUM-Smk51	*k*	n/a[3]	n/a	K. Takada[4]
NUM-Smk52	*k*	n/a	n/a	K. Takada
NUM-Smk89	*k*	n/a	n/a	K. Takada
OR22P1	*k*	-	+	K. Nakano
SA31	*k*	+	-	K. Nakano
SA53	*k*	-	+	K. Nakano
TLJ106-1	*k*	-	-	K. Nakano
TLJ11b	*k*	+	-	K. Nakano
TLJ60a	*k*	-	+	K. Nakano
TLJ85d	*k*	+	-	K. Nakano
YT1	*k*	+	-	K. Nakano
107-1-PP3-07-02*	*c*	+	-	S. Momeni, N. Childers
173-1-PP3-06-05	*c*	-	-	S. Momeni, N. Childers
196-1-PP3-07-04	*c*	-	+	S. Momeni, N. Childers
219-1-PP30-07-07*	*c*	-	+	S. Momeni, N. Childers
247-1-PP30-07-03	*c*	+	+	S. Momeni, N. Childers
531-1-PBI-07-06	*c*	+	-	S. Momeni, N. Childers
582-1-PBB-06-02	*c*	+	+	S. Momeni, N. Childers
586-1-PBT-07-02	*c*	+	-	S. Momeni, N. Childers
608-1-PBB-06-01	*c*	+	+	S. Momeni, N. Childers
UA159 *	*c*	-	-	ATCC 700610

[1]University of Alabama School of Dentistry, Birmingham, AL, USA

[2]Osaka University Graduate School of Dentistry, Osaka, Japan

[3] Not available (n/a)

[4]Nihon University School of Dentistry at Matsudo, Chiba, Japan

* Denotes strains used in CLSM analyses

Table 3.2 The effects of *S. mutans* serotype on biofilm responses to arginine (10 mg/ml) and nicotine (4 mg/ml).

		Serotype				
		c		***k***		
		OD 490nm		OD 490nm		
Arginine (10 mg/ml)	**Nicotine (4 mg/ml)**	**mean**	**SD**	**mean**	**SD**	**P-value**
Crystal Violet – Biofilm Mass						
-	-	0.717	0.217	0.671	0.225	0.1987
+	-	0.689	0.198	0.628	0.209	0.1127
-	+	0.938	0.308	1.019	0.397	0.6341
+	+	0.930	0.363	1.066	0.463	0.1884
XTT – Biofilm Metabolic Activity						
-	-	0.461	0.144	0.518	0.291	0.2479
+	-	0.464	0.131	0.508	0.297	0.2836
-	+	0.542	0.159	0.657	0.372	0.2308
+	+	0.573	0.170	0.680	0.391	0.5271

Table 3.3 The effects of *S. mutans* CBP genotype on biofilm mass measured by crystal violet in response to 10 mg/ml arginine and 4 mg/ml nicotine.

		Genotype (n)		vs. arginine	vs. nicotine	vs. arginine + nicotine	vs. *cbm+ cnm+*	vs. *cbm+ cnm-*	vs. *cbm- cnm+*
		OD 490nm							
Arginine	**Nicotine**	**mean**	**SD**	**p**	**p**	**p**	**p**	**p**	**p**
		cbm-/cnm- (7)							
-	-	0.606	0.212	0.4014	0.0000	0.0000	0.6737	0.0666	0.0032
+	-	0.584	0.228		0.0000	0.0000	0.3106	0.3165	0.0070
-	+	0.872	0.388			0.7210	0.1125	0.016	0.0000
+	+	0.862	0.365				0.9594	0.1026	0.0000
		cbm+/cnm+ (3)							
-	-	0.626	0.259	0.4596	0.1876	0.0000		0.3535	0.0554
+	-	0.661	0.206		0.2028	0.0000		0.7251	0.2650
-	+	0.688	0.309			0.0001		0.0004	0.0000
+	+	0.837	0.285					0.2379	0.0006
		cbm+/cnm- (13)							
-	-	0.712	0.233	0.0001	0.0000	0.0000			0.1204
+	-	0.647	0.201		0.0000	0.0000			0.0340
-	+	1.029	0.344			0.0481			0.0167
+	+	1.020	0.440						0.0006
		cbm-/cnm+ (7)							
-	-	0.777	0.133	0.1199	0.0000	0.0000			
+	-	0.746	0.181		0.0000	0.0000			
-	+	1.190	0.243			0.3527			
+	+	1.284	0.308						

Table 3.4 The effects of *S. mutans* CBP genotype on biofilm metabolic activity in response to 10 mg/ml arginine and 4 mg/ml nicotine.

Arginine	Nicotine	Genotype (n) OD 490nm mean	SD	vs. arginine p	vs. nicotine p	vs. arginine + nicotine p	vs. *cbm+ cnm+* p	vs. *cbm+ cnm-* p	vs. *cbm- cnm+* p
		cbm-/cnm- (7)							
-	-	0.355	0.267	0.2801	0.0008	0.0000	0.5376	0.0002	0.0000
+	-	0.341	0.257		0.0000	0.0000	0.2480	0.0001	0.0000
-	+	0.419	0.34			0.5754	0.1894	0.0000	0.0000
+	+	0.43	0.347				0.0708	0.0000	0.0000
		cbm+/cnm+ (3)							
-	-	0.419	0.144	0.1775	0.0000	0.0000		0.0359	0.0040
+	-	0.464	0.095		0.0000	0.0000		0.1080	0.0172
-	+	0.517	0.119			0.0023		0.0214	0.0022
+	+	0.572	0.113					0.1518	0.0032
		cbm+/cnm- (13)							
-	-	0.593	0.232	0.4294	0.0000	0.0000			0.1702
+	-	0.573	0.194		0.0000	0.0000			0.1894
-	+	0.745	0.282			0.9997			0.1742
+	+	0.745	0.285						0.0172
		cbm-/cnm+ (7)							
-	-	0.654	0.136	0.4162	0.0000	0.0000			
+	-	0.65	0.156		0.0000	0.0000			
-	+	0.814	0.206			0.0001			
+	+	0.914	0.205						

Table 3.5 Pairwise comparisons of the effects of genotype on *S. mutans*, EPS and EPS:*S. mutans* ratio in biofilm as measured by CLSM.

		Direction of effect		**p-value**
S. mutans				
	cbm+/cnm-		*cnm-/cnm+*	n.s.
	cbm+/cnm-		*cbm-/cnm-*	n.s.
	cnm-/cnm+		*cbm-/cnm-*	n.s.
EPS				
	cbm+/cnm-	**<**	*cnm-/cnm+*	<0.0001
	cbm+/cnm-		*cbm-/cnm-*	0.9396
	cnm-/cnm+	**>**	*cbm-/cnm-*	<0.0001
EPS : *S. mutans*				
	cbm+/cnm-	**<**	*cnm-/cnm+*	0.0001
	cbm+/cnm-		*cbm-/cnm-*	0.4316
	cnm-/cnm+	**>**	*cbm-/cnm-*	<0.0001

n.s. = not significant

Table 3.6 Pairwise comparisons of the effects of treatment on S. mutans, EPS and EPS:S. mutans ratio in biofilm as measured by CLSM.

		Direction of effect		p-value
S. mutans				
	TSBS		arginine	0.2437
	TSBS	**<**	nicotine	0.0007
	TSBS		arginine + nicotine	0.1748
	arginine	**<**	nicotine	0.0202
	arginine		arginine + nicotine	0.8213
	nicotine	**>**	arginine + nicotine	0.0404
EPS				
	TSBS		arginine	0.1374
	TSBS		nicotine	0.3669
	TSBS	**>**	arginine + nicotine	0.0061
	arginine	**<**	nicotine	0.0227
	arginine		arginine + nicotine	0.1783
	nicotine	**>**	arginine + nicotine	0.0006
EPS : *S. mutans*				
	TSBS	**>**	arginine	0.0022
	TSBS	**>**	nicotine	0.0332
	TSBS	**>**	arginine + nicotine	0.0001
	arginine		nicotine	0.3356
	arginine		arginine + nicotine	0.3469
	nicotine		arginine + nicotine	0.0649

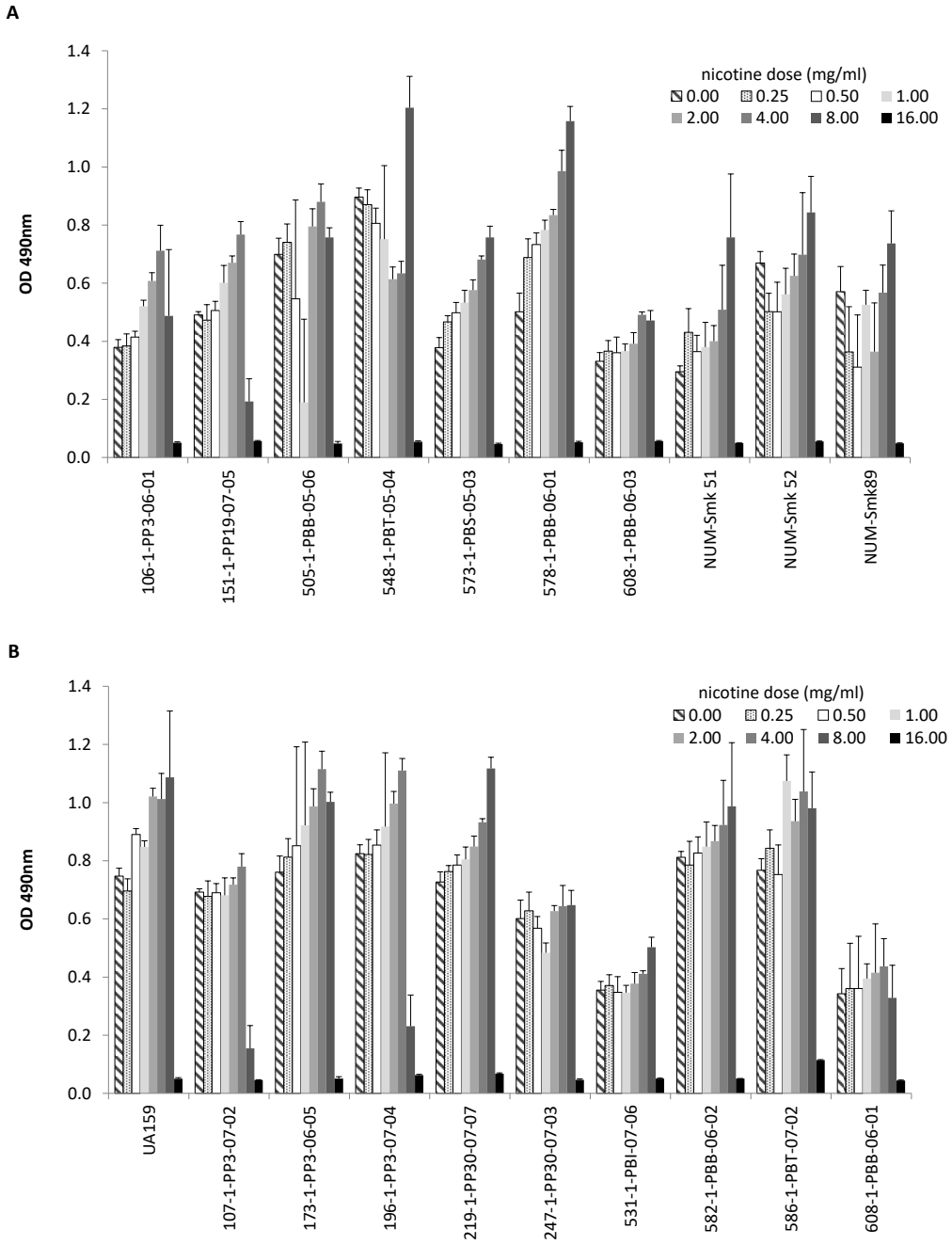


Figure 3.1 Effects of nicotine on *S. mutans* biofilm formation. Nicotine was added to TSBS in doubling doses from 0.25 to 16.0 mg/ml prior to addition of *S. mutans* into quadruplicate wells of a 96-well plate and incubation at 37ºC, 5% CO_2 for 24 hours. The 24-hour biofilm from 10 serotype *k* (A) and 10 serotype *c* (B) strains was stained with crystal violet. The mean absorbance for each dose is shown. The error bars represent the standard deviation of quadruplicates. The bars with diagonal lines represent TSBS without nicotine, and bars on the right represent increasing doses of nicotine.

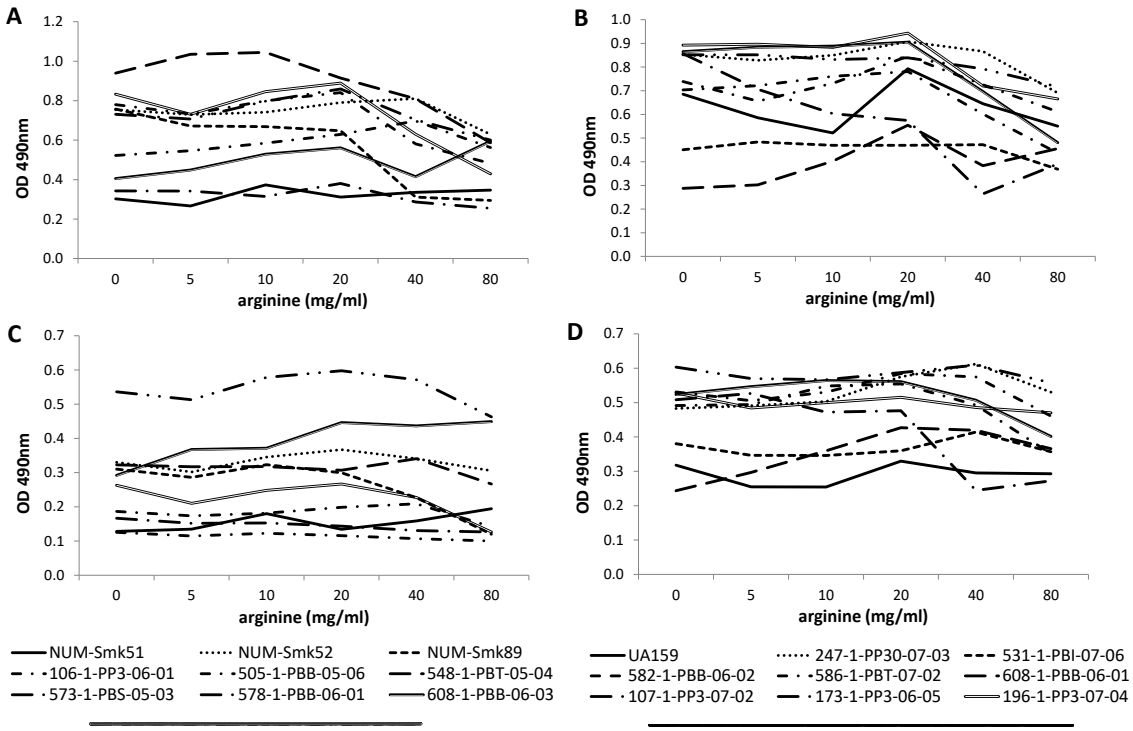
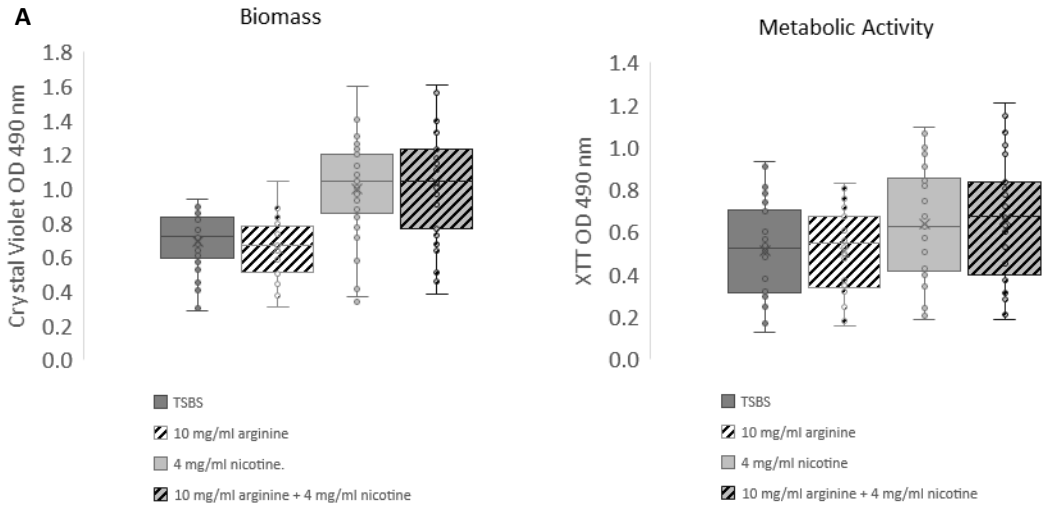


Figure 3.2 Selected strains of *S. mutans* serotypes *k* and *c* biofilm in studies of dose response to arginine. Biomass of serotype *k* (A) and *c* (B) strains stained with crystal violet. Metabolic activity of serotype *k* (C) and *c* (D) strains measured by the XTT assay.



C

	10 mg/ml arginine p	4 mg/ml nicotine p	10 mg/ml arginine + 4 mg/ml nicotine p
Biomass			
TSBS	0.0561	0.0000	0.0000
10 mg/ml arginine		0.0000	0.0000
4 mg/ml nicotine			0.0028
Metabolic Activity			
TSBS	0.6107	0.0000	0.0000
10 mg/ml arginine		0.0000	0.0000
4 mg/ml nicotine			0.0021

Figure 3.3 Bar and whiskers graphs showing the effects of arginine and nicotine on *S. mutans* 24-hour biofilm growth and metabolic activity. Biofilm from all 33 strains was grown for 24-hours in TSBS (dark gray), 10 mg/ml arginine (black and white diagonal), 4 mg/ml nicotine (light gray), or 10 mg/ml arginine plus 4 mg/ml nicotine (gray and black diagonal) and then (A) stained with crystal violet to measure biomass or (B) tested for metabolic activity by the XTT assay. Statistical differences are shown in (C). Each data point represents the average of quadruplicate biofilm values from 3 independent experiments.

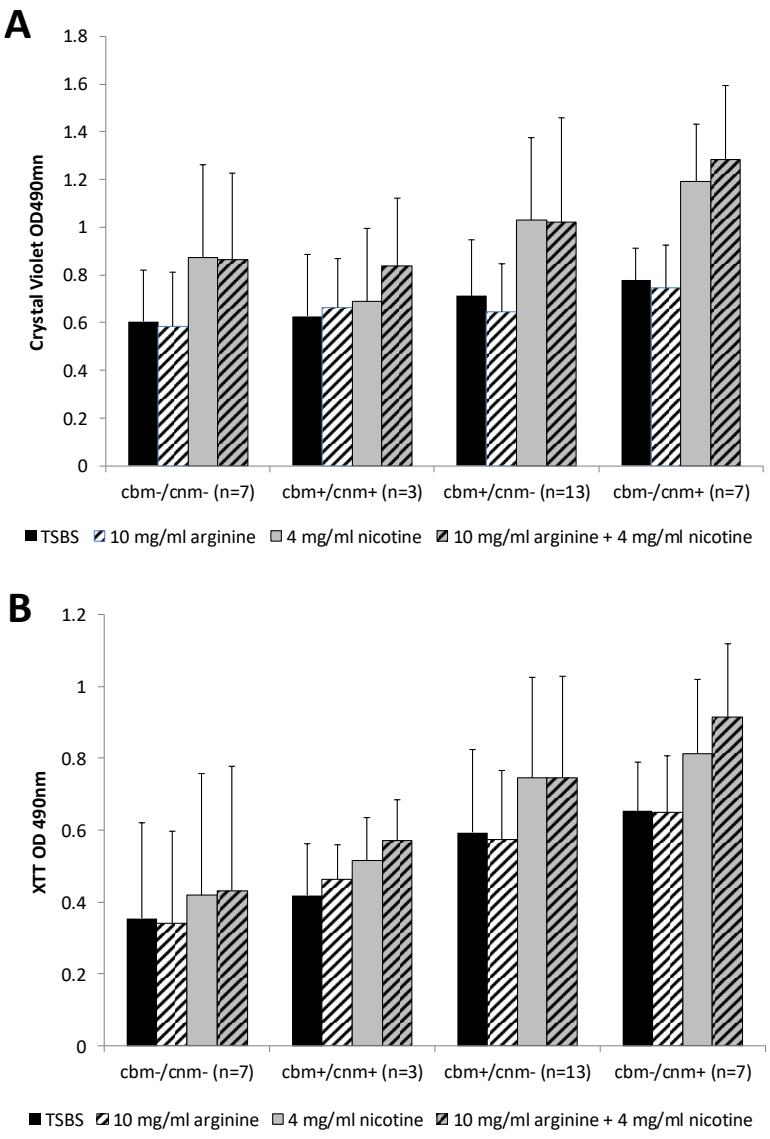


Figure 3.4 Effects of *S. mutans* genotype on biofilm production in the presence of arginine (10 mg/ml), nicotine (4 mg/ml), and arginine plus nicotine. Arginine and nicotine were added separately and together to TSBS prior to addition of *S. mutans* cultures into quadruplicate wells of a 96-well plate and incubation at 37ºC, 5% CO_2 for 24 hours. The 24-hour biofilm was stained with crystal violet (A) to determine biomass, and the XTT reduction assay (B) was performed to determine metabolic activity of each biofilm. The mean absorbance in TSBS is shown by black bars, 10 mg/ml arginine in black and white diagonals, 4 mg/ml nicotine in gray, and 10 mg/ml arginine + 4 mg/ml nicotine is shown as black diagonals on a gray background. Error bars represent the standard deviation of quadruplicates from three separate experiments. N indicates the number of strains.

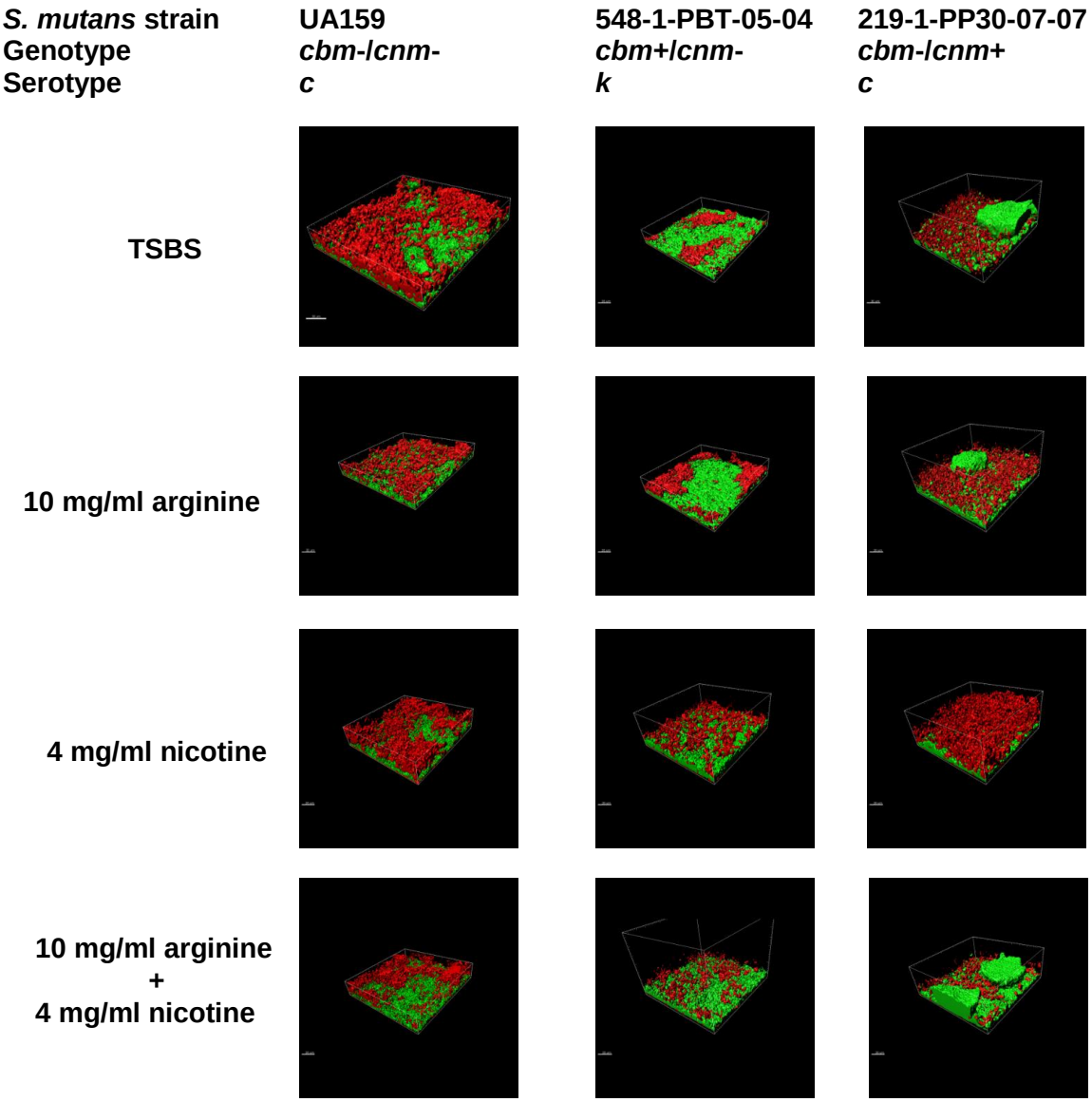


Figure 3.5 Representative processed Z-stack images from CLSM analyses of *S. mutans* biofilm grown in TSBS alone, 10 mg/ml arginine, 4 mg/ml nicotine, or 10 mg/ml arginine + 4 mg/ml nicotine. *S. mutans* bacterial cells are labeled green with Syto9 and the EPS labeled with Alexa Fluor 568 is red.

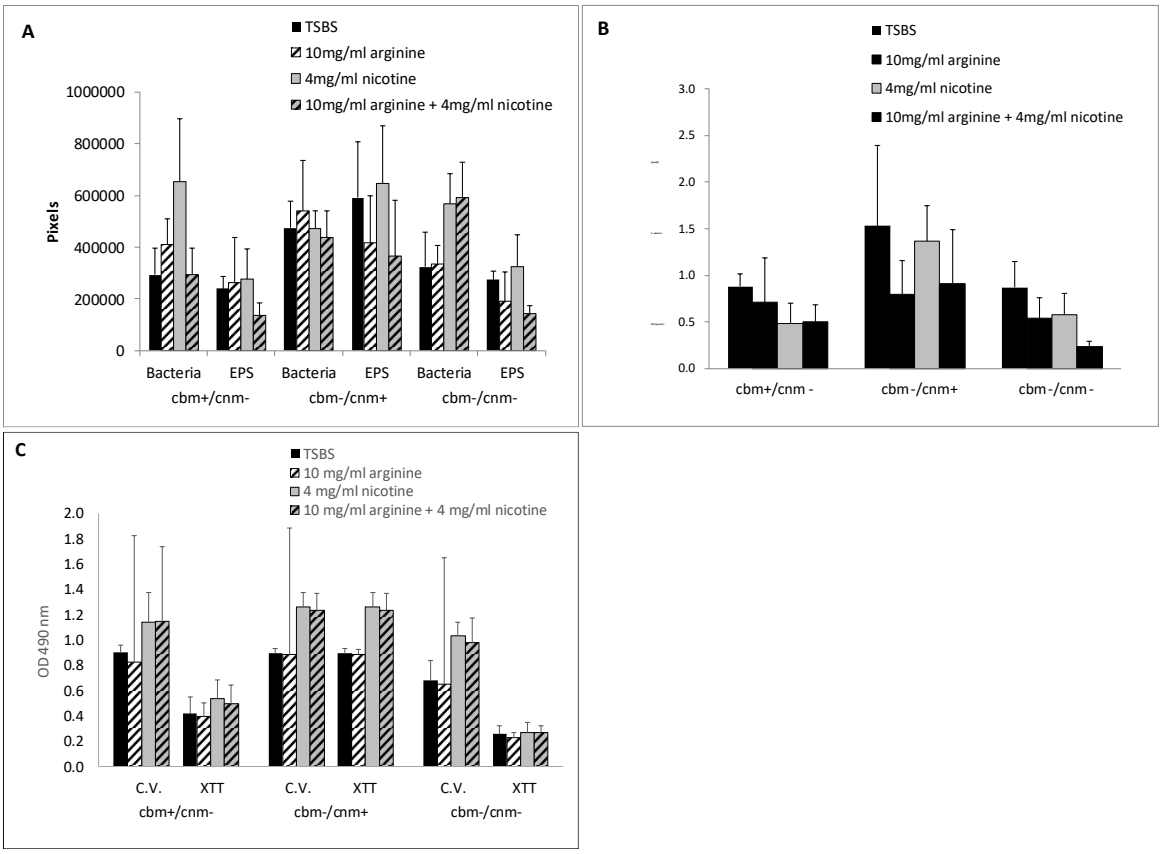


Figure 3.6 *S. mutans* bacterial cells and EPS from 24-hour biofilm measured by CLSM. The effects of 10 mg/ml arginine, 4 mg/ml nicotine and the combination on *S. mutans* biofilm were quantified by CLSM. Alexa Fluor 568 labeled dextrans were added at the beginning of biofilm formation to label the EPS as it was synthesized. Arginine and nicotine were added simultaneously with the Alexa Fluor 568 labeled dextrans. Prior to imaging the 24-hour biofilm, *S. mutans* DNA was labeled with Syto 9. Biofilm grown in TSBS alone (black) was compared to biofilm grown with 10 mg/ml arginine (black and white diagonal), 4 mg/ml nicotine (gray), and 10 mg/ml arginine plus 4 mg/ml nicotine (black and gray diagonal). (A) Total pixels representing bacterial cells and EPS for each CBP genotype of *S. mutans* tested by CLSM. (B) Ratio of EPS to *S. mutans* bacterial cells. (C) Crystal violet and XTT analyses of the specific subset of strains used in the CLSM experiments. Each bar represents analyses of 3 to 7 fields from each biofilm and error bars represent standard deviation.

Chapter Four

ENDOTHELIAL CELL INVASION BY *S. MUTANS* GROWING IN PLANKTONIC CULTURE

INTRODUCTION

S. mutans, a native resident of oral plaque, is the causative agent of dental caries. Carbohydrate fermentation by *S. mutans* within the oral plaque creates an acidic environment at the tooth surface causing demineralization and erosion of dental hard tissues. When it escapes the oral cavity, *S. mutans* can cause bacteremia and invade extra-oral soft tissue leading to IE, atherosclerotic plaques and cerebrovascular stroke (Nomura, Nakano et al. 2006, Nakano, Nemoto et al. 2009, Nakano and Ooshima 2009, Nomura, Naka et al. 2013, Miyatani, Kuriyama et al. 2015, Inenaga, Hokamura et al. 2018).

Strains of *S. mutans* are defined by serotype *c, e, f* or *k* according to the structure of cell surface polysaccharides that are comprised of a rhamnose backbone with glucose side chains. The ratio of glucose to rhamnose is drastically decreased in serotype *k* strains, which may contribute to the increased infectivity of serotype *k* strains (Nakano and Ooshima 2009). In the oral cavity, more than 70% of *S. mutans* strains are of serotype *c,* serotype *e* strains represent approximately 20%, and the frequencies of serotypes *f* and *k* are less than 5% of oral *S. mutans* (Nakano, Nemoto et al. 2007, Nomura, Nakano et al. 2012). Serotype *k* strains represent roughly 1.5% of the *S. mutans* isolated from the oral cavities of healthy subjects; however, within the oral cavities of patients with CVD, 10.3% of *S. mutans* strains were found to be serotype *k* ($p<0.05$) (Nakano, Nemoto et al. 2007). In addition, the oral cavities of patients with CV diseases are more likely to harbor *S. mutans* of multiple serotypes compared to healthy subjects (53.8% versus 25.0%, respectively) (Nakano, Nemoto et al. 2007). *S. mutans* serotype *k* strains were reported in 9.1% of heart valve and 25% of atheromatous tissue specimens, and are increased in prevalence in the cardiovascular tissue compared to the oral cavities of the same patients (Nakano, Nemoto et al. 2007). These data suggest that serotype *k* strains of *S. mutans* may adhere to, or interact with, endothelial cells at greater frequency than strains of serotype *c,* the most prevalent serotype in the oral cavity.

Blood isolates of *S. mutans* serotype *k* from patients with bacteremia are less susceptible to phagocytosis by human polymorphonuclear leukocytes which may explain

the longer duration of bacteremia caused by *S. mutans* serotype *k* compared to strains of other serotypes (Nakano and Ooshima 2009). *In vitro* studies demonstrated that isogenic *S. mutans* mutants lacking surface antigens were less susceptible to phagocytosis than parent strains (Nakano and Ooshima 2009). The diminished presence of glucose side chains of serotype *k S. mutans* may be similar to the isogenic mutants lacking surface antigens and enable serotype *k* strains to evade phagocytosis. Serotype *k* strains may persistent longer in blood than serotype *c* strains, and this persistence may contribute to the increased frequency of serotype *k* strains in cardiovascular tissue compared to oral plaque (Nagata, Okayama et al. 2006, Nakano and Ooshima 2009).

Expression of CBPs, SpaP (protein antigen I/II, PA I/II), WpaA, Cbm and Cnm on the surface of *S. mutans* impart this bacterium with the ability to interact with the extracellular matrix of tissues and organs (Aviles-Reyes, Miller et al. 2017). The CBP Cbm and Cnm are produced from the *cbm* and *cnm* genes, respectively (Abranches, Miller et al. 2011, Nomura, Nakano et al. 2012, Aviles-Reyes, Miller et al. 2017). Studies have demonstrated that *cbm* and *cnm* confer upon *S. mutans* the ability to invade non-phagocytotic mammalian cells leading to chronic and systemic infections (Abranches, Zeng et al. 2009, Abranches, Miller et al. 2011, Lapirattanakul, Nakano et al. 2011, Nomura, Nakano et al. 2012, Miller, Aviles-Reyes et al. 2015). The *cnm* gene is present in approximately 20% of *S. mutans* strains but is concentrated in serotype *f* strains such that 80% of serotype *f* strains bear the gene (Abranches, Miller et al. 2011). In contrast, the *cbm* gene is found in roughly 2% of *S. mutans* and is usually identified in serotype *k* strains (Aviles-Reyes, Miller et al. 2017). Many studies of endothelial cell invasion have focused on *S. mutans* serotypes *e* and *f* strains (Abranches, Zeng et al. 2009, Abranches, Miller et al. 2011, Aviles-Reyes, Freires et al. 2018). Studies of endothelial cell adhesion and invasion by serotype *k* strains have been performed with isolates from Japan, Finland and Thailand (Nomura, Nakano et al. 2012, Lapirattanakul, Nomura et al. 2013). To date, no endothelial cell invasion studies have been performed with serotype *k* strains from the United States.

We investigated the effects of *S. mutans* CBP genes, *cbm* and *cnm*, on endothelial cell invasion by 20 serotype *k* strains of *S. mutans* in planktonic culture. For this, I used cultured bovine aortic endothelial cells (BAEC), which synthesize and secrete types III and IV collagen that are immunologically and biochemically analogous to basement membrane collagen (Howard, Macarak et al. 1976, Jaffe, Minick et al. 1976, Sage, Crouch et al. 1979). The strains represent clinical isolates obtained from

pediatric subjects in rural Alabama in the United States as well as previously characterized strains from Japan. Serotype *c* and *e* strains with and without *cbm* and *cnm* genes were included in the study in order to investigate the separate effects of serotype and CBP on endothelial cell invasion by *S. mutans*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. mutans strains used in this study are listed in Table 4.1. All strains were oral isolates of known serotype and *cbm*/*cnm* genotype. Most of the strains used in this study were kind gifts of Drs. Noel Childers and Stephanie Momeni at University of Alabama School of Dentistry and Dr. Kazuhiko Nakano, Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, Osaka, Japan. *S. mutans* strains OMZ175 and UA159 were included as well-characterized control strains. Frozen stock of each strain was inoculated into tryptic soy broth (TSB, Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37^{o}C in 5% CO_2 for 18 to 48 hours prior to each experiment. One ml of each overnight culture of *S. mutans* was washed three times with sterile 0.9% saline and resuspended in 1.0 ml of sterile saline. The OD 595nm of each *S. mutans* strain was adjusted to 0.500 +/- 0.150 before diluting 1:100 in BAEC growth media without penicillin and streptomycin. The diluted *S. mutans* cultures were held at 37^{O}C while the endothelial cells were washed in preparation for addition of the *S. mutans*.

BAEC Culture

BAEC were grown in Dulbecco's modified Eagle's medium (DMEM, Corning Life Sciences, New York, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA), 2 mM L-glutamine (Corning Life Sciences), 100 IU penicillin (Corning Life Sciences) and 100 ug/ml streptomycin (Corning Life Sciences) at 37^{o}C with 5% $C0_2$. Cultured BAEC were removed from the surface of the flask by a five-minute incubation with 0.25% trypsin protease (Hyclone, GE Healthcare Life Sciences, Pittsburgh, PA, USA). After washing the detached cells with DMEM growth media, the BAEC were seeded into 24-well plates or into T-75 flasks for further culture. The BAEC used for invasion assays were between passages 6 and 13.

BAEC Invasion Assay

The endothelial cell invasion assay was modified from that developed by Abranches et. al. (Abranches, Miller et al. 2011). Briefly, each well of a 24-well plate was seeded with 0.5, 1.0 or 2.0 x 10^5 BAEC in 1.0 ml of BAEC growth media with antibiotics, and the plate was incubated for 22 hours at 37^{O}C, 5% CO_2. After incubation, the BAEC were washed 3 times with warm (37^{O}C) Dulbecco's phosphate buffered saline (DPBS, GE Healthcare, Life Sciences). After discarding the final wash, 1.0 ml of prepared *S. mutans* in BAEC growth media without antibiotics was added to duplicate wells to facilitate the attachment and invasion of the bacteria into the BAEC and the plate was incubated for 2 hours at 37^{O}C, 5% CO_2. During the incubation, each *S. mutans* inoculum was diluted and plated onto blood agar plates in order to determine the number of colony forming units (cfu) per ml of inoculum. After the 2-hour incubation, the BAEC were washed three times with warm DPBS followed by addition of 1.0 ml of BAEC growth medium with antibiotics and incubation at 37^{O}C, 5% CO_2 for 2 hours to kill any bacteria that attached but did not invade the BAEC. Following the second incubation, the BAEC were washed three times with warm DPBS. After discarding the final wash, 1.0 ml of ice-cold sterile H_2O was added to each well of BAEC followed by a 20-minute incubation at room temperature to lyse the BAEC. After BAEC lysis, each well was scraped to remove BAEC from the plastic surface and the contents of each well was transferred into a separate sterile tube. The lysate from each well was plated onto blood agar plates to determine the cfu of *S. mutans* invading the BAEC. For strains with high invasion rates, 1:10 and 1:100 dilutions of the lysate were required to obtain accurate cfu counts.

The blood agar plates were incubated at 37^{O}C, 5% CO_2 for two days before counting cfu to determine the number of *S. mutans* bacterium in the inoculum and the number that invaded the BAEC in each well. The percent of *S. mutans* invading BAEC in each well was calculated as: cfu/ml invading BAEC divided by cfu/ml in the inoculum times 100. Each strain of *S. mutans* was tested in duplicate in three biological replicate experiments.

Statistical Analyses

Basic statistics (mean, standard deviation) were computed before statistical evaluation by using analyses of ranks and analysis of variance (ANOVA). When non-normality was present, a rank transformation was performed prior to the analysis.

RESULTS

Initial invasion assays using 0.5 x 10^5 BAEC per well in the 24-well plate yielded very low to no invasion by the previously untested *S. mutans* strains from the University of Alabama. Since OMZ175 invasion of endothelial cells has been well documented, titration experiments of BAEC in 24-well plates were performed with OMZ175 prior to continuing the BAEC invasion assays with the untested strains. To do this, BAEC diluted to 0.5 x 10^5, 1.0 x $10^5$and 2.0 x 10^5 were seeded into duplicate wells the day before the invasion experiment with OMZ175. There was no difference in the percentage of OMZ175 invading 0.5 x 10^5 and 1.0 x 10^5 BAEC (Figure 4.1). However, OMZ175 invasion doubled when 2.0 x 10^5 BAEC were seeded into the wells; therefore, subsequent experiments were performed with 2.0 x 10^5 BAEC in each well of the 24-well plate to ensure adequate BAEC for experiments using strains with low invasion rates.

The results of BAEC invasion assays are shown in Figure 4.2 and Table 4.2. Planktonic cultures of serotype *c* and *e* strains demonstrated very low BAEC invasion (range 0.0001% - 0.0011% of the *S. mutans* inoculated). The invasion rate for OMZ175 was 0.0506%. BAEC invasion by serotype *k* strains was highly variable and ranged between 0.0002% for strain 608-1-PBB-06-03 to 0.5911% for NN2323M-1. Overall, serotype had a significant effect on BAEC invasion ($p<0.0001$). Serotype *k* strains had significantly higher rates of invasion than serotypes *c* and *e* strains (Table 4.3). There was no difference in BAEC invasion between OMZ175 and the serotype *k* strains collectively ($p=0.5563$).

Endothelial cell invasion by *S. mutans* has been attributed to the presence of CBP genes *cbm* or *cnm*. OMZ175 bears the *cnm* gene and is a prototype for studying *S. mutans* invasion into endothelial cells. As in prior studies, OMZ175 invaded BAEC. No invasion was seen among five serotypes *c* and *e* strains, four of which possess genes for CBP. Thirteen of 20 serotype *k* strains demonstrated BAEC invasion by at least 0.01% of the bacterial cells in the inoculum. Serotype *k* strain TLJ106-1 displayed significant BAEC invasion although it lacks both CBP genes. Six of the nine serotype *k* strains with the *cbm* gene had measurable BAEC invasion as did three of four *cnm* positive strains. The presence of *cbm* had a significant positive effect on BAEC invasion by planktonic cultures of *S. mutans* ($p=0.0030$); however, the *cnm* gene did not ($p=0.1014$). Significantly increased BAEC invasion was observed for *cbm*+/*cnm*- strains compared to strains lacking the *cbm* gene (Table 4.3). Analysis of the combined effects of serotype and genotype found that the mean invasion rate for serotype *k* strains was

similar between *cbm* positive and *cnm* positive strains (0.1586% and 0.1636%, respectively; Table 4.4).

PA I/II is a well characterized virulence factor of *S. mutans*. Previous studies reported increased adhesion and endothelial cell invasion in strains lacking PA I/II. Figure 4.3 shows the BAEC invasion abilities of the thirteen strains, including OMZ175, typed for expression of PA I/II. All eight of the PA I/II negative strains possess one of the CBP, and all strains demonstrated BAEC invasion. Five PA I/II positive strains, two without CPB and three with *cbm*-/*cnm*+ were tested. In this group, low level BAEC invasion was observed for two of the *cbm*-/*cnm*+ strains. Interestingly, TLJ106-1, a PA I/II positive strain with no CBP gene had the second highest rate of BAEC invasion among all strains tested for BAEC invasion by planktonic cells of *S. mutans*.

Excluding UA159 and OMZ175, eleven of the strains were clinical isolates from children living in the state of Alabama in the United States, and twelve were clinical isolates from Japan. The strains from Japan demonstrated significantly increased BAEC invasion compared to the strains from Alabama ($p<0.0000$). Four of the strains from Alabama were serotype *c* or *e* and possessed CBP genes; BAEC invasion was predicted due to the presence of the CBP genes but was not observed for these strains.

DISCUSSION

Oral streptococci, including *S. mutans*, have been identified in lesions of IE and atherosclerotic plaques (Kozarov, Sweier et al. 2006, Nakano, Inaba et al. 2006, Nakano, Nemoto et al. 2007, Abranches, Miller et al. 2011, Lapirattanakul, Nomura et al. 2013, Fernandes, Oliveira et al. 2014). Additional evidence suggests a role for *S. mutans* in hemorrhagic stroke, ulcerative colitis and inflammatory bowel disease (Nakano, Hokamura et al. 2011, Kojima, Nakano et al. 2012, Kojima, Nomura et al. 2014). At the nexus of these diseases, bacterial invasion of vascular endothelial cells is believed to occur at a site of endothelial cell injury triggering initiation of a local inflammatory response by the host (Nagata, de Toledo et al. 2011, Kozarov 2012). Certain strains of *S. mutans* have been shown to invade endothelial cells, whereas other strains do not (Abranches, Zeng et al. 2009, Lapirattanakul, Nomura et al. 2013). Characteristics of endothelial cell invasive *S. mutans* strains include both the serotype and the presence of certain bacterial cell surface proteins. Serotypes *f* and *k* were more frequently found in CVD lesions than the most common serotype, *c* (Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009). Furthermore, the presence of the CBP, Cbm and

Cnm, on the bacterial cell surface are key elements for endothelial cell invasion (Abranches, Miller et al. 2011, Nomura, Nakano et al. 2012, Otsugu, Nomura et al. 2017). Surveys of large numbers of clinical *S. mutans* isolates have revealed that *cnm* is frequently observed in serotype *f* isolates and that *cbm* is most prevalent in serotype *k* strains (Nomura, Nakano et al. 2012).

Our study focused on BAEC invasion of 20 serotype *k* strains with and without genes for CBP. Serotype *c, e* and *f* strains with CBP and well characterized strains UA159 and OMZ175 were investigated for comparisons. The highest rate of *S. mutans* invasion was observed with the serotype *k* strains compared to seven strains of serotypes *c, e,* and *f*. There were only two serotype *f* strains included in this study; however, the invasion by these strains was not statistically different from the serotype *k* strains. In addition to serotype, cell surface CBPs are key factors in endothelial cell invasion. Without regard for serotype, the *cbm*+/*cnm*- strains demonstrated significantly higher rates of BAEC invasion than strains lacking the *cbm* gene. Increased endothelial cell invasion by *cbm* positive compared to *cnm* positive strains of *S. mutans* is consistent with previous reports (Lapirattanakul, Nakano et al. 2011, Nomura, Nakano et al. 2012). Interestingly, as has been reported by others I found no invasion with two non-serotype *k* *cbm*+/*cnm*- strains (Lapirattanakul, Nomura et al. 2013). This suggests that perhaps serotype *k* in combination with a gene for CBP is necessary for *S. mutans* invasion of endothelial cells.

The presence of a gene for CBP was not enough to confer endothelial cell invasion upon *S. mutans* as evidenced by eight strains with CBP genes that did not invade BAEC. Three of the non-invasive CBP positive strains were serotype *k*. Lapirattanakul, et al. reported that non-invasive *S. mutans* strains with CBP also displayed decreased binding to collagen in addition to lower rates of endothelial cell invasion compared to invasive strains (Lapirattanakul, Nakano et al. 2011). The CBP genes in the strains used in the present study were defined by DNA sequencing; thus, a possible explanation for lack of BAEC invasion by certain CBP gene positive strains is decreased CBP gene expression in these strains compared to the invasive strains. Additional testing by RT-PCR or Western-blot with *cbm* and *cnm* specific antibodies may be helpful to unravel this conundrum.

Another *S. mutans* surface protein, PA I/II, has been proposed to play a role in endothelial cell invasion. Otsugu et. al. recently reported that CBP positive *S. mutans* strains lacking PA I/II aggregated, adhered to bovine heart valves, and in a rat model of

IE, generated bacterial masses on cardiac valves whereas strains positive for both CBP and PA I/II, or CBP negative, PA I/II positive strains did not display these traits (Otsugu, Nomura et al. 2017). PA I/II expression defects resulting from mutations in the gene and transcriptional processing, defects in the *S. mutans* sortase gene or low mRNA expression are more prevalent in serotypes *f* and *k* than in the most common serotype, *c* (Nakano, Nomura et al. 2008, Lapirattanakul, Nomura et al. 2015). *S. mutans* with PA I/II defects are less susceptible to phagocytosis, and as observed in an animal model, the duration of bacteremia as well as the severity of systemic inflammation was increased for PA I/II defective strains compared to PA I/II expressing isolates (Nakano, Nomura et al. 2004, Nakano, Inaba et al. 2006, Nomura, Hamada et al. 2007, Lapirattanakul, Nomura et al. 2015). Thirteen of the strains used in this study have been typed for PA I/II expression (five were PA I/II positive and eight were PA I/II negative). Each of the eight PA I/II negative strains also had a CBP gene, and all demonstrated BAEC invasion of at least 0.05%. In contrast, two of three *cbm*-/*cnm*+ strains expressing PA I/II demonstrated low levels of BAEC invasion; no invasion was observed with the third strain. One of these *cbm*-/*cnm*+ strains, OMZ175, expresses low levels of PA I/II, which may explain BAEC invasion at a similar rate to the least invasive PA I/II negative strain (Palmer, Miller et al. 2013).

TLJ106-1, a serotype *k* strain which lacks both *cbm* and *cnm* but expresses PA I/II, demonstrated the second highest rate of *S. mutans* invasion in my study. Only one of six other *cbm*-/*cnm*- strains demonstrated modest (0.0121%) BAEC invasion; the PA I/II phenotype of this strain is unknown. Fifteen CBP-/PA I/II+ clinical strains in an *ex vivo* adherence model failed to adhere to bovine heart valve specimens demonstrating that PA I/II without CBP is insufficient for adherence, and likely invasion as well (Otsugu, Nomura et al. 2017). Strain TLJ106-1 raises the possibility of an additional unidentified endothelial cell binding protein(s) in this and perhaps in other strains.

In conclusion, significant factors for endothelial cell invasion by planktonic *S. mutans* are the presence of *cbm*, serotypes *k* or *f*, and lack of PA I/II expression. Inasmuch as this was a study of serotype *k* strains, it is not unexpected that *cbm* was identified as a significant factor compared to *cnm* given that the *cbm* gene is predominantly found in serotype *k* strains (Nomura, Nakano et al. 2012). The *cnm* gene is predominant in serotype *f* strains (Nomura, Naka et al. 2013). Complete genetic analysis of strain TLJ106-1 and other CBP negative serotype *k* and *f* invasive strains of

S. mutans would help define other factors promoting endothelial cell invasion by the *S. mutans* strains which escape the oral cavity and host immune surveillance.

Table 4.1 *S. mutans* strains used in this Chapter.

Strain	Serotype	Collagen Binding Protein genes		Source
		cbm	***cnm***	
107-1-PP3-07-02	*c*	+	-	S Momeni, N Childers[1]
196-1-PP3-07-04	*c*	-	+	S Momeni, N Childers
219-1-PP30-07-07	*c*	-	+	S Momeni, N Childers
UA159	*c*	-	-	S Momeni, N Childers
241-1-PP3-07-09	*e*	-	+	S Momeni, N Childers
119-1-PBP-01-01	*f*	+	-	S Momeni, N Childers
OMZ175	*f*	-	+	B Guggenheim
106-1-PP3-06-01	*k*	-	-	S Momeni, N Childers
151-1-PP19-07-05	*k*	-	-	S Momeni, N Childers
505-1-PBB-05-06	*k*	-	-	S Momeni, N Childers
548-1-PBT-05-04	*k*	+	-	S Momeni, N Childers
573-1-PBS-05-03	*k*	-	-	S Momeni, N Childers
578-1-PBB-06-01	*k*	+	-	S Momeni, N Childers
608-1-PBB-06-03	*k*	+	-	S Momeni, N Childers
AT1	*k*	+	-	K Nakano[2]
FT1	*k*	-	-	K Nakano
LJ23	*k*	-	+	K Nakano
NN2193-1	*k*	+	-	K Nakano
NN2323M-1	*k*	+	-	K Nakano
OR22P1	*k*	-	+	K Nakano
SA31	*k*	+	-	K Nakano
SA53	*k*	-	+	K Nakano
TLJ106-1	*k*	-	-	K Nakano
TLJ11b	*k*	+	-	K Nakano
TLJ60a	*k*	-	+	K Nakano
TLJ85d	*k*	+	-	K Nakano
YT1	*k*	+	-	K Nakano

[1]University of Alabama School of Dentistry, Birmingham, AL, USA

[2]Osaka University Graduate School of Dentistry, Osaka, Japan

Table 4.2 Planktonic *S. mutans* invasion of bovine aortic endothelial cells (BAEC).

Strain	% *S. mutans* invading BAEC	
	mean	**SD**
107-1-PP3-07-02	0.0004%	0.0000
196-1-PP3-07-04	0.0001%	0.0000
219-1-PP30-07-07	0.0005%	0.0000
UA159	0.0009%	0.0000
241-1-PP3-07-09	0.0011%	0.0000
119-1-PBP-01-01	0.0013%	0.0000
OMZ175	0.0506%	0.0006
106-1-PP3-06-01	0.0005%	0.0000
151-1-PP19-07-05	0.0008%	0.0000
505-1-PBB-05-06	0.0012%	0.0000
548-1-PBT-05-04	0.0005%	0.0000
573-1-PBS-05-03	0.0121%	0.0001
578-1-PBB-06-01	0.0103%	0.0000
608-1-PBB-06-03	0.0002%	0.0000
AT1	0.1505%	0.0011
FT1	0.0005%	0.0000
LJ23	0.0006%	0.0000
NN2193-1	0.3449%	0.0010
NN2323M-1	0.5911%	0.0047
OR22P1	0.0151%	0.0001
SA31	0.0538%	0.0003
SA53	0.2143%	0.0014
TLJ106-1	0.4402%	0.0025
TLJ11b	0.1550%	0.0007
TLJ60a	0.4244%	0.0023
TLJ85d	0.0778%	0.0010
YT1	0.0580%	0.0005

Table 4.3 Effects of serotype and genotype on invasion of bovine aortic endothelial cells (BAEC) by planktonic cultures of *S. mutans*.

Serotype	n	*S. mutans* invasion mean	*S. mutans* invasion SD	vs. serotype *e* p	vs. serotype *f* p	vs. serotype *k* p
c	4	0.0005%	0.0000	0.7947	<0.0001	<0.0001
e	1	0.0011%	0.0000		<0.0001	<0.0001
f	2	0.0351%	0.0005			0.5563
k	20	0.1547%	0.0026			

Genotype	n	*S. mutans* invasion mean	*S. mutans* invasion SD	vs. *cbm+/cnm-* p	vs. *cbm-/cnm+* p
cbm-/cnm-	7	0.0920%	0.0024	0.0125	0.6897
cbm+/cnm-	12	0.1330%	0.0024		0.0125
cbm-/cnm+	8	0.0795%	0.0016		

Table 4.4 Combined effects of genotype and serotype on BAEC invasion by planktonic *S. mutans*.

		Strains	*S. mutans* invasion	
Genotype	Serotype	n	mean	SD
cbm-/cnm-	*c*	1	0.0009%	0.0000
	k	6	0.1112%	0.0011
cbm+/cnm-	*c*	1	0.0004%	0.0000
	f	1	0.0013%	0.0000
	k	10	0.1586%	0.0026
cbm-/cnm+	*c*	2	0.0003%	0.0000
	e	1	0.0011%	0.0000
	f	1	0.0464%	0.0005
	k	4	0.1636%	0.0022

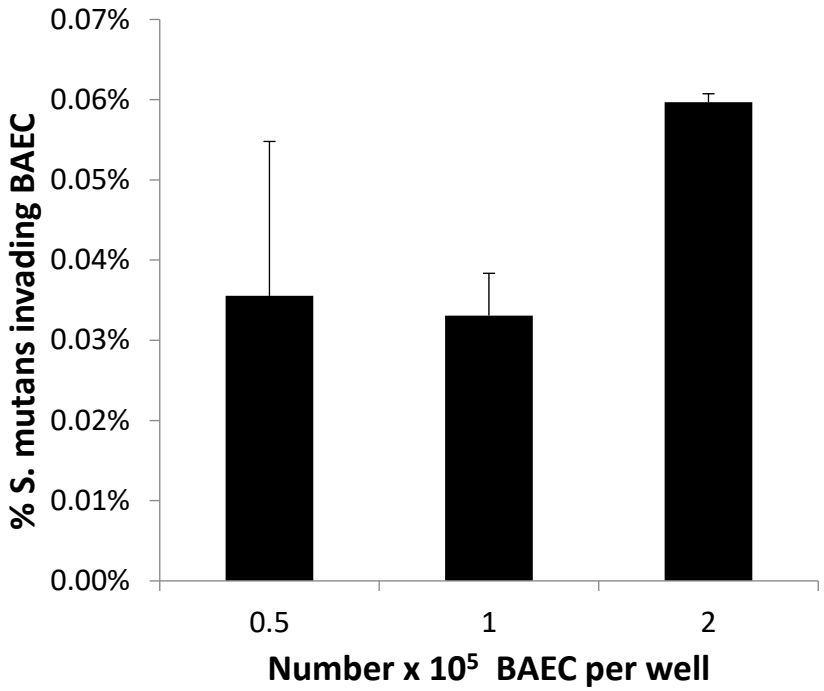


Figure 4.1 Titration of bovine aortic endothelial cells (BAEC) in 24-well plates used for BAEC invasion experiments. BAEC were counted, seeded into 24-well plates and incubated at 37^{o}C, 5% CO_2 for 22 hours. *S. mutans* strain OMZ175 was added to each well of BAEC for two hours prior to addition of fresh BAEC culture media containing penicillin and streptomycin. After a two-hour incubation, the BAEC were washed to remove the antibiotics followed by cold sterile dH_2O added to each well to lyse the BAEC. The inoculum and each lysate were plated on blood agar plates and incubated for 2 days before counting cfu to determine the percentage of the OMZ175 inoculum that invaded the BAEC. The error bars represent standard deviations of the mean for each data point.

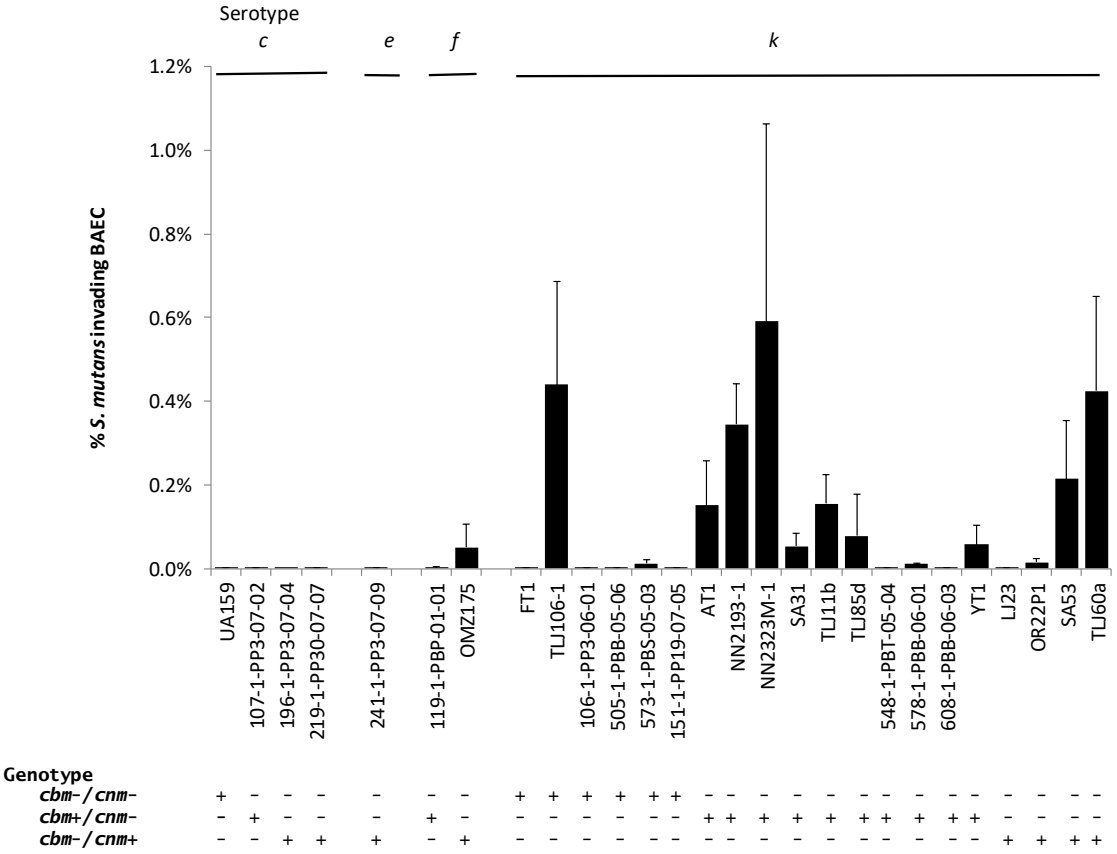


Figure 4.2 Bovine aortic endothelial cell (BAEC) invasion by planktonic cells of *S. mutans*. Overnight planktonic cultures of *S. mutans* grown in TSB were incubated with BAEC in the absence of antibiotics for 2 hours followed by a 2-hour incubation with penicillin and streptomycin to kill *S. mutans* on the exterior of the BAEC. After washing to remove the antibiotics, the BAEC were lysed with sterile ice-cold dH_2O. The lysate was diluted and plated on blood agar plates to determine the cfu of invasion for each strain. The invasion assays were performed in duplicate, and each assay was repeated three times. The serotype of each strain is indicated above the graph, and the CBP genotype of each strain is shown at the bottom of the graph. The error bars represent standard deviations of the mean for each strain.

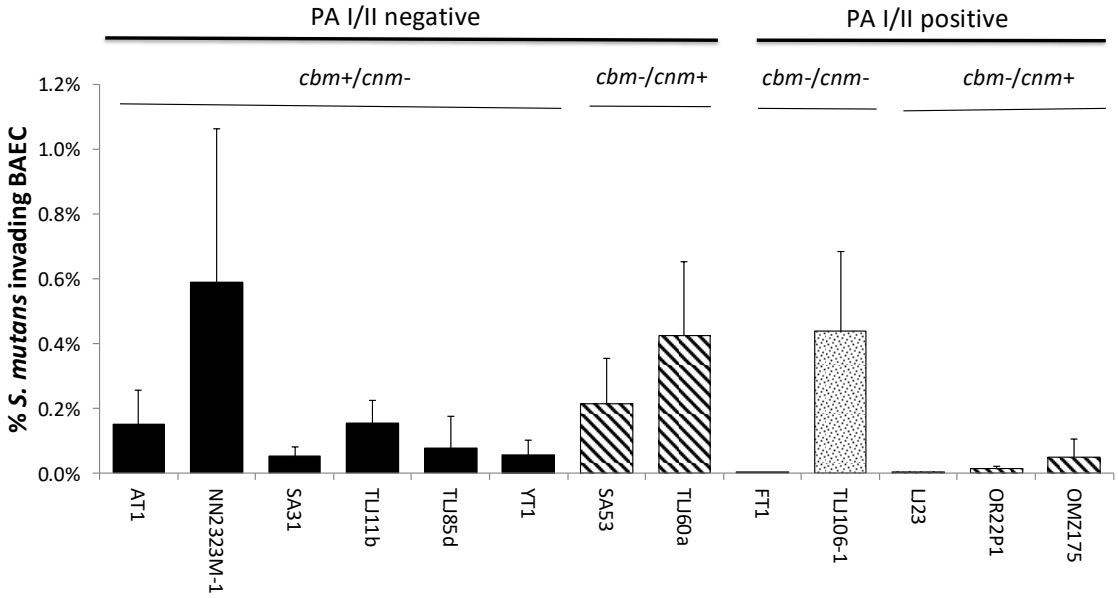


Figure 4.3 Bovine aortic endothelial cell (BAEC) invasion by planktonic *S. mutans* serotype *k* strains typed for surface expression of PA I/II and collagen binding protein genes (*cbm* and *cnm*). OMZ175, a serotype *f* strain is included for comparison. Overnight cultures of planktonic *S. mutans* grown in TSB were incubated with BAEC in the absence of antibiotics for 2 hours followed by a 2-hour incubation with penicillin and streptomycin to kill *S. mutans* on the exterior of the BAEC. After washing the BAEC to remove antibiotics, the BAEC were lysed with ice cold water, and the lysates were diluted and plated on blood agar plates to determine the cfu invading BAEC. The invasion assays were performed in duplicate, and testing for each strain was repeated three times. The black bars represent *cbm*+/*cnm*- strains, hashed bars represent *cbm*-/*cnm*+ strains and the stippled bars represent *cbm*-/*cnm*- strains. PA I/II surface expression for each strain is indicated at the top of the graph.

Chapter Five

ENDOTHELIAL CELL INVASION BY *S. MUTANS* GROWING IN A BIOFILM LIFESTYLE

INTRODUCTION

S. mutans, a common inhabitant of the human oral microbiota, is considered a major pathogen in the destruction of mineralized tooth tissue known as dental caries (Hamada and Slade 1980, Hajishengallis, Parsaei et al. 2017). In addition, *S. mutans* is detected in extra-oral pathologies such as IE and AT (Nakano, Inaba et al. 2006, Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009). The specific composition of cell-surface polysaccharides, rhamnose backbone with glucose side-chains, define *S. mutans* into serotypes *c, e* ,*f* and *k* (Nakano and Ooshima 2009). Notably, serotype *k* has a deficit of glucose side-chains on the rhamnose backbone which may contribute to lower cariogenicity and increased blood virulence of these strains (Nakano, Nomura et al. 2004, Nakano, Nomura et al. 2010).

Surface proteins of *S. mutans* have also been associated with virulence. Expression of CBPs such as PA I/II (also named SpaP, AgB, P1 and PAc), WpaS, Cbm and Cnm on the surface of *S. mutans* impart this bacterium with the ability to interact with the extracellular matrix of tissues and organs (Brady, Maddocks et al. 2010, Aviles-Reyes, Miller et al. 2017). PA I/II is also correlated with sucrose-independent attachment to salivary components on tooth surfaces (Koga, Okahashi et al. 1990). Strains with PA I/II expression defects are frequently serotype *k,* resistant to phagocytosis by polymorphonuclear leukocytes, and more likely to invade endothelial cells in the presence of other CBP (Nakano, Nomura et al. 2010, Lapirattanakul, Nomura et al. 2015, Otsugu, Nomura et al. 2017). The CBP Cbm and Cnm are instrumental in *S. mutans* virulence by enabling attachment to collagen at the site of dental caries as well as collagen in the extracellular matrix of endothelial cells, which facilitates *S. mutans* invasion into the endothelial cells (Abranches, Zeng et al. 2009, Nomura, Nakano et al. 2012, Aviles-Reyes, Miller et al. 2017, Otsugu, Nomura et al. 2017). The genes for these CBP, *cbm* and *cbm*, are predominant in serotypes *f* and *k* strains of *S. mutans* (Abranches, Miller et al. 2011, Aviles-Reyes, Miller et al. 2017).

The native habitat of *S. mutans* is within oral biofilm known as dental plaque. In addition to bacteria, the biofilm consists of a matrix composed of EPS mostly produced by the resident bacteria. Accounting for over 90% of the dry mass of the biofilm, the EPS

consists of exopolysaccharides, proteins, extracellular DNA (eDNA) and other polymeric substances, which collectively form the matrix within which the bacteria grow (Flemming and Wingender 2010). *S. mutans* produces biofilm in the presence of sucrose and other dietary sugars. In transitioning from planktonic growth into a biofilm community, bacteria undergo major transcriptional and proteomic changes, which differentiate the bacteria within the biofilm from its planktonic form (Klein, DeBaz et al. 2010, Wright, Burns et al. 2013). By using *in vitro* comparative transcriptome analyses of biofilm and free-living *S. mutans* cells, Shemesh et. al. reported significant differential expression in approximately 12% of *S. mutans* genes (Shemesh, Tam et al. 2007). Consequently, bacteria within a biofilm behave differently from their planktonic counterparts and properties observed in biofilm are often not predictable by study of the same bacteria in planktonic culture (Bowen and Koo 2011, Flemming, Wingender et al. 2016). One example is the loss of phenotypic antibiotic tolerance when bacteria disperse from biofilm to a planktonic state (Flemming, Wingender et al. 2016).

Tobacco use is associated with increased risk for CVD and oral diseases, including caries (Wagenknecht, Balhaddad et al. 2018). Nicotine, a bioactive agent in tobacco, increases the *in vitro* growth and metabolism of *S. mutans* strains grown in biofilm and planktonic cultures (Huang, Li et al. 2012). The basic amino acid, arginine, is present in saliva where the concentration of arginine is higher in caries-free compared to caries-susceptible subjects (Van Wuyckhuyse, Perinpanayagam et al. 1995, Sharma, Lavender et al. 2014). Free arginine is also found in the glycocalyx of endothelium, which is the point of *S. mutans* invasion into cardiovascular tissues (Tarbell and Pahakis 2006, Flam, Eichler et al. 2007). *In vitro* studies demonstrate that the adhesive and structural properties of *S. mutans* biofilm are altered, biomass is increased and EPS is decreased in the presence of arginine (Sharma, Lavender et al. 2014, Zheng, Cheng et al. 2015). Arginine also causes downregulation of genes encoding virulence factors responsible for attachment, competence development and bacteriocin production (Chakraborty and Burne 2017). The effects of nicotine and arginine on *S. mutans* biofilm were discussed in Chapter 3 of this dissertation.

Certain *S. mutans* serotype *k* strains and strains bearing *cbm* and *cbm* genes have been shown to bind and invade endothelial cells *in vitro* and *in vivo* in tissue and whole animal models. All previous studies of endothelial cell invasion were performed with planktonic cultures of *S. mutans*. Considering the biological differences between bacteria in planktonic and biofilm phases, I investigated endothelial cell invasion by *S.*

mutans in single-species sucrose-induced biofilm. The effects of CBP, PA I/II and serotype *k* were examined and compared to endothelial cell invasion by the same *S. mutans* strains grown in planktonic conditions and was presented in Chapter 4 of this tome. In addition, the effects of nicotine and arginine on *S. mutans* biofilm invasion of endothelial cells were also surveyed.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The strains of *S. mutans* used in this study are listed in Table 5.1. Frozen stock of each strain was inoculated into tryptic soy broth (TSB, Becton Dickinson and Co., Sparks, MD, USA) and incubated at 37ºC in 5% CO_2 overnight prior to inoculating into TSBS (Sigma-Aldrich, St. Louis, MO, USA) for biofilm formation in 6-well plates. In certain experiments, 10 mg/ml arginine (Sigma-Aldrich) or 4 mg/ml nicotine (Sigma-Aldrich) was added to the TSBS. Biofilm formation occurred over 20 hours at 37ºC, 5% CO_2. Biofilm was washed three times with sterile 0.9% NaCl before scraping the biofilm from the plastic and transferring to a sterile microfuge tube. The harvested biofilm was centrifuged at 13,000 rpm for 5 minutes and washed once with sterile 0.9% NaCl before sonication at 30 amps for 10 seconds per cycle for up to four cycles. Subsequent to sonication, the *S. mutans* biofilm was diluted 1:100 in BAEC growth media without penicillin and streptomycin. The diluted *S. mutans* was held at 37^{O}C while the BAEC were washed in preparation for addition of the inoculum.

BAEC Culture

BAEC growth media consisted of DMEM (Corning Life Sciences, New York, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 2 mM L-glutamine (Corning Life Sciences), 100 IU penicillin (Corning Life Sciences) and 100 ug/ml streptomycin (Corning Life Sciences) at 37ºC with 5% $C0_2$. Cultured BAEC were removed from the surface of the flask by a five-minute incubation with 0.25% trypsin protease (Hyclone, GE Healthcare Life Sciences, Pittsburgh, PA, USA). After washing the detached cells with BAEC growth media, the BAEC were seeded into 24-well plates or into T-75 flasks for further culture. The BAEC used for invasion assays were between passages 6 and 13.

BAEC Invasion Assay

The BAEC invasion assay was modified from that developed by Abranches et. al. (Abranches, Miller et al. 2011). Briefly, each well of a 24-well plate was seeded with 2.0 x 10^5 BAEC in 1.0 ml of BAEC growth media with antibiotics, and the plate was incubated for 20 hours at 37^{O}C, 5% CO_2. After incubation, the BAEC were washed 3 times with 37^{O}C DPBS (GE Healthcare, Life Sciences). After discarding the final wash, 1.0 ml of diluted *S. mutans* in BAEC growth media without antibiotics was added to duplicate wells of BAEC, and the plate was incubated for 2 hours at 37^{O}C, 5% CO_2. During the incubation, the residual *S. mutans* inoculum was diluted and plated onto blood agar plates to determine the number of colony forming units (cfu)/ml in the inoculum. After the incubation, the BAEC were washed three times with warm DPBS followed by addition of 1.0 ml of BAEC growth media with antibiotics, then incubation at 37^{O}C, 5% CO_2 for 2 hours. The BAEC were again washed three times with warm DPBS to remove the antibiotics before adding 1.0 ml of ice-cold sterile H_2O to each well of BAEC and incubating at room temperature for 20 minutes to lyse the BAEC. After BAEC lysis, each well was scraped to remove BAEC from the plastic surface, and the lysate from each well was plated onto blood agar plates to determine the cfu of invading *S. mutans*. For strains with high invasion rates, 1:10 and 1:100 dilutions of the lysate were required to obtain accurate quantitation.

The blood agar plates were incubated at 37^{O}C, 5% CO_2 for two days before colony counting by using the ProtoCOL automatic colony counting instrument and software (Symbiosis, Fredrick, MD, USA). The percent of *S. mutans* invading BAEC for each well was calculated as: cfu/ml invading BAEC divided by cfu/ml in the inoculum times 100. Each strain of *S. mutans* was tested in duplicate in three separate experiments. Positive BAEC invasion was defined as invasion by > 0.01% of the inoculum.

Statistical Analyses

Basic statistics (mean, standard deviation) were computed before statistical evaluation by using analyses of ranks and analysis of variance (ANOVA). When non-normality was present, a rank transformation was performed prior to the analysis.

RESULTS

Previously published endothelial cell invasion assays have been performed with planktonic cultures of *S. mutans*. In order to adapt biofilm cultures to the BAEC invasion assay, I washed and then scraped 24-hour *S. mutans* biofilm from the wells of a 6-well plate before sonicating the biofilm to disrupt it further. Figure 5.1 shows BAEC invasion by OMZ175 biofilm after two, three and four 10-second cycles of sonication at 30 amps. BAEC invasion after 2 cycles of sonication was not different from biofilm without sonication. Increasing BAEC invasion was observed with subsequent sonication cycles. All subsequent BAEC invasion experiments were performed with biofilm after four cycles of sonication.

BAEC invasion was calculated as the percentage of the inoculum from each strain that invaded BAEC. As shown in Table 5.2, BAEC invasion by biofilm ranged from 0.0001% to 0.8718% with 10 strains demonstrating invasion rates greater than 0.01%. Figure 5.2 displays the BAEC invasion data with the strains grouped by serotype and CBP genotype. Compared to the strains obtained from Osaka University Graduate School of Dentistry, the strains isolated from subjects at the University of Alabama School of Dentistry did not invade BAEC ($p<0.0001$).

Nine of the 20 serotype *k* strains had invasion rates of greater than 0.01%. There was no difference in invasion between the serotype *k* strains and the serotype *f* strain, OMZ175 (Table 5.3). Similarly, the serotype *c* and *e* strains were not statistically different. The serotype *k* strains and OMZ175 demonstrated statistically greater endothelial cell invasion than UA159 and 241-1-PP3-07-09.

When analyzed by CBP genotype (Table 5.3), *cbm*+ and *cnm*+ were significant predictors of BAEC invasion compared to *cbm*- strains ($p<0.0001$ and 0.0220, respectively). Invasion by *cbm*+/*cbm*- strains was not significantly different from *cbm*-/*cnm*+ strains. Strains without CPB demonstrated significantly less BAEC invasion than strains with either *cbm* or *cbm* ($p<0.0001$). BAEC invasion by TLJ60a biofilm of 0.87% was the highest of all strains tested and may skew the statistical analysis of the *cbm*-/*cbm*+ group of strains, wherein BAEC invasion of the remaining strains averaged 0.0380%.

The PA I/II phenotype was known for a subset of 15 strains. Figure 5.3 displays the BAEC invasion data grouped by PA I/II phenotype and CBP genotype. Strains expressing PA I/II were less invasive than strains that did not express PA I/II ($p=<0.0001$). PA I/II$^-$, *cbm*$^-$, *cbm*$^+$ strains averaged 0.407% BAEC invasion and PA I/II$^-$,

cbm^{+}, *cbm*$^{-}$ strains averaged 0.109% invasion (Table 5.4). Invasion by the PA I/II^{+} strains was not different between *cbm*$^{-}$/*cnm*$^{-}$ and *cbm*$^{-}$/*cbm*$^{+}$ strains.

We next compared BAEC invasion by biofilm to the invasion of planktonic cultures presented in the previous chapter. Planktonic cultures of 14 strains and biofilm from 10 strains demonstrated invasion of BAEC by at least 0.01% of the *S. mutans* inoculum (Figure 5.4, Table 5.2). Overall, there was no difference in BAEC invasion between planktonic cultures and biofilm (p=0.7635). There were 10 strains in which both biofilm and planktonic cultures invaded EC. Biofilm from 4 of the strains invaded better than planktonic cultures from the same strain, but the difference was significant for only TLJ60a (p=0.0484). Although invasion by FT1 biofilm was lower than the 0.01% threshold, invasion by planktonic cultures of FT1 was significantly lower than the biofilm invasion (p<0.0001). For six strains, planktonic cultures demonstrated better BAEC invasion than biofilm with significantly increased invasion with planktonic cultures of AT1, NN2193-1, SA31 and 578-1-PBB-06-01 compared to the respective biofilm. For biofilm, the presence of CBP was associated with increased BAEC invasion, and there was no difference between *cbm*$^{+}$ vs *cmn*$^{+}$ strains (Table 5.3). In contrast for planktonic cultures, *cbm*$^{+}$ increased BAEC invasion (p=0.01) compared to *cmn*$^{+}$ and CBP^{-} strains (Table 4.3). Greater BAEC invasion occurred for PA I/II^{-} strains than for PA I/II^{+} strains for both biofilm and planktonic cultures (p<0.0001, Figure 5.5, Table 5.4). In comparisons between biofilm and planktonic culture, only PA I/II-,*cbm*+, *cbm*- strains exhibited significantly different invasion rates between cells in biofilm and planktonic culture (p=0.0182).

In order to determine whether the EPS in biofilm affected BAEC invasion by *S. mutans*, the biofilm was washed three times with sterile saline after sonication. Eight strains were selected for comparison (Figure 5.6). Post-sonication, the biofilm from each strain was divided into two microfuge tubes, and one of the tubes received three sterile saline washes. The washed and not washed biofilm were separately diluted into BAEC growth media and tested in parallel BAEC invasion assays. Additional washing of the biofilm post-sonication resulted in increased BAEC invasion for all strains. Increases in BAEC invasion varied among the strains and ranged from two-fold OMZ175 to 147-fold for biofilm.

We previously demonstrated that 4 mg/ml nicotine significantly increased biofilm biomass and metabolic activity, whereas 10 mg/ml arginine had no significant effect compared to biofilm grown in TSBS alone. When nicotine and arginine were combined,

the biomass and metabolic activity significantly increased albeit to a lesser extent than for nicotine alone. Nine strains were selected to measure the effects of nicotine and arginine treatment of *S. mutans* biofilm on endothelial cell invasion (Figure 5.7). Nicotine and arginine were added during *S. mutans* biofilm formation, and since the biofilm was washed prior to *S. mutans* inoculation, the cultured BAEC were not exposed to these supplements. Across the nine strains, adding nicotine and arginine to the TSBS growth media had no significant effect on BAEC invasion by *S. mutans* biofilm. While *S. mutans* biofilm biomass and metabolic activity increased with addition of nicotine or arginine plus nicotine to TSBS (Chapter 3), BAEC invasion by YT1 and TLJ60a decreased when the biofilm was grown in the presence of nicotine. In contrast, invasion by OMZ175 biofilm grown in arginine plus nicotine was more than three-fold greater than biofilm grown in any of the other growth media.

DISCUSSION

S. mutans is an obligate human pathogen naturally habituating the dental plaque, a multispecies biofilm formed on tooth hard surfaces (Lemos, Palmer et al. 2019). The cariogenic potential of *S. mutans* is attributed to three core traits: (1) the ability to synthesize large quantities of EPS from sucrose, (2) ability to metabolize carbohydrates into organic acids which reduce the local pH in the biofilm (acidogenicity) and (3) the ability to thrive in conditions of environmental stress, especially low pH (aciduricity) (Lemos, Palmer et al. 2019). *S. mutans* adherence to the tooth surface and initiation of biofilm formation is dependent upon surface proteins of *S. mutans*, including glucosyltransferases, glucan-binding proteins, PA I/II and CBP (Palmer, Miller et al. 2013). In addition to the role in adherence to tooth surfaces, *S. mutans* strains with CBP and defects in PA I/II expression have been implicated in a variety of extra-oral systemic diseases such as IE, AT, IgA nephropathy, and in the brain, hemorrhagic stroke, cerebral microbleeds and cogitative impairment (Nakano, Nomura et al. 2008, Nakano, Nemoto et al. 2009, Nomura, Naka et al. 2013, Misaki, Naka et al. 2016, Watanabe, Kuriyama et al. 2016, Otsugu, Nomura et al. 2017, Inenaga, Hokamura et al. 2018). The proposed mechanism of infection after *S. mutans* enters the bloodstream is that in the absence of PA I/II expression, CBP facilitate *S. mutans* invasion of endothelial cells lining the blood vessel walls. Previous studies of *S. mutans* invasion of endothelial cells have been performed with planktonic cultures (Stinson, Alder et al. 2003, Abranches, Zeng et al. 2009, Abranches, Miller et al. 2011, Nagata, de Toledo et al. 2011,

Lapirattanakul, Nomura et al. 2013, Nomura, Naka et al. 2013). To my knowledge, there have been no endothelial cell invasion studies performed with *S. mutans* biofilm.

I assessed biofilm from 21 clinical isolates and two type strains of *S. mutans* for capacity to invade endothelial cells. Because early studies of extra-oral infections reported increased prevalence of serotype *k* strains in IE and AT tissues, I focused my study on serotype *k* strains with known CBP genotype. Endothelial cell invasion by *S. mutans* biofilm was diverse across the strains and ranged from essentially non-invasive to invasion by almost 0.9% of the *S. mutans* inoculum. The presence of CBP genes was significant for endothelial cell invasion, but whether the strain carried the *cbm* or the *cbm* gene was not significant. Interestingly, the presence of *cbm* or *cnm* was not sufficient to confer the ability to invade as a number of *cbm*+ and *cnm*+ strains demonstrated minimal or no endothelial cell invasion. In a study of planktonic *S. mutans*, Lapirattanakul et. al. reported low levels of collagen binding, extremely low levels of endothelial cell adhesion and lack of invasion for certain strains bearing *cbm* and *cnm* genes (Lapirattanakul, Nomura et al. 2013). The variations in collagen binding and interactions with endothelial cells among strains with CBP genes may result from differences in levels of *cbm* and *cnm* protein expression. Currently, expression levels of these CBP is unknown, but this knowledge would be beneficial for understanding of the mechanisms involved in the strain-to-strain variability and is worthy of additional investigation.

PA I/II expression defects are the result of point and frameshift mutations in the *spaP* gene encoding PA I/II as well as a frameshift mutation in the sortase gene which is responsible for anchoring the PA I/II protein to the cell surface (Lapirattanakul, Nomura et al. 2015). PA I/II expression defects were reported in approximately 7% of clinical isolates with expression defects found in 39% of serotype *f* strains and in 100% of serotype *k* strains isolated from subjects in Thailand (Nakano, Tsuji et al. 2006, Lapirattanakul, Nomura et al. 2015). Nomura and colleagues reported that *S. mutans* strains lacking PA I/II (CBP+/PA I/II-) expression demonstrated increased endothelial cell adhesion and invasion rates compared to CBP+/PA I/II+ strains, while CBP-/PA I/II+ strains demonstrated negligible adhesion and invasion (Nomura, Naka et al. 2013). In my study, *S. mutans* strains lacking PA I/II expression demonstrated significantly higher endothelial cell invasion than strains expressing PA I/II for both biofilm and planktonic cultures. This observation was striking when comparing *cnm*+ strains with and without PA I/II expression. Because the strains with PA I/II expression did not include *cbm*+ strains, one can only hypothesize a similar comparison for PA I/II+/*cbm*+ strains. The

crystal structure of *S. mutans* PA I/II reveals unique elongated fibrillar architecture forming an extended stalk on the bacterial cell surface (Brady, Maddocks et al. 2010). Stearic hindrance by PA I/II may block CBP access to the endothelial cell surface and this may explain the enhanced invasion by CBP+/PA I/II- strains. At this time the crystalline structures of Cbm and Cnm have not been determined; thus, speculation of stearic hinderance of CBP is merely a theoretical explanation of the observations.

In the present study, PA I/II expressing strains from planktonic cultures had a non-significant increase in invasion compared to biofilm *S. mutans*. Shemesh et. al. reported a 56-fold increase in PA I/II mRNA in the presence of 1% sucrose compared to planktonic culture. Thus increased PA I/II expression in biofilm compared to planktonic *S. mutans* cells might be predicted to diminish endothelial cell binding and invasion by biofilm (Shemesh, Tam et al. 2007). In addition, it raises into question the decrease in invasion by *S. mutans* biofilm observed in seven strains reported to lack PA I/II expression. Inasmuch as reported PA I/II expression defects were likely not due to deletion of the gene, one might speculate that in the biofilm phase, PA I/II expression was increased sufficiently to inhibit endothelial cell invasion by at least some of the seven PA I/II^{-}, *cbm*+ strains. Indeed PA I/II expression by these strains was detected in cells grown without sucrose; thus, it is not known whether growth in sucrose would increase PA I/II expression to a detectable level in these strains (Nakano, Tsuji et al. 2006, Lapirattanakul, Nomura et al. 2015, Otsugu, Nomura et al. 2017).

The presence of nicotine during sucrose-induced biofilm production results in increases of both *S. mutans* biomass and EPS. Not surprisingly, nicotine significantly increases expression of the glucosyltransferase gene *gtfC*, which accounts for increased EPS production (Li, Huang et al. 2013). Nicotine upregulates certain virulence associated genes (*ldh* and *nlmC*) as well as genes associated with the phosphotransferase system of *S. mutans* (Li, Huang et al. 2016). In contrast, nicotine had no effect on expression of PA I/II or sortase A, which controls PA I/II expression through a LPXTGX motif (Li, Huang et al. 2013). The invasion of BAEC by *S. mutans* biofilm was not increased when the biofilm was grown in the presence of nicotine, and invasion was significantly suppressed for two strains that demonstrated high invasion by biofilm grown in TSBS alone. The suppression was observed for both *cbm*+ (YT1) and *cbm*+ (TLJ60a) strains. These data suggest that nicotine may downregulate expression of the CBP thereby decreasing the ability of the bacteria to invade endothelial cells. Additional experiments investigating CBP expression in the presence of sucrose and

nicotine such as using qRT-PCR or western blot analyses to quantitate CBP expression may help explain this observation.

Diverse genotypic and phenotypic heterogeneity is observed between strains of *S. mutans*, and not all strains are equally virulent (Palmer, Miller et al. 2013, Song, Wang et al. 2013, Lemos, Palmer et al. 2019). This study of 23 clinical and type strains found, great heterogeneity in endothelial cell invasion across all strains and among strains bearing the same CBP genotype and PA I/II phenotype. Strains capable of endothelial cell invasion had varied invasion rates between planktonic and biofilm cells. For some strains, increased endothelial cell invasion was observed for planktonic cells ($p=0.0000$), whereas for other strains markedly better invasion was observed for biofilm cells ($p=0.0480$). Based on PA I/II expression, *cbm*-/*cbm*- genotype and extremely low collagen binding reported for TLJ106-1, this strain was not predicted to invade endothelial cells; however, endothelial cell invasion was observed for planktonic and biofilm cells from this strain (Lapirattanakul, Nakano et al. 2011). These data suggest a yet to be unidentified virulence factor or mechanism is employed by strain TLJ106-1 for endothelial cell invasion. Screening of additional clinical strains from diverse geographical areas may reveal similar or additional virulence mechanisms for endothelial cell invasion. Indeed, I observed significant differences in endothelial cell invasion from clinical strains collected in rural Alabama, Japan and Thailand.

The increased endothelial cell invasion observed after washing sonicated biofilm was an unanticipated observation, which suggests that elements of the biofilm matrix may negatively influence *S. mutans* invasion of the endothelial cell. Bacteria remained after washing as evidenced by the endothelial cell invasion, suggesting that extracellular components of the biofilm matrix were removed by the additional washing steps. The biofilm matrix, or EPS, is composed of a collection of polysaccharides, proteins, extracellular DNA (eDNA) and lipids produced by the bacteria within the biofilm (Flemming and Wingender 2010, Okshevsky and Meyer 2015). The eDNA is associated with bacterial membrane vesicles trapped within the biofilm (Jakubovics and Burgess 2015). The effect of the EPS on endothelial cell invasion may be passive or active. Adsorption of the long loop structure of eDNA, which can extend up to 300 nm from the bacterial cell surface, may create steric hinderance, and membrane vesicles with surface CBP may block intact *S. mutans* from binding (Okshevsky and Meyer 2015). One example of active interference is changes in the hydrophobicity of the bacterial cell surface caused by eDNA (Okshevsky and Meyer 2015). Alternatively, the components of

the EPS in the inoculum may affect the endothelial cell directly making it less receptive to *S. mutans* invasion. Certainly, more investigation is needed to understand the influence of washing biofilm after sonication upon endothelial cell invasion by *S. mutans*.

These data and investigations by others demonstrate that the presence of *cbm* or *cbm* concurrently with absence of PA I/II expression is not alone sufficient to predict endothelial cell invasion by *S. mutans*. Studies by Shemesh et al. indicate that bacteria in biofilms express different genes according to the substrate (polystyrene, hydroxyapetite, titanimu and composite) as part of the adjustment to the microenvironment (Shemesh, Tam et al. 2010). This leads to speculation that gene expression upon *S. mutans* encounter with endothelial cells may be quite different from gene expression upon contact with the tooth, or in the case of the present study, polystyrene surface, leading to upregulation of genes for a different set of attachment and virulence factors. Studies comparing mRNA from *S. mutans* adhered to ploystyrene, tooth and cardiac valve surfaces would begin to address the effect of these different surfaces on *S. mutans*. Additional studies are necessary to further elucidate differences among diverse strains of *S. mutans* and to to define those capable of binding and invading endothelial cells leading to extra-oral disease.

Table 5.1 *S. mutans* strains used in this Chapter.

Strain	serotype	*cbm*	*cnm*	Source
106-1-PP3-06-01	*k*	-	-	S. Momeni, N. Childers[1]
151-1-PP19-07-05	*k*	-	-	S. Momeni, N. Childers
505-1-PBB-05-06	*k*	-	-	S. Momeni, N. Childers
548-1-PBT-05-04	*k*	+	-	S. Momeni, N. Childers
573-1-PBS-05-03	*k*	-	-	S. Momeni, N. Childers
578-1-PBB-06-01	*k*	+	-	S. Momeni, N. Childers
608-1-PBB-06-03	*k*	+	-	S. Momeni, N. Childers
AT1	*k*	+	-	K. Nakano[2]
FT1	*k*	-	-	K. Nakano
LJ23	*k*	-	+	K. Nakano
NN2193-1	*k*	+	-	K. Nakano
NN2323M-1	*k*	+	-	K. Nakano
OR22P1	*k*	-	+	K. Nakano
SA31	*k*	+	-	K. Nakano
SA53	*k*	-	+	K. Nakano
TLJ106-1	*k*	-	-	K. Nakano
TLJ11b	*k*	+	-	K. Nakano
TLJ60a	*k*	-	+	K. Nakano
TLJ85d	*k*	+	-	K. Nakano
YT1	*k*	+	-	K. Nakano
UA159	*c*	-	-	ATCC 700610
241-1-PP3-07-09	*e*	-	+	S. Momeni, N. Childers
OMZ175	*f*	-	+	B. Guggenheim

[1]University of Alabama School of Dentistry, Birmingham, AL, USA

[2]Osaka University Graduate School of Dentistry, Osaka, Japan

Table 5.2 Invasion of bovine aortic endothelial cells (BAEC) by *S. mutans*.

Strain	BAEC Invasion (% *S. mutans*)				Biofilm vs. Planktonic
	Biofilm		Planktonic		
	mean	SD	mean	SD	p-value
FT1	0.00%	0.00%	0.00%	0.00%	0.1534
TLJ106-1	0.08%	0.09%	0.44%	0.25%	**0.0018**
106-1-PP3-06-01	0.00%	0.00%	0.00%	0.00%	0.4960
505-1-PBB-05-06	0.00%	0.00%	0.00%	0.00%	0.5989
573-1-PBS-05-03	0.00%	0.00%	0.01%	0.01%	0.1581
151-1-PP19-07-05	0.00%	0.00%	0.00%	0.00%	0.2799
NN2193-1	0.08%	0.06%	0.34%	0.10%	**0.0009**
NN2323M-1	0.03%	0.01%	0.59%	0.47%	**0.0465**
SA31	0.01%	0.01%	0.05%	0.03%	**0.0001**
TLJ11b	0.10%	0.05%	0.16%	0.07%	0.4608
TLJ85d	0.03%	0.01%	0.08%	0.10%	0.5339
548-1-PBT-05-04	0.00%	0.00%	0.00%	0.00%	**0.0087**
578-1-PBB-06-01	0.00%	0.00%	0.01%	0.00%	0.1805
608-1-PBB-06-03	0.00%	0.00%	0.00%	0.00%	0.5786
YT1	0.53%	0.91%	0.06%	0.05%	0.5088
LJ23	0.00%	0.00%	0.00%	0.00%	0.4306
OR22P1	0.02%	0.02%	0.02%	0.01%	**0.0196**
SA53	0.06%	0.06%	0.21%	0.14%	0.5776
TLJ60a	0.87%	0.22%	0.42%	0.23%	0.5608
OMZ175	0.12%	0.22%	0.05%	0.06%	0.2823
AT1	0.00%	0.01%	0.15%	0.11%	**0.0000**
UA159	0.00%	0.00%	0.00%	0.00%	0.1382
241-1-PP3-07-09	0.00%	0.00%	0.00%	0.00%	0.9012

Table 5.3 Effects of serotype and genotype on invasion of bovine aortic endothelial cells (BAEC) by *S. mutans* biofilm cells.

Serotype	Strains (n)	*S. mutans* invasion mean	SD	vs. serotype *e* p	vs. serotype *f* p	vs. serotype *k* p
c	1	0.0013%	0.0000	0.2561	0.0614	0.0416
e	1	0.0001%	0.0000		0.0015	0.0004
f	1	0.1294%	0.0022			0.8282
k	20	0.0881%	0.0028			

Genotype	Strains (n)	*S. mutans* invasion mean	SD	vs. *cbm+/cnm-* p	vs. *cbm-/cnm+* p
cbm-/cnm-	7	0.0150%	0.0004	0.0009	0.0220
cbm+/cnm-	10	0.0759%	0.0029		0.3709
cbm-/cnm+	6	0.1654%	0.0031		

Table 5.4 Effects of PA I/II phenotype and CBP genotype on biofilm and planktonic *S. mutans* cell invasion of BAEC.

	strains (n)	% *S. mutans*		*vs.* PA I/II-, *cbm+, cnm-*	*vs.* PA I/II+, *cbm-, cnm-*	*vs.* PA I/II+, *cbm-, cnm+*
		mean	SD	p-value	p-value	p-value
Biofilm						
PA I/II-, *cbm-*, *cnm+*	2	0.407%	0.442%	0.0516	0.0003	<0.0001
PA I/II-, *cbm+*, *cnm-*	7	0.109%	0.356%		0.0109	0.0002
PA I/II+, *cbm-*, *cnm-*	3	0.032%	0.063%			0.3642
PA I/II+, *cbm-*, *cnm+*	3	0.074%	0.169%			
Planktonic						
PA I/II-, *cbm-*, *cnm+*	2	0.319%	0.211%	0.9947	0.001	<0.0001
PA I/II-, *cbm+*, *cnm-*	7	0.222%	0.283%		<0.0001	<0.0001
PA I/II+, *cbm-*, *cnm-*	3	0.188%	0.328%			0.5475
PA I/II+, *cbm-*, *cnm+*	2	0.031%	0.031%			

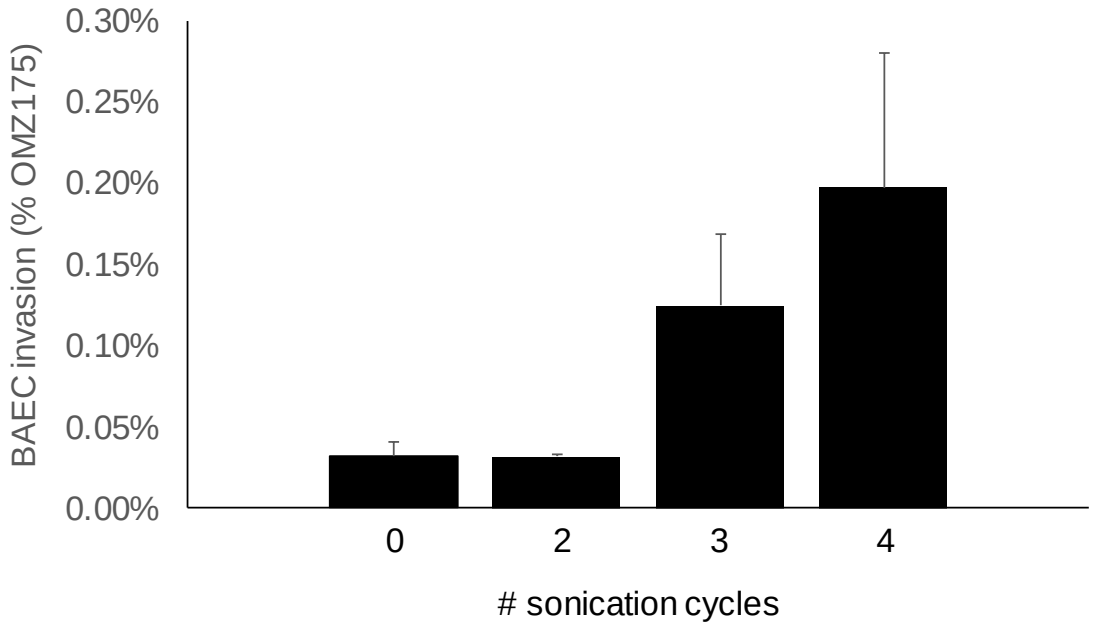


Figure 5.1 The effect of sonication on *S. mutans* OMZ175 biofilm invasion of bovine aortic endothelial cells (BAEC). Planktonic cultures of OMZ175 were inoculated into TSBS and seeded into 6-well plates. After a 20-hour incubation at 37º, 5% CO_2, the biofilm was washed, harvested by scraping, and then divided into 4 microtubes. The disrupted biofilm was sonicated 0, 2, 3 or 4 times with 30 amps for 10 seconds per cycle and stored on ice for at least 1 minute before each subsequent cycle. The error bars represent the standard deviation of duplicates from two biological replicate experiments.

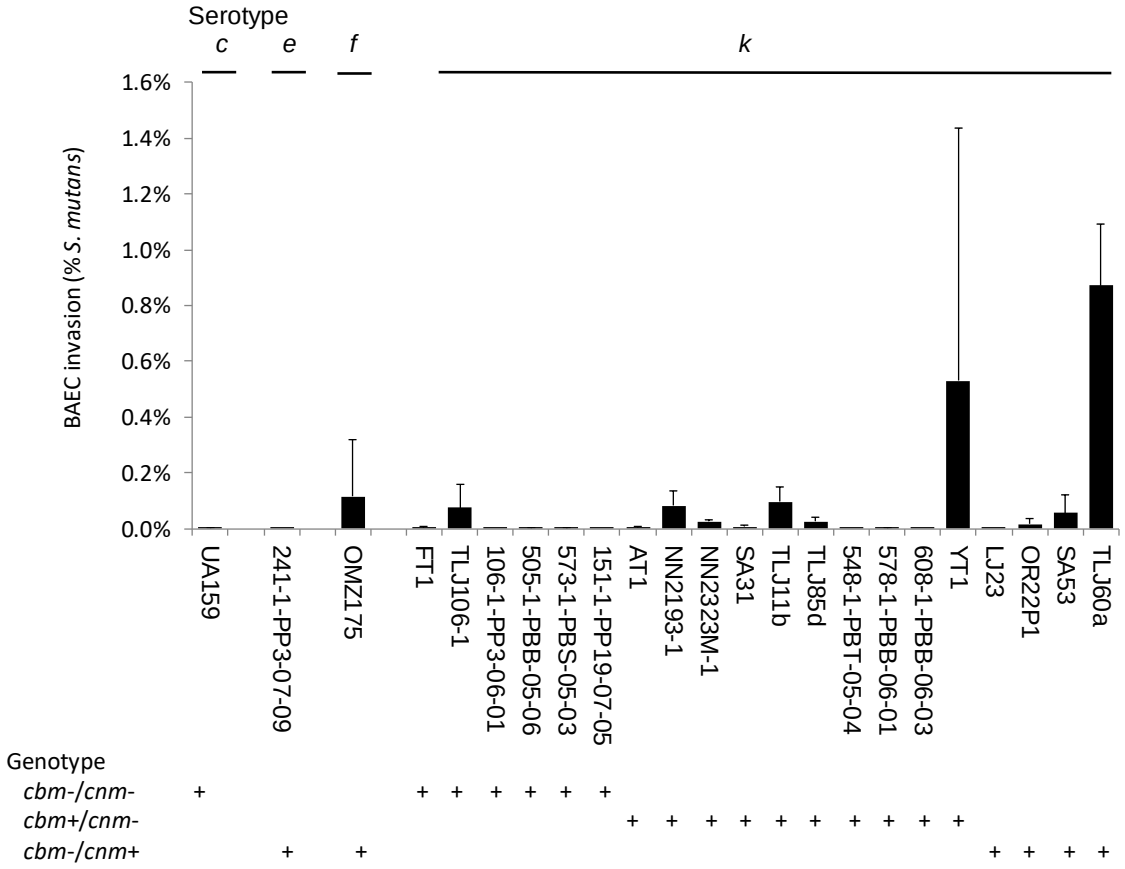


Figure 5.2 Bovine aortic endothelial cell (BAEC) invasion by *S. mutans* biofilm divided by serotype and CBP. Overnight biofilm cultures of *S. mutans* grown in TSBS were sonicated, then incubated with BAEC in the absence of antibiotics for 2 hours followed by a 2-hour incubation with penicillin and streptomycin to kill *S. mutans* cells that did not invade the BAEC. After washing the BAEC to remove antibiotic, the BAEC were lysed with ice cold water. The lysate was diluted and plated on blood agar plates to determine the number cfu of *S. mutans* that invaded BAEC. The invasion assays were performed in duplicate, and each assay was repeated three times. The serotype and genotype of the strains are shown on the graph. Error bars represent standard deviations.

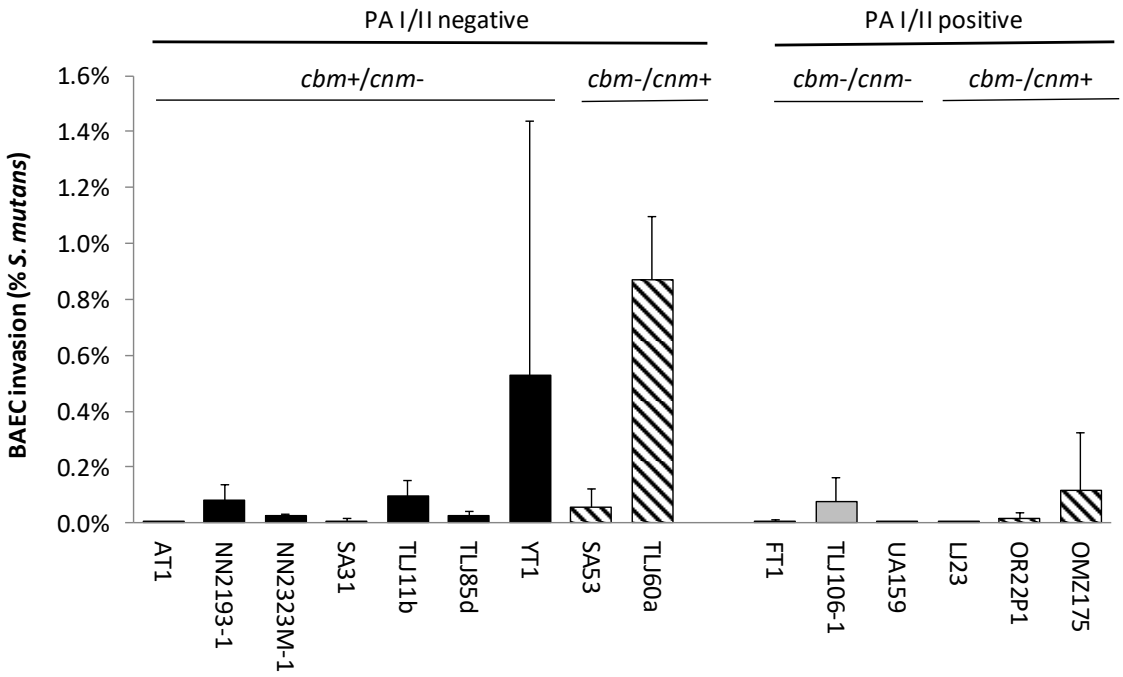


Figure 5.3 Bovine aortic endothelial cell (BAEC) invasion by *S. mutans* biofilm divided by PA I/II expression and CBP. Overnight biofilm cultures of *S. mutans* grown in TSBS were sonicated, then incubated with BAEC in the absence of antibiotics for 2 hours followed by a 2-hour incubation with penicillin and streptomycin to kill *S. mutans* cells that had not invaded the BAEC. After washing the BAEC to remove the antibiotics, the BAEC were lysed with ice cold water and then plated on blood agar plates to determine the number of colony forming units from each strain that invaded the BAEC. The invasion assays were performed in duplicate, and each assay was repeated three times. The phenotypes and genotypes of the strains are shown at the top of the graph. Black bars represent *cbm*+/*cbm*- strains, bars with black and white diagonal lines are *cbm*-/*cbm*+ strains and grey bars represent *cbm*-/*cbm*- strains. Error bars represent standard deviations.

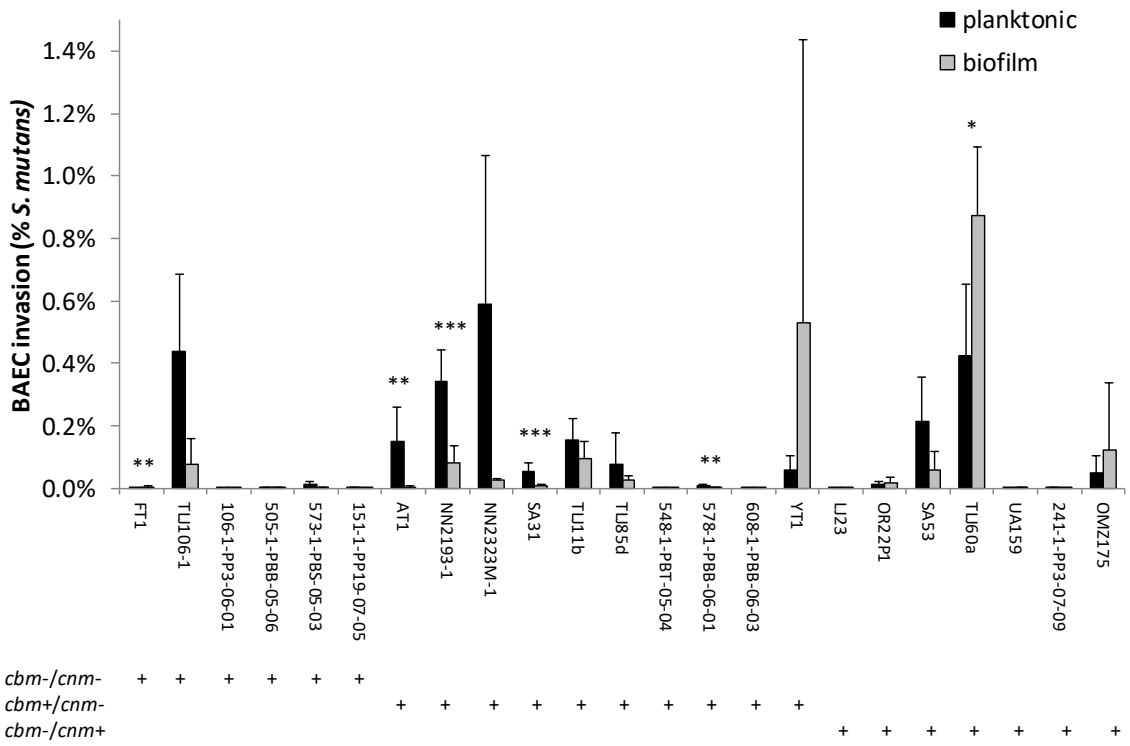


Figure 5.4 Paired comparison of biofilm and planktonic cultures of *S. mutans* in ability to invade bovine aortic endothelial cells (BAEC). Planktonic cultures were grown in TSB overnight, and then washed 3 times with sterile 0.9% NaCl before addition to 24-well plates of BAEC without antibiotics. Biofilm was grown for 24-hours in TSBS followed by 4 cycles of sonication before addition to BAEC cultures without antibiotics. The BAEC and *S. mutans* were incubated for 2 hours at 37^{o}C, 5% CO_2. After washing to remove unbound *S. mutans*, BAEC growth media with penicillin and streptomycin was added to the BAEC for two hours followed by washing to remove the antibiotics. Lastly, the BAEC were lysed with ice cold water, and the lysate was plated onto blood agar plates to determine the number of colony forming units of invading *S. mutans*. Planktonic *S. mutans* invasion is shown by the black bars, and the gray bars represent invasion by *S. mutans* grown in biofilm. The genotype ofeach strain is shown on the graph. Error bars represent the standard deviation of duplicates from three biological replicates. * $p<0.05$, **$p<0.01$, ***$p<0.0001$

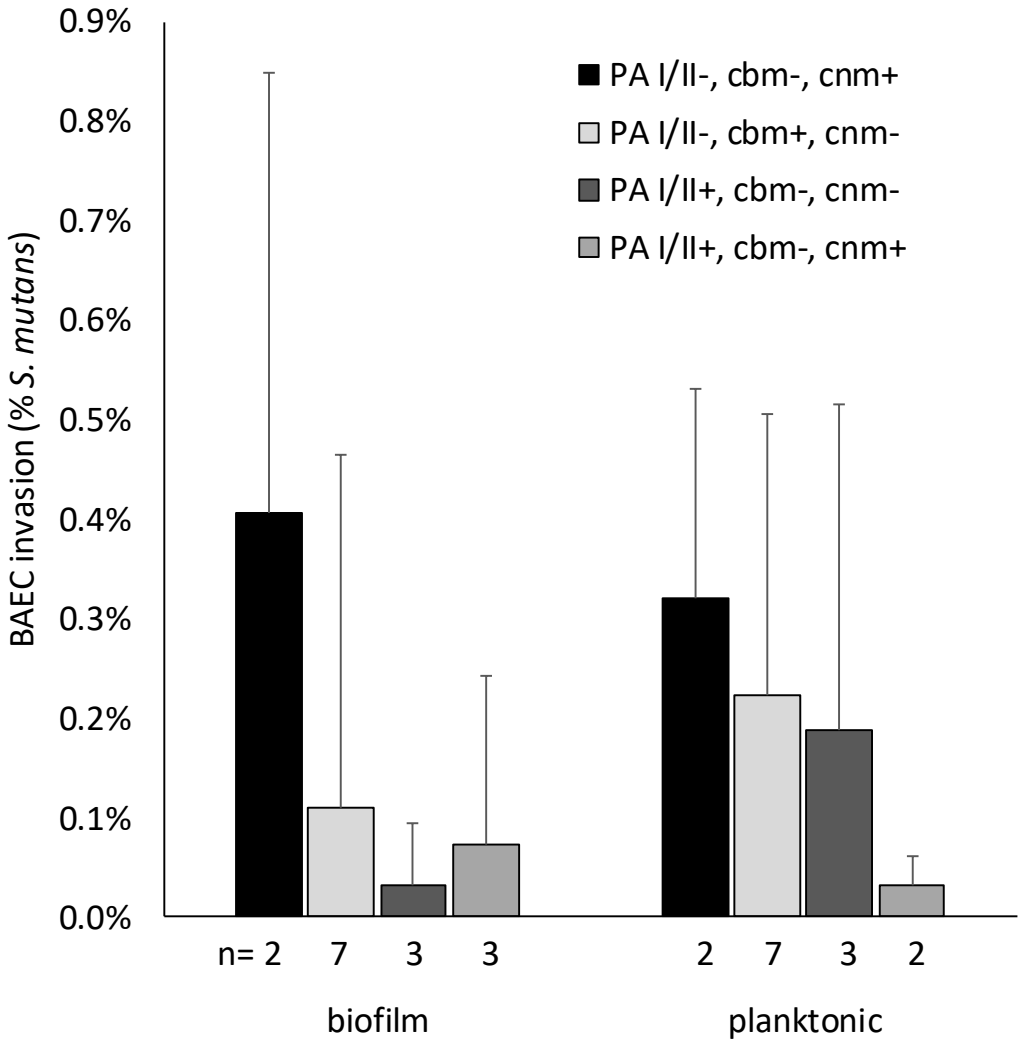


Figure 5.5 Comparison of *S. mutans* biofilm and planktonic cell invasion of bovine aortic endothelial cells (BAEC). The effects of PA I/II expression and the presence of the collagen binding protein genes *cnm* and *cbm* are shown in the bars. Error bars represent the standard deviation of all strains with the stated phenotypes and genotypes. Each strain was tested in duplicate, and in three biological replicates.

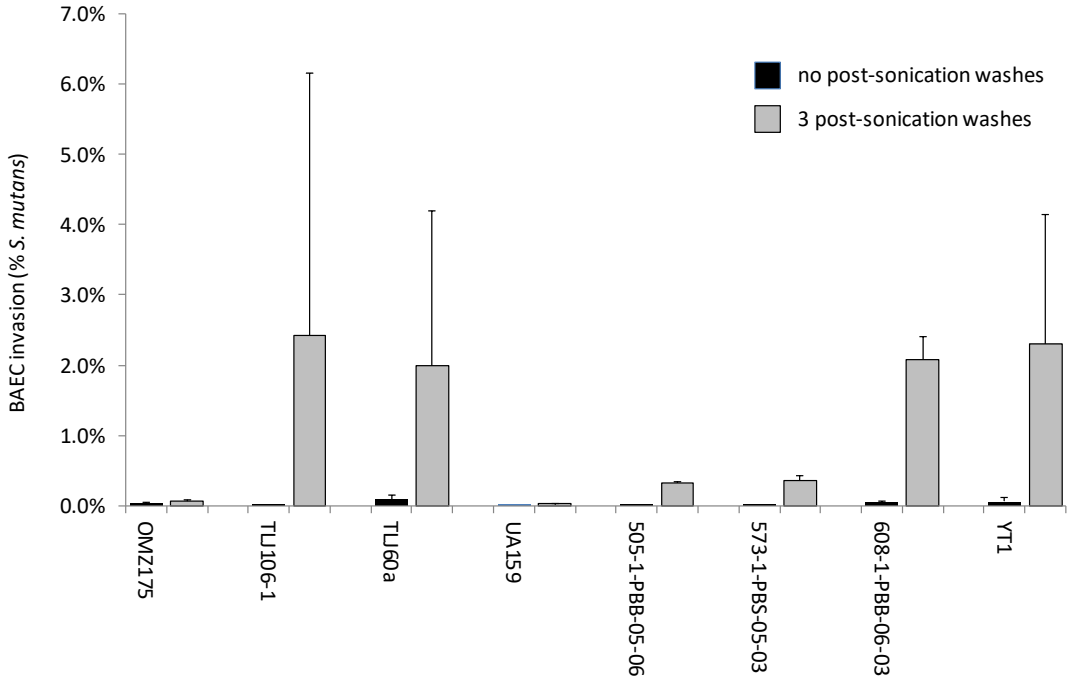


Figure 5.6 The effects of post-sonication washes on invasion of bovine aortic endothelial cells by *S. mutans* biofilm. *S. mutans* 24-hour biofilm grown in TSBS was sonicated for four cycles of 30 amps, 10 seconds. After sonication, the *S. mutans* was nor washed or washed 3 times with sterile 0.9% NaCl ,prior to BAEC invasion assays. The black bars represent *S. mutans* that was not washed post-sonication and gray bars represent biofilm washed 3 times after sonication. Strains TLJ106-1, TLJ60a and YT1 were tested in two separate experiments whereas all other strains were tested in one experiment. The error bars represent the standard deviation of replicates.

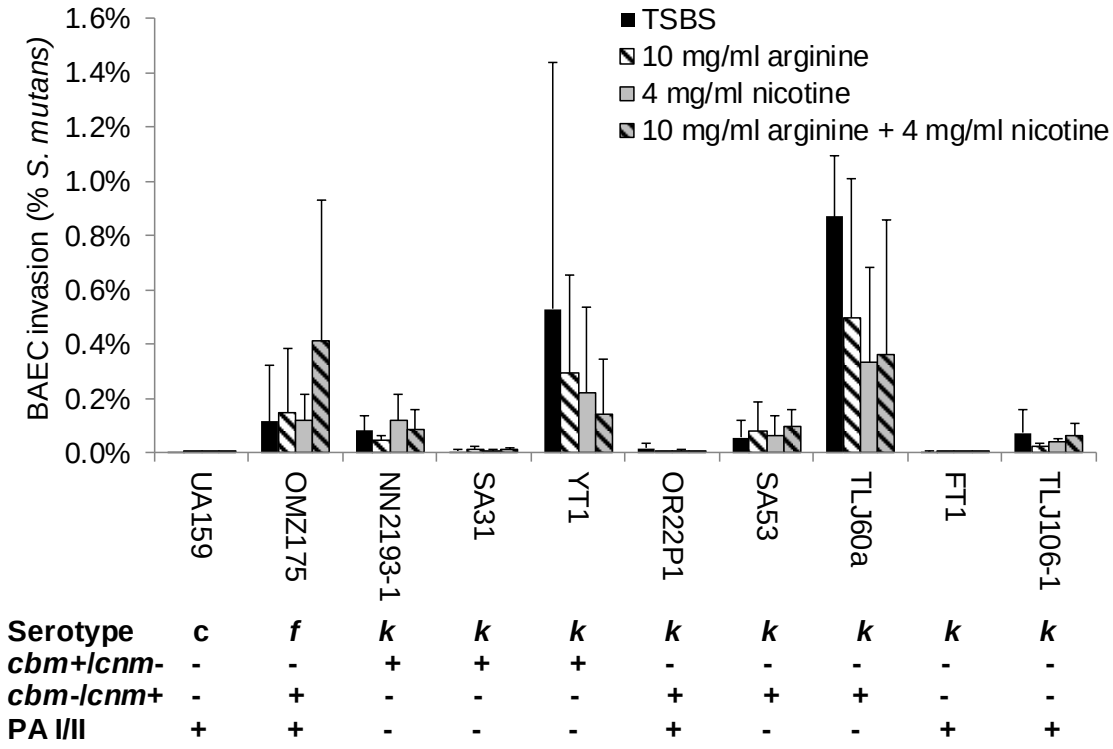


Figure 5.7 The effects of arginine (10 mg/ml) and/or nicotine (4 mg/ml) treatment of *S. mutans* biofilm on the ability of *S. mutans* to invade bovine aortic endothelial cells (BAEC). Biofilm was grown for 24-hours in the presence of 10 mg/ml arginine (black and white diagonal), 4 mg/ml nicotine (gray), combined arginine and nicotine (black and gray diagonal) and TSBS alone (black). After washing to remove growth media, each biofilm underwent 4 cycles of sonication before a 2-hour incubation with BAEC in the absence of antibiotics. After washing to remove unbound *S. mutans*, BAEC growth media with penicillin and streptomycin were incubated with the BAEC for two hours followed again by washing. Lastly, the BAEC were lysed with ice cold water, and the lysate was plated onto blood agar plates to determine the number of cfu of invading *S. mutans*. The error bars represent standard deviations of duplicate wells from 3 biological experiments.

Chapter Six

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation is a study of endothelial cell invasion by *S. mutans* conceived based upon earlier investigations that reported evidence of *S. mutans* in atherosclerotic plaques and lesions of IE (Nakano, Inaba et al. 2006, Nomura, Nakano et al. 2006, Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009, Nakano and Ooshima 2009, Nakano, Nomura et al. 2010, Nomura, Naka et al. 2013, Otsugu, Nomura et al. 2017). Endothelial cell invasion by *S. mutans* is associated with the CBP Cbm and Cnm which are predominantly found in serotypes *f* and *k* strains of *S. mutans*. (Abranches, Miller et al. 2011, Lapirattanakul, Nakano et al. 2011, Nomura, Nakano et al. 2012, Lapirattanakul, Nomura et al. 2013). Previous studies demonstrating endothelial cell invasion by *S. mutans* were performed with planktonic cultures as opposed to the dental plaque habitat wherein *S. mutans* maintains a biofilm lifestyle. Separate studies of *S. mutans* serotypes *c, e* and *f* documented the effects of nicotine and arginine on biofilm formation, but no study addressed *S. mutans* serotype *k* strains (Huang, Li et al. 2012, Li, Huang et al. 2014, Huang, Li et al. 2015, Li, Huang et al. 2016, Huang, Zhang et al. 2017). Inasmuch as nicotine, a component of tobacco, is associated with both compromised cardiovascular and oral health, I wondered whether nicotine treatment of *S. mutans* would influence its ability to invade endothelial cells. Prior studies demonstrated that *S. mutans* biofilm grown in the presence of arginine produced a more fragile EPS matrix compared to biofilm grown in the absence of arginine (Sharma, Lavender et al. 2014, Huang, Zhang et al. 2017). Given this prior knowledge, I investigated the effects of nicotine and arginine on biofilm formation by 33 serotyped and CBP genotyped strains of *S. mutans* with a focus on serotype *k* strains. In order to put my investigations into the context of the previous investigations, comparisons of endothelial cell invasion by free-living (planktonic) and biofilm *S. mutans* were undertaken. The following are my conclusions from these studies.

A ROLE FOR BACTERIA IN THE EVOLUTION OF ATHEROSCLEROSIS

AT, a chronic inflammatory disease of the arterial wall, is found early in life and early in human history (McGill, Herderick et al. 2002, Badimon and Vilahur 2014, Wann and Thomas 2014). Atherosclerotic plaque formation is a distinct process with defined stages of development: (i) cholesterol deposition (fatty streak formation), (ii)

inflammation, (iii) formation of the extracellular atherosclerotic matrix and (iv) thrombosis (Libby 2002). Fatty streaks composed of macrophage foam cells are present in the arteries of about one-third of children under 9 years of age, and by their late 20s, one-third of young adults will have well-developed raised lesions with large extracellular lipid cores and fibromuscular caps (McGill, McMahan et al. 2000). The multi-center Pathobiological Determinants of Atherosclerosis in Youth (PDAY) research study found atherosclerotic lesions in all aorta and half of the right coronary arteries of 15 to 19 year-olds who died of external causes (PDAY 1993). AT is not unique to modern societies as the presence of AT has been documented in mummies from multiple cultures and from multiple millennia (Clarke, Thompson et al. 2014, Piombino-Mascali, Jankauskas et al. 2014, Wann, Thompson et al. 2014, Wann and Thomas 2014). The earliest record of AT is found in the 5,300 year old mummified remains of the Tyrolean “Iceman” who was also found to have several single nucleotide polymorphisms known to predispose modern humans to AT (Wann, Thompson et al. 2014). Evidence of AT in the mummified remains of other hunter-gather societies raises doubt to the assertion that cardiovascular disease arose with the advent of agriculture (Ridker Paul 2002, Wann and Thomas 2014).

The renown 19th century pathologist, Rudolph Virchow, was the first to contend that inflammation plays a key role in the development of AT (Wann, Thompson et al. 2014). With the use of modern technology, evidence of parasitic, viral and microbial infection has been detected in ancient mummified remains (Thomas, Wann et al. 2014). An emerging paradigm in modern medicine implicates infection as a contributor to atherogenesis either through direct infection of vascular cells or by the direct effects of cytokine induced inflammation resulting from infection at non-vascular sites (Rosenfeld and Campbell 2011, Kozarov 2012, Jonsson and Backhed 2017). Both oral and gut microbiota have been associated with AT (Slocum, Kramer et al. 2016, Jonsson and Backhed 2017, Aarabi, Heydecke et al. 2018). A meta-analysis of 63 studies confirmed the presence of 23 oral bacterial species in AT plaque samples (Chhibber-Goel, Singhal et al. 2016). *S. mutans* is among the oral bacteria found in AT plaques and in cardiac valves in cases with IE (Nakano, Inaba et al. 2006, Nakano, Nemoto et al. 2007, Nakano, Nemoto et al. 2009, Nomura, Naka et al. 2013, Chhibber-Goel, Singhal et al. 2016, Otsugu, Nomura et al. 2017, Inenaga, Hokamura et al. 2018). The earliest documented cases of *S. mutans* associated IE were published by Abercrombie and Scott in the 1920’s, and subsequent confirmatory reports appear in more modern times

(Lockwood, Lawson et al. 1974, Aviles-Reyes, Miller et al. 2017). In addition to *S. mutans*, other streptococci, *Staphylococcus aureus*, enterococci and other microorganisms have been linked to IE (N'Guyen, Duval et al. 2017). Although *S. mutans* has been associated with AT and IE, it is not the sole microbe found in these diseased tissues, which suggests that perhaps AT and IE result from repeated infections by single or multiple members of the microbial community.

NICOTINE ENHANCES *S. MUTANS* BIOFILM GROWTH

Chapter 2 of this work is a review of the effects of nicotine on oral microorganisms and oral tissues. Nicotine enhances biofilm production by *S. mutans* and many other oral bacteria as well as the oral yeast, *Candida albicans* (Table 2.1). The effect of nicotine on biofilm is not universal as Li et. al. reported it had no effect on *Streptococcus sanguinis* biofilm production (Li, Huang et al. 2014). The *S. mutans* strains in the present collection produced peak biofilm mass and metabolic activity in the presence of 4 or 8 mg/ml nicotine, and there were no significant differences between serotypes *k* and *c*. The nicotine doses producing peak biofilm mass and metabolic activity are identical to those previously reported by Huang et al. for *S. mutans* serotype *c, e* and *f* strains (Huang, Li et al. 2012, Huang, Li et al. 2015). The CLSM studies with six strains of *S. mutans* showed nicotine significantly increased bacterial mass but did not have a significant effect on EPS production. This is in contrast to previous studies of *S. mutans* strain UA159 in single- and dual-species biofilm that reported increases in both bacterial mass and EPS in the presence of nicotine (Li, Huang et al. 2014, Huang, Li et al. 2015). A possible explanation for the discrepancy is that perhaps the nicotine induced increase in EPS production is a characteristic of UA159, which is not evident in all strains of *S. mutans*.

A new perspective on the effects of nicotine on *S. mutans* is consideration of the combined effects of nicotine and presence of CBP on *S. mutans* biofilm. The presence of 4 mg/ml nicotine and either *cbm* or *cnm* resulted in increased biofilm mass and metabolic activity compared to strains with neither or both CBP. There was no difference in metabolic activity between *cmn*+ and *cbm*+ strains. The biofilm mass of *cnm*+ strains was increased compared to *cbm*+ strains, and the increase appears to be the result of an increase of EPS rather than bacterial mass. Exposure to nicotine during biofilm development had no effect on the ability of *S. mutans* to invade endothelial cells regardless of the presence or absence of CBP. Nicotine upregulates certain *S. mutans*

virulence genes, increases lactate production and upregulates sortase A, a transpeptidase that covalently links surface proteins with LPXTG domains to the cell wall of gram-positive bacteria (Li, Huang et al. 2013, Li, Huang et al. 2016). Concomitant with sortase A upregulation, UA159 expression of PA I/II is increased. Cbm and Cnm also have LPXTG domains (Aviles-Reyes, Miller et al. 2017); thus, increased surface expression of Cbm and Cnm was predicted based upon upregulation of sortase A in the presence of nicotine. Preliminary collagen-binding assays indicate that nicotine does upregulate collagen binding by *S. mutans* UA159 and many of the other strains used here. Exposure to nicotine during biofilm development had no significant effect on endothelial cell invasion by any of the 10 *S. mutans* strains tested regardless of the presence or absence of CBP or PA I/II. It is possible that increased expression of CBP does not enhance the ability of *S. mutans* to invade endothelial cells. Alternatively, upregulation of sortase A may not significantly increase CBP surface expression. Additional studies by both RT-PCR and western-blotting methods measuring Cbm and Cnm expression subsequent to nicotine exposure might shed light on the effects of nicotine on CBP expression.

ARGININE AFFECTS *S. MUTANS* BIOFILM STRUCTURE

The amino acid, arginine, is present in saliva and is found within the endothelial glycocalyx in cardiovascular tissues (Van Wuyckhuyse, Perinpanayagam et al. 1995, Tarbell and Pahakis 2006, Flam, Eichler et al. 2007, Sharma, Lavender et al. 2014). Studies of sucrose-induced biofilm of *S. mutans* serotype *c* strains found that addition of arginine resulted in increased biomass but altered adhesive and structural properties, which may be partly due to decreased EPS production (Sharma, Lavender et al. 2014, Zheng, Cheng et al. 2015). Arginine causes down regulation of *S. mutans* UA159 genes responsible for attachment including PA I/II (SpaP) and decreases environmental stress tolerance (Chakraborty and Burne 2017). The 33 strains in this study showed no significant difference in biomass or metabolic activity when 10 mg/ml arginine was added to TSBS (Chapter 3). CLSM studies of *S. mutans* biomass and EPS production support these observations. When the presence or absence of CBP were considered in the analysis, biofilms grown in the presence of arginine were like biofilms produced in TSBS alone.

The most interesting effects of arginine on *S. mutans* biofilm occurred when it was combined with nicotine. Simultaneous addition of 10 mg/ml arginine and 4 mg/ml

nicotine resulted in significantly decreased *S. mutans* and EPS compared to nicotine alone for the six strains used in the CLSM studies. In the larger study, *S. mutans* strains with the *cmn* gene showed significantly enhanced biofilm mass and metabolic activity when arginine was added with nicotine compared to biofilm grown in nicotine alone. In contrast, for the *cbm*+/*cnm*- and *cbm*-/*cnm*- strains, the addition of arginine to nicotine had essentially no effect on nicotine increased biofilm mass and metabolic activity. As the CLSM study contained a single *cnm*+ strain, the effects of this gene on biofilm mass and metabolic activity may not be apparent in the CLSM study.

As with nicotine alone, addition of arginine with or without nicotine had no overall effect on endothelial cell invasion by 10 selected strains of *S. mutans*. A significant reduction in endothelial cell invasion was observed for only TLJ60a and TLJ106-1 when grown with arginine, and addition of nicotine negated the statistical significance. The effects of arginine and arginine with nicotine observed in the biofilm mass, metabolic activity and CLSM experiments did not translate to changes in endothelial cell invasion.

The effects of post-sonication washes of *S. mutans* prior to the endothelial cell invasion assay are intriguing (Figure 5.6). Certain strains displayed significant increases in endothelial cell invasion with the addition of post-sonication washes. Inasmuch as endothelial cell invasion is based upon the percentage of the individual inoculum that invaded the endothelial cells, loss of bacteria is not a likely explanation for the increase in invasion associated with the additional washes. It is likely that removal of components of the biofilm EPS matrix contributed to the increased ability of certain strains to invade endothelial cells. The EPS matrix is composed of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender 2010). The nucleic acids, or eDNA, in the matrix adsorb to the surface of individual bacterial cells in long loop structures that extend up to 300 nm from the surface and influence the hydrophobicity of the bacterial cell (Okshevsky and Meyer 2015). Removal of eDNA by washing the inoculum might positively affect endothelial cell invasion by removing steric hinderance posed by long loops of eDNA adhered to the *S. mutans* or by normalizing hydrophobicity of the bacterial cell. Residual proteins and rigid EPS structure in the unwashed post-sonication inoculum may represent additional physical barriers that prevent direct interaction between the *S. mutans* cell wall and the endothelial cell. Wu and colleagues demonstrated that biofilm formation, autolysis and eDNA production by *Streptococcus epidermidis* were all enhanced in the presence of nicotine in similar doses as used in this study (Wu, Ma et al. 2018). This observation may explain the lack of increased

endothelial cell invasion by nicotine treated *S. mutans* biofilm cells such that nicotine increased eDNA production interfered with endothelial cell invasion. Addition of post-sonication washes or DNase treatment of the *S. mutans* prior to introduction to the endothelial cells might clarify the role of *S. mutans* eDNA in endothelial cell invasion.

S. MUTANS BIOFILM DIFFERS FROM THE PLANKTONIC STATE

Bacterial cells growing in planktonic and biofilm states express unique phenotypes characterized by differences in gene expression between planktonic and biofilm cells (Shemesh, Tam et al. 2007, Shemesh, Tam et al. 2010, Khan, Islam et al. 2011, Flemming, Wingender et al. 2016, Florez Salamanca and Klein 2018). In transitioning from planktonic to biofilm growth, microorganisms undergo major transcriptional and proteomic changes (Wright, Burns et al. 2013). In microarray analyses of strain UA159, Shemesh and colleagues discovered differential expression of 12% of *S. mutans* genes between biofilm and planktonic cells (Shemesh, Tam et al. 2007). In the biofilm lifestyle, gene expression varies by the surface colonized, whether grown in flow or static conditions and as the biofilm matures with increasing thickness (Motegi, Takagi et al. 2006, Shemesh, Tam et al. 2007, Shemesh, Tam et al. 2007, Shemesh, Tam et al. 2008, Shemesh, Tam et al. 2010). Further, the variability in expression of each gene is higher for biofilm than planktonic cells reflecting the inherent heterogeneity of biofilms and the difficulties in obtaining repeatable results with biofilm cells (Svensater, Welin et al. 2001). Consequently, bacteria within a biofilm behave differently from planktonic bacteria, and properties observed in biofilm are often not predictable by study of the same bacteria in planktonic culture (Bowen and Koo 2011, Flemming, Wingender et al. 2016).

Previous studies of endothelial cell invasion by *S. mutans* cells were performed with planktonic cultures grown in a variety of media (Stinson, Alder et al. 2003, Abranches, Zeng et al. 2009, Abranches, Miller et al. 2011, Nagata, de Toledo et al. 2011, Lapirattanakul, Nomura et al. 2013, Nomura, Naka et al. 2013). This is the first study to investigate endothelial cell invasion by sucrose-induced *S. mutans* biofilm. Within-strain comparisons demonstrated different rates of endothelial cell invasion dependent upon the lifestyle of the invading *S. mutans*. For six strains, increased endothelial cell invasion was obtained by free-cell state *S. mutans*. In contrast, four strains demonstrated superior invasion by *S. mutans* biofilm. These observations exemplify the diversity among strains of *S. mutans* and suggest the need for

comparative gene expression studies of planktonic and biofilm lifestyles across a larger number of *S. mutans* strains. In particular, focus on genes associated with endothelial cell invasion such as *cnm*, *cbm*, PA I/II and others may be useful toward understanding why strains with similar genetic profiles display different rates of endothelial cell invasion and how lifestyle influences the rate of invasion.

S. mutans is a highly genetically and phenotypically diverse species with a core genome consisting of 1,370 to 1,490 genes (Cornejo, Lefebure et al. 2013, Song, Wang et al. 2013). In a study of 57 strains, Cornejo et. al. identified an average of 1,636 genes per strain leading to an estimated pan-genome of 3,296 genes (Cornejo, Lefebure et al. 2013). Similar to many other *Streptococcus* species, different strains of *S. mutans* have highly dynamic genomes with the overall gene composition differing from one isolate to another as a result of lateral gene transfer and possibly other mechanisms. The distribution of putative virulence genes in 183 *S. mutans* isolates did not correlate with caries status, indicating that other factors play a role in caries development (Meng, Lu et al. 2017). Meng and colleagues state that the SpaP gene (PA I/II) is dispensable as it is found in isolates from oral cavities with and without caries (Meng, Lu et al. 2017). Inasmuch as *cbm* and *cnm* are not present in all *S. mutans* strains, these must be considered dispensable, or non-core, genes as well. As exemplified in the strains used in this dissertation, the presence of *cbm* or *cnm* alone is not sufficient to confer the ability to invade endothelial cells upon a particular strain (e.g., AT1, LJ23, 548-1-PBT-05-04). Similarly, absence of PA I/II in the presence of *cbm* or *cnm* (e.g., SA31, AT1) does not necessarily allow a strain to invade endothelial cells. To the contrary, TLJ106-1 (PA I/II+, *cbm*-, *cnm*-) demonstrated endothelial cell invasion whether this strain was free-living or in a sucrose-dependent biofilm. In a previous study, TLJ106-1 demonstrated extremely low binding to type I collagen, indicating that a mechanism other than binding to type I collagen is involved in the endothelial cell invasion by this strain (Lapirattanakul, Nakano et al. 2011). These findings suggest that TLJ106-1 possesses an, as yet unidentified, virulence factor(s) which does not depend on collagen binding to invade endothelial cells.

FUTURE DIRECTIONS

Research is an investigative process in which known facts inform new questions leading to novel experiments with which to reach additional conclusions. As such, research is a continuous cycle of study, formulation of questions and design of

experiments to seek answers or the next questions. The research described herein is not different, many questions and opportunities for additional query have evolved through the course of my studies. A short, and most likely incomplete, summary of “next questions” follows.

1. Larger scale comparative investigations of *S. mutans* in planktonic and biofilm states using serotypes *c, e* and *f* strains would help to determine whether the differences in endothelial cell invasion are serotype and lifestyle related.
2. My studies and those of others show strain-to-strain differences in EPS production by *S. mutans* biofilm. Nicotine increases biofilm mass and arginine creates a more fragile EPS matrix. These findings lead to questions of whether strain specific components of the EPS (i.e., eDNA, glucans, lipids or proteins) affect the ability of each strain to invade endothelial cells. Washing *S. mutans* biofilm post-sonication to remove EPS components before commencing with the endothelial cell invasion assay will begin to address this question.
3. Strain TLJ106-1 affords the opportunity to identify additional virulence factors involved in endothelial cell invasion.
4. This dissertation focused on the effects of nicotine and arginine on *S. mutans*; however, the effects of the same on the endothelial cell may prove to reveal important mechanisms within the endothelial cell that affect its receptivity to invasion by bacteria.

This dissertation is not an ending. It opens the door to additional investigations and new pursuits. I look forward to where this work will lead me!

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Zonuz, A. T., A. Rahmati, H. Mortazavi, E. Khashabi and R. M. Farahani (2008). "Effect of cigarette smoke exposure on the growth of *Streptococcus mutans* and *Streptococcus sanguis*: an in vitro study." Nicotine Tob Res **10**(1): 63-67.

CURRICULUM VITAE

Dawn R. Wagenknecht

EDUCATION

2019 Ph.D. Indiana University

Dissertation: “Exploration of endothelial cell invasion and responses to nicotine and arginine by *Streptococcus mutans* serotype *k* strains in a sucrose-induced biofilm lifestyle”

1993 M.S. Department of Biology; Purdue University, earned at Indiana University-Purdue University

Thesis: “Interactions of ß$_2$GPI with anionic phospholipids”

1982 B.A. in Microbiology; Southern Illinois University at Carbondale

HONORS AND AWARDS

April 2018 – Second Place for Research Poster Presentation, Ph.D. Division, Indiana Branch American Society for Microbiology, University of Indianapolis, Indianapolis, IN.

April 2018 – King Saud University Ph.D. Student Travel Award, Ph.D. Division for Research Poster Presentation at Indiana University School of Dentistry Research Day, Indianapolis, IN.

April 2018 – Elite 50 Award for Graduate and Professional Students, Indiana University – Purdue University Indianapolis, IN.

February 2017 – Graduate-Professional Education Grant, Graduate and Professional Student Government, Indiana University – Purdue University Indianapolis, IN.

October 2015 – Graduate – Professional Education Grant. Graduate and Professional Student Government, Indiana University-Purdue University Indianapolis, IN.

September 2015 – Outstanding Technologist Award, American Society for Histocompatibility and Immunogenetics, Savannah, GA.

PROFESSIONAL EXPERIENCE

1999 – 2017 HLA-Vascular Biology Laboratory; Franciscan Health Indianapolis (a.k.a. Franciscan St. Francis Health 2011 – 2016, St. Francis Hospital prior to 2011); Indianapolis, IN

Manager/General Supervisor

1997 – 1999 Transplantation Immunology Laboratory; Clarian Health Partners; Indianapolis, IN.
Lead Molecular Research Biotechnologist

1986 – 1996 Center for Reproduction and Transplantation Immunology; Methodist Hospital of Indiana; Indianapolis, IN.
8/86 to 3/93 Histocompatibility/Research Technologist
3/93 to 12/96 Lead Molecular Research Biotechnologist

1983 – 1986 Immunotransplant Laboratory; Memorial Medical Center; Springfield, IL.
1/83 to 5/83 Immunology Technician
5/83 to 7/86 Immunology/Perfusion Technologist

1981 – 1982 Dr. Michael Madigan's Laboratory; Department of Microbiology; Southern Illinois University at Carbondale; Carbondale, IL.
Laboratory Assistant

CONFERENCES ATTENDED AND PRESENTATIONS

27th Annual Indiana University School of Dentistry Research Day, Indiana Branch American Society for Microbiology, Nashville, IN
Poster presentation: Wagenknecht, D.R., Moser, E.A.S. and Gregory, R.L. (2019) Endothelial cell invasion by *Streptococcus mutans* biofilm.

44th Annual Meeting of the American Society for Histocompatibility and Immunogenetics, 2018, Baltimore, MD

2018 American Association for Dental Research Annual Meeting, Ft. Lauderdale, FL; Indiana Branch American Society for Microbiology, Indianapolis, IN; 26th Annual Indiana University School of Dentistry Research Day
Poster presentation: Wagenknecht, D.R., Moser, E.A.S. and Gregory, R.L. (2018) Nicotine Increases *Streptococcus mutans* Biofilm and Arginine Negates the Effect.

95th General Session and Exhibition of the International Association for Dental Research, San Francisco, CA; Indiana Branch American Society for Microbiology, Marshall, IN; 25th Annual Indiana University School of Dentistry Research Day
Poster presentation: Wagenknecht, D.R., Monemi, S., Childers, N. and Gregory, R.L. (2017) Nicotine and arginine increase biofilm growth of *Streptococcus mutans* serotype *k* strains.

Indiana University School of Dentistry Research Day, Indianapolis, IN; Indiana Branch of the American Society for Microbiology, Indiana University-Purdue University

Poster presentation: Wagenknecht, D.R. and Gregory, R.L. (2016) Nicotine and arginine increase biofilm growth of *Streptococcus mutans* serotype *k* strains.

41st Annual Meeting of the American Society for Histocompatibility and Immunogenetics, 2015, Savannah, GA

Indiana University School of Dentistry Research Day, Indianapolis, IN

Poster presentation: Wagenknecht, D.R., Kowolik, M.J., Galli D.M. (2015) Racial Differences in Neutrophil Response.

Indiana University School of Dentistry Research Day, Indianapolis, IN

Poster presentation: Wagenknecht, D.R., Guptill, L.F., Galli D.M. (2014) Modification of the Limulus Amoebocyte Lysate assay for endotoxin detection in canine blood specimens.

PUBLICATIONS

1. Coulam, C.B., Johnson, P.M., Ramsdan, G.H., Wagenknecht, D.R., Faulk, W.P., McIntyre, J.A. and Annegars, J.F.: Occurrence of ectopic pregnancy among women with recurrent spontaneous abortion. Am J Reprod Immunol 21:105-107, 1989.
2. Hasselaar, P., Triplett, D.A., LaRue, A., Derksen, R.H.W.M., Bolkzijl, L., de Groot, P.G., Wagenknecht, D.R., and McIntyre, J.A.: Heat treatment of serum and plasma induces false positive results in the antiphospholipid ELISA. J Rheumatol 17:186-191, 1990.
3. Coulam, C.B., McIntyre, J.A., Wagenknecht, D.R. and Rote, N.: Interlaboratory inconsistencies in detection of anticardiolipin antibodies. Lancet 335:865, 1991.
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5. Coulam, C.B., Wagenknecht, D.R., McIntyre, J.A., Faulk, W.P. and Annegars, J.F.: Occurrence of other reproductive failures among women with recurrent spontaneous abortion. Am J Reprod Immunol 25:96-98, 1991.
6. Wagenknecht, D.R. and McIntyre, J.A.: Interaction of heparin with B2-glycoprotein I and phospholipid antibodies in vitro. Thromb Res 68:495-500, 1992.
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8. Wagenknecht, D.R. and McIntyre, J.A.: Changes in B2-glycoprotein I antigenicity induced by phospholipid binding. Thromb Hemostas, 69:361-365, 1993.
9. McIntyre, J.A., Wagenknecht, D.R. and Triplett, D.A.: Detection of antiphospholipid

antibodies in heat inactivated normal human sera. Thromb Res, 69: 489-490, 1993.

10. McIntyre, J.A. and Wagenknecht, D.R.: Effect of storage conditions on the activity of antiphospholipid antibodies. Thromb Haemostas, 71(5):676, 1994.
11. McCarthy, J.M., Wagenknecht, D.R. and McIntyre, J.A.: Activity of antiphospholipid antibody ELISA cofactor in different animal sera. J Clin Lab Anal, 8:167-171, 1994.
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21. Wagenknecht, D.R., Green, K.M., Getty, R.R., Britton, R.M., Faucett, S.T., Flowers, R., Kincade, M.A., Lewis-Johnson, S. and McIntyre, J.A.: Caucasian US Bone Marrow Disease. In HLA 1997, P.I. Terasaki and D.W. Gjertson, eds., p. 362-363.
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frequency of antiphospholipid antibodies than non-exposed recipients. ASAIO Journal, 47(5):537-540, 2001.

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44. Sokol, D.K., McGuire, L.M., Johnson, N.S., Wagenknecht, D.R. and McIntyre, J.A. Obsessive-compulsive disorder and central nervous system autoimmunity. Br J Psychiatry, 189:190-1, 2006.
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Transplant 23, 1989–1997, 2017 doi: 10.1016/j.bbmt.2017.07.007.

BOOK CHAPTERS AND REVIEWS

1. Wagenknecht, D.R. and McIntyre, J.A.: Immunological findings in unexplained recurrent spontaneous abortion. ASHI Quarterly 13:13-14, 1989.
2. Wagenknecht, D.R. and McIntyre, J.A.: Immunogenetics of reproduction. SEOPF Technical Manual, A9, 1 - 10, 1993.
3. Wagenknecht, D.R., Sugi, T. and McIntyre, J.A.: The evolution, evaluation and interpretation of antiphospholipid antibody assays. Clin Immunol Newsletter 15(2/3):28-38, 1995.
4. McIntyre, J.A. and Wagenknecht, D.R.: Anti-phosphatidylethanolamine (aPE) antibodies: a survey. J of Autoimmun, 15:185-193, 2000.
5. McIntyre, J.A. and Wagenknecht, D.R.: Antiphospholipid antibodies (aPL): Risk assessments for solid organ, bone marrow and tissue transplantation. Rheum Dis Clin North Am., 27(3):611-31, 2001.
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